ONE-STEP SYNTHESIS AND CHARACTERIZATION OF BIODEGRADABLE ‘CLICK-ABLE’ POLYESTER POLYMER FOR BIOMEDICAL APPLICATIONS

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ONE-STEP SYNTHESIS AND CHARACTERIZATION OF BIODEGRADABLE ‘CLICK-ABLE’ POLYESTER POLYMER FOR BIOMEDICAL APPLICATIONS

A Thesis Submitted to the Graduate School
in Partial Fulfillment of the Requirements
For the Degree of
Master of Science

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Pittsburg State University
Pittsburg, Kansas
December, 2017
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One-Step Synthesis and Characterization of Biodegradable ‘Click-able’ Polyester Polymer for Biomedical Applications

An Abstract of the Thesis by
Wadha Alqahtani

In this study, we have synthesized linear polyester polymers from bio-based small molecules including sorbitol, glutaric acid and others. A propargylic acid derivative was used to make the resulting polymer “click”able. Melt polymerization technique was used for this polymerization reaction using a novel lipase enzyme catalyst, NOVO 435. This reaction was conducted between 90-95 °C for 72 hours. The polymer samples were collected within 24 h for detailed characterizations and polymerization reaction monitoring. The resulting polymer was purified and characterized using various spectroscopic methods such as NMR, GPC, FTIR, DSC and TGA. Next, these polymers were used for the formulation of polymeric drug delivery system using the solvent diffusion method and taxol as drug. The results were analyzed by using the cytotoxicity (MTT) assay using prostate cancer cells, and absorbance and fluorescence microscopy. The drug delivery experiments and the cytotoxic tests and showed that nanoparticles developed in this work and successfully effects in drug delivery and can be potentially used for cancer treatment, that was conducted by using in vitro prostate cancer cell model.

Keywords: nanoparticle, click chemistry, prostat cancer, cytotoxicity
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CHAPTER I

INTRODUCTION

There are only few things which come across the mind of a common man when the word “Polymer” is discussed. This thought includes the traditional things such as table, chair, bench etc. Anyhow, if this term is discussed in depth, then its scope is beyond the traditional household items and production items. Polymer science is an amazing branch of science and it has influenced the way of living especially in the past few decades. It has reshaped the lives. Polymer is helping to solve critical issues related to the traditional materials. This is the core reason that the Polymer science is further divided into sub-branches such as linear and branched polymer to make it simple and more useful.

Polymers have revolutionized almost every walk of life and the most affected department or field in this regard is the field of medicine or the medicine science. One of the most recent innovations in this context is the invention of “Polymeric nanoparticles”. In the same manner another contribution is “Dendrite polymers”. These polymers can easily be made with the help of common chemicals available in the market. These polymers are used in the important applications of the biomedical field such as surgical system and tissue engineering. This synthesis require a high level of care. So that the intake of such polymers do not harm the body.\footnote{1}

Nanotechnology is the other field of science which is greatly influenced by the polymers. The deadly and notorious diseases such as cancer are now curable. Polymers have enabled to
find such methods of treating these diseases which have made the cure predictable and easy. The traditional techniques such as “Radiation therapy” and “Chemotherapeutics” are replaced by new technology. These techniques had a lot of side effects and limitations. These are covered by the use of polymers in the new techniques. The major side effects of these methods were loss of hair, skin problems, appetite loss, weight loss etc. These all problems are solved by the innovation and introduction of nanoparticles in the field of medicine. Nanotechnology is playing a vital role in this context because these particles can be engineered and designed to package and transport drugs directly to where they’re needed. This targeted approach means the drugs cause most harm in the particular, and intended, area of the tumour they are delivered to. This minimises collateral damage to surrounding healthy tissues, and therefore the side effects.²

The essence of this thesis is the synthesis of two important and frequently used polymers named as “Hexanediol” and “Decanediol”. Some of the monomers were also used in this regard termed as “Sorbitol”, “Glutaric Acid” and “Hexyniocacid”. Sorbitol and Gultaric acid were selected for the process due to their ease of metabolism in the living cell and the hexynoic acid was used to ensure the surface uniformity during the conversion of alkyne to these nanoparticles. This conversion helps the spontaneous up gradation of these particles using the “Click” Chemistry.

In order to perform this kind of polymeric nanoparticles synthesis, anticancer medicine and optical dye were encapsulated inside the polymers. These two were “Taxol” and “Dil Optical Dye”. These both were encapsulated and placed inside the polymers in case of the current “Drug delivery system”. The incubation process for these polymeric nanoparticles was then completed with the help of normal prostate cells and LNCaP “prostate Cancer Cells”. The effectiveness of this nanoparticle as an anti-cancer delivery system is assessed was tested on
prostate cancer cells using the MTT cytotoxicity assay. Also in order to show the high affinity ratio for the folic acid, a PSMA is used. This membrane displays the high affinity because it is absent in the normal cells. The “Prostate-Specific Membrane Antigen” is expressed by the LNCaP cells. Maybe you can use the following statement as an ending statement for this section. "The research presented in this thesis is aimed at the development of a polymeric nanoparticle that can be used to specifically target cancer cells, thus providing a better anticancer therapy than the therapeutic methods that are currently being used."
CHAPTER II

LITERATURE REVIEW

2.1. History of Polymer

The word polymer has been derived from Greek words “Poly” meaning “many” and “Meres” meaning “parts”. It is commonly defined as a large molecule formed by a combination of small repeating or chemical units. These units are called monomers and they combine in thousands to form a polymer. The polymers are considered as the building blocks of life because of their presence in all classes of the living organisms. They are not only found in nature (such as chitosan, cellulose, collagen) but can also be synthesized through a process of polymerization. Renowned synthetic polymers include polyethylene, polypropylene, polystyrene, polyvinyl chloride, and so on.

