Biodegradable polymers for controlling/studying material-cell interaction

Jereme Raphael Doss
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SYNTHESIS OF FUNCTIONAL NANOSCALE BIODEGRADABLE POLYMERS
FOR CONTROLLING/STUDYING MATERIAL CELL INTERACTIONS

Advisor: Dr. Ishrat Khan

Dissertation dated November 2012

Functionalized nanomaterials have an expansive range of potential uses in biomedical applications. Functionalized synthetic (biocompatible and/or biodegradable) polymers that control or monitor cell signaling can be effective antagonists and promising drug candidates. We have developed a series of biodegradable functional polymer systems (with dimensions in the nanoscale) for creating allergy-effective drugs, using RBL mast cells and anti-2,4 dinitrophenyl (DNP) IgE antibodies that sensitize these cells by binding to high affinity IgE receptors (FceRI); creating polymers which are effective inhibitors of degranulation of mast cells stimulated by a potent allergen. The inhibition is possible because of the specific interaction of the functional polymers with the proteins (IgE) on the mast cell surfaces to control cell-signaling i.e. intelligent design of functional materials to manipulate cellular functions. The functional polymer system is based on biodegradable poly(lactides) carrying two, three or four 2,4-dinitrophenyl (DNP) groups. Although these DNP-functionalized ligands do not stimulate the granule exocytosis
response, they have the potential to inhibit the robust response stimulated by other multivalent DNP ligands. These polymeric ligands are effective inhibitors of degranulation of mast cells stimulated by a potent allergen and thus are a potential model drug system. Additionally these lactide based polymers bind and achieve steady state binding with solution IgE within a few seconds at low concentrations; achieving about 75% of the binding below 0.2 uM. The preparation, characterization, processing and effectiveness of the functional polymers to control material-cell interactions will be discussed
BIODEGRADABLE POLYMERS FOR CONTROLLING/STUDYING MATERIAL CELL INTERACTIONS

A DISSERTATION

SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

JEREME RAPHAEL DOSS

DEPARTMENT OF CHEMISTRY

ATLANTA, GA

MAY 2013
ACKNOWLEDGEMENTS

I would like to first thank my mother Michelle Berry-Anthony, my sister Portia Blanchette, and my grandparents Howard Berry Sr. and Arletha Berry. Your love and support is the driving force that made this possible. Words cannot express how much I love and appreciate you. This degree is just as much yours as it is mines.

Dr. Ishrat Khan, I appreciate the opportunity, funding, and guidance throughout this journey. I also would like to thank my committee members Dr. Cass Parker, Dr. James Reed, Dr. Myron Williams, and Dr. James Bu. Each of you has provided me knowledge and support, and your guidance has been invaluable. It is something that will stay with me as I move forward with my career. I would also like to thank Mrs. Joyce Lockhart, Ms. Debra Ware, Mrs. Carolyn Taylor, and the various administration and staff throughout Clark Atlanta University for their role in making this process of acquiring my degree as seamless as possible; in particular, members of the Center for Functional Nanoscale Materials, the Department of Chemistry, and MBRS-RISE.

I owe a great deal of gratitude to Dr. LaTonia Taliaferro-Smith, Dr. Olayinka Ogunoro, Ian Stubbs, and Dr. Michelle Gaines, for your advice, training and sincerity.
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LIST OF ABBREVIATIONS

IgE IMMUNOGLOBULIN E
FCεRI FC EPSILON RECEPTOR I
FAB FRAGMENT ANTIGEN SEGMENT
DNP DNP-ε-AMINO-N-CAPROIC ACID
ASTM AMERICAN SOCIETY FOR TESTING MATERIALS
ISO INTERNATIONAL STANDARD ORGANIZATION
PCL POLY(ε-CAPROLACTONE)
PLA POLY(LACTIDE)
PGA POLY(GLYCOLIDE)
PLGA COPOLYMER OF POLY(LACTIDES)/POLY(GLYCOLIDES)
PEG POLY(ETHYLENE GLYCOL)
P2MS POLY(2-METHOXY STYRENE)
T\text{G} GLASS TRANSITION TEMPERATURE
PDLA POLY( D-LACTIDE)
PLLA POLY(L-LACTIDE)
LLA L-LACTIC ACID
ROP RING OPENING POLYMERIZATION
Sn(Oct)\textsubscript{2} STANNOU OCTOATE OR TIN(II) 2-ETHYLHEXANOATE

LIST OF ABBREVIATIONS

FDA FOOD AND DRUG ADMINISTRATION
RES RETICULOENDOTHELIAL
MW MOLECULAR WEIGHT
# LIST OF ABBREVIATIONS

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<td>ABA</td>
<td>TRIBLOCK COPOLYMER</td>
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<td>DCT</td>
<td>DNP-AMINOCAPRYOL-L-TYROSINE</td>
</tr>
<tr>
<td>RBL</td>
<td>RAT BASOPHIL LEUKEMIA CELLS</td>
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<tr>
<td>NMR</td>
<td>NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY</td>
</tr>
<tr>
<td>GLY</td>
<td>GLYCEROL</td>
</tr>
<tr>
<td>PENT</td>
<td>PENTAERYTRITOL</td>
</tr>
<tr>
<td>¹H NMR</td>
<td>PROTON NUCLEAR MAGNETIC RESONANCE</td>
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<tr>
<td>MHZ</td>
<td>MEGAHertz</td>
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<tr>
<td>PPM</td>
<td>PARTS PER MILLION</td>
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<tr>
<td>M&lt;sub&gt;N&lt;/sub&gt;</td>
<td>NUMBER AVERAGE MOLECULAR WEIGHT</td>
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<tr>
<td>IR</td>
<td>INFRARED SPECTROSCOPY</td>
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<tr>
<td>-OH</td>
<td>HYDROXYL GROUP</td>
</tr>
<tr>
<td>cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>RECIPROCAL CENTIMETER</td>
</tr>
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<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>NITROGEN DIOXIDE</td>
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<td>TGA</td>
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<td>DIFFERENTIAL SCANNING CALORIMETRY</td>
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<td>GLY-PLA</td>
<td>GLYCEROL-POLY(LACTIDE)</td>
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<td>P2MS57</td>
<td>POLY(2-METHOXY STYRENE)</td>
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LIST OF ABBREVIATIONS

FITC  FLUORSCEIN ISOTHIOCYANATE
K_D  DISSOCIATION CONSTANT
BSA  BOVINE SERUM ASSAY
IC50  HALF MAXIMAL INHIBITORY CONCENTRATION
μM  MICROMOLAR CONCENTRATION
NG  NANOGRAMS
nM  NANOMOLAR CONCENTRATION
UHP  ULTRA HIGH PURITY
CHAPTER 1

INTRODUCTION

The clustering of IgE-FcεRI on mast cells surface by bivalent or multivalent ligands controls the allergic reactions mediated by the IgE antibody. This clustering is the key step for initiating signal transduction resulting in the granule exocytosis. There are two Fragment antigen binding (Fab) segments that contribute to the ligand/antigen binding with IgE. Previous studies have shown that ligands end-functionalized by DNP-ε-amino-n-caproic acid (DNP) have the ability to control the crosslinking of anti-DNP IgE-FcεRI complex and, furthermore, the degree or activity level of crosslinking can be controlled by the size (i.e. length), composition, and DNP valency.\cite{1,2} Earlier studies demonstrated that monovalent ligands, in most instances, do not stimulate mast cells because the monovalent ligands do not result in significant crosslinking. Crosslinking by multivalent ligands were quite effective and stimulates cell activation or degranulation. On the other hand, certain bivalent ligands have the ability to inhibit granule exocytosis. The inhibition or inhibitory activity is dependent on the structure of the bivalent ligand and also whether the binding is intermolecular or intramolecular.\cite{1,3} The development of biodegradable polymers from renewable sources will make excellent inhibitors of cell activation.
A large fraction of common polymeric components in the market and in most applications are produced from non-renewable fossil resources. While many synthetic polymers can be recycled and reused, the use of polymers synthesized from non-renewable sources on such a large scale has had many negative effects that have altered the world’s environment, including the change in its climate. Waste from nondegradable polymeric solids or polymers which are not recycled and reused can generate greenhouse gases during various steps of processing raw materials, manufacturing of products, waste management etc. These materials, commonly referred to as plastics are used for building material, packing, and many other non-degradable consumables. Even with all the drawbacks of using synthetic polymers, the annual production of synthetic polymers from petroleum products is increasing. It is reported that the reserve to production ratio for oil in the world is around 40 years. This information has increased the push to develop polymer systems whose development does not rely on petroleum or natural gas sources.

In addition to developing products whose production does not rely on fossil fuels, the development of consumable products that degrade has become a priority. Non degradable products end up in a landfill after a short shelf life. Even though recycling has decreased lingering waste products, it only accounts for 30% of the plastics discarded yearly. Furthermore, successive melting/recasting of the plastics diminish the physical properties, leaving it unfit for consumer products.

