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SYNTHESIS AND CHARACTERIZATION OF ENZYME CATALYZED BIODEGRADABLE “CLICK-ENE” POLYMERS FOR TARGETED CANCER THERAPY

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SYNTHESIS AND CHARACTERIZATION OF ENZYME CATALYZED BIODEGRADABLE “CLICK-ENE” POLYMERS FOR TARGETED CANCER THERAPY

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An Abstract of the Thesis by
Elaf Alattas

In this study, we report various biodegradable polymers with tunable physical properties and their possible drug delivery applications. These polymers were designed in such a way that bio-based starting materials (for example, sorbitol, hexanediol, glutaric acid) were used in order to obtain double-bond functionalized biopolymers in one-pot, and the polymerization reaction was catalyzed using an enzyme catalyst, Novozyme 435. In addition, a novel “Click-ene” chemistry was used to functionalize the resulting polymers in order to target specific cancer cells. The resulting polymers were purified using solvent precipitation method and characterized using spectroscopic techniques such as NMR, FT-IR, GPC, DSC and TGA, and the results are summarized in this thesis. In addition, to evaluate the potential biomedical applications of the DiI-encapsulating polymeric nanoparticles (PNPs), we assessed their potential cytotoxicity by the MTT assay. Finally, these functional polymers were used to synthesize anti-tumor drug encapsulating polymeric drug delivery systems for the targeted therapy of cancer. Including synthesis and characterization results, various cell-based assays for cancer therapy will be highlighted in this work.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Cancer of all types is currently the second most common cause of death in the U.S., according to the American Cancer Society,[1] and is predicted to cause approximately 12 million deaths globally in 2030 according to the World Health Organization.[1] In 2011, 571,950 Americans died due to cancer, 1500 cancer deaths per day.[2] There are various types of cancer such as breast cancer, lung cancer and brain cancer, however, prostate cancer is the focus of this study.

In the western world, prostate cancer is the second most common type of cancer in men after lung cancer.[3,4] In the U.S., one in seven men will be diagnosed with prostate cancer during their lifetime,[5,6] and worldwide, 307,000 men die each year of prostate cancer (LNCaP).[5,6]

After indicating all these estimates of deaths in the U.S. and around the world, scientists found some varies treatments for LNCaP cancer. Current treatments for prostate cancer include surgery, radiation, chemotherapy, and hormone therapy.[2,7,8] Unfortunately, while these treatments improve patients' survival, they cause damage and toxicities in other organs, tissues and normal cells. To clarify, chemotherapy is distributed everywhere in a patient's body, not just to the specific cancer cells. Therefore, in 1980$_{s.}$ to try to reduce harm to healthy cells, it is hoped that nanoparticles (NPs) that target only cancer cells can be used for cancer drug delivery.[2,5] To reach this goal, very small size of NPs about (1-100 nm) have been formed using different materials including polymers, lipids, inorganic materials and biological materials.[2-4]

In our research, we focused on polymers and polymeric nanoparticles (PNPs) particularly. Targeting cancer cells by using PNPs is one of the most significant and effective ways to treat these cancer cells without toxicity to normal cells. Using PNPs for cancer drug delivery has advantages, such as increasing drug efficacy, lowering drug toxicity, solubility of hydrophobic drugs, ability to specifically target the cancer cells, pH-sensitivity, and temperature-sensitive system.[8,9]

Polyester polymers have unique properties, such as their biocompatibility, biodegradability, multivalence and well-defined molecular weight that make them promising new scaffolds for drug delivery. In this study, we have synthesized two types of biodegradable linear polymers from four biocompatible and biodegradable monomers: sorbitol, glutaric acid, hexanediol, and decanediol. Hexenoic acid was chosen in order to obtain an alkene (C=C) surface functionality when turned into polymeric nanoparticles. The resulting polymers were purified using diffusion method and characterized using several of spectroscopic analysis, including NMR, FT-IR, GPC, TGA, and DSC.