In scientific research, the word “polymer” was first introduced by Jons Jacob Berzelius in his book “Jahres-Bericht”, in the year 1833. One of the earlier attempts to successfully synthesize a polymer was made by A.V.Lourenco in the year 1860. He made a series of compounds by using ethylene dihalide and ethylene glycol. In the early 20th century, a considerable work on polymerization was done by Hermann Staudinger. He studied a wide range of polymeric compounds and discovered a class of organic compounds known as ketenes. In the later years, a huge variety of polymers (such as thermoplastic polymers, thermosetting polymers) were studied, developed and commercialized for wide-scale applications.
Polymers can be classified in a variety of ways based on their origin, polymer structure, polymerization method, and processing technique. In terms of their structure, polymers can be classically identified as linear, branched, and cross-linked polymers. A linear polymer is formed from a bifunctional monomer and is consisted of a long chain of repeating units. Typical examples include polyethylene, polystyrene, and polyvinyl chloride are the typical examples of linear polymers.\(^3\)

The advances in polymer chemistry have led to the development of novel structures such as the dendritic polymers. These polymers are consist of a central core and a hyper branched (tree-like) structure containing a large number of terminal groups.\(^7\) A structure of an aromatic polyether dendrimer is shown in the figure 2-1.

![Figure 2-1. Structure of an aromatic polyether dendrimer](image)

### 2.2. Biodegradable Polymers

#### 2.2.1. Introduction

The history of biodegradable polymers can be traced back to the second half of the 20\(^{th}\) century, when the applications of synthetic biodegradable polymers came into limelight. In the first half, research on polymer systems such as glycolic acid and hydroxy acids was abandoned
because of the short usable life of the resulting polymers. This limited lifespan was attributed to the biodegradability of those polymers. However, in the later decades, this biodegradability was found to be significantly important for their applications in the biomedical field.\textsuperscript{8,9} The first ever biodegradable sutures were developed in the year 1960. Since then, in the early 21\textsuperscript{st} century, a paradigm shift took place from biostable polymers to biodegradable polymers for applications in medical and biomedical fields. The biodegradable polymers have been used in a variety of applications such as drug delivery systems, tissue engineering, therapeutic devices, orthopedic applications.\textsuperscript{4,10-11} There classification is given in the following section.

\textbf{2.2.2. Classification of Biodegradable Polymers}

Biodegradable polymers can be classified in a number of ways such as on the basis of their raw material (bio derived or synthetic monomers) or biodegradation levels (fully or partially biodegradable).\textsuperscript{12} In the Figure 2-2, the biodegradable polymers have been classified on the basis of the monomer origin.
2.2.3. Biodegradation Routes

The biodegradable polymers can undergo degradation through simple hydrolytic process or enzymatic degradation. In chemical hydrolysis process, the hydrolytically unstable chemical bonds in the backbone of the polymer are responsible for degradation. In case of semi-crystalline polymers such as polylactides, poly(caprolactone), and polydioxanone, this process occurs in two stages. The first stage involves the penetration of water to the bulk of polymeric material, leading to the shortening of long polymer chains through bonds cleavage. These changes occur only in the amorphous phase of the polymer and the material is held together by the crystalline phase. Later on, a reduction in molecular weight takes place by the water-assisted fragmentation of the material. In the second stage, enzymes metabolize the fragments and lead to complete loss of the polymer mass. Polymers such as polysaccharides and poly (amino acids) are more susceptible to enzymatic degradation. Some polymers such as
Polyolefins undergo degradation through photochemical process. The bulk of the material is broken down photochemically and complete degradation is accomplished through microorganism degradation. 14

2.3. Biomedical Application of Polymers

Polymers can be effectively utilized in biomedical applications because of their ability to conform to living tissues. They can also be tailor made with specific properties such as mechanical and specific biodegradation capability. Both natural and synthetic polymers can be used for such applications. 13 Their biomedical applications include orthopedic devices, surgical systems, pharmaceutical applications, tissue engineering applications, ophthalmic applications, and so on. 4,10,13,15-16 The specific polymers used in the above-mentioned applications are often referred to as polymeric biomaterials or biopolymers.
2.3.1. Desired Properties of Biopolymers

A biopolymer must possess a number of desirable characteristics for its utilization in the biomedical field. These are listed as follows.\textsuperscript{10,11-17}

**Biocompatibility:** it should be non-toxic and noninflammatory upon its implementation into a human body

**Biodegradation:** the biodegradation time of the polymer should match the natural healing time of the body. In addition, its degradation products should undergo complete metabolism and they should not interfere with the body functions.

**Shelf life:** the shelf life of material should be reasonable enough for its storage.

**Processability and performance:** the processability of the biopolymer should be easy and cheap. In addition, it should serve the intended purpose and possess adequate functionality.

**Sterilization:** it should be sterilizable by using any of the conventional methods such as dry heating, irradiation, and chemical treatment.

2.3.2. Biopolymer Applications

The biomedical applications of some of the important biopolymers are given as follows,

- 9as biomedical implants. It is a crystalline biopolymer and it undergoes complete degradation in nearly 3 months. Its major applications include surgical sutures and bone fixation devices.\textsuperscript{10,13}

- Polylactides are semi-crystalline in nature and they are widely used for fracture fixation, augmentation of ligaments, and surgical sutures.\textsuperscript{13}

- Poly(caprolactone) is a semi-crystalline polymer and is highly processable because of its solubility in a variety of organic solvents. It possesses excellent biocompatibility and that is why it is used in a number of applications such as contraceptive devices, nano-sized drug delivery systems, and as a scaffold in bone tissue engineering.\textsuperscript{11,13}
• Polydioxanone is semi-crystalline in nature and it possesses a degradation time of nearly 7 months. The first commercial monofilament suture was made from polydioxanone in the year 1980. It finds application in orthopedic devices such as osteochondral fragments and bone fixation screws.$^{4,13}$

Figure 2-3. Some biopolymer applications, (a)-Commercial polyester-based meniscal repair devices,$^{17}$ (b)-Poly(caprolactone) based spacer and reinforcement screw fixation $^{17}$, (c)- Carbon fibre Poly-ether-ether-ketone based osteosynthetic plates,$^{18}$ (d)- Poly-ether-ether-ketone interference screw $^{19}$
2.4 Polymeric nanoparticles and their Role in Cancer Treatment

