4. Biotin-PEG-PLA

4.1 Polymers for Antigen Delivery

Polymeric nanoparticles have shown a certain degree of success for the delivery of proteins and vaccines to the systemic circulation and to the immune system. Association of protein based antigen with particulate drug carrier system may facilitate its localization in mucosal cell lining and stimulate M cells for the generation of antibody against the antigen (Giri 2008). By controlling polymer properties, nano-particles formulated with polymer can potentially deliver antigens to the desired location at predetermined rates and durations to generate an optimal immune response. The intention of use of biodegradable polymer in delivering drug and antigen at specific site is to enable them to bind to specific receptor/ antigen presenting cells for the desired output. For the given purpose, there has been an increasing need for biomaterials that can present biological molecule at specific receptors or APCs. Many existing biodegradable polymers lack reactive groups that can be used to couple biological molecules to fabricated surfaces (Desai 1991, Barrera 1993, Langer 1993, Langer 1998). Even if such reactive groups are available, covalent strategies can not be applied in all circumstances due to harsh reaction conditions, in-efficient coupling or purification methods. Hence, there is absolute need for biocompatible, biodegradable polymers having interactive surface where mild chemical reactions are used for covalent coupling of antigens without compromising antigenicity of the antigens.

The common theme in degradable polymer systems is the presence of cleavable bonds along the polymer backbone. The most widely studied degradable materials are polylactic acid (PLA), polyglycolic acid (PGA) and their co-polymers Poly- Lactide-co-Glycolide (PLGA). PLA is typical of commonly used polymer systems in its synthesis and degradation schemes. Degradation of these systems is characterized by bulk erosion from random hydrolytic cleavage of ester bonds along the polymer backbone. Schematic diagram is as shown in figure 4.1 (Atala 1997).

The biocompatibility of the degradation products often defines the bio-compatibility for the polymer. The hydrolysis of PLA leads to degradation of the polymer back to the lactic acid repeat unit. As a natural metabolite of the body, lactic

acid is a non-toxic degradation product. PLA of high molecular weight is produced from the lactide monomer by ring-opening polymerization using most commonly a stannous octoate catalyst. This mechanism does not generate additional water, and hence, a wide range of molecular weights are accessible (Kulkarni 1966).

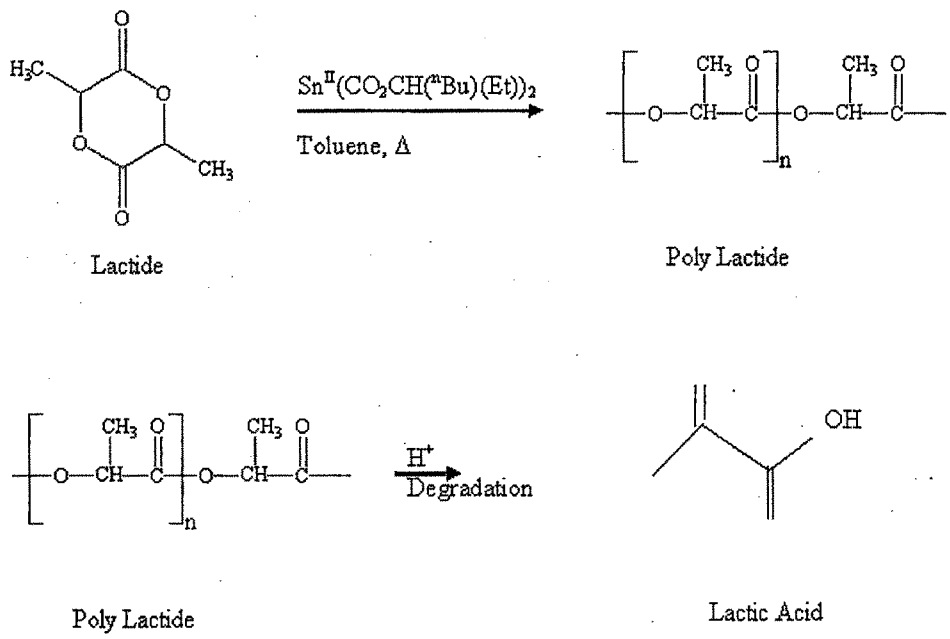

Figure 4.1 Scheme for synthesis of Poly Lactide from Lactide and biodegradation into Lactic Acid

Polyethylene glycol (PEG) also referred to as polyethylene oxide (PEO) at high molecular weights, represents another widely used biomaterials that differs from polystyrene systems insofar as PEG is resorbable but not degraded (Shield 1994). PEG is ubiquitous in biomaterial applications. It has been extensively researched in controlled-release systems and as a coating for medical devices. The hydrophilic nature of PEG leads to its ability to resist protein deposition (Kingshott 1999). Much of recent work on self assembled mono layers (SAMs) has been invaluable in defining the behavior of molecules presented at surfaces.

The PEG acts as hydrophillic component. It is included to reduce non-specific interactions between the biomaterials and living environment. (Shalaby 1994, Atala 1997).

To provide interactive surface to the polymer systems, biotin ligand was added to the bulk polymer. Poly (Lactic acid)- poly(ethylene glycol)-biotin (b-PEG-PLA) is a biodegradable polymer. The goal of synthesis of b-PEG-PLA is to maintain bulk property of PLA-PEG copolymers and provide a facile surface modification with biological ligands. Biotin moiety allows surface chemical engineering achieved by avidin-biotin interaction. Avidin possesses a tetrameric structure with four binding sites for biotin. Avidin binds to the biotin at the end of PEG chains using one of these sites. The other free binding sites are then available for attachment of Biotinylated ligand motifs (Cannizzaro 1998).