The anticancer drug (Taxol) and DiI optical dye were encapsulated within the polyester polymer in order to create a polymeric nanoparticle solution for the drug delivery system. In addition, to measure and examine the cytotoxicity of the PNPs, LNCaP (cancer cells) and PC3 (normal cells) were incubated with polymeric nanoparticles. PC3 has locking receptors in it surface for folic acid, while LNCaP has prostate-specific membrane antigen (PSMA) receptors and a high affinity for folic acid. In this study, we synthesized two polyester polymers and we characterized their properties and examined their ability to be used as dynamic polymeric nanoparticles for the treatment of cancer cells.

CHAPTER II

REVIEW OF THE LITERATURE

Historical Information about Polymer:

According to Wallace Carothers, polymers are chemical compounds which are composed of large molecules built of one or more types of atomic groups that constitute basic structural units which are connected between themselves and which repeat in some regular manner many times within each molecule.[10] The word polymer was presented to the science world by a Swedish chemist, J.J Berzelius. For instance, he showed that repeating unit of ethane ($C_2 H_2$) produce a polymer that is benzene ($C_6 H_6$).[10] Later in the 20th century, the chemistry Herman Studinger, also known as the father of polymer chemistry due to his substantial contribution to polymer science, presented many chemical reaction that have high molecular weights. Furthermore, polymers are repeating small units (monomers) of large molecules and it known also by macromolecule.[10-13] Polymers could be natural and synthetic (man-made). Natural polymers include proteins, enzymes, silk, wool, DNA and nucleic acid. On the other hand, there are synthetic polymers such as plastics, fibers, polystyrene, silicone, nylon and elastomers.[10,12] Additionally, there are two main types of linking in polymers; brunched such as dendrimer and hyper brunched and linear such as alternative, block, random and grafted. Furthermore, polymers are now routinely synthesized with functionality aimed at improving their chemical, physical mechanical and thermal properties.

Microstructure, identity, high resistance, low density, high molecular weight, adaptation, low cost, flexibility and tacticity are properties of polymers.[11,14] As a result, polymers have great and endless applications in food, medical products, plastic materials, and packaging.[11]

Aliphatic Polyesters:

Aliphatic polyester polymer are concedered as the most significant type of polymers. There are two main types of aliphatic polyester polymers: homopolymers, such as polyglycolic acid (PGA) and poly-ε-caprolactone (PCL), and copolymers such as polyethylene adipate (PEA) and polybutylene succinate (PBS).[11,12,15] There are also different types of molecular architecture aliphatic polyesters such as hyperbranched aliphatic polyester and grafted linear polyester. Due to their solubility, biocompatibility and biodegradability, aliphatic polyesters are the most significant type of polymers in biomedical applications.[16,17] Aliphatic polyesters are also considered prime synthetic biomaterial by the US Food and Drug Administration (FDA).[18,19] There are also many applications of aliphatic polyesters in biomaterial, for example in drug delivery systems, tissue-engineering, and temporary bone repair.[18] (Figure1).

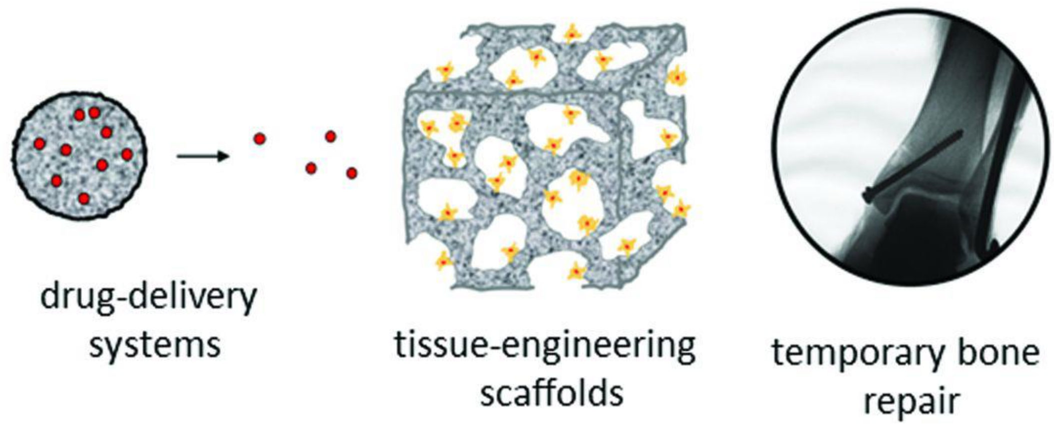

Figure 1. Examples of biomaterial applications using aliphatic polyesters[18]