2.4.1. Introduction to Nanoparticles

In the last decade, nanoparticles have gained interest of the researchers in the medical field because of their targeted drug delivery capabilities.\textsuperscript{20-23} These particles are specifically engineered to have dimensions in the nanometre range (10\textsuperscript{-9}). They can be distinctively categorized into layers, tubes, and spheres on the basis of their dimensions in the nanometre range. For example, the particles having at least two dimensions in the nanometre range and possessing elongated structure are referred to as nanotubes.\textsuperscript{22,24} These nanoparticles can be read from a variety of materials such as noble metals (gold, silver),\textsuperscript{21} nonmetals (carbon, silica),\textsuperscript{24} and polymers.\textsuperscript{25} There are variety of material designs of polymer nanoparticles that can be effectively used for drug delivery in any medical treatment such as cancer therapy. These include nanospheres, nanocapsules,\textsuperscript{26} and liposomes.\textsuperscript{27} There self-explanatory structures are given in the figure 2-4.
Figure 2-4. Structures of polymer nanoparticles, (a)-Nanosphere, (b)-Nanocapsule, (c)-Liposome design

2.4.2. Preparation of Nanoparticles

There are a number of methods for the preparation of polymer nanoparticles such as solvent evaporation, solvent diffusion, and nanoprecipitation. The solvent diffusion method (as shown in the Figure 2-) is also referred to as emulsification solvent diffusion method and it is a modified form of the solvent evaporation method. It involves the dissolution of encapsulating polymer in a solvent (partially soluble in water) and then saturating the mixture with water. This mixture is then emulsified by using an emulsion stabilizer. As a result, the solvent diffuses to the external face and formation of nanoparticles takes place.

![Organic solution: Polymer + Drug in partially water soluble solvent](image)

![Aqueous solution: Stabilizer in water](image)

![Solvent Elimination](image)

Figure 2-5. Illustration of solvent diffusion method

2.4.3. Characterization of Nanoparticles

There are a number of techniques used for the characterization of size, morphology, and surface characteristics of nanoparticles. The commonly used techniques are scanning electron microscopy, transmission electron microscopy, and atomic force microscopy. The different properties of nanoparticles and their respective characterization tools or methods are given in the given table 2-1.
Table 2-1. Characterization techniques and methods for nanoparticles

<table>
<thead>
<tr>
<th>Property</th>
<th>Characterization Method/Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size &amp; distribution</td>
<td>Laser diffractometry</td>
</tr>
<tr>
<td></td>
<td>Photon correlation spectroscopy</td>
</tr>
<tr>
<td></td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td></td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Surface charge determination</td>
<td>Laser Doppler Anemometry</td>
</tr>
<tr>
<td></td>
<td>Zeta potentiometer</td>
</tr>
<tr>
<td>Drug stability</td>
<td>Bioassay of drug extracted from Nanoparticles</td>
</tr>
<tr>
<td></td>
<td>Chemical analysis of drug</td>
</tr>
<tr>
<td>Release profile</td>
<td>In vitro release characteristics (physiologic &amp; sink conditions)</td>
</tr>
<tr>
<td>Surface hydrophobicity</td>
<td>Water contact angle measurement</td>
</tr>
<tr>
<td></td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
</tbody>
</table>

2.4.4. Drug release

An important area in drug delivery or discovery is the targeting of a drug to infected cells or tissues without interfering with the healthy cells. Advancement in the drug targeting systems have made it possible to estimate about the ultimate fate of a drug in the human body. The drug can be targeted to the desirable site by using any of the two mechanisms such as active or passive targeting. Active targeting involves the surface functionalization of carrier nanoparticles with ligands. The receptors on the surface of targeted cells are able to selectively recognize these drug carriers. In passive targeting, in cancer treatment example, the chemotherapeutic agents are accumulated in infected cells or tumors.²⁹

In the area of site-specific drug delivery, polymer nanoparticles offer a number of advantages. They can effectively deliver drugs to various organs (large or small) of the body by
overcoming the physiological barriers. Their physical attributes (such as particle size, surface features) can be engineered for a specific treatment system. They offer improvement in bioavailability and can reduce the side effects or toxicity of a drug to other organs. A number of drugs such as Cisplatin, Doxorubicin, and Cyclosporine-A can be delivered through polymeric nanoparticles for treatment of multiple cancers.\(^{29,30}\)

### 2.4.5. Targeted drug delivery

At present, there is a limited variety of drug delivery nanoparticles which have been approved by the United States Food and Drug Administration (US-FDA). The first ever nanoparticles-based drugs for cancer therapy were the Liposomal drugs. They were used in two formulations such as Doxil\(^\oplus\) (pegylated liposomal doxorubicin) and DaunoXome\(^\oplus\) (pegylated liposomal daunorubicin).\(^{22}\) For treatment of breast cancer, albumin-based nanoparticle drug delivery system (Abraxane\(^\oplus\)) was also established and approved by the US-FDA for commercial application.\(^{31}\) Myocet\(^\oplus\) is a commercial name of nonpegylated liposomal doxorubicin.

Doxorubicin is an important medication used for treatment of a variety of cancers such as leukemia, breast cancer, lung cancer, Hodgkin's disease, and so on. This drug has been studied and approved by the European Medicines Agency for treatment of metastatic breast cancer in women above 18 years of age.\(^{32}\)

### 2.5 Evaluation of cancer treatment

#### 2.5.1. Introduction to cytotoxicity assays

The cell cytotoxicity is generally referred to as the ability of mediator cells or certain chemicals to destroy healthy living cells. By determining the cytotoxic nature of compounds (especially pharmaceuticals), their impact on human health can be identified and end-user safety could be ensured. In order to choose a cytotoxicity assay, an in-depth understanding of
the measured endpoint, correlation of measurement with cell viability, and knowledge of assay chemistry is very essential.33

2.5.2. Various Cytotoxicity Assays

The commonly used cytotoxicity assays34-35 are as follows.

- MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-
  tetrazolium)
- MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- SRB (Sulforhodamine B)
- XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)
- ATP (adenosine triphosphate) formation
- [³H]-thymidine incorporation

2.5.3. Cytotoxicity Applications

The cytotoxic compounds can be effectively used for inducing programmed (apoptosis) or accidental death (necrosis) to the living cells. For example, targeted delivery of a therapeutic protein (such as Cytochrome-c encapsulated in polymeric nanoparticles) to cancer cells can lead to a selective apoptosis in the cells. On the other hand, the healthy cells are not affected by the therapeutic protein. As a result, such cancer therapies hold considerable importance in modern medical research.36-37
CHAPTER III

RESULTS AND DISCUSSION

3.1. Polymers Synthesis and Characterization

Highly biocompatible and hydrophilic compounds were selected for polymer synthesis. To enable the “click” chemistry after the conversion of polymer to nanoparticle suspension, the reagent (hexynoic acid) was chosen so that the surface chemistry of the polymer can be tuned.\textsuperscript{39}

The polymer was synthesized by the melt polymerization process with Novozyme-435 used as enzyme catalyst. Sorbitol, hexanediol, glutaric acid, and hexynoic acid were used as reagents for polymer synthesis in the molar ratio of 1:0.9:2:0.4, respectively. Polyols were provided in equimolar ratio, whereas glutaric acid was provided in excess to ensure the esterification between polyols and glutaric acid. On the other hand, the chances of reaction between hydroxyl groups and hexynoic acid were reduced by providing hexynoic acid in molar deficit. In another reaction, decanediol was used as one of the reagents under similar conditions. Figure 3-1 shows the proposed reaction scheme for the synthesis of polyester polymers 1 and 2.
3.1.1. Nuclear Magnetic Resonance Spectroscopy (\(^1\)H NMR) of Polyester-1: Figure 3-2 shows the proton NMR spectra of the starting materials used in this study. The peak at 0 ppm was assigned as TMS reference peak. In each spectrum, the peak at 2.5 ppm was assigned to DMSO-\(d_6\) solvent. The aliphatic secondary protons in sorbitol were found in the peak cluster at 3.3 -3.8 ppm while the aliphatic primary protons were found in the peak cluster at 4.1-4.9 ppm. The peaks at 1.7 ppm in glutaric acid and hexynoic acid spectra was indicative of methylene protons. The alkyne peak of hexynoic acid was observed at 2.9 ppm. The proton peak in hexanediol was observed at 3.5 ppm. The characteristic peak for the carboxyl hydrogen which is usually found at 10-12 ppm was not observed in the spectra of glutaric acid and hexynoic acid due to the hygroscopic nature of the solvents used in the sample preparation for \(^1\)H NMR spectra. The shallow peak at 3.40 ppm is a clean indication of hydroxyl group in the sample of glutaric acid.\(^{40}\)
The spectrum **Figure 3-3** of the synthesized polyester-1 after 48 h of the reaction is compared with the spectra of monomers in **Figure 3-2**. The characteristics peaks of the monomers were discussed in the previous section. All the important peaks were observed in the $^1$H NMR spectra of polyester polymer-1. For example, aliphatic protons of sorbitol structure were found at peak clusters of 4.1 and 4.9 ppm. The proton peak in hexanediol was observed 3.8 ppm in the spectra of polyester polymer-1. The alkyne peak of hexynoic acid was observed at 2.23 ppm in the polymer-1 spectra. These analyses indicated the successful polymerization of reagents under current experimental conditions.
Figure 3-3. $^1$H NMR spectrum for monomers and 48 h of polyester polymer-1

The $^1$H NMR spectra of the synthesized polyester polymer-1 at different time intervals is shown in Figure 3-4. The spectra were compared after 24, 48, and 72 hours. No change in the $^1$H NMR spectra was observed after 48 hours which shows the completion of polymerization reaction after 48 hours.
3.1.2. $^{13}$C NMR Polyester-1

$^{13}$C NMR spectra of monomers and polyester polymer-1 are shown in Figure 3-5, Figure 3-6, and Figure 3-7. In each spectrum, a strong solvent peak for DMSO-d$_6$ was observed at 40 ppm. The peak at 173 ppm in hexynoic acid and glutaric acid represented carbonyl carbon. At 68.7 ppm, the peak represented the terminal alkyne carbon of hexynoic acid. The peak at 60 ppm was assigned to carbons of hexanediol in $^{13}$C NMR spectra hexanediol. Aliphatic carbons of
sorbitol were observed at 61-65 ppm. At 33 ppm, the carbon next to carbonyl groups in hexynoic acid and glutaric acid was manifested. The carbon next to alkyne bond in hexynoic acid was observed at 21 ppm.

All these peaks with small shifts were observed in the $^{13}$C NMR spectra of polyester polymer-1 as shown in Figure 3-6 which shows polymerization of monomers. The spectra of polyester polymer-1 at different intervals is shown in Figure 3-7. At 48 h, distinct spectrum was observed which shows the completion of polymerization reaction.
Figure 3-5. $^{13}$C NMR Spectra of Starting Compounds
Figure 3-6. $^{13}$C NMR Spectrum of monomers and 48 h of polyester polymer-1
Figure 3-7. $^{13}$C NMR Spectrum of different times of polyester polymer -1
3.1.3. $^1$H NMR of Polyester-2

$^1$H NMR spectra of the polyester polymer-2 along with its monomers is displayed in Figure 3-8 and Figure 3-9. The spectra of monomers have been discussed in detail in the previous section. For polyester polymer-2, the spectrum was found to be identical to polyester polymer-1 and its identification is similar to the aforementioned as well.

Figure 3-8. $^1$H NMR spectrum for monomers and 48 h of polyester polymer-2
Figure 3-9. $^1$H NMR spectrum of different times for polyester polymer-2
3.1.4 $^{13}$C NMR of Polyester 2

Similarly, the $^{13}$C NMR spectra of polyester polymer-2 are displayed in Figure 3.10 and Figure 3.11. The $^{13}$C NMR spectrum of polyester polymer-2 matched the spectrum of polyester polymer-1. Hence, it confirmed the successful polymerization of the monomers.

Figure 3-10. $^{13}$C NMR Spectrum of monomers and 48 h of polyester polymer-2
Figure 3-11. $^{13}$C NMR Spectrum of different times for polyester polymer-2
3.1.5. Fourier transform infrared spectroscopy (FTIR):

Figure 3-12 and Figure 3-13 show the FTIR spectra of polyester polymers 1-2 after 48 h of reaction compared with monomer materials. Most importantly, the ester carbonyl (C=O) bond was observed at a 1712 cm\(^{-1}\) in the form of a distinctive strong peak for both polymers.\(^4\) This peak is a clear indication of successful polymerization of the monomers. Furthermore, a strong peak at around 1146 cm\(^{-1}\) shows C-O stretching from ester and aliphatic ether linkages (from diols) is also a confirmation of successful polymerization of the monomers.