When compared to the conditions used in covalent coupling strategies, avidin-based surface engineering of b-PEG-PLA is rapidly completed in mild aqueous environment, with simple washing and purification steps. Therefore, it may eliminate the potential damage to ligand. Surface modification is a post fabrication event under mild conditions allowing for any architecture to be rapidly modified with a ligand, without damage to either. As PLA block molecular weight is decreased; the amount of PEG per total amount of block polymer is increased (Cannizzaro 1998).

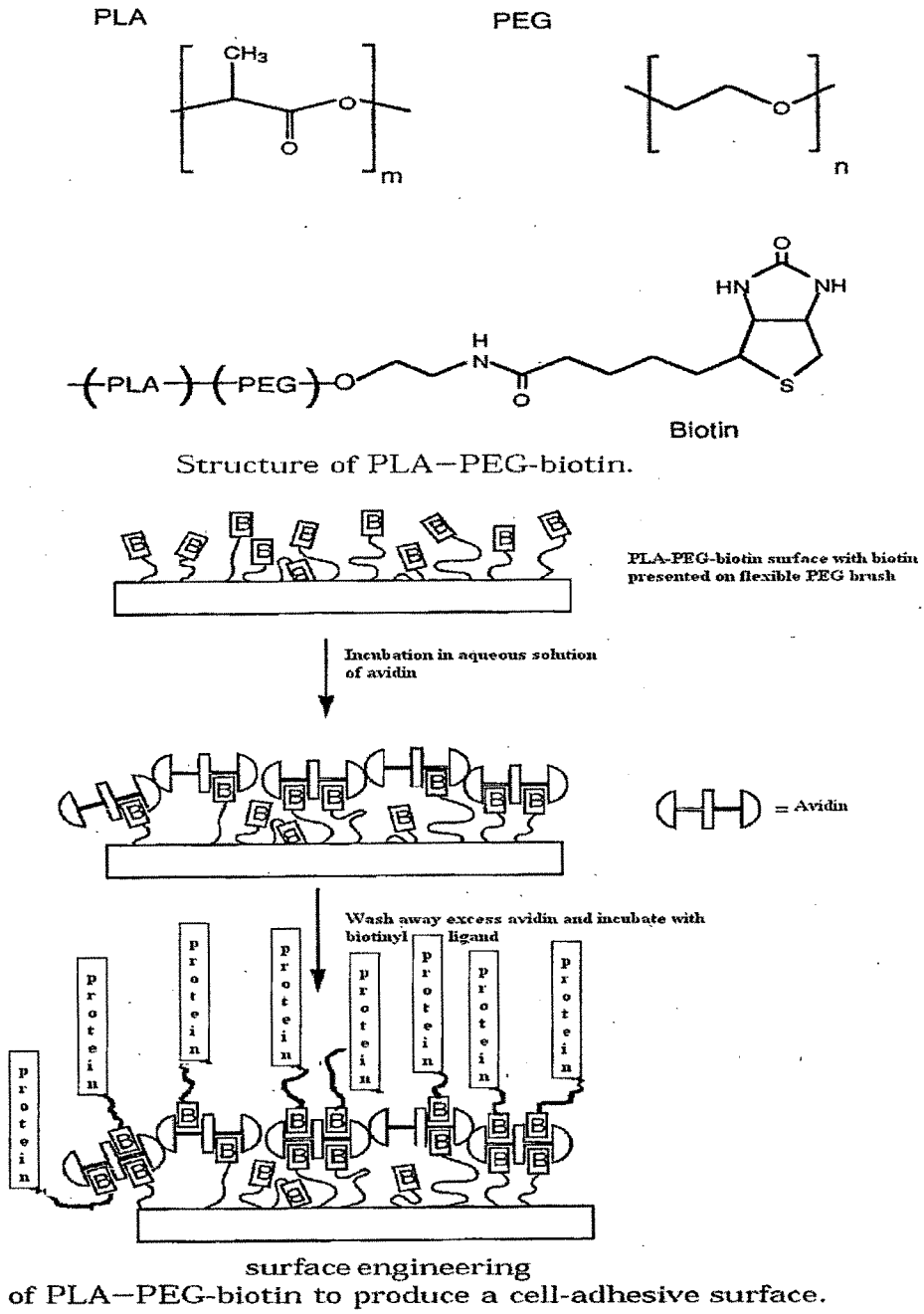

Figure 4.2 Surface engineering of B-PEG-PLA for protein conjugation

Figure 4.2 shows the schematic presentation of covalent coupling of proteins by using biotin-avidin chemistry. This chemistry revealed and confirmed by fluorescent confocal microscopy (Reches and Gazit 2007). Particles made from these materials

were first incubated with avidin fluorescently labeled with Texas red. Subsequent incubation with biotin labelled with fluorescein isothrocyanate revealed an nearly exact distribution to the surface-bound avidin, in contrast, PLA-PEG (no biotin) particles showed no surface fluorescence when incubated with labeled avidin or biotin (Cannizzaro 1998).