Biodegradable aliphatic polyesters:

Recently, researchers have given considerable attention to improve and develop biodegradable polymers such as polyamide, polyester, and polyurethane due to their dynamic applications in many different fields.20,21 For example, biodegradable polymers are recognized in surgery as greater consumption especially for tissues, sealing and hemostasis.21,22 In 1960's, there was a small number of chemists who knew about biodegradable polyesters. Bowman was the first researcher who published on biodegradable polyester in 1961.16 Biodegradable aliphatic polyesters are considered the most significant sort of biodegradable polymers due to their biodegradability, bioabsorbility, mechanical resistance, and biocompatibility.20-24 However, even though there are a huge number of biodegradable polyesters, only a few of them are available commercially, such as polyglycolic acid (PEG), poly-ε-caprolactone (ε-CL), poly-ε-caprolactone (PCL) and polylactic acid (PLA).20 Biodegradable aliphatic polyesters have have important properties that make them ideal for medical and pharmaceutical applications, such as high molecular weight, short degradation time, low melting point, tacticity and stability.20,23,25 Drug delivery and nanomedicine are the most useful applications of biodegradable aliphatic polyesters in the medical field.15 For instance, biodegradable drug delivery systems (DDS) have great potential due to their ability to carry the drug to the target, and release it in a specific area in the human body, and then degrades to nontoxic materials.[25-27] Even though biodegradable polyesters have benefits in medical applications, they cannot be used clinically because of their toxicity. However, there is a small number of non-toxic aliphatic polyesters, such as polyethylene and silicone.[22,28]

Advance Drug Delivery System:

Drug delivery systems (DDS) are the way to transport pharmaceutical compounds in the body. The role of DDS is to deliver a drug at a specific and controlled rate, slow delivery and targeted delivery site.[29,30] This selectivity is a significant difference between traditional drug delivery and advanced drug delivery. Advanced drug delivery has the potential to deliver the drug more easily; reduce fluctuations in drug concentration; offer more specific, less frequent treatment; and decrease toxic metabolites.[31] A number of materials have been used to develop the drug-loaded nanoparticles such as polymeric micelle, dendrimers, liposomes, solid lipid nanoparticles and polymeric nanoparticles.[32-35]

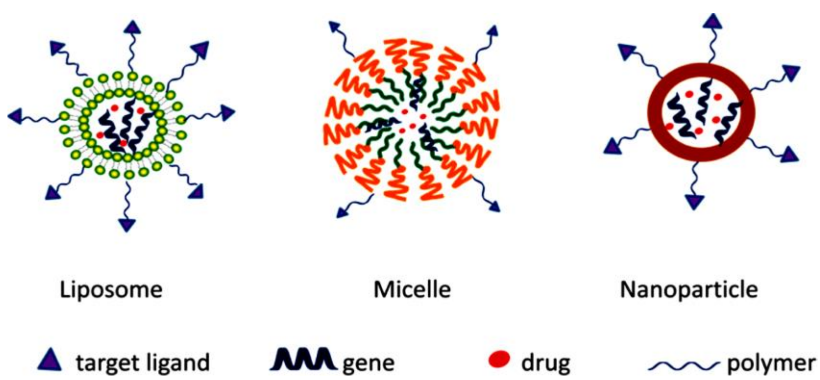

Figure 2. Different drug delivery systems for drug delivery[32]

In our study, the drugs are usually sparse within polymeric nanoparticles or conjugated with the polymeric backbone. Moreover, the biggest advantage of encapsulating drugs in a polymeric nanoparticles is that the drugs are gradually released from the polymer matrix by diffusion.[36] Other features of nanoparticles as drug delivery systems involve controlled drug release, enhanced bioavailability, and drug targeting.[37] Delivery system has three factors that can be targeted by nanotechnology: anti-cancer drug, a carrier, and moiety-penetration enhancer.[1,38]