![FTIR spectra of monomers and 48 h of polyester polymer-1](image)

**Figure 3-12.** FTIR spectra of monomers and 48 h of polyester polymer-1
The aliphatic alkyl bond stretching (C-H) was observed at 2900 cm\(^{-1}\). The hydroxyl (O-H) groups of the sorbitol monomer were observed at stretching band of 3350-3580 cm\(^{-1}\) in the form of broad and shallow peak. Alkyne stretching from (C≡C) of hexanoic acid was observed at 2180-2220 cm\(^{-1}\) in the form of weak peaks due to low concentration of hexynoic acid in the reaction mixture. The presence of these characteristics peaks is an indication of successful polymerization of polyester polymers 1-2 after 48 h.

**Figure 3-13.** FTIR spectra of monomers and 48 h of polyester polymer-2
3.1.6. Gel permeation chromatography (GPC):

The results obtained from GPC analysis of each monomer sample are seen in Figure 3.14. The elution of each monomer was observed at the characteristic time of each monomer in the form of a single distinct peak. This shows the purity of the monomers. Figure 3-15 shows the molecular weight of polyester polymer-1 after 48 hrs. of reaction compared with molecular weight of monomers in Figure 3-14 founding that high molecular weight was observed at 31 minutes. GPC spectra of polyester polymer-1 after different time intervals is displayed in Figure 3-16. Elution of high molecular weight product was observed in the spectra at around 30-35 minutes for various reaction times. With the increase of reaction time, elution of polymer traces was observed at 30 minutes for 72 h showing that the product was of higher molecular weight range compared to 24h and 48 h.
Figure 3-15. GPC of monomers and 48 h of polyester polymer-1

Figure 3-16. GPC of different times of polyester polymer-1
This was further analyzed by the estimation of number average molecular weight “Mn,” weight average molecular weight “Mw”, and Polydispersity Index “PDI” of each polymer sample as shown in Table 3-1. Thus, the value for high molecular weight polyester was observed for the 72 h product. However, the PDI was lower for the 24 polymer sample. Therefore, 48 h was found to be the optimal time for the synthesis of polyester polymer-1.

<table>
<thead>
<tr>
<th>Time</th>
<th>Mn</th>
<th>Mw</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>15,698</td>
<td>23,547</td>
<td>1.5</td>
</tr>
<tr>
<td>48 h</td>
<td>19,434</td>
<td>23,320</td>
<td>1.2</td>
</tr>
<tr>
<td>72 h</td>
<td>23,341</td>
<td>32,677</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Table 3-1. Progress of the polymerization reaction as mentioned by GPC of polyester polymer-1

Based on this observation, polyester-2 was synthesized at 48 h conditions and GPC spectrum is shown in Figure 3-17. The spectrum was also compared with monomers in Figure 3-14. High molecular weight polyester was observed at 27 minutes in GPC spectra of polyester polymer-2 synthesized by decanediol. This shows that when subjected to equal reaction conditions, polyester polymer-2 was higher in molecular weight compared to polyester polymer-1 synthesized with hexandiol. This difference was also observed in MALDI analysis which confirms higher molecular weight of polyester polymer-2.
3.1.7. Matrix Assisted Laser Desorption Ionization (MALDI-TOF):

Figure 3-18 and Figure 3-19 show the MALDI-TOF scanning of the synthesized polyester samples. For polyester-1 sample, large fragments with Mn of 4654 were observed in Figure 3-18. However, the MALDI-TOF results of polyester-1 sample shows that the polymer was of poly-dispersed nature. In case of polyester-2 sample, fragments with Mn values of 27675 were observed as can be seen in Figure 3-19. Polyester-2 sample was also found to be poly-dispersed.
Mw based on MALDI-TOF results was calculated by using polydispersity index (PDI) of 1.2 and 1.7 obtained from GPC analysis. PDI was found to be lower than the typical value of synthetic polyesters which might be due to uniform chain length of the synthesized polyesters.\textsuperscript{42,43} Using the equation (PDI=Mw/Mn), Mw was found to be 23,320 and 58,772 for polyester-1 and polyester-2, respectively. Polymer-2 exhibited high molecular weight compared to polymer-1 which is in-line with GPC analysis and can be attributed to higher chain length of decanediol.
Figure 3-18. MALDI-TOF of 48h polyester polymer-1

Figure 3-19. MALDI-TOF of 48h polymer polyester-2

Mn = 34583

Mn = 19554
3.1.8. Thermogravimetric analysis (TGA):

As shown in Figure 3-20 and Figure 3-21 display the TGA analysis of the two polymer samples. Both polymers displayed a continuous weight loss starting from 25°C until 370°C before the onset of thermal degradation of polymer backbone. This is an indicative of the presence of moisture in the polymer samples showing hydrophilicity of the material. Nevertheless, the polymers were found to be thermally stable at biological temperature (37 °C) which shows the suitability of the synthesized polymers in biomedical applications.

Figure 3-20. TGA of 48h polyester polymer-1
3.1.9. Differential scanning calorimetry (DSC):

Differential Scanning calorimetry analysis of the synthesized polymers was performed and transition temperatures were found out. Differential scanning calorimetry is a technique that is used to find out the glass transition temperature (Tg), crystallization temperature (Tc) and melting temperature after cooling or crystallization (Tm). A typical curve of differential scanning calorimetry shows the heat-cool cycle. As shown in Figure 3-22 both the polyester polymers were heated and cooled again between the range of -80 °C and 120 °C. A glass transition temperature (Tg) was observed for first and second polymers at -35.16 °C and -37.35 °C respectively. The lower Tg show the samples are biodegradable, also low Tgs conformed that both polyester polymers are amorphous. The polymers were then cooled, leading to crystallization in the structure. Following crystallization, polymers were again heated. The melting temperature of first and second polymers was found to be 19.45 °C and 25.89 °C respectively. The type of polymer-1 had a lower
Tg as compared to type of polymer-2, this means their mechanical strength was less as compared to second type, and they also may degrade earlier as compared to second type of polymers.