4.2 Synthesis of B-PEG-PLA

B-PEG-PLA can be synthesized in two easy steps: biotin attachment to bi-functional PEG followed by PLA attachment to PEG- biotin by ring opening polymerization of the lactide. The graft polymerization of Lactide is possible using 2 methods; solution polymerization or melt polymerization. Surface Plasmon resonance (SPR) and Fluorescent Spectroscopy prove to be useful methods of assessing the surface engineering process and whether the biotin unit is degraded or biological activity is reduced by synthesis (Salem 2001).

Solution polymerization is a method of industrial polymerization. In this procedure, a monomer is dissolved in a non-reactive solvent that contains a catalyst. The heat released by the reaction is absorbed by the solvent, and so the reaction rate is reduced. The melt polymerization is a little different than what is described for solution polymerization where the contents were not diluted with toluene. The round-bottom flask with lactide (2 g) and biotin-PEG-OH (0.35 g) was sealed under Nitrogen and heated at 140°C for 16 h before being re-precipitated (Salem 2001).

4.2.1 Chemicals and reagents

α- hydroxy -ω-amine PEG (-NH2-PEG-OH-) MW 3000 Da was purchased from REPP polymere, Germany, N-hydroxysuccinamide-biotin, Stannous Octanoate, methyltrichlorosilane were from Sigma Aldrich, USA, Diethyl ether anhydrous, Isopropyl Alcohol were purchased from Baker, USA,. Lactide was a kind gift from PolyScience, the Netherlands.

4.2.2 Procedure

The Solution polymerization method was used for these studies (Salem et al 2001).

PART 1 Boitinylation of PEG

1 gm of α- hydroxy -ω-amine PEG (-NH2-PEG-OH-) was dissolved into acetonitril 2 mL methylene chloride 1 mL and pyiridine 80 µl and stirred for one minute. 0.250 gm of N-hydroxysuccinamide-biotin was added to it and stirred at RT under Nitrogen overnight. Diethyl ether, anhydrous (about 40 mL) was added slowly to precipitate the polymer and filtered on a Buchner funnel with Whatman filter paper and washed with diethyl ether. The isolated material was dissolved in IPA (70^0C) and re-precipitated polymer by cooling. The product was analyzed for biotin attachment by H^1-NMR spectroscopy and gel permeation chromatography as described later.

Part II Removing water and aqueous impurities

About 350 mg of polymer was dissolved into toluene and refluxed with a Dean-Stark trap and a condenser and toluene then removed by distillation. Remaining polymer was isolated on a rotary evaporator to get as dry as possible. Diethyl ether was then added to the polymer and washed with ether. The product was dried under vacuum for 2 days.

Part III Purification of Lactide

Lactide (20 gm, L- Lactide = 0.14 mol) was re-crystallised from a minimum amount of toluene (15 mL for 20 gms of L- Lactide). Precipitated crystals were filtered using Buchner funnel and Whatman filter paper. Crystallization and filtration were repeated at least three times. The crystals were dried for 24 hours under reduced pressure (0.01 mm Hg) and kept over P_2O_5 in vacuum at 4 ^{0}C before use to avoid the absorption of water.

Preparation of $Sn(Oct)_2$

Stannous Octanoate ($Sn(Oct)_{2)}$ (10 gm, 2.5 X 10^{-2} mol, 1.25 specific gravity) was added into one-neck round bottomed flask with magnetic stirring bar equipped with distilling head and condenser. The flask was placed into oil bath at 175 ^{0}C and distilled under reduced pressure (0.1 mm Hg) using vacuum pump. The product was dried under reduced pressure (0.01 mm Hg) and stored at -20 ^{0}C in an evacuated, sealed glass tube. $Sn(Oct)_2$ was dissolved in anhydrous toluene (1.0M).

Preparation of Anhydrous Toluene

Toluene was distilled under reduced pressure (0.01 mm Hg) for 30 minutes and the flask was sealed.

PART IV Preparation of PLA-PEG-Biotin

The graft polymerization of lactide on Biotin-PEG-OH by solution polymerization: Glassware were silanized by rinsing with 5% methyltrichlorosilane (Aldrich, 38543-3) solution in toluene, rinsed with acetone and left overnight at 130 ^{0}C. The round bottom flask was charged with biotin-PEG-OH. 2.0 gm of Lactide was transferred and diluted with 10 mL of toluene, closed with cap and kept in desiccator. The round bottom flask was taken out and heated at 60 ^{0}C until contents disperse into the solution. $Sn(Oct)_2$/ toluene (0.1gm in 1 mL) was then added and reaction allowed at 110 ^{0}C for 6-8 hours under nitrogen. Dean-Stark trap was attached to remove solvent and any of remaining solvents were removed by vacuum rotary evaporator. Remaining viscous material was heated to 140 ^{0}C to melt and left for 1 hour under Nitrogen. Reaction mixture was then cooled and dissolved in approximately 10 mL of Dichloromethane. This polymer solution was added drop wise to diethyl ether. The final product was isolated by vacuum filtration and lyophilize overnight. White powder was the final product.

Product was analysed for molecular weight and attachment of biotin and polyethylene glycol to Poly Lactide by Gel Permeation Chromatography and NMR.