Biodegradable Polymeric Nanoparticles:

The first polymeric nanoparticles for therapeutic applications was first developed in the period of 1960-1970.[39] Some common methods used in the preparation of biodegradable polymeric nanoparticles include, solvent evaporation, solvent diffusion method, solvent displacement, dialysis, electrospraying and salting-out.[39-41] There are two types of polymeric nanoparticles, natural and synthetic including dendrimers and micelles, both of which have been used in the preparation of nanoparticles used for drug delivery.[40-42] Furthermore, some of the most useful properties of polymeric nanoparticles that make them suitable for drug delivery include, their particle size (1-100 nm), surface properties, special material, biocompatibility and biodegradability. [43,44] Researchers found that there are two major types of nanoparticles that be utilized for cancer treatment and diagnosis. On the contrary, inorganic nanoparticles such as metallic nanoparticles, magnetic nanoparticles, and silica based nanoparticles, and quantum dots.[44,45] However, going through their applications the researchers had limited them because they found some harmful impact of using metal nanoparticles (gold nanoparticles, iron oxide nanoparticles and quantum dots). The issues were their instability, toxicity and difficulty of selectivity.[44-

[46] On the other hand, inorganic nanoparticles such as metallic and magnetic nanoparticles, silica based nanoparticles, and quantum dots have limited use in medicine due to their instability, toxicity and lack of selectivity. However, new promising research is aimed at improving the biocompatibility of metallic nanoparticles by attaching biodegradable and biocompatible polymers to the surface of the metallic nanoparticles. Even though polymeric nanoparticles are concedered as an optical nanoparticle because of their size which allow them to reach the harm cells optically, there are some unique advantages such as surface functional groups, release behavior, biodegradability and biocompatibility.[47]

Nanoparticles and cancer treatment:

Cancer is known as an uncontrolled and serious disease due to its harmful and horrible impacts on a human body.[48,49] It is a serious disease that kills millions of people world wild every year. Therefore, due to the cancer complexity, it needs a perfect and stable treatment that has high selectivity for the cancerous cells.[50] The complexity and this disease has prompted researchers to develop multiple methods in an attempt to find a cure for this mostly fatal illness. These include radiation therapy, hormonal therapy and chemotherapy.[2,51] Although these treatment methods proved successful in combating many types of cancers, their lack of selectivity in targeting only cancer cells makes them inefficient and most of the time detrimental to the immediate health of the patient.[50] Recently, there are great results of nanotechnology with selective cancer targeting drug delivery. Polymeric nanoparticle is the most important branch of nanotechnology. In addition, polymeric nanoparticles are particles have made of polymers. Polymerization is the process of linking several monomers to create either natural or synthetic polymers.[52] These polymers divided into natural hydrophilic such as polysaccharides and proteins and synthetic hydrophobic such as polymerization in process and pre-polymerization. Moreover, the significance of PNPs come from their ability to control the drug delivery establishments and control their chemical and physical proprieties.[52] Polymeric NPs have phenomenal proprieties include high selectivity, size, shape, and biodegradability physical and chemical proprieties.[53,54] Furthermore, polymeric nanoparticle has the ability to prepare and design a require drug with all desirable proprieties such as molecular weight, polymer stricter, functions and compositions.[37,50,55]