Figure 3-22. DSC of 48 polyester polymers 1-2
3.2. polymeric nanoparticle synthesis and characterization:

Following characterization, the polymers were converted into polymeric nanoparticles (PNPs) for drug and dye encapsulation utilizing a “solvent diffusion” and “encapsulation” methods. Solvent diffusion method is an aqueous method in which the solid lipid nanoparticles are effectively prepared. While the encapsulation method is the process in which the nanoparticles are covered with a coating. This method is used to incorporate tiny particles in the new particles to form the nanoparticles. Figure 3-23 explains the process of synthesis of nanoparticles followed by characterizations of these nanoparticles.

![Diagram of nanoparticle synthesis and characterization](image)

**Figure 3-23.** Conversion of polymer to nanoparticles and surface ligand modification
3.2.1. Dynamic light scattering (DLS):

Dynamic light scattering is a characterization techniques used to find out the size of nanoparticles, the size distribution range of particles. Figures 3-24 and 3-25 show Dynamic Light scattering analysis of polymeric nanoparticles synthesized using hexanediol (first) and decanediol (second) gave the size distribution for both types of polymeric nanoparticles. Size distribution curve shows that first type of polymeric nanoparticles had an average size of 78.82 nm. On the other hand, second type of polymeric nanoparticles had an average size of 189.5 nm. The difference in the size distribution of the two types of polymeric nanoparticles can be attributed to the different types of diols used. In case of first type, hexanediol was used, which has a shorter carbon chain i.e. the diol carbon length is 6, whereas for second type, in which decanediol was used, the diol carbon length is longer, containing 10 carbons, this can result in larger particle size. First type of synthesized polymeric nanoparticles had smaller size range as compared to the second type of polymeric nanoparticles. However these sizes make nanoparticles capable of crossing in-vitro cells.

![Size Distribution by Intensity](image)

**Figure 3-24. Dynamic light scattering of PNP-1**

78.82 nm
3.2.2. Zeta Potential Determination

In these studies, Zeta Potential measures the charge repulsion or attraction between functional groups on the surface of the nanoparticles. For the Zeta potential measurements to be valid, the nanoparticles must be placed in a conducting medium such as water. This difference is calculated by Zeta potential. Figure 3-26 shows the zeta potential for the first type of polymeric nanoparticles, synthesized using hexanediol. Zeta potential is the electrostatic charge on the synthesized nanoparticles. For hexanediol polymeric nanoparticles, the average zeta potential was found to be -18.4mV. These particles are highly negatively charged as shown by the results. While Figure 3-27 shows the zeta potential of second type of polymeric nanoparticles synthesized using decanediol. Zeta potential of these particles ranged from -1.24mV to -3.27mV. These particles are also negatively charged, but not as strongly negatively charged as first type of polymeric nanoparticles. These particles are less negatively charged as compared to first type of synthesized nanoparticles. This can be attributed to the different lengths of the diols used in both polymer types. The overall charge on nanoparticles effects cellular uptake ability and hence the

![Graph showing dynamic light scattering of PNP-2 with peak at 189.5 nm](image)
drug delivery function. In this case, the synthesized polymeric nanoparticles will be further functionalized to make them clickable with folate groups, so the surface charge will be covered.

Figure 3-26. Average surface zeta potential of PNP-1

Figure 3-27. Average surface zeta potential of PNP-2
3.2.3. Absorbance and Fluorescence:

Following encapsulation of Dil and surface decoration with folic acid, the nanoparticles were analyzed by absorbance and fluorescence spectroscopy. This was performed to determine the presence of Dil dye and folic acid after the completion of synthesis and dialysis, as well as to determine if they maintained their activities after encapsulation. The results of these analyses are shown in Figure 3-28 and 3-29.

The absorbance spectra for the PNP 1-2 presented in Figure 3-28 showed a peak at 370 nm which corresponds to folic acid and two peaks between 510-570 nm which correspond to Dil dye. This indicated that the cargo molecules were present within the nanoparticles and the folic acid ligands conjugated to the surface maintained their absorbance through the synthesis and purification. The somewhat broadened absorbance for Dil was likely due to the polymer matrix and the media (deionized water) in which the nanoparticles were suspended, which can cause absorbance to deviate slightly from the expected values.

![Absorbance of PNPs 1-2 with encapsulated Dil Dye and Folic Acid](image-url)

**Figure 3-28.** Absorbance of PNPs 1-2 with encapsulated Dil Dye and Folic Acid
**Figure 3-29** represents the fluorescence emission spectra of PNP 1 and 2 with encapsulated Dil Dye. The spectra show characteristic emission spectra of dye encapsulating polymeric nanoparticles. Fluorescence intensity maxima of the polymeric nanoparticles around 570 nm shows and confirms the presence of Dil dye in both PNPs. This shows that Dil dye was successfully encapsulated in the polymeric nanoparticles. High fluorescence intensity is observed in both the cases, showing strong fluorescence emissions. Both the Dil encapsulating polymeric nanoparticles can be used for in vivo imaging. Cells can be effectively tracked using these nanoparticles.

**Figure 3-29.** Fluorescence emission of PNPs 1-2 with encapsulated Dil Dye
3.2.4. MTT assay

MTT assay displays the cytotoxic effect of the nanoparticles as they are incubated with the cells. Using a 96 well-plate, LNCaP and normal prostate cells were cultured in 96-well plate. 50 uL of each (1) propargyl-PNP and (2) PNP-folic and (3)PNP-taxol for both nanoparticles samples were incubated with the cells for 24h at 37°C and 5% CO₂ atmosphere. After the incubation, the cells were treated with the MTT/ Phosphate-buffered saline (PBS) solution and incubated for an additional 4-6 hours. **Figure 3-30** gives the cytotoxicity analysis of the synthesized polymers using MTT assay. The control group displayed no cytotoxicity and was used as reference to measure cytotoxicity of the synthesized polymeric nanoparticles. It was observed that the second type of polymeric nanoparticles showed higher cytotoxicity as compared to first type of polymeric nanoparticles. For the propargylated polymeric nanoparticles, cell viability was observed as compared to the control for first and second type of polymeric nanoparticles synthesized using hexanediol and decanediol showed slight cytotoxicity. For folate-labeled PNPs significant toxicity was observed compared to all other types of PNPs used on the assay. These results indicate the ability of folate PNPs to be uptaken by cells greater as compared to propargyl loaded PNPs. For Taxol labeled PNPs, high cytotoxicity was observed for synthesized polymeric nanoparticles. Second types of PNPs labelled with Taxol were the most cytotoxic. The results show that polymeric nanoparticles labelled with folate, taxol, and synthesized using hexanediol killed cancer cells less efficiently than those synthesized with decanediol, though only by approximately 20%. Taxol encapsulated PNPs are the best for use in anticancer drug delivery as shown by the results.
Figure 3-30. Cytotoxicity Effects of PNPs 1-2 on LNCaP Prostate Cancer Cells
CHAPTER IV