4.3 Analysis of Polymer

4.3.1 Gel Permeation Chromatography

Gel permeation chromatography (GPC) is a term used when the separation technique separates analytes on the basis of size, is applied to polymer in particular. As a technique, GPC was first developed in 1955 by Lathe and Ruthven. While polymers can be synthesized in a variety of ways, it is often necessary to separate polymers, both to analyze them as well as to purify the desired product. When characterizing polymers, it is important to consider the polydispersity index (PDI) as well the molecular weight. Polymers can be characterized by a variety of definitions for

molecular weight including the number average molecular weight (M_n), the weight average molecular weight (M_w)., the size average molecular weight (M_z), or the viscosity molecular weight (M_v). GPC allows for the determination of PDI as well as M_v and based on other data, the M_n, M_w, and M_z can be determined (Pickett 1966).

GPC separates based on the size or hydrodynamic volume (radius of gyration) of the analytes. This differs from other separation techniques which depend upon chemical or physical interactions to separate analytes (Skoog 2006). Separation occurs via the use of porous beads packed in a column.

The smaller analytes can enter the pores more easily and therefore spend more time in these pores, increasing their retention time. Conversely, larger analytes spend little if any time in the pores and are eluted quickly. All columns have a range of molecular weights that can be separated (Skoog 2006).

If an analyte is either too large or too small it will be either not retained or completely retained respectively. Analytes that are not retained are eluted with the free volume outside of the particles (V_o), while analytes that are completely retained are eluted with volume of solvent held in the pores (V_i). The total volume can be considered by the following equation, where V_g is the volume of the polymer gel and V_t is the total volume: $Vt = Vg + Vi + VoI$ (Sandler 1998)

As can be inferred, there is a limited range of molecular weights that can be separated by each column and therefore the size of the pores for the packing should be chosen according to the range of molecular weight of analytes to be separated. For polymer separations the pore sizes should be on the order of the polymers being analyzed. If a sample has a broad molecular weight range it may be necessary to use several GPC columns in tangent with one another to fully resolve the sample (Helmut 1969).

4.3.3.1 Materials and Equipment

Polymer molecular weight was determined by gel permeation chromatography (JASCO PU-980 intelligent HPLC pump, 1560 intelligent column thermoset and RI-1530 intelligent refractive index detector). Samples were filtered and eluted in

chloroform through a series of Styragel columns (guard, HR4 and HR3 Styragel columns, Waters). The Polystyrene was purchased from

4.3.3.2 Sample preparation

GPC studies were conducted using degassed chloroform as the eluent with a sample concentration of 0.1% w/v, an injection volume of 150 µL injected with auto sampler AS-950, separated with CO-1560 columns, flow rate was maintained at 0.3 mL/ min with pump (PU 980 Analytical pump). Studies were carried out at ambient temperature and pressure of 50 Bar. The detector used was DRI 1530. The known molecular weight polymer polystyrene (Easical PS-2) (0.1% w/v) was run along with the sample to calibrate the system. Borwin 1HSS-1500 was the software for data collection and Microsoft Excel was used for calculations of number average and weight average molecular weight.

4.3.2 NMR analysis of Polymer

One of the challenges in polymer evaluation is molecular weight (avg. chain length). While membrane osmometry, gel permeation chromatography, viscosity analysis and mass spectrometry are typically used for molecular wt. determination, the techniques can be time consuming, inaccurate for the molecular wt. ranges involved or require specialized instrumentation. End group analysis by NMR offers an easy alternative method using an instrument commonly found in many analytical labs. In addition, NMR analysis can also be used to accurately determine monomer ratios for various copolymer molecules.

^{1}H NMR is also a useful tool for calculating the monomer ratio in copolymers. This method can be used even in situations where repeating unit proton signals overlap, as long as there is a clearly distinguishable signal from one of the repeating unit.

NMR polymer analysis helps to understand (Tyszka 2005):

- Monomer Type, Level and Distribution, Block or Random
- Polymer Chain Branching
- Polymer Sequencing
- Polymer Tacticity

- Polymer Molecular Weight
- Polymer End-groupings

4.3.2.1 Materials and Equipment

Biotin-PEG-NH2 was purchased from RAPP polymere, Germany. Biotin-PEG and Biotin-PEG-PLA prepared at the Hanes Lab, Johns Hopkins University, Baltimore, USA. Chloroform and Chloroform- d were purchased from Aldrich, USA. The Proton H^1 NMR spectra of Biotin-PEG-PLA were recorded on a Bruker NMR *TOPSPIN* V 2.1 (As shown in figure 4.3) spectrometer at 250 Hz. H^1NMR chemical shifts was measured in parts per million (ppm) relative to $CDCl_3$.