Estimates by the United States Environmental Protection Agency suggest that nearly 25% of the municipal landfill space is occupied by plastic and the incineration of polymeric materials contribute significantly to carbon dioxide in the atmosphere. This
concern particularly affects Europe and Japan as there is less room for land fill than there is in the United States. Many widely used polymers like polystyrene and poly(vinylchloride) are made from toxic monomers. The stability of these polymers has led to the commercial success of these hazardous polymers. However, due to the toxicity of the monomers used to prepare these polymers, there is a need for environmentally friendly methods of producing renewable commodity plastics.

The problems associated with synthetic polymers have led to increased interest in developing polymers that are derived from non-petrochemical sources and also has the additional characteristic of natural degradation. Ideally these polymers can be made from renewable resources and will be suitable for products with a short shelf life. Degradation occurs in one of two ways. The polymers will degrade naturally from interaction with micro-organisms such as fungi or bacteria. Or polymers will degrade via hydrolytic degradation with the byproducts of CO₂, water, biomass, and inorganic material at the same rate while leaving no toxic residue.

1.1 Biodegradable Polymers

Biodegradable polymers continue to hold great promise for applications in agriculture, industry, marine, biomedical, and pharmaceuticals applications. Bio-based polymers are polymers that are made from natural resources while biodegradable polymers can be synthesized from either natural or synthetic monomers. Bio-based degradable plastics are defined by the American Society for Testing of Materials (ASTM) and the International Standards Organization (ISO) as those polymers which can undergo a significant change in the chemical structure under specific environmental conditions.
Biodegradable plastics or polymers are those that degrade with the help of microorganisms such as algae, fungi, and bacteria. These measurable changes effect the mechanical and physical properties of the polymer.

Biodegradable aliphatic polyesters with great promise, such as poly(ε-caprolactone) (PCL), poly(lactide) (PLA), poly(glycolide) (PGA), and copolymer of poly(lactides)/poly(glycolides) (PLGA) have been widely adopted in biomedical applications.\(^9\),\(^10\) Of these biodegradable polymers, PLA has received significant attention in biomedical application due to the polymers biodegradability, biocompatibility, and non-toxic nature.

1.2 Biodegradable Polymers for drug Delivery.

The ability to develop controlled and specific interactions with biological materials are of great interest. These biospecific interactions may be utilized in therapeutics such as specific drug delivery, and also monitoring and controlling cellular performance. Furthermore, the possibility of preparing such materials from biodegradable polymers is of significant interest because once the polymers carry out its function and the polymer is no longer required, it would be ideal for the polymer to degrade into non-toxic small molecules.

The established and desirable properties of many biodegradable polymers have led to the belief that these polymers are excellent alternatives to both biocompatible and nonbiocompatible polymers. Therefore, this area of research has shown impressive strides in advancement over the past 35 years and the research has resulted in well-established synthesis, manufacturing, and processing of these materials.\(^11\),\(^12\) The main advantage of biodegradable polymers used for drug delivery and other biological
applications (such as tissue engineering) is their reduced toxicity. This has given rise to
the use of biodegradable polymers in a broad range of practical applications that range
from packaging to advanced medical devices. Of the many different classes of
biodegradable polymers, linear aliphatic polyesters are particularly attractive and most
used, especially those derived from lactic acid (PLA), glycolic acid (PGA), and their
copolymers (PLGA)\cite{11}{13}(Figure 2.1). Aliphatic polyesters such as poly(L-lactide)
(PLLA), polyglycolide (PGA), poly(ε-caprolactone) (PCL) and copolymers of
PLLA/PGA have been widely used for biomedical applications such as drug delivery,
pharmaceutical, and tissue-engineering applications because of their biodegradability,
low immunogenicity, and excellent biocompatibility.\cite{14}

1.3 Polymer Biospecific Binding

The synthesis, characterization and biospecific binding capacities of monovalent
and multivalent 2, 4-dintrophenyl(DNP) functionalized synthetic ligands has been
previously reported.\cite{1,15} DNP functionalized poly(ethylene glycol)s have demonstrated
the ability to react with DNP-specific IgE and either stimulate and inhibit mast cell
degranulation. Mast cells are primarily located in the mouth, nose, lungs, and digestive
tract. When a mast cell is degranulated granules rich in histamines and heparin are
released via a sequence of biological reactions initiated by the clustering of high affinity
receptors (FceRI) on the surface of the mast cell. This response is mediated by the
crosslinking of immunoglobulin E by a specific antigen. Inhibitory and stimulatory
functions have been determined to be dependent on molecular weight and degree of
functionality of the polymers.\cite{1} DNP, when attached to a polymer, provides the ability to
directly bind to the anti-DNP-IgE receptors on the surface of mast cells. Studies
previously conducted by Khan et al have shown that lower molecular weight DNP functionalized poly(2-methoxystyrene) have the ability to specifically bind to the IgE-FeRI receptor proteins in solution. \cite{16} While binding was achieved, the hydrophobic nature of poly(2-methoxystyrene) caused the binding constant in aqueous solution to be dramatically lower than the water soluble DNP-PEG system. However, because the poly(2-methoxystyrene) is hydrophobic, higher molecular weight (50,000 or higher) polymers were electrospun to form nanofibers decorated with DNP groups on the fiber surface. The DNP on the fiber surface can specifically bind with IgE in solution and also IgE on surfaces of mast cells. \cite{17}

In the present study, the synthesis and characterization of DNP functionalized poly(lactide)s is reported and additionally, the binding properties of these polymers with IgE and IgE on mast cell have been evaluated.
BACKGROUND

CHAPTER 2

Polylactide, also known as poly(lactic acid) (PLA), is a biodegradable polymer which can be produced from renewable sources such as sugarcane and corn. Lactic acid is harvested from the renewable resources and formed into a lactide via cyclic dimerization by condensation (Fig 2.2). The degradation of PLA results in products that are either excreted by the kidneys or eliminated as carbon dioxide and water via metabolic pathways. This efficient degradation coupled with the fact that PLA is a renewable source has made PLA one of the leading renewable source polymer systems. Polylactide has made its way into a variety of applications in the pharmaceutical industries, medical industries, and the plastics industry as a whole. Among biomedical applications, PLA is an important polymer for drug delivery and tissue engineering. Hydrophobic drugs can be encapsulated into polylactides and delivery both with targeted effectiveness and controlled release has been demonstrated. Controlled and targeted drug delivery is essential for newer drugs, growth factors, and antibodies that need to be locally delivered to induce tissue regeneration, and for these applications PLA is proving to be an important polymer.

2.1 History of Poly(lactides)

Wallace Carothers was the first to report the synthesis of polylactides in the early 1900’s. However, the early polylactides produced had relatively low molecular
weights and poor mechanical properties. Further research by Dupont to improve on the properties in the 1950's resulting in patented technology that allowed the production of higher molecular weight polylactides.\textsuperscript{[25]} Despite the early interest in polylactides, applications were limited based on reports of hydrolytic instability of the polymer.
Photosynthesis

\[ CO_2 + H_2O \]

Corn

Fermentation

\[ \text{Lactic Acid} \]

HIT

HOA

Aerobic Bacteria

\[ \text{CH}_3, \text{H}_2/\text{O} \]

Hydrolytic Degradation

[\text{Lactic Acid}]

Enzymatic Breakdown

Dehydration

\[ \text{Lactide} \]

Ring Opening

Polymerization

Figure 2.1 Lactic Acid life cycle
Figure 2.2 Chemical structures of L-, D-, and meso-lactides.
Eventually in the 1970's, the use of polylactides as a medical bioresorbable suture was introduced by Ethicon.\[26\] This opened the door for a variety of drug delivery and medical applications based on polylactide polymers. High production cost limited the early industrial adoption of polylactides because the polymer was unable to compete with the cheaper petrochemical derived resources. In 1994, Cargill and Dow launched a joint venture that resulted in the development of technology that significantly reduced the high production cost of polylactides.\[27\] The availability of cheaper polylactides allowed the polymer to become more widely adopted as a renewable replacement for petroleum derived polymers. In the following years a variety of companies have patented technologies based on polylactide or poly(lactic acid). \[27,28\]

Polylactides have played an important role in many applications in pharmaceuticals.\[29\] The polymers are used as macromolecular pharmaceutical substances, blood substituent, auxiliary materials and excipients in the production of macromolecular prodrugs, drug delivery systems and therapeutic systems.\[30-33\]

Polylactides are becoming a favored commodity product because it is renewable, conserves energy, and degrades easily.

The molecular weight and chirality of PLA affect its melting point, crystallinity and mechanical strength of the resulting polymer.\[34-36\] The glass transition temperature (T_g) of PLA is in the range of 50-80°C and the melting temperature ranges from 130-180°C.\[34\] Copolymerization of lactic acid with glycolic acids to produce PLGA is utilized to obtain polymers with improved processability and mechanical properties.