EXPERIMENTAL METHODS

4.1. Materials:

Five compounds were used as biodegradable monomers to prepare both polyester polymers. Sorbitol, glutaric acid, hexynoic acid, hexanediol, and decanediol were purchased from Sigma Aldrich and used as received. Deuterated dimethyl sulfoxide (DMSO-d₆) was purchased from Sigma-Aldrich and used in ¹H NMR and ¹³C NMR spectroscopy. Variety of solvents utilized to determine solubility of the polymers. Methanol (MeOH), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), and other chemicals were purchased from Sigma-Aldrich and used without further purification. The fluorescent probe dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) used as a surrogate for hydrophobic drugs and chemotherapy drug (taxol) to target cancer cells and causes these cells to die were obtained from thermofisher. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 4'6-diamidino-2-phenylindole (DAPI) were purchased from Biotium. LNCaP and PC3 prostate cancer cells were obtained from the American Type Culture Collection (ATCC) organization and cultured per their supplied protocol.
4.2. Synthesis of polyester polymer:

The experimental setup for the raw materials was done in a round bottle flask of 50ml capacity. One of three components were added and melted at temperature of 140°C which included the Sorbitol, glutaric, hexynoic and either hexanediol or decanediol. All of them were considered in quantities of 3.44g, 5g, 1.58, 3, or 3.29g in mmol. Another component, novozyme-435 was then added in quantity of 400mg after cooling initial melt till 95°C. this element was added to act as catalyst for esterification reaction. Inert atmosphere was created for the flask with the help of Schlenk line containing the nitrogen gas in pure form 12 hours and then entire vacuum process was begun for the next 72 hours at very high value of 4x10^-4 mm/Hg. While the compounds were added in quantities of 1.5 to 2g, there were three samples collected for 24, 48 and 72 hours in vacuum. Each of the treated samples was then purified with the help of methanol dissolving and filtering with P8-grade filter paper. This was done for separating the polymer solution from catalyst. The methanol was then removed from the 50 ml flask by placing it in the evaporator at 60°C. additional vacuum treatment was given to ensure complete elimination of methanol component. The structure of both polymer samples were different, first was similar to wax and second was similar to molasses in terms of texture and viscosity. While they both showed insolubility in water, contrary was observed when treated with dimethyl sulfoxide (DMSO), methanol (MeoH), tetrahydrofuran (THF), and toluene.
4.3. Synthesis of Polymeric Nano-particle:

For this synthesis the 30 mg of the polymer was put in the “Eppendorf Tube” and it was then dissolved in the 250 micro liter DMSO solution. Then 2 micro liter of Taxol and 2 micro liter of Dil Optical Dye were added to this suspension. This solution or the mixture was then vortexed at 1500 revolutions per minute for 3 minutes. A 15 milli liter centrifuge tube was then used to carry the resultant mixture. The mixture was dropped slowly and the constant vortexing was done. The tube was carrying 4 milli liters of H20 in itself.

The centrifuge tube was locked after the complete addition of the suspension and the speed of vortexing was increased from 1500 rpm to 2500 rpm. And the dye and drugs having nanoparticles encapsulated in them were turned into faint pink color. After the completion of the process the suspension was then poured into a “Porous Dialysis Sleeve”. The MWCO=3.6 kDa was used in this context. This was used for the dialytic purification process. And in the presence of the de-ionized water for about one hour.

4.4. Folic Acid Conjugation:

The selected treatment of the LNCap prostate cancer cells could only be done after changing the functional group found on surface. The high chemistry with the Azide functional groups in folic acid could be explained on the basis of triple carbon bonds found in extracted functional groups. For the selective performance of the LNCaP cells, folic ligands were combined with them which enhanced the expression of folate receptors at surface level of cells. The next section gives brief intro to synthesis and preparation of Fol-N3.
The first step in modification was to prepare aminopropl azide which was done by combination of 3-bromopropyl amine (7 g, 0.051 mol) and sodium azide (14.23 g, 0.219 mol) in a round bottom flask of total volume 100 mL. The flask was initially filled with deionized water and after addition of other compounds, was heated at 80°C for next 20 hours. The next steps included the removal of solvent by rotary evaporator under vacuum, followed by reaction with potassium hydroxide and extraction perform with petroleum ether.

The next step in the synthesis of Folic acid was its dissolving in DMSO (2 mL). In another apparatus a combination was performed for 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.02 g, 0.129 mmol) and N-Hydroxysuccinimide (NHS) (0.013 g, 0.113 mmol) with in 2-(N-morpholino) ethanesulfonic acid (MES) Buffer (0.5 mL, pH 5.0). This combination was followed by incubation for 3 minutes, after which dissolving occurred in PBS (0.025 mL). This reaction was completed in duration of 3 hours with drop wise addition of PBS. The last step was centrifugal process at 5000 RPM where the azide functionalized folic acid was collected.

The azide functionalized folic acid was then dissolved and combined with DMF (0.02 mol) and nanoparticle solution of bicarbonate buffer, respectively. This was done to ensure bonding of acid with nanoparticles. The reaction of acid was done with copper iodide (0.001 mmol) in DMF (5 µL) followed by incubation process occurring at table mixture of duration of 12 hours. The process of dialysis was performed for acid with deionized water and its was finally stored at 4°C.
4.5. Instrumentation:

$^1$H NMR: either (1 mL) of chloroform or DMSO-d6 was used for dissolving monomer or polymer samples in quantities of 15-20 mg or 50 mg, respectively. The process of scanning was performed in DPX-300 M Hz spectrometer with help of TOPSIN 1.3 system to obtain 25 scans. Drying process then occurred in vacuum for making it suitable to dissolve in deuterated solvent.