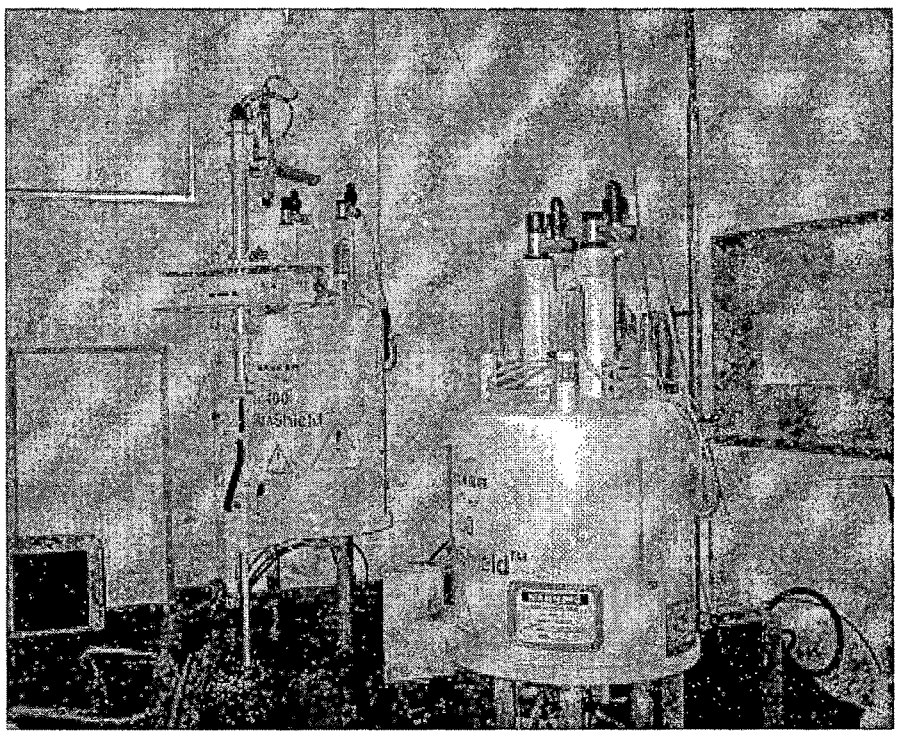

Figure 4.3 Bruker NMR *TOPSPIN* v 2.1 spectrometer equipment

4.3.2.2 Sample Preparation

Biotin-PEG and Biotin-Peg-PLA were dissolved in $CDCl_3$ in concentration range of 0.1gm/mL. Transfer this content in NMR tubes and covered properly. Along with

these products, starting material NHS-biotin and NH_2-PEG- OH were also dissolved in CDCl3 in 0.1gm/mL concentration. NMR spectra were then obtained following the procedure provided with the Bruker NMR *TOPSPIN* v 2.1 spectrometer equipment. Figure 4.5 shows the picture of the Bruker equipment for ^{1}HNMR spectroscopy.

4.4 Results and Discussions

Biotin-PEG-PLA was synthesized by ring opening polymerization. The polymer chain length determined the molecular weight of the polymer and hence it is necessary to evaluate molecular weight of the polymer. Gel Permeation chromatography is a useful tool to determine molecular weight of polymer when it was run with standard polystyrene polymer. The polystyrene polymer (Easical PS-2) in the concentration of 0.1 % w/v was run with the sample B-PEG-PLA polymer. Polystyrene has known 4 different peaks 1000, 3000, 10000 and 100,000. The software supplied with the instruments gave data on retention time and refractive index of the polystyrene (Figure 4.4), which were processed with Microsoft Excel software (table 4.1) and GPC calibration was plotted as per shown in figure 4.5.

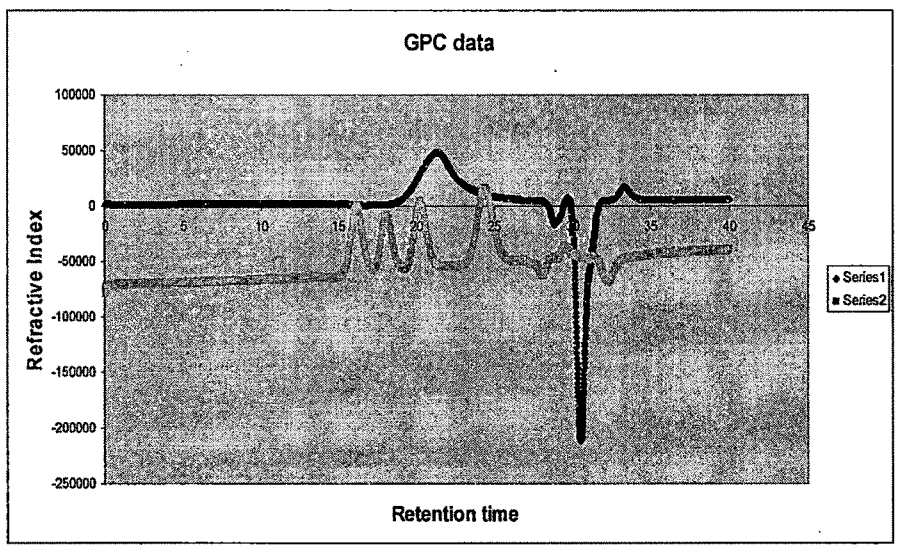

Figure 4.4 Calibration of GPC with PS beads (mixture of known mol weight polymer)

Peak number	Retention Time ± SD	Mol Weight
1.	16.18 ± 0.23	100000
2.	18.11 ± 0.35	30000
3.	20.3 ± 0.46	10000
4.	24.42 ± 0.53	1000
B-PEG-PLA	19.23 why no SD?	~20000