CHAPTER III

RESULT AND DISCUSSION

1. Synthesis and Characterizations of Polymers:

1.1 **Synthesis:**

In our study, we synthesized and characterized two different types of polyester polymer. The first Polyester polymer include four main biodegradable monomers which are, sorbitol ($C_6H_{14}O_6$), glutaric acid ($C_5H_8O_4$), hexanediol ($C_6H_{14}O_2$), and hexenoic acid ($C_6H_{10}O_2$). Those monomers were in a molar ratio of 1.4: 2.0: 0.90: 0.44, respectively. On the other hand, the second polymer contains of four biodegradable monomers that were sorbitol ($C_6H_{14}O_6$), glutaric acid ($C_5H_8O_4$), decanediol ($C_{12}H_{26}O_2$) and hexenoic acid ($C_6H_{10}O_2$). In addition, those monomers were in a molar ratio of 3.44: 5.0: 3.29: 1.60, respectively. The reasons behind using sorbitol and glutaric acid are that both of them renewable resources, nontoxic and biobased material. Hexandiol and decandiol were chosen due to their aliphatic chain. The benefit of having aliphatic chin in a polymer is that higher in molecular weight and higher in hydrophobicity. Moreover, hexenoic acid was selected to create functionalities amenable to "click-ene" chemistry later to synthesize polymeric nanoparticle. In addition, the polymerization reactions were catalyzed by using an enzyme biocatalyst, (Novozyme- 435) in both polyester polymer samples. The reasons behind choosing and using this type of biocatalyst enzyme are its high efficiency performance for polymerization, under alike condition Novozyme 435 induce higher polymerization rate than other commercially catalysts, nontoxic, stable at low temperature and keeps polymerization result hard to change even with five cycles of using. The polymers were synthesized under 95 °C and that was a great challenge. Also, N_2 and high vacuum were applied. N_2 gas was applied to remove the O gas, and high vacuum was applied to remove the byproduct and to avoid getting oligomers. The synthesis for both polyester polymers are detailed in the following **(Figures 3-4)**.

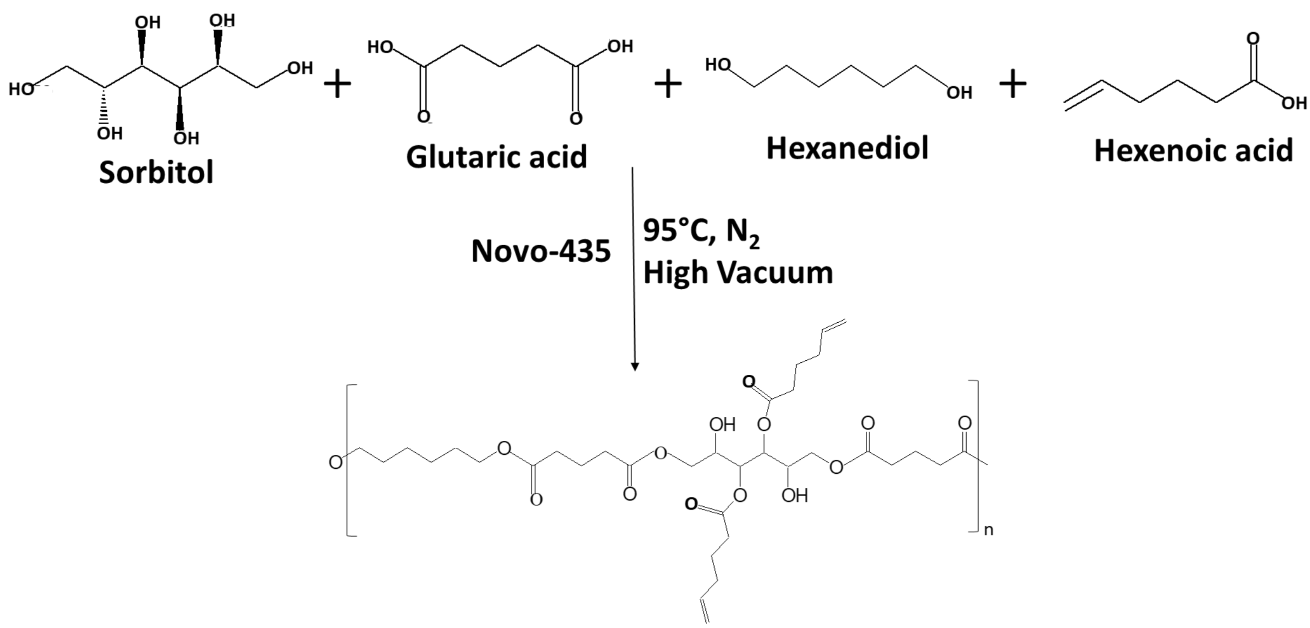

Figure 3. Synthesies of polyester **polymer-1** that consist four biodgradable monomers