The lactide monomer is a cyclic dimer produced by the dehydration of lactic acid. It contains two stereocenters (Figure 2.2) and can be homopolymerized from either pure
D (PDLA) or L (PLLA) enantiomer. The D and L enantiomers can also be copolymerized. Polymers made from a racemic mix of alternating D- and L- lactide create a completely amorphous polymers, leading to a polymer with a faster degradation rate.\textsuperscript{[37, 38]} Homopolymers prepared from the enantiomeric pure D- or L- lactide have a semi-crystalline structure and a much lower rate of degradation. Research on lactides is often focused on the L- enantiomer since LLA is more commonly found in vivo. PLLA is also a fairly crystalline and is also quite hygroscopic.\textsuperscript{[8]} Polylactides take on one of three forms; poly(L-lactide), poly(D-lactide), and poly(DL-lactide) and the three forms are shown in figure 2.2.\textsuperscript{[38, 39]} Polymerization with L-lactide as the monomer produces poly(L-lactide), polymerization with D-lactide produces poly(D-lactide), and polymerization with both D-lactide and L-lactide produces poly(DL-lactide). Poly(L-lactide) and poly(D-lactide) are both semi-crystalline and identical in properties with the exception of their stereochemistry.\textsuperscript{[40]}

2.2 Properties and Characteristics of PLA

Commercial PLA polymers are usually copolymers of L-lactide and D-lactide. Optical purity, which is defined as the absolute value of the L%-D%, strongly affects the properties of the polymer. Optically pure PLA (having all L configuration) is isotactic and highly crystalline.\textsuperscript{[36]} A decrease in the optical purity reduces the degree of stereoregularity and the crystallinity. It has been shown that PLA with more than 15% D-lactide is amorphous.\textsuperscript{[27]} Molecular and physical characteristics of PLA polymers are determined by the crystallinity, crystalline thickness, degree of chain orientation, and spherulite size. Purity of the enantiomeric monomer has a strong influence on the physical properties of polylactides. Poly (D-lactide) or Poly (L-lactide) homopolymers, as
well as, the racemic DL copolymer that has either high L- or D- content have regular structures and form a crystalline phases/domains. When PLA has more than 90% L-lactide, it is semicrystalline. When the polymer is below 50-percent L-lactide, the polymer is strictly amorphous. Lactide polymerization will always have at least a small percentage of racemization and this contributes to its amorphous phases in the polymer matrix.[41]

The melting temperature of semicrystalline polylactide is a function of the polymer stereochemistry and also the processing conditions of the polymers. Singh et al reported the temperature range corresponding to properties of semicrystalline PLA having high molecular weight. The presence of mesolactide in the polymer affects the crystalline melting temperature and results in the depression of the melting temperature.[34]

In PLA applications utilizing semicrystalline polymers, both appropriate glass transition temperature and melting points are required. Therefore, the overall stereochemistry of the PLA is most important since stereochemistry affects the overall physical properties. Stereochemistry of PLA is easily determined using NMR spectroscopy.

2.3 Synthesis of PLA

2.3.1 Polycondensation

There are two primary methods for synthesizing PLAs. The first approach is the polycondensation of lactic acid and the second approach entails the ring opening polymerization of the lactide monomer. Polycondensation polymerization is a step growth approach and is possible via an esterification reaction (Fig 2.3). PLA synthesis
via condensation is a multi-step process. The disadvantage of polycondensation is its difficulty in obtaining high molecular weight polymers due to complications with removing impurities and the by-product, water. Often the resulting polymer is brittle and unusable unless additives are introduced to increase its chain length. Other shortcomings of this technique are the need for a large reactor, evaporation, recovery of solvent and high racemization.

\[
\begin{align*}
\text{CH}_3 & \quad \text{HO} \left( \frac{\text{C} - \text{C} - \text{O}}{\text{H}} \right)_n \text{H} \quad \xrightarrow{\text{H}_2\text{O}} \quad \text{HO} \left( \frac{\text{C} - \text{C} - \text{O}}{\text{H}} \right)_n \text{H} \\
\text{H} & \quad \left( \frac{\text{O} - \text{C} - \text{C}}{\text{H}} \right)_n \text{OH} \quad + \quad \text{H}_2\text{O}
\end{align*}
\]

Figure 2.3 Equilibria observed during polycondensation of LA to PLA.

### 2.3.2 Ring Opening polymerization

In ring opening polymerization (ROP), an addition polymerization, the terminal end of a polymer acts as a reactive center. The reactive center in a nucleophilic fashion attacks the lactide ring resulting in ring-opening and addition of repeat units to the polymer chain. Each macromolecule formed generally contains one chain end terminated with a functional group originating from the termination reaction and one terminus end capped with a functional group originating from the initiator. For PLA, the alkoxide of a metal having d-orbitals such as aluminum or tin are considered to be effective initiators for ring opening polymerization of lactide.

Anionic ROP (Fig 2.4) of cyclic ester monomers takes place by the nucleophilic attack of the negatively charged initiator. The anion attacks the carbonyl carbon atom with acyl-oxygen scission. This results in an alkoxide species that is negatively charged.
and is counter balanced with a positive ion. While anionic ROP produces polymers with high molecular weight, there is also extensive back biting and in some cases only low molecular weights can be achieved.\textsuperscript{[42],[46]} The back biting reactions can be minimized by using appropriate initiators and polymerization conditions.

![Anionic Ring Opening Polymerization of lactide.](image)

Various anionic initiators have been studied. Studies by Kricheldorf et al have shown that initiators with higher nucleophilicity are required to efficiently initiate Poly(lactide) synthesis.\textsuperscript{[47]} Zinc salt, potassium phenoxide, and other weaker bases initiate only at temperatures of 120°C and higher. Bulk polymerization at high temperatures exhibit racemization of the polymer and is accompanied with significant back biting reaction which interferes with polymer propagation.\textsuperscript{[39]} Initiators such potassium butoxide and n-,sec-,tert-butyl lithium rapidly initiate polymerization at low temperatures, but also are excellent deprotonation agents. When lactides experiences deprotonation, the result is racemization, low molecular weights, and unpredictable polymerization.\textsuperscript{[47,48]} Lithium based initiators also have concerns regarding toxicity, so some prefer to use sodium or potassium metal ions because they are less toxic.\textsuperscript{[44]}
2.3.3 Coordination-insertion ring-opening polymerization

Coordination-insertion ring-opening polymerization has been considered the pseudo-anionic ROP,\cite{49} because propagation is believed to proceed by the coordination of the monomer to the active species. This is followed by the insertion of the monomer into the metal-oxygen bond by rearrangement of electrons.\cite{42} The alkoxide bond formed during the propagation steps keeps the growing chain attached to the metal. Termination of the reaction occurs through hydrolysis forming a hydroxyl group.

Stannous Octoate, also known as tin (II) 2-ethylhexanoate (Sn(Oct)$_2$) is commonly used as a catalyst in the bulk polymerization ROP of lactide because of its solubility in the monomer, low rate of racemization, and good catalytic activity.\cite{8,50} Sn(Oct)$_2$ as a catalyst allows greater than 90% conversion of the monomer to the polymer and this coupled with racemization levels of lower than 1%, leads to the formation of high molecular weight polymers. Sn(Oct)$_2$ is also FDA approved as a food additive.\cite{8,51} Different mechanisms for coordination-complex have be presented, however it is commonly accepted that the stannous alkoxide formed is the main catalyst in the reaction. The presence of water converts the stannous alkoxide into a dormant hydroxyl which is less reactive.\cite{52}

2.4 Poly(ethylene glycol)

Poly(ethylene glycol) (PEG) has earned a deserving reputation as an attractive polymer in biomedical applications. PEG is neutral, crystalline, thermoplastic polymer that is highly soluble in aqueous solutions, PEGs aqueous properties has led to a number of useful applications in biomedical, biotechnical, physiochemical and pharmaceutical communities.\cite{53,54}
(PEG) is ideal for biomedical applications due to its lack of toxicity and is also FDA approved for being injectable in humans without adverse side effects. It is currently used in many drug delivery and biomedical applications. The attachment of PEG for chemical modification of various polymers to make them more stable and suitable for pharmaceutical applications has increased in recent years.\cite{55,56} The attachment of PEG to many polymers increases the water solubility of the polymer. PEG conjugation reduces reticuloendothelial (RES) clearance and recognition by the immune system, in turn reducing degradation by proteolytic enzymes.\cite{53} These distinguished properties of PEG have been explained by its chain’s high mobility associated with conformational flexibility and water binding ability.\cite{57-59}

In water, PEG has a high exclusion volume, allowing the polymer to have optimal interaction with water molecules, making it suitable material for water-based applications. PEG’s excellent hydrophilicity is due to the double free electron pair on the oxygen atom, and the flexibility of its linear structure.\cite{58,60}

Poly(ethylene glycol) is solely comprised of ethylene oxide monomer. As the molecular weight of PEG increases, the viscosity increases as well. When PEG has a molecular weight of below 800, it is fluid like. When the molecular weight (MW) range reaches between 800-1500, the consistency becomes more like petroleum jelly. From MW of 2000-6000 PEG is waxy. Once the MW reaches 20000 and above, PEG is brittle and crystalline at room temperature. PEG had good solubility in water; however, solubility decreases as MW increases.
2.5 Copolymers

PLA and its copolymers suffer from some disadvantages and these include strong
hydrophobicity, poor cell affinity, high crystallinity, and difficulty in controlling the
polymer degradation.\cite{22, 61} Poly(ethylene glycol) (PEG) has received great attention for
its hydrophilicity and water solubility compared to most polyethers.\cite{1, 62} In order to
increase hydrophilicity and decrease crystallinity, PLLA is often copolymerized with
more amorphous and hydrophilic polymers such as PEG. PEG has gained much notoriety
for its exceptional physiochemical and biological properties which include hydrophilicity,
water solubility and lack of toxicity.\cite{63} The terminal hydroxyl of PEG is readily reactive
with a variety of compounds.