N = 3

Table 4.1 Data for GPC calibration curve

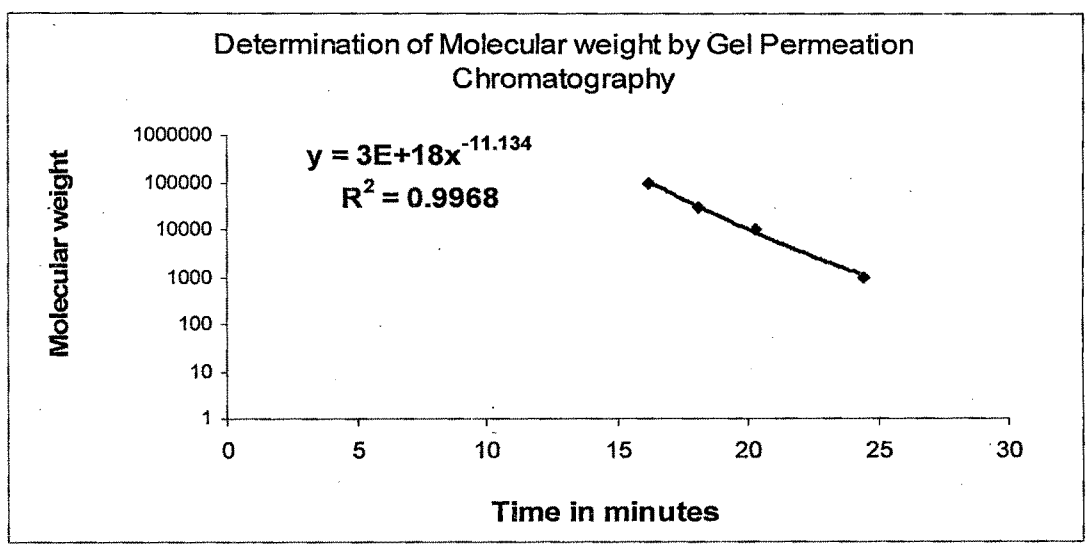

Figure 4.5 Calibration curve for determination of mol wt of B-PEG-PLA by GPC

Molecular weight of B-PEG-PLA was calculated from the quadratic equation obtained from the standard curve. Calculated average molecular weight of the polymer was 20,000. This was also indicating polymerization of the monomer lactide. Molecular weight of the polymer can be controlled by varying the amount of PEG and PLA used for the polymerization. Higher the amount of PEG, lower would be the molecular weight of the PLA. For our purpose where antigens molecular weights are 9.8 kDa and 32 kDa, we decided to keep polymer weight at about 20K.

Another parameter which is very necessary to evaluate in polymer characterization is the attachment of ligand. This was done by H^1NMR spectroscopy (Metters 2001). Here biotin is the ligand. The attachment of biotin to NH_2-PEG-OH was achieved through N-hydroxy- succinimide chemistry. The graft polymerization of Lactide to the HO-PEG-biotin proceeds through a ring opening polymerization mediated by Sn(Oct)2 complex suggested by Kricheldorf 1995.

Bruker NMR *TOPSPIN* v 2.1 spectrometer was used to take H^1NMR spectra of the sample. Spectra for α- hydroxy -ω-amine PEG (-NH_2-PEG-OH-) and N-hydroxysuccinamide-biotin were also taken along with PEG-biotin and B-PEG-PLA. Figure 4.6 and 4.7 show the spectra for PEG-biotin and B-PEG-PLA respectively.

Analysis of PEG-biotin by H^1NMR spectroscopy showed the appearance of a triplet at 2.05 ppm that can be assigned to the methylene from the biotin chain α to the amide and appearance of a broad singlet belonging to the free amino proton at 7.85 ppm. These signals were not present on the NMR spectra of NHS-biotin. The biotin group was identified through the two methane protons from the cyclic biotin structure at 4.3 and 4.2 ppm and two urea protons from cyclic biotin structure at 6.45 and 6.35 ppm. In NMR spectra's of PLA-PEG-biotin, biotin signals could not be seen as the signal from the PLA overwhelmed the biotin signal. (CH; 1.53 ppm and CH_3, 5.22 ppm).

The data of GPC and NMR clearly indicated that polymerization had occurred with the graft polymerization reaction and there was proper attachment of PEG and Biotin to the backbone of the polymer PLA.