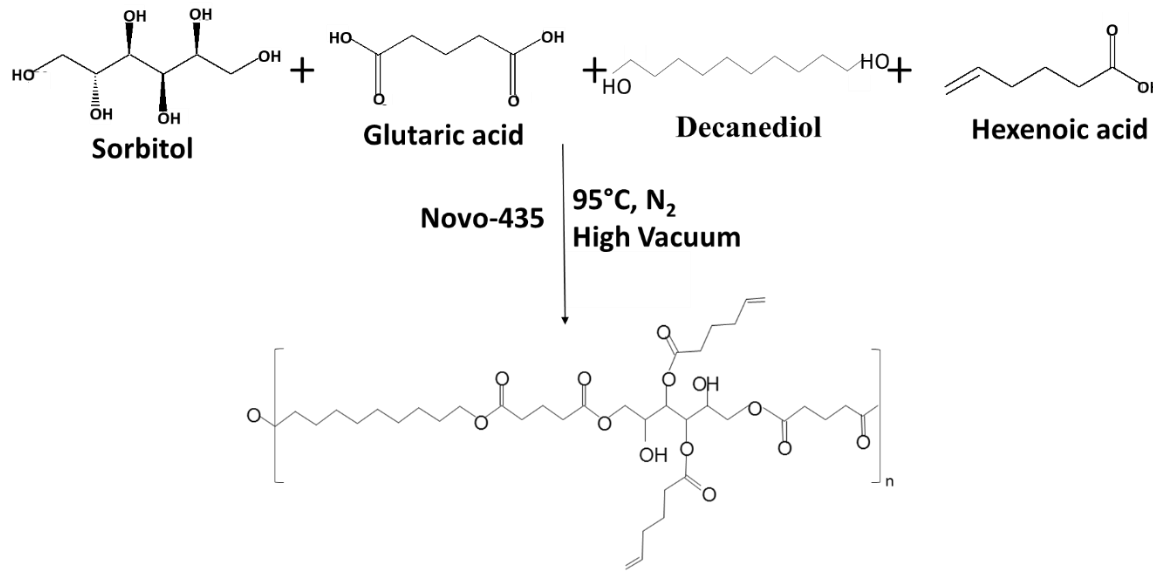

Figure 4. Synthesies of polyester **polymer-2** that consist four biodegradable monometrs

1.2 Characterizations of polyester polymers:

1.2.1 Nuclear Magnetic Resonance Spectroscopy:

I. ^{1}H NMR: The proton NMR spectra for all four monomers and polymer sample are shown in (**Figure 5, 7)**. Moreover, the solvent peak for DMSO-d_6 was observed as a singlet around 2.5 ppm in each of the spectra. TMS reference peak was also observed at 0 ppm.

In both polyester polymer samples there are six main types of hydrogen and we used Spectral Database and ChemDraw to analyze the NMR spectra of the polymers. First, at 5.7 ppm there is a proton peak represent an ethylene (C=C-H). In addition, there is multiple peaks observed at 5.3 ppm that represent ethylene ($C=CH_2$).

Between (4.1-4.8 ppm) a broad peak represents methine protons (aliphatic chain CH) found in the sorbitol. Moreover, the fourth type of proton is also aliphatic methylene (CH_2) which observed between (1.2-4.2 ppm) in sorbitol and glutaric acid monomers as well. (CH_2-C=O) can be observed between (2.2 ppm) and they indicate to hexenoic acid and glutaric acid monomers. The last proton that can be recognized in both polyester polymers is (CH_3) methyl proton that represented between (0.96-1.11ppm) and these clustering peaks indicate to hexanediol, and decanediol. These were the major chemical shifts of proton NMR. There are also time dependent study for 24, 48 and 72 hours of both polyester polymers 1&2 (Figure 6, 8).