Amphiphilic block copolymers containing covalently bound hydrophilic and
hydrophobic blocks have been materials of great interest in the development of
nanomaterials in biomedicine and biotechnology.\cite{64} Copolymerization is used to alter
the crystallinity and hydrophillicity, and the processability of degradable polymers.\cite{9}

2.6 Biodegradable Block Copolymer

ABA copolymers of lactide and poly(ethylene glycol) have been previously
synthesized as a potential biodegradable polymers, but have not been functionalized in
order to achieve specific binding. Previous block copolymers using PLA and PEG have
been used as compatibilizers and precursor polymers for the synthesis of biodegradable
elastomers and thermosets.\cite{8, 65, 66}

Biodegradable polymers are defined as having the ability to be converted into
carbon dioxide under microbial, hydrolytic, photodegradable, or oxidative degradation;
the ability to disintegrate via fragmentation and a visible loss of mass of the product in
the final compost, and the absence of negative effects in the final compost according to
industrial standard EN13432.[6] These actions are reflected in decrease in molecular weight of the polymer as the polymer degrades, disappearance of crystalline regions within the polymer, and diminished physical properties. Biodegradation is controlled by either microorganisms like bacteria and fungi, or by enzymes in vitro or hydrolytic conditions.

Polycaprolactone, aliphatic copolyesters, aromatic copolyesters, poly(vinyl alcohol), and aliphatic polyesters are some of the more common biodegradable polymers. Aliphatic polyesters are well-known bioresorbable polymers which can possibly replace nondegradable bioresistant polymers in many applications.[67] The use of nondegradable polymers produces an increasing amount of waste that has long been an ecological problem.[68] Furthermore nondegradable polymers are generally produced from nonrenewable resources such as crude oil and natural gas.[22,68] Linear aliphatic polyesters are the most used biodegradable polymers with the majority of the attention focused on polymers derived from lactic acid, glycolic acid and their copolymers.

The polylactides are both biodegradable and bioassimilable. PLA hydrolysis in physiological environments produces lactic acid and glycolylic acid. Both of which are nontoxic and eliminated via the Krebs Cycle as water and carbon dioxide. Lactic acid can be collected by fermentation of renewable resources such as corn and sugar beets.[68]

2.7 DNP mediated receptor clustering

Ligand-mediated receptor clusterings that modify intracellular signaling are prevalent in nature.[15] In depth immunological studies on the activation of the cell signaling, causing the allergic response and inflammation have been done.[69] This receptor clustering can be observed in processes from viral entry into a host cell to
antigen induced stimulation of signal transduction in host immune response. More specifically, the phosphorylation of the receptor in active mast cells is initiated by the antigen induced cross-linking of receptor bound anti-2,4-dinitrophenyl-immunoglobulin E (IgE), which leads to the secretion of allergy and inflammation mediators. Figure 5 provides a schematic representation of the important steps in the IgE-receptor signaling leading to cell degranulation. Previously therapeutics for allergies have focused on decreasing the effects of inflammation, vasodilation, and congestion. However, many scientist changed direction and began to focus on targeting the molecules directly involved in initiating the allergic response.
Figure 2.5. IgE mediated cell stimulation
The production of IgE occurs in response to the body's exposure to foreign substances (allergens).\textsuperscript{[73, 74]} Once produced, IgE circulates in the body in both the free form and bound to cell-surface receptors.\textsuperscript{[74]} FceRI, a high-affinity IgE receptor, is expressed mainly on the surface of mast cells and basophils as a multimeric complex consisting of an \( \alpha \)-subunit, a \( \beta \)-subunit, and a disulfide-linked homodimeric \( \gamma \)-subunit.\textsuperscript{[1, 73]} The binding of allergens to cell surface bound IgE leads to receptor aggregation, triggering a cascade of events leading to an allergic response and ultimately to the release of histamine and other immune mediators.\textsuperscript{[75]}

Postner et al reported that the symmetrical bivalent ligand N,N\( \varepsilon \)-bis-\( [\_-(2,4\text{-dinitrophenyl})amino]\)caproyl]-L-tyrosyl]-L-cystine ((DCT)\( 2 \)-cys) stably crosslinks anti-\( 2,4\text{-dinitrophenyl} \)-immunoglobulin E bound to high affinity receptors FceRI on the surface of RBL-2H3 cells (mast cells).\textsuperscript{[76]} At low concentrations (DCT)\( 2 \)-cys binding formed mostly cyclic dimers containing two IgE-FceRI and two (DCT)-2cys, that didn't trigger mast cell degranulation. Conversely, (DCT)\( 2 \)-cys at higher concentrations forms linearly crosslinked IgE-FceRI that does activate cellular degranulation.\textsuperscript{[77]}

Baird et al embarked on studies whose primary objective was to determine the architectural features of ligands that enhance their capacity to occupy the sites of a bivalent receptor (anti-DNP IgE) and their capacity to inhibit binding and cellular activation caused by an antigen that stimulates intermolecular cross-linking.\textsuperscript{[1, 77]} This was achieved by the synthesis, characterization of univalent, trivalent and multivalent PEG polymers functionalized with DNP\textsuperscript{[1, 77]}. In addition Baird et al investigated the role of valency and polymeric spacer length via a series of PEG-based ligands.
Baird et al investigated the relationship between ligands structural features and its ability to function as inhibitors of antigen stimulated degranulation. Results of the research showed that PEG based bivalent ligands will successfully bind to the IgE complex without stimulating cellular degranulation and are 100-fold more potent than small monovalent ligands when it comes to inhibition of degranulation. It was also shown by Baird and company that varying the ligand of molecular weights can used as a method to tune the binding capability of the inhibitor.
3.1 Reagents

(3S)-cis-3,6-Dimethyl-1,4-dioxane-2,5-dione

(3S)-cis-3,6-Dimethyl-1,4-dioxane-2,5-dione: (Sigma-Aldrich, FW. Wt. 144.13; mp 92-94°C.) was recrystallized in toluene under ultra high purity N₂ gas.

Stannous Octoate

Stannous Octoate: (Sigma-Aldrich, Mol. Wt. 405.12; d 1.251 at 25°C.) was used as received.

Poly (Ethylene glycol)

Poly(ethylene glycol): (Sigma- Aldrich, Av. Mol. Wt. 2000., mp 52-54°C) was dried in a vacuum oven for 24 hours at -634 mmHg.

Glycerol

Glycerol: (Sigma- Aldrich, FW. 92.09; d 1.25) was azeotropically distilled with toluene.

Pentaerythritol

Pentaerythritol: (Sigma- Aldrich, FW. 136.15, mp 253-258 °C) was dried in a vacuum oven for 48 hours under -634mmhg pressure. Needs correction pressure (the pressure is not correct)
Chloroform

Chloroform: ( Sigma- Aldrich, FW. 119.38; d 1.492; bp 60.5-61.5 °C ) was used as received.

Methanol

Methanol: ( Sigma- Aldrich. FW. 32.04; d 0.791; bp 64.7 °C) was used as received.

Toluene

Toluene: ( Sigma –Aldrich. FW. 92.14 ; d 0.865; bp 110-111 °C) was dried over 3A molecular sieves for 48 hours before use.

DNP-e-amino-n-caproic acid ( Sigma-Aldrich. FW 297.3) was used as received.

Benzene

Benzene: (Sigma-Aldrich: ACS Reagent, ≥99%, FW 78.11, fp -11°C, bp 80-80.2°C/760mmHg, mp 113°C) ws used during freeze drying

Calcium Hydride

Calcium Hydride: (Sigma-Aldrich: FW 42.1, bp 600°C, mp 816°C, d 1.9) was used to dry solvents.

Hexane

Hexane: (Sigma-Aldrich: anhydrous, 95%, FW 86.18, bp 69°) was used to precipitate polymers.

Nitrogen (N₂)

Airgas: ultra high purity, compressed, FW 28.02, fp -210°C, bp -195.8°C, d 0.072

Airgas: refrigerated liquid, FW 28.02, fp -210°C, bp -195.8°C, d 0.072
3.2 Procedure

3.2.1 Recrystallization of L-lactide

In a 250ml Erlenmeyer flask, L-Lactide was fully dissolved in a minimal amount of dry toluene over heat under UHP nitrogen gas. Once dissolved the flask was removed from heat and allowed to cool to ambient temperature. The resulting crystals were vacuum filtered to remove excess solvent and impurities. The crystals were dried in a vacuum oven for 2 days under reduced pressure at 45°C. $^1$H NMR analysis was done on the recrystallized L-lactide to confirm the absence of water and toluene.

3.3.2 Synthesis of PLA

The selected alcohol Poly(ethylene glycol), glycerol, pentaerythritol) and recrystallized L-lactide were added into a 100-ml round bottom flask containing a Teflon-coated magnetic stirring bar. Catalytic amounts of stannous octoate was added, followed by sealing the flask with a stop cock adapter. The flask was purged under nitrogen at 90°C for 20 minutes in an oil bath. The flask was then sealed under vacuum and the heated to between 135-145°C for 6 hours with stirring. Figure 3.6a shows the general polymerization scheme.