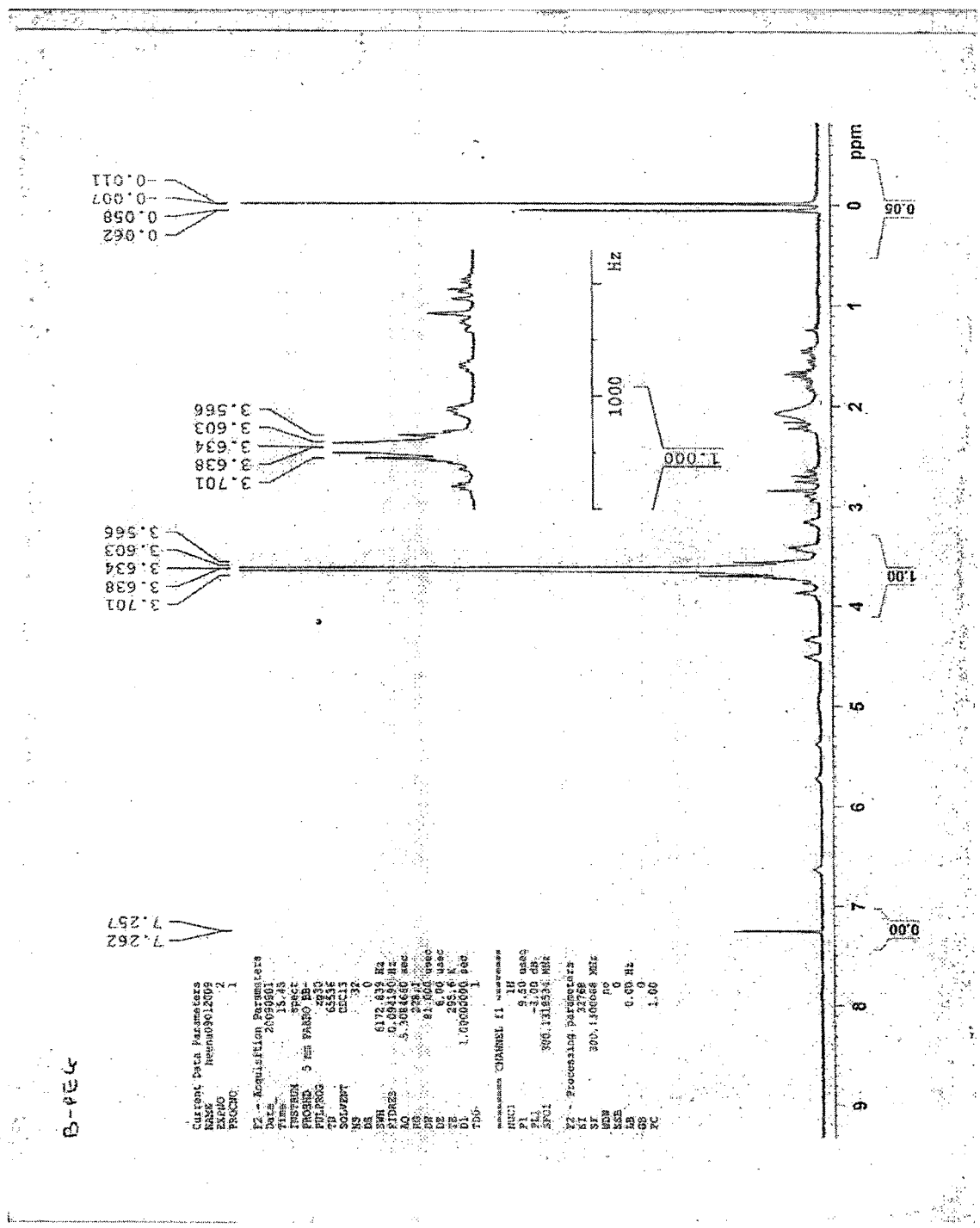

Figure 4.6 H^1 NMR Spectra for B-PEG

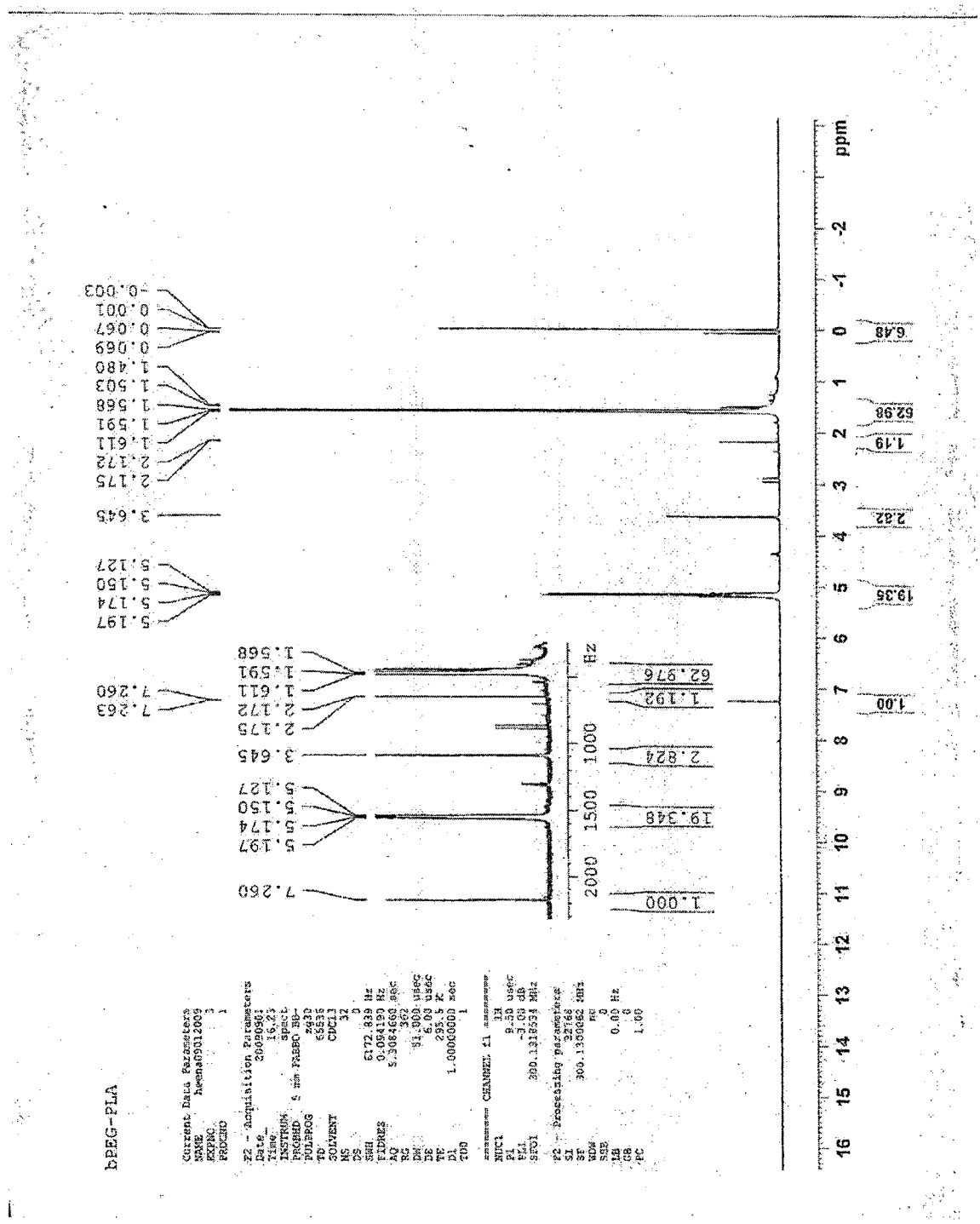

Figure 4.7 H^1 NMR Spectra for B-PEG-PLA

4.5 References

Atala, M. (1997). Synthetic biodegradable polymer scaffolds: Tissue Engineering. Birkhaser, Boston.

Barrera, D. A.; Zylstra, E.; Lansbury, P. T., Jr.; Langer, R. Synthesis and RGD peptide modification of a new biodegradable copolymer: poly(lactic acid-co-lysine). J. Am.Chem. Soc. 1993, 115, 11010-11011.

Cannizzaro, S., Padera, R., Langer, R., Rogers, R., Black, F., Davies, M., Tendler, S., Shakesheff, K (1998). "A novel biotinylated degradable polymer for cell-interactive applications", Biotechnology and Bioengineering; 58: 529-535.

Desai, N.P., Hubbel, J.A. (1991). Solution technique to incorporate polyethylene oxide and other water-soluble polymers into surfaces of polymeric biomaterials. Biomaterials, 12, 144-153.

Giri P.K., Khuller G.K. (2008). Is intranasal vaccination a feasible solution for TB? *Expert Rev. Vaccines*, 7(9), 1342-1356.

Helmut, D. (1969). Gel Chromatography, Gel Filtration, Gel Permeation, Molecular Sieves: A Laboratory Handbook; Springer-Verlag.

Kingshott, P., Griesser H.J. (1999). Surfaces that resist bioadhesion. *Current opinion in solid state and materials science*, **4 (4)**, 403-4121.

Kricheldorf, H. R.; Kreisersaunders, I.; Boettcher, C. (1995). Polylactones .31. Sn(II)Octoate-Initiated Polymerization of L-Lactide - A Mechanistic Study Sn (II) Octoate-Initiated Polymerization of L-Lactide - A Mechanistic Study*Polyme*, *36*, 1253-1259.

Kulkarni, P.K., Pani, P.C., Newman, C., Leonard, F. (1966). PolyLactic Acid for surgical implants. *Arch, Surg*, **93**, 839-844.

Langer, R., (1998). Drug Delivery and taregeting" Nature, 392 (Supp.), 5-10.

Langer, R., Vacanti, J. "Tissue Engineering", Science; 260: 920-926, 1993.

Lathe, G.H., Ruthven, C.R.J. (1956). The Separation of Substance and Estimation of their Relative Molecular Sizes by the use of Columns of Starch in Water. *Biochem J.*, **62**, 665–674.