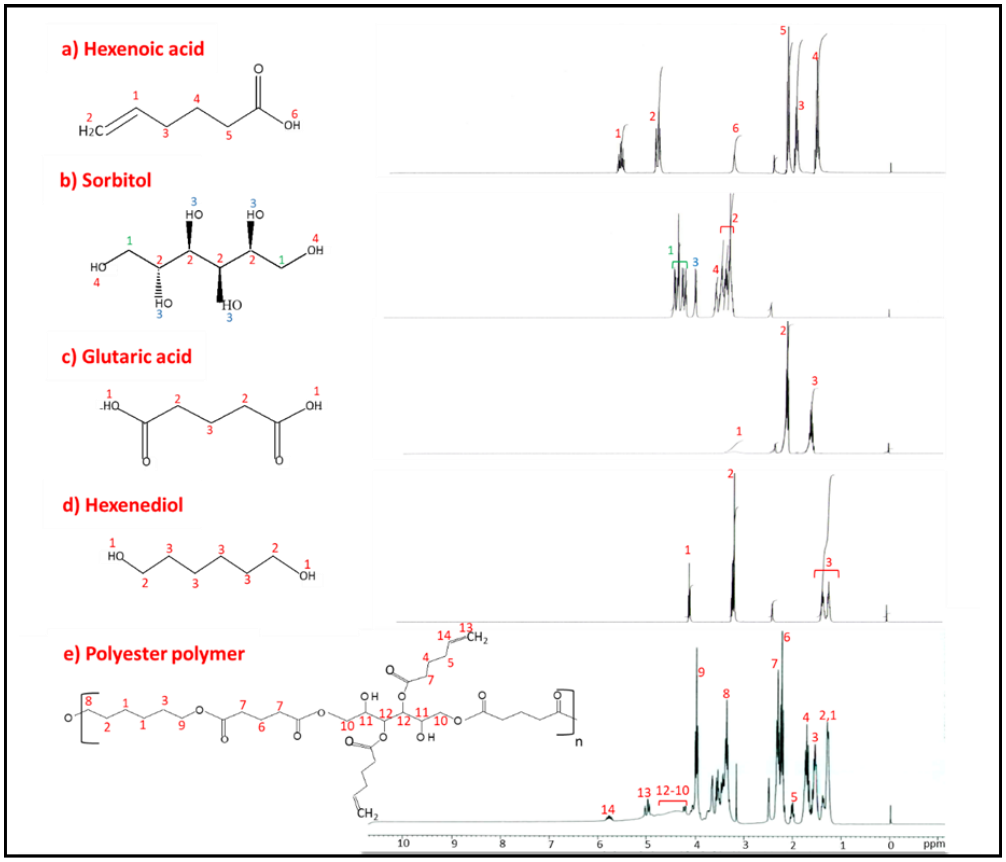

Figure 5. ^{1}H NMR Spectra of four monomers and the **polymer-1**

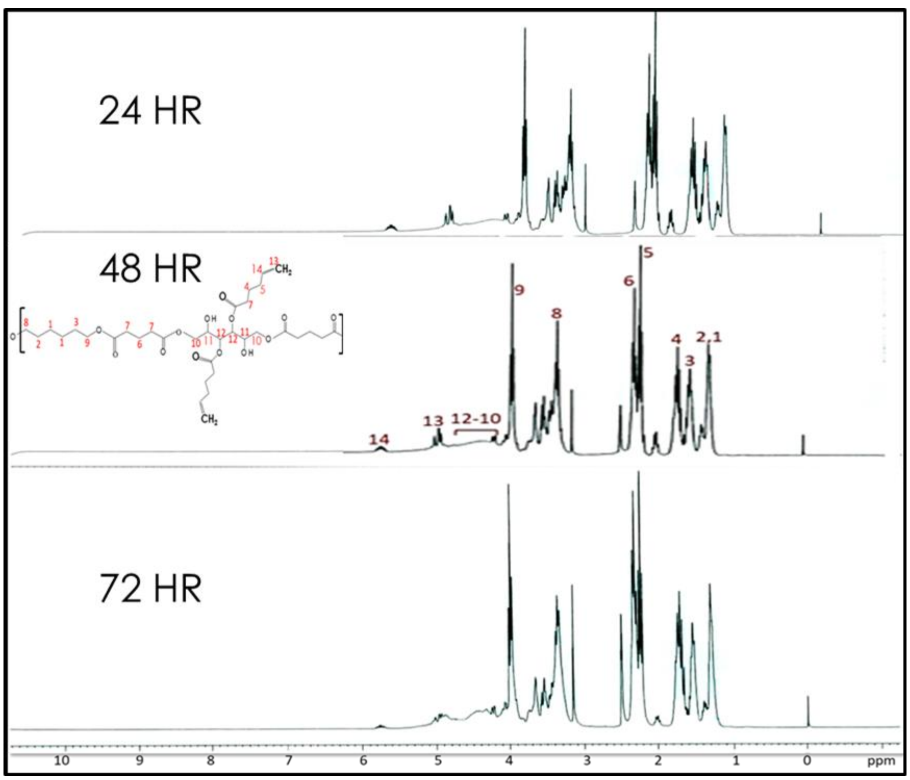