Upon completion of the reaction, the flask was taken out of the oil bath and allowed to cool to room temperature. The product was dissolved in chloroform and microfiltered. The polymer was precipitated into hexanes, filtered and dried under vacuum for 2 days. The polymers was then dried in a vacuum over under reduced pressure ($10^{-3}$-$10^{-5}$ Torr) for 3 days at 45°C. $^1$NMR (CDCl$_3$ δ relatives to TMS, 1.56(CH$_3$), 5.8(CH), 3.64(CH$_2$))
3.2.3 Preparation of $\alpha,\omega$-bis[2,4-dinitrophenyl][Poly(L-lactic acid)-b-poly(ethylene glycol)-b-poly(L-lactic acid)]

$\alpha,\omega$-bis[hydroxyl][Poly(L-lactic acid)-b-poly(ethylene glycol)-b-poly(L-lactic acid)] were dried under vacuum for 4 hours at 50°C. To a 50 ml flask 10 ml of dry dichloromethane, 0.5 g (0.2mmol) of polymer, 0.149 g (0.5 mmol) of N-2,4-DNP-$\epsilon$-amino caproic acid, and 0.001 g (0.02mmol) of 4-(dimethylamino)pyridine (DMAP) were added (Fig 3.6b). The solution was stirred and cooled in an ice bath to 0° while 0.62 g (0.3 mmol) of DCC was added over a 5 min period. The solution was allowed to stir for an additional 5 mins then removed from the ice bath, and further allowed to stir at room temperature for 16 hours. The solution was then filtered through a fritted buchner funnel,
and the filtrate was precipitated into hexanes. The functionalized polymer was filtered and dried in the oven for two days at 50°C.

3.3 Characterization

3.3.1 $^1$H Nuclear Magnetic Resonance (NMR)

$^1$NMR spectra were obtained using a Bruker ARX 500 NMR spectrometer with CDCl$_3$ as the solvent and tetramethylsilane (TMS) was used as the internal standard. The number average molecular weights were determined by $^1$H NMR.

3.3.2 Differential Scanning Calorimetry (DSC)

Thermal analysis was performed with a TA instruments Q2000 Differential Scanning Calorimeter with a heating rate of 15° per minute and reported values were obtained after quench cooling the sample. The $T_g$'s were taken at the midpoints of the heat capacity changes, the $T_m$'s were taken at the maximum of the enthalpy endothermic peaks.

3.3.3 Characterization of DNP functionalized lactides

$^1$H NMR spectra were obtained using a Bruker Avance III 500 NMR spectrometer in CDCl$_3$. Number average ($M_n$) molecular weight was determined by end group analysis using $^1$H NMR spectroscopy. TMS (tetramethylsilane) was used as internal standard. Percent functionalization was determined from NMR analysis.

FTIR spectra were recorded on a Nicolet 510P FT-IR spectrometer with an accuracy band of ± 2 cm$^{-1}$. FT-IR spectra of P2MS-SO$_3$Na samples were obtained as KBr pellets.
Thermal analysis was carried out with a TA Q2000 Differential Scanning Calorimeter (DSC). The samples, between 6-9 mg, were run at a 15°C min⁻¹ heating rate and 5°C min⁻¹ cooling rate. Samples underwent two heating cycles heating and one cooling cycles in a nitrogen atmosphere.

Thermal Gravimetric Analysis (TGA) was conducted using a TA Instruments TGA 50. Data was analyzed using TA Instruments Universal Analysis. Samples were heated at a rate of 10° C/min from room temperature to 500°C under a nitrogen purge.

3.3.4 Dynamic Light Scattering

Static and dynamic light scattering (DLS) was carried out on a Goniometer system (ALV) based on a rotary disk allowing finest angular steps to be performed. The solvent used was CHCl₃ with a flow rate of 1.0 mL/min at 35°C performing simultaneous measurements of static and dynamic light scattering. DLS was also conducted using a Zetasizer Nano (Malvern). On this instrument toluene was used at a flow rate of 1.0 mL/min at 25°C.

3.4 Protocol for sensitizing cells with a solution of IgE and DNP functionalized polymers.

The first step was to calculate the necessary amount of IgE to saturate the plated cells. The binding data, on average, shows that there are 1.65 nM of IgE on roughly 1 million cells. Therefore, for a flask of 10 mL and 10⁶ cells/mL, use 7.4nM of IgE. Next the amount of polymer necessary to crosslink the IgE was calculated. This concentration was close to the K_d value. On average the polymer concentration per binding site (IgE) estimated by the binding analysis was 0.4. The cells were then harvested Tripsin, and
quenched with RBL media. Cells were then spun down and re-suspended in Tyrode-BSA (10mL). The cells were spun one more time for a wash step, and re-suspended again in Tyrode-BSA (10mL).

The calculated amount of IgE (16mL of 0.8mg/mL of IgE stock solution) in 10mL of 10^6 cells/mL flask was added to the solution. Test tubes were used to aliquot the cell solution to prepare different polymer dilutions for the assay. Enough cell solution was left over for the control wells with no polymer. The 96 well plate was plated with the prepared cell solution mixed with IgE and polymer, 150mL in each well. For controls, some wells with a cell solution mixed with IgE alone were plated. Incubation of the cells with the polymer/IgE solution went on for 1hr, sensitizing the cells. The wells were plated with 50 mL more of a sensitive concentration of DNP-BSA (1ng/mL) and incubated for 30mins. Once completed the inhibition assay for fluorescence detection was performed.

3.5 Binding analysis

3.5.1 Equilibrium Binding of DNP Ligands to FTIC-IgE

The polymers affinity to bind was assessed by fluorescence quenching of FTIC-IgE in solution and on RBL-cells, following addition of the polymer under equilibrium. A binding model was used to determine the dissociation constant.

Anti-DNP FTIC in media was used to sensitize the RBL-2H3 cells for one hour. The RBL cells were then washed with Tyrodes-BSA solution to increase specificity and remove excess IgE. Tyroded-BSA was used to dilute to a final concentration of 10 million cells in 2ml in an acrylic cuvette and placed into a SLM 8000 fluorimeter in time...
based acquisition mode. The solution was continually mixed with a magnetic stirrer at a constant temperature (37° C). Excitation and emission wavelengths of FTIC were 490nm and 520nm respectively. Varying amounts of polymer solution were added to the cuvette and the binding to IgE was monitored with the decrease of FTIC fluorescence intensity.

3.5.2 Equilibrium Binding of DNP Ligands to FITC- IgE

Equilibrium binding equations were used to determine the dissociation constant for DNP binding to IgE. Modeling the binding at equilibrium was used to determine the hyperbolic equation for the dissociation constant. The equilibrium binding equation to determine the fraction of bound sites is as follows:

\[ \text{L} = \text{Free ligand concentration} \]

\[ \text{R} = \text{Free IgE binding sites} \]

\[ \text{R}_{\text{tot}} = \text{Total IgE binding sites (bound to ligand + free)} \]

\[ \text{K}_{a} = \text{Apparent association constant} \]

\[ f = \text{fraction of IgE binding sites occupied by the ligand at equilibrium} \]

\[ L + R \leftrightarrow LR \]

The dissociation equilibrium constant is the ratio of the binding rate over the separation rate.

\[ K_{a} = \frac{k_{on}}{k_{off}} = \frac{[LR]}{[L][R]} = \frac{1}{K_{d}} \]
The fraction of bound sites can be defined as the ratio of the total sites occupied over the total sites available. After substituting in the mathematical definition for the association constant, the equilibrium equation becomes a symmetric hyperbolic with the association constant as the parameter.

\[
R_{\text{tot}} = [R] + [LR]
\]

\[
f = \frac{[LR]}{[R] + [LR]}
\]

\[
f = \frac{K_a[L]}{1 + K_a[L]}
\]

The fluorescence titration data from the binding experiment was plotted as the ligand concentration vs. the fraction of bound sites, and the resulting graph was fit to the calculated symmetric hyperbolic equation. The inverse of the fit parameter defined the dissociation constant for the binding data. The \(K_d\) from all the experiments are listed in Table 4.5.