Metters, A.T., Anseth, K.S., Bowman, C.N. (2001). A Statistical Kinetic Model for the Bulk Degradation of PLA-*b*-PEG-*b*-PLA Hydrogel Networks: Incorporating Network Non-Idealities. *J. Phys. Chem. B*, **105 (34)**, 8069-8076.

Pickett, H. E., Cantow, M. J. R. and Johnson, J. F. (1966), Column fractionation of polymers. VII. Computer program for determination of molecular weight distributions from gel permeation chromatography. Journal of Applied Polymer Science, 10: 917–924.

Reches, M. and Gazit, E. (2007). Biological and chemical decoration of peptide nanostructures via biotin-avidin interactions. *J. Nanosci, Nanotechnol*, 7, 2239-2245.

Salem, A.K., Cannizzaro, S.M., Davies, M.C., Tendler, S.J.B., Roberts, C.J., Williams, P.M., Shakesheff, K.M. (2001). Synthesis and Characterisation of a Degradable Poly(lactic acid)-Poly(ethylene glycol) Copolymer with Biotinylated End Groups. Biomacromolecules 2001, 2, 575-580.

Sandler, S.R., Karo, W. Bonesteel, J., Pearce, E.M. *Polymer Synthesis and Characterization: A Laboratory Manual*; Academic Press: San Diego, 1998.

Shakesheff, K.M., Evora, C., Soriano, I., Langer, R (1997). The Adsorption of Poly(vinyl alcohol) to Biodegradable Microparticles Studied by X-Ray Photoelectron Spectroscopy (XPS). J. Colloid Interface Sci. 185: 538–547.

Shalaby, S.W. (1994). Biomedical Polymer: Designed –to- Degrade Systems. Hanser/ Gardner, Cincinnati, OH.

Shields, R., Harris, J., and Davies, M. W. (1968). Suitability of polyethylene glycol as a dilution indicator in the human colon. Gastroenterology, 54, 331-333.

Skoog, D.A., Holler, F.J., Crouch,S.R. (2006). Principles of Instrumental Analysis. Sixth Edition, Thompson Brooks/Cole: Belmont, CA, 2006, Chapter 28.

Tyszka, J.M., Fraser, S.E., Jacobs, R.E. (2005). "Magnetic resonance microscopy: recent advances and applications". *Current Opinion in Biotechnology,* 16 (1), 93-99.

Reviewer's Comments