$^{13}$C NMR: this process contained dissolving of monomer and polymers in DMSO-d6 (1 mL). In this method the same TOPSIN 1.3 system was used while processing of samples was done with Bruker DPX at 300 M Hz to get 1000 scans. Similar vacuum drying process was used to make it suitable for dissolving in deuterated solvent.

FT-IR: the Perkin Elmer Spectrum, 2 FT-IR spectrometers were used 15 mg for treating the monomers and polymers. They were each scanned for getting their spectrums. Similar vacuum drying process was used.

Gel Permeation Chromatography (GPC): Water 2410 DRI gel permeation chromatograph was used for this technique. The apparatus and materials consisted of four phenogel columns that was containing the polystyrene-divinylbenzene (PSDVB) beads in a cross linked manner. After drying polymer with vacuum drying process, they were dissolved in THF (1 mL) before transferring them to GPC vial. The flow rate of THF used for this process was 1ML/min to occur at 25 degree Celsius for 50 minutes.

Thermogravimetric Analysis (TGA): TA instruments Q50 thermogravimetric analyzer was used to study the thermal stability of polymer. Samples were taken in 5 mg quantities and then equilibrated followed by a heat treatment through nitrogen conducted by heat changing rate of 10°C per minute. The entire procedure required 60 minutes for completion where the temperature ranged from 25°C to 600°C.
**Differential Scanning Calorimetry (DSC):** Q100 differential scanning calorimeter was used with required gauge parameters for this process. Weighted samples of polymers were used in 7-8 mg. the process completed in 3 cycles where temperature varied from -70°C to 120°C with rate of change of 10°C/minute. The samples was kept at constant temperature in isothermal period before initiation of next cycle.

**Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF):**

Bruker microflex™ LRF MALDI-TOF, system was used for this process. Matrix samples were self-prepared. The initial step was to prepare 100 µL TA30 solvent, with 30:70 ratio of acetonitrile to 0.1% trifluoroacetic acid. The matrix was obtained by dissolving the prepared TA30 solvent with 2,5-dihydroxybenzoic acid (2 mg). Next step included moisturization by vacuum dryer, desiccated and solution with methanol. Eppendorf tube was then used for combining polymer solution and TA30 matrix. Steel MALDI target plates were used for spotting the solution which were left for drying for about 6 hours and then spectrometric scans were observed under mass spectrometer.

**Dynamic Light Scattering and Zeta Potential:** polymer solution was mixed with the deionized water in quantities of 10 µl. and 1 milli liter respectively. standard and electrode containing cuvettes were used for the DLS and zeta potential analysis. Malvern ZS90 zetasizer was used for the cuvette examination in which program was programmed for obtaining data at rate of 50 readings in 3 cycles.

**Uv/Vis and Fluorescence Analysis:** Tecan infinite M200 Pro microplate readers were used as apparatus for UV and fluorescence examination. 96-well plate contained 70 µl of the samples in volumetric quantities and examined through spectrophotometer. The scans were done for 300 to 800 nm wavelength range for absorption and range of 600 to 900 nm was set for the fluorescence
scanning. Data was obtained after differential increase of 5 nm with 10 flashes in single data acquisition. Comparison of the two plots obtained was done on Microsoft excel.

4.6. Cell Studies

**Cell Culturing:** There was a special formulation created for growth of LNCaP and PC3 cancer cells. The formulation contained three components in different ratios by volume 85% RPMI-1640 media, 10% fetal bovine serum, and 5% Penicillin/Streptomycin antibiotic. The compounds were kept intact at 4°C after filtering by vacuum. Cryo cells were kept in this medium, then placed onto 7-mL flask and incubated at 37°C. for achieving longevity and to avoid overcrowding of cells, they were divided into two different flasks containing same medium. Those cells were used for examination that had been in media for more than 24 hours or had achieved confluency of 80%.

**MIT Assay:** The medium for this examination contained fresh cells placed on 96-well plate which already contained 50 µL amount of polymer nanoparticle formulations for 24 h. The cells were then washed with addition of 50 µL of 1X PBS after incubation process. Further incubation for 4 to 6 hours was then done for cells by addition of 25 µL of MIT solution. Followed by incubation was addition of 30 µL isopropanol after removing MIT solution from wells. Now, TECAN infinite M200 PRO multi-detection microplate reader was used for analysis of cells in order the analyze the effectiveness of nanoparticle processing.
CHAPTER V

CONCLUSION

The biocompatible monomers were used in the thesis for the synthesis of a “Hydrophobic Polyester Polymer”. Different features of the polymers were considered. These features which include thermo stability, molecular weight and the alkyne surface uniformity were modified to form a drug delivery system. The experiment was successful and the polymers were converted to the “Polymeric Nanoparticle Suspensions” effectively. And the resultant suspensions included the alkyne surface functionality and biologically effective Nano-scale diameters. The surface functionality was successfully introduced using using “Click” chemistry. The anti-cancer drug and the optical dye encapsulation within the polymer was also successfully accomplishe. The specificity and the efficacy of the nanoparticles were shown by Cytotoxicity assay using prostate cancer cells. After 24-hour incubation of Taxol-nanoparticles with prostate cancer cells, polymeric nanoparticles PNP-1 and PNP-2 resulted in 60% and 80% cell death, respectively.

Future work will involve the use of Fluorescence microscopy as a tracking tool to trace the optical dye incapaculated in the nanoparticles to determine the efficiency of their accumulation in cancer cells, a neccessary step needed in order to ultimately be able to market these products as an effective drug delivary system. Moreover, more work is needed to quantatively determine the optimal concentration of the optical dye needed to obtain the clearest image and the optimal amount of taxol that will lead to maximum cell death. Other
nanoparticles with more improved properties that will make them more effective as drug delivery systems will be explored. Ultimately, experiments using animal models will be used to test the efficacy of these nanoparticles as drug delivery systems.
REFERENCES


13 Nair, L. S.; Laurencin, C. T., Biodegradable Polymers as Biomaterials. Progress in polymer science 2007, 32 (8), 762-798.


22 Haley, B.; Frenkel, E. In *Nanoparticles For Drug Delivery in Cancer Treatment*, Urologic Oncology: Seminars and original investigations, Elsevier: 2008; pp 57-64.


32 B.V., T. Myocet (Doxorubicin).


60