3.6 Granule Exocytosis assay

Under sterile conditions, RBL cells were sensitized with 1 \(\mu\)g/ml anti-DNP IgE and plated overnight at a density of \(2.5 \times 10^5\) cells in a 96-well plate, in order to measure the granule exocytosis response triggered by multivalent DNP-BSA or DNP-functionalized polymers. The DNP ligands along with the RBL cells were incubated together for 1 h at 37° C and the exocytotic release of the granule marker \(\beta\)-hexosaminidase was used to quantify the extent of degranulation.\(^{[78]}\)

Determining the inhibition of degranulation was achieved by measuring the \(\beta\)-hexosaminidase release from incubating IgE-sensitized RBL cells with varying
concentrations of DNP-polymer for 30 min at 37° C followed by stimulation with 2 ng/ml DNP-BSA for 30 minutes.
RESULTS AND DISCUSSION

CHAPTER 4

4.1 Synthesis

The synthesis of the lactide based polymers was carried out by coordination-insertion ROP of L-lactide with stannous octoate (Sn(Oct)₂) as the catalyst. Different alcohols were used as coinitiators in the polymerization. ROP polymerization using Sn(Oct)₂ has been shown to be an effective catalyst/initiator when dealing with cyclic esters. Furthermore, Sn(Oct)₂ is accepted as a food additive and is FDA approved for use in pharmaceutical applications. The exact mechanism for the ROP with Sn(Oct)₂ is still up for debate. However, the most accepted mechanism involves the alkoxide mechanism, where stannous octoate reacts with the OH-bearing species (i.e. the alcohol coinitiator) to form an alkoxide which is the initiating species of the polymerization. Three and four arm PLLA were synthesized using glycerol and pentaerythritol respectively via Sn(Oct)₂ catalyzed ROP. The reaction vessel was slowly heated until the desired reaction temperature was met. All polymerizations were carried out in bulk with continuous stirring between 130°-140° C for 6-9 hours under reduced pressure. The melt solution turned white once the polymerization began. These conditions allowed for high conversions of the monomer to the polymer. The overall scheme is shown in Figure 3.6. Polymerizations carried out above 160° C causes the
system to turn brown. The brown coloration at elevated temperatures is due to undesired side reaction and decomposition of monomer and polymer.

The lactide to alcohol molar feed ratio determines the theoretical molecular weight of the polymers.\(^{[9,80]}\) Poly(ethylene glycol) (PEG) 2000 was used as a co-initiator to synthesize the linear, difunctional PLLA (Table 1). The lactide based polymers synthesized with PEG are the only triblock copolymers in this series of polymers. The central PEG segment results in a fairly water soluble system. The triblocks were significantly more water soluble than the star polymer systems. The use of combining PEG with PLLA has been of considerable interest because its adds much needed water solubility to PLLA.\(^{[62]}\) In addition PEG stimulates virtually no biological response, has high chain mobility, and steric stabilization effects makes it a desirable addition to copolymers used in biological environments.\(^{[82-84]}\) Star shaped polymers using glycerol (Table 4.2) and pentaerythritol (Table 4.3) were synthesized in order to increase the end group concentration, and are considered to be star PLA polymers. Each series of polymers were made with the target molecular weights of 5k, 10k, and 15k.

When using glycerol to initiate the ring opening polymerization of lactides, it is certain that the two primary hydroxyl groups are expected to initiate, while the initiation by the secondary hydroxyl is still debatable due to possible steric hindrance.\(^{[85]}\) NMR can be used to investigate whether all of the hydroxyl groups have been activated. Polymerization temperatures using glycerol as a coinitiator range from 110-190°C.

It is believed that steric hindrance plays a role in end-functionalizing the hydroxyl terminated polymer with DNP. The series of polymers, i.e. diblock copolymers, utilizing
PEG was able to achieve up to 75% functionalization via steglich esterfication (Table 4.1). However the star polymers prepared using glycerol (Table 4.2) and pentaerythritol (Table 3) were only functionalized up to 35% and 23%, respectively. The hydroxyl ends of the triblock copolymers are most likely more accessible due to the increased flexibility of the PEG central segment or block. The 3-arm and 4-arm polymer were more rigid in that they were mostly comprised of PLLA plus one unit of the initiating core.
Table 4.1 Polymerization conditions for triblock copolymers, molecular weights and % functionalization

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mole Ratio(PLA:PEG)</th>
<th>Time(hr)</th>
<th>Temp(°C)</th>
<th>Expected MW</th>
<th>Mₙ</th>
<th>% Functional</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA-PEG-PLA</td>
<td>21:1</td>
<td>5</td>
<td>140</td>
<td>5k</td>
<td>5100</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>56:1</td>
<td>7</td>
<td>140</td>
<td>10k</td>
<td>9800</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>91:1</td>
<td>10</td>
<td>140</td>
<td>15k</td>
<td>14,900</td>
<td>70</td>
</tr>
</tbody>
</table>
Table 4.2 Polymerization conditions for three arm polymers, molecular weights and % functionalization

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Molar Ratio</th>
<th>Time (hr)</th>
<th>Temp (°C)</th>
<th>Expected MW</th>
<th>M_n</th>
<th>% Functional</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA-Gly-PLA</td>
<td>70:1</td>
<td>5</td>
<td>150</td>
<td>5k</td>
<td>4900</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10k</td>
<td>9900</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>15k</td>
<td>15,200</td>
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Table 4.3 Polymerization conditions for four arm polymers, molecular weights and % functionalization

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mol. Ratio</th>
<th>Time (hr)</th>
<th>Temp (°C)</th>
<th>Expected MW</th>
<th>Mₐ</th>
<th>% Functional</th>
</tr>
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<tbody>
<tr>
<td>PLA-PENT-PLA</td>
<td>35:1</td>
<td>5</td>
<td>140</td>
<td>5k</td>
<td>4800</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>70:1</td>
<td>7</td>
<td>140</td>
<td>10k</td>
<td>9900</td>
<td>23</td>
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<tr>
<td></td>
<td>104:1</td>
<td>10</td>
<td>140</td>
<td>15k</td>
<td>14,700</td>
<td>19</td>
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</tbody>
</table>
4.2 NMR and IR Analysis

$^1$H NMR spectroscopy was used to determine the structures of the polymers synthesized. The lactide based polymers were characterized by solution 500MHz $^1$HNMR. The 500MHz $^1$H NMR spectra for the triblock copolymer (Fig 4.7) pre-DNP functionalization showing the following resonances: δ 1.6 ppm (-CH$_3$); δ 3.7 ppm (-CH$_2$-CH$_2$-O-) and δ 5.2 ppm (-CH-). Once the triblock copolymers were functionalized with DNP-$\epsilon$-amino-$\eta$-caproic acid, resonances at δ 9.2 ppm, δ 8.6 ppm, and δ 8.3 ppm represent the two hydrogens in the meta position and the one hydrogen in the ortho position respectively (Fig 4.9). There is a shift in the methylene peak upon the conjugation of the DNP, suggesting that the DNP group may have non-covalent interaction with PEG block.

The 3-arm polymers (Fig 4.11) synthesized around glycerol show resonances of: δ 5.2 ppm (-CH-), δ 4.4 (-CH$_2$-), δ 3.6 ppm (-CH-), and δ 1.6 ppm (-CH$_3$). The resonance at δ 5.2 ppm is representative of the (-CH-) peak within the lactide block, while the signal at 3.6 ppm is represents the (-CH-) in the center of the glycerol unit. This is confirmed by published information as well as by studying the $^1$H NMR of the glycerol and homopolymer PLLA individually.$^{[85]}$ Once functionalized, the 3-arm polymers exhibited the same DNP resonances as the triblock copolymers. The 4-arm polymers (Fig 4.13) synthesized around pentaerythritol show $^1$H NMR resonances of: δ 5.1 ppm (-CH-) , δ 3.6 ppm (-CH$_2$-), and δ 1.6 ppm (-CH$_3$) (Fig 4.13). Number average molecular weight (M$_n$) was calculated using $^1$H NMR according to equations provide by Izunobi et al.$^{[86]}$ The peak assignments allowed for the calculation of the number average molecular weight (M$_n$) via numerical integration of the of the peak.$^{[86]}$
Further confirmation was obtained by running IR of the polymers both pre and post DNP functionalization. Prior to DNP functionalization, the triblock (Fig 4.8), the three-arm (Fig 4.10), and the 4-arm (Fig 4.12) polymers all exhibit and broad peak around 3300 cm\(^{-1}\) indicating the presence of −OH groups. In addition an absorption band at 1750 cm\(^{-1}\) confirms the presence of −O−C=O ester bonds in all of the polymers. Once functionalized a peak around 1520 cm\(^{-1}\) indicates the presence of NO\(_2\) confirming DNP functionalization.
Figure 4.7 500 MHz $^1$H NMR spectra of $\alpha$-$\omega$-[2,4-dinitrophenyl][Poly(L-lactic acid)-b-poly(ethylene glycol)-b-poly(L-lactic acid)] triblock copolymer in CDCl$_3$. 
Figure 4.8 a) IR spectra of α-ω-bi[2,4-dinitrophenyl][Poly(L-lactic acid)-b-poly(ethylene glycol)-b-poly(L-lactic acid)] b) IR spectra of DNP functionalized α-ω-bi[2,4-dinitrophenyl][Poly(L-lactic acid)-b-poly(ethylene glycol)-b-poly(L-lactic acid)]
Figure 4.9. 500 MHz $^1$H NMR spectra of a) $\alpha,\omega$-bi[2,4-dinitrophenyl][Poly(L-lactic acid)-b-poly(ethylene glycol)-b-poly(L-lactic acid)] (b) $\alpha,\omega$-di-hydroxy[Poly(L-lactic acid)-b-poly(ethylene glycol)-b-poly(L-lactic acid)]
Figure 4.10 a) IR spectra of 3-arm PLLA b) IR spectra of DNP functionalized PLLA
Figure 4.11500 MHz $^1$H NMR spectrum of three arm PLLA polymer in CDCl$_3$
Figure 4.12 a) IR spectra of 4-arm PLLA b) IR spectra of DNP functionalized PLLA
Figure 4.13 500 MHz $^1$H NMR spectrum of a) four arm PLLA polymer in CDCl$_3$ b) DNP functionalized four arm PLLA polymer in CDCl$_3$
4.3 Thermal Analysis

Thermal studies were carried out using Differential Scanning Calorimetry (DSC) and Thermal Gravimetric Analysis (TGA). TGA experiments were carried out on all samples under nitrogen. Two transitions can be seen for the triblock in the TGA (Fig 4.14). The first transition at slightly above 250°C is the start of the degradation of the PLLA, and the transition is dependent on the ratio of the PLLA and PEG in the triblock copolymers. The PEG breakdown starts around 350°C and higher and once again is dependent on the composition of the copolymers. The triblock copolymer with MW 5K having the lowest PLLA:PEG ratio shows transition at 275°C for the degradation of the PLLA. As the molecular weight increase to 10 K and 15K, and along with it the fraction of the PLLA in the copolymer increases, the first transition is lowered around 240°C. This suggest that the triblock copolymer degradation is governed by the polymer composition. The first transition begins at 235°C, 201°C and 187°C for the 5K, 10 K and 15K, respectively. Upon completion of the lactide degradation, the PEG segment then begins to degrade at 380°C, 351°C and 343°C, again for the 5K, 10 K and 15K, respectively. Pure PEG-2000 begins thermal degradation at 361°C, while pure lactide homopolymer begins degradation at 214°C, with complete degradation occurring at 524°C. Once the second transition begins, the polymer continues to degrade to almost to completion with very little residual mass left at 500°C. Both PLA and PEG are fairly hydroscopic, therefore even though the polymers were dried in a vacuum over prior to the TGA studies, some initial weight loss (a few percent) is observed and this loss can be attributed to loss of water molecules associated with the polymer, most likely with the
PEG segment. TGA also confirms that the two polymers are not miscible and undergo phase separation during heating of the polymer.

Thermal degradation of both the 3-arm (Fig 4.15) and 4 arm (Fig 4.16) PLLA show only one transition due to the degradation of PLLA. The degradation temperature increases slightly with increasing molecular weight.
Figure 4.14 TGA thermogram of triblock PEG-PLLA copolymer as a function of molecular weight.
Figure 4.15 TGA thermogram of three arm-PLLA star polymers as a function of molecular weight.
Figure 4.16 TGA thermogram for four arm PLLA star polymers as a function of molecular weight.
The DSC thermograms for the triblock copolymers are shown in figure 4.17. The $T_m$ for the polymer of 5K show a broad crystalline peak with two nodes at 18°C and 24°C indicating that the formation of two crystalline regions within the polymer. The $T_m$ is low compared to both pure PEG-2000 and homopolymer PLA which have $T_m$ transitions at 51°C and 150°C respectively. This is due to the loose packing of crystalline regions between both the PEG and PLA. The amorphous affect of PEG keeps the lactide regions from forming tight crystalline regions, while the crystalline lactide regions prevent the amorphous PEG from ordering. As the lactide content increases in the polymer, there is an increase in the $T_m$ as shown by the DSC thermograms for TB10k and TB15k which have $T_m$'s of 118°C and 141°C respectively. Both melting peaks have nodes indicating that there is more than one crystal size was formed during the $T_c$. Previous studies have discussed how polymer miscibility is affected by polymer composition, leading to varied crystal structure formation during crystallization. These varied crystal structures affect the melting and crystallization thermal transitions. Also as PLA content increased, the expression of the $T_g$ decreased, as shown in comparison between TB10K and TB15k. The $T_c$ for TB5k, TB10K, and TB15K at 0.51°C, 78°C, and 99°C respectively occur during the cooling phase of the DSC.
Figure 4.17 DSC thermogram of triblock PEG-PLLA
The bimodal peaks shown in the DSC thermogram for GLY-PLA polymer series are due to the formation of crystals of more than one size (Fig 4.18). This possibility occurs when all the arms of the glycerol have not initiated, making the polymer a mix of 2 arm and 3 arm PLLA. The bimodal peaks are seen for all the polymer synthesized with glycerol and pentaerythritol. As the molecular weight increase, there is a greater intensity in the $T_m$ peak. The 3-arm polymer shows a clearly defined glass transition, cold crystallization, and two melting peaks at 37°C, 98°C, and 116°C and 126°C respectively. It is seen that the maximum $T_m$ of the 3-arm PLLA is lower than that of the triblock copolymers. However the melt transitions peaks for the 3-arm PLLA are more clearly defined indicating greater crystal alignment that that of the triblock.

The $T_g$ for the 3-arm polymers increased and with increasing molecular weight. The glass transition values are lower than reported values for linear homopolymer PLLA and those of the previously synthesized triblocks.
Figure 4.18 DSC thermogram of Glycerol-PLLA
Figure 4.19 DSC thermogram of PENT-PLLA series
4.4 IgE Binding Studies by PLLA Series vs. 2-vinyanisol Ligands

Lactide based polymers have an advantage of being both flexible and biodegradable, while exhibiting a high binding affinity to the IgE receptor complex on mast cells; especially when compared to previously tested rigid 2-vinylanisol based polymers (P2MS57) that were also DNP functionalized and tested for their binding affinity to the IgE receptors on mast cells. Of the two systems compared, P2MS57 (FIG 18b) has the highest DNP functionalization at greater than 90% while the TRB10k (Fig. 4.22) have around 75% functionalization (Table 4).

Table 4.4 Polymer Comparison: Biodegradable vs. Biocompatible

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MW(g/mol)</th>
<th>DNP Functionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2MS57</td>
<td>20100</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>TRB10k</td>
<td>10500</td>
<td>75%</td>
</tr>
</tbody>
</table>
Figure 4.20 Structure of aTB10k b) P2MS57
4.5 Equilibrium Binding of DNP Ligands to FITC-IgE

Equilibrium titrations with RBL cells sensitized with FITC-IgE were performed in order to test the specific binding of the polymers with anti-DNP IgE by fitting the data to a binding equation plotting the binding sites in solution vs the functionalized polymers. This resulted in a hyperbolic curve from which the dissociation constant \( (k_d) \) was determined.

The binding of these polymers was first assessed by determining the total available binding sites with a monovalent ligand DNP-aminocaproyl-L-tyrosine (DCT) (Fig 4.23). The initial data points were extrapolated to determine the number of binding sites from the DCT binding curve.

Figure 4.21 shows the quenching data for the triblock copolymer. The data was adjusted for volume and concentration changes, leading to the fraction of the binding sites being plotted against the ligand concentration in solution. Fitting the curve to a hyperbolic trend made it possible to find the parameter for the dissociation constant based on equilibrium steady state binding.

Fluorescence quenching experiments carried out with DCT was used as a binding standard in order to gain better understanding of the polymer binding. The experiments were performed with the same cell stock.

Titration data from the fluorescence binding experiments was plotted as the ligand concentration vs. the fraction of bound sites. This resulted in a graph fit to a calculated symmetric hyperbolic equation. Figure 4.22 shows these plots for both the P2MS-7 and
the triblock. Table 5 summarizes the final values for the dissociation constant ($K_a$) of the triblock, P2MS57, and DCT.
Fluorescence Quenching with Triblock Addition

Figure 4.21 Fluorescence quenching of FITC-IGE in time based acquisition mode. Each dip in the line represents an addition of ligand at a certain concentration.
Figure 4.22 Fluorescence binding data Fraction of binding sites vs concentration. A) Triblock b) P2MS57.
Table 4.5 Summary of $K_d$ (nM) values in IgE solution for the TB10K, P2MS57, and DCT

<table>
<thead>
<tr>
<th>$K_d$ (nM): IgE in Solution</th>
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<tbody>
<tr>
<td>TRB10K</td>
</tr>
<tr>
<td>5.81</td>
</tr>
<tr>
<td>1.97</td>
</tr>
<tr>
<td>3.4</td>
</tr>
<tr>
<td>5.5</td>
</tr>
</tbody>
</table>

Table 4.6 Summary of $K_d$ (nM) values in IgE on RBL cells for the TB10K, P2MS57, and DCT

<table>
<thead>
<tr>
<th>$K_d$ (nM): IgE on RBL cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRB10K</td>
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</tr>
<tr>
<td>2.02</td>
</tr>
<tr>
<td>3.3</td>
</tr>
<tr>
<td>4.35</td>
</tr>
</tbody>
</table>
Extrapolation of the initial binding slope of the equilibrium binding curve to meet the complete fraction of bound sites was performed to further identify the binding mechanism for Triblock and P2MS57. This works with the assumption that bivalent binding is occurring and all of the polymer binding sites are initially occupied without dissociation occurring. The concentration to which the slope was extrapolated was compared to the total concentration of the IgE solution. DCT, being a well-studied chemical, binds monovalently to IgE receptor and was used a standard to calculate the number of binding sites via a fluorescence quenching experiment. This was used to calculate both the number of IgE binding sites in solution as well as the number of binding sites available on the RBL mast cells, under the assumption that the DCT/binding site ratio is 1:1.
Figure 4.23 Total available IgE binding sites with a monovalent ligand, DNP-aminocaproyl-L-tyrosine (DCT).
4.6 Degranulation and Inhibition Analysis

The capacity of the DNP-functionalized polymers to excite granule exocytosis in RBL cells was examined (Fig 4.24). Prior to testing the polymers granule exocytosis response, the K_d of DCT was determined to be 1.5 ± 0.7 nM with IgE attached to cells and 4.6 ± 1.0 nM for IgE in solution. RBL mast cells showed a less than 1% granule exocytosis response, measured by the release of β-hexosaminidase from the cells, when incubated with P2MS57 polymer at various concentrations. Under similar conditions, multivalent antigen DNP-BSA shows a granule exocytosis response of approximately 55%. Additional testing with the P2MS57 polymer looked into the polymers capacity to inhibit granule exocytosis when stimulated by multivalent DNP-BSA. This was accomplished by pre-incubating the P2MS57 with IgE-sensitized cells followed by incubation with a concentration of 2ng/ml of DNP-BSA. Results showed that the IC_{50} inhibitory concentration (i.e. 50% inhibitory concentration of ligand) of P2MS57 polymer was 7±2.1 μM (n=4)(Fig 4.25). When the concentration of the antigen was decreased to a concentration of 1ng/ml with an incubation time of 10 minutes in order to optimize the assay, the IC_{50} concentration observed was 4 μM (Fig 4.25)

Using fluorescence quenching assay to determine the K_d for triblock binding to IgE-FceRI on cells, the K_d was determined to be 2.8 ± 1.1 nM (n=5)(Fig 4.26a). For IgE in solution the dissociation constant was calculated to be 4.2 ± 1.8 nM(n=4). When comparing the dissociation constants for P2MS57 to the triblock, there is more than a tenfold decrease in the K_d of the lactide based polymer in both the IgE solution as well as the IgE-FceRI on cells. This is an indication that the triblock has a tighter binding to IgE
than P2MS57. This is possible due to the smaller molecular weight and architecture of the polymer.

The triblock was also shown not to initiate granule exocytosis in IgE- sensitized RBL cells just as P2MS57 (Fig 4.24). To determine the triblock polymers ability to inhibit DNP-BSA stimulated granule exocytosis, IC₅₀ test were conducted. The IC₅₀ were determined by measuring the polymer concentration that inhibit 50% of degranulation stimulated by the DNP-BSA at a concentration of 2ng/ml. The triblock showed IC₅₀ values of 123 nM, proving to bind to IgE and inhibit DNP-BSA more efficiently than P2MS57 which showed IC₅₀ an value of 4 µM. Even though the lactide based polymer show improvement in binding over P2MS57, neither at this time compare to the binding efficiency of DCT (Fig 4.26b).

Previous studies performed by Baird et al have show that the most effective inhibitors are bivalent ligand that are capable of stable intramolecular binding between the two binding sites of IgE-FceRI. However the unstable binding exhibited by P2MS57 to the receptor may be the cause for IC₅₀ values with micromolar concentration. The triblock show IC₅₀ values similar to that of DCT.
Figure 4.24 Dose response of degranulation of a) TB10K b) P2MS57, normalized to spontaneous degranulation levels.
Inhibition of Degranulation

Figure 4.25 Inhibition of Degranulation of P2MS57 at 1ng/ml and 2ng/ml DNP-BSA concentrations
Figure 4.26 Inhibition of Degranulation with a) TB10K b) DCT at 3 different DNP-BSA concentrations
Figure 4.27 a) TB10K b) P2MS 57 inhibition of degranulation at 1 ng/ml DNP-BSA at various time points.
The lactide based copolymer also inhibits the granule exocytosis response stimulated by multivalent DNP-BSA. The IC$_{50}$ concentration of the polylactide copolymers was between 45-50 nM after optimization of inhibition conditions as shown in Fig 4.26a. Under similar conditions, the monovalent ligand DCT has a lower IC$_{50}$ value indicating that its still the more potent in inhibiting granule exocytosis that the polymeric ligand (Fig4.25b).

4.7 Polymer-IgE Binding Analysis

DNP functionalized polylactides have proven to bind specifically to anti-DNP IgE immobilized on RBL mast cells. In addition to binding to IgE, the polymers did not activate a detectable response as determined by the lack of Ca$^{2+}$ mobilization, PKC activation, or granule exocytosis.

It's believed that the polymers are binding monovalently or bivalently intramolecularly, because there is no detectable crosslink of IgE-FceRI initiating cell signaling. To further investigate the question of if the polymers are binding monovalently or bivalently, monovalent binding studies in parallel with the monovalent ligand DCT were performed. Using DCT it is possible to gain an approximate estimate of the number of IgE binding sites available. Comparing this to the binding data of the polymer made it possible to assess if the polymer is binding bivalently. The data indicated that the polymers are not binding bivalenty as determined to the ratio of polymer/binding sites. However, the DNP functionality was not taken into account for determining the dissociation constants of bivalency.
Data obtained from the binding experiments via fluorescence titration was plotted as the ligand concentration vs the fraction of bound sites (Fig 4.24). The $K_d$ values show that the lactide based polymer is achieving 50% binding of the IgE, in both solution and attached to cells, sites in solution in the nanomolar ranges. Table 5 shows that the $K_d$ for these polymers was consistently less than 6nM. This is a definite improvement over the previously studied systems developed to bind to the IgE complex.

To further identify the binding mechanism for the polylactides, the initial slope of the equilibrium binding curve was extrapolated to meet the fullest fraction of bound sites. This method assumes that all the polymer binding sites are initially occupied with no dissociation affect; therefore this binding is most likely to be bivalent. The concentration to which this slope was extrapolated was compared to the total concentration of IgE in solution. This number of binding sites was calculated from a similar fluorescence quenching experiment with DCT. This well-studied chemical binds monovalently to IgE receptors. The same initial slope analysis for the DCT binding experiments estimates the number of binding sites (IgE) in solution under the assumption that the DCT per binding site ratio is 1. Analysis is shown is summarized in Table 5.3. When the polymer per binding site ratio is greater than 1, it suggest that the polymers are not binding bivalently and have some other complex binding mechanism. Although the experiments with FITC-IgE sensitized on cells does not show conclusively that the polylactides are binding bivalently, this interaction is seen in the experiments with FITC-IgE in solution, where the polylactide 3 has a ratio of polymer per binding site that is lower than
<table>
<thead>
<tr>
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<th>IgE in Solution Initial Binding Extrapolated to Saturation (nM)</th>
<th>Average</th>
<th>Polymer/Binding Site</th>
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<tr>
<td>DCT</td>
<td>11.4  3.5  1.59</td>
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<td>1</td>
</tr>
<tr>
<td>Triblock</td>
<td>4.4   0.98  0.81</td>
<td>2.06</td>
<td>0.38</td>
</tr>
<tr>
<td>P2MS57</td>
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<td>2.68</td>
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<table>
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<th>IgE on Cells Initial Binding Extrapolated to Saturation (nM)</th>
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</thead>
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</tr>
<tr>
<td>Triblock</td>
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<td>2.13</td>
<td>1.29</td>
</tr>
<tr>
<td>P2MS57</td>
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<td>17.41</td>
<td>10.53</td>
</tr>
</tbody>
</table>

Table 5.3 Binding analysis based on the assumption: [DCT Bound]=[Binding Sites Available]
CHAPTER 5

CONCLUSIONS

Lactide based polymers with 2, 3, and 4-arm arms were synthesized using coordination-insertion complex polymerization. It was decided that stannous octoate would be used as the primary catalyst due to its approval by the FDA as a food additive. The composition and structure of the polymers and monomers were determined by IR spectroscopy, NMR spectroscopy, TGA, DLS, and DSC. Functionalization of the polymers with DNP-ε-amino-γ-caproic acid via steglich esterfication gave polymers capable of specifically binding with anti-DNP IgE in solution and also on the surface of RBL mast cells.

The triblock copolymers was functionalized at least 70% or higher. Because of steric hindrance, it was not possible to functionalize the three- and the four-arm polymers to a high degree and functionalization did not reach above 40 percent. The biodegradable triblock copolymers (i.e. the difunctional polymer) successfully bound to anti-DNP IgE immobilized on RBL cells. In addition the polymers did not activate a detectible signal response as determined by the lack of stimulated Ca^{2+}, PKC activation or granule exocytosis. The polymers were binding intramolecularily, because there was no detectable crosslinking of IgE-FceRI that initiated cell signaling.
The difunctional polymer, being the only triblock copolymer, had an advantage of being both flexible and biodegradable when compared to the previously tested P2MS57 (polystyrene based system) polymers that are more rigid in nature. The triblocks flexibility because of the middle soft poly(ethylene glycol) block most likely contributes to its ability to bind to the IgE receptor complex at lower concentrations than P2MS57. This is quite interesting because the biodegradable polymers are also significantly lower in percent functionalization compared with the P2MS57.

Overall, the difunctional polymers have biofunctional properties which are extremely interesting and the polymers provide potentially a platform for anti-allergic therapeutics.
REFERENCES

REFERENCES


REFERENCES

[67] Vert, M., *Biomacromolecules*, (2005) 6,

REFERENCES
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