Figure 6. ^{1}H NMR Spectra of the **polymer-1** time dependent study

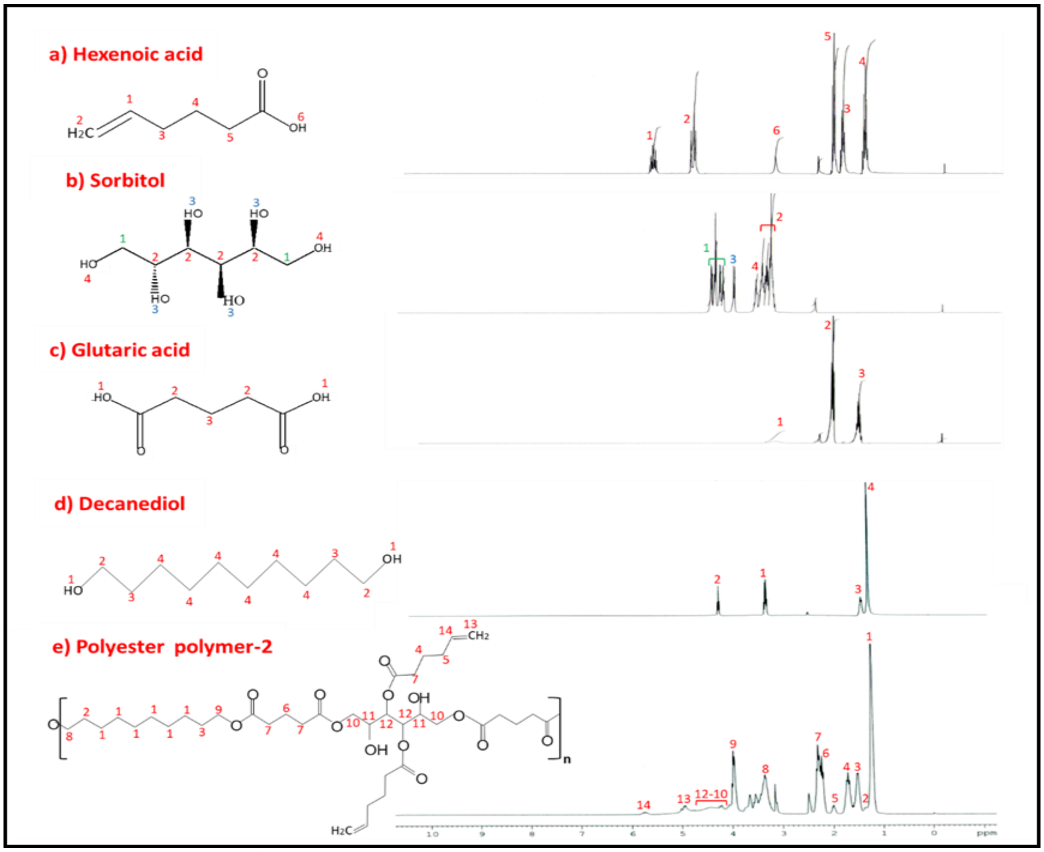

Figure 7. ^{1}H NMR Spectra of four monomers and the **polymer-2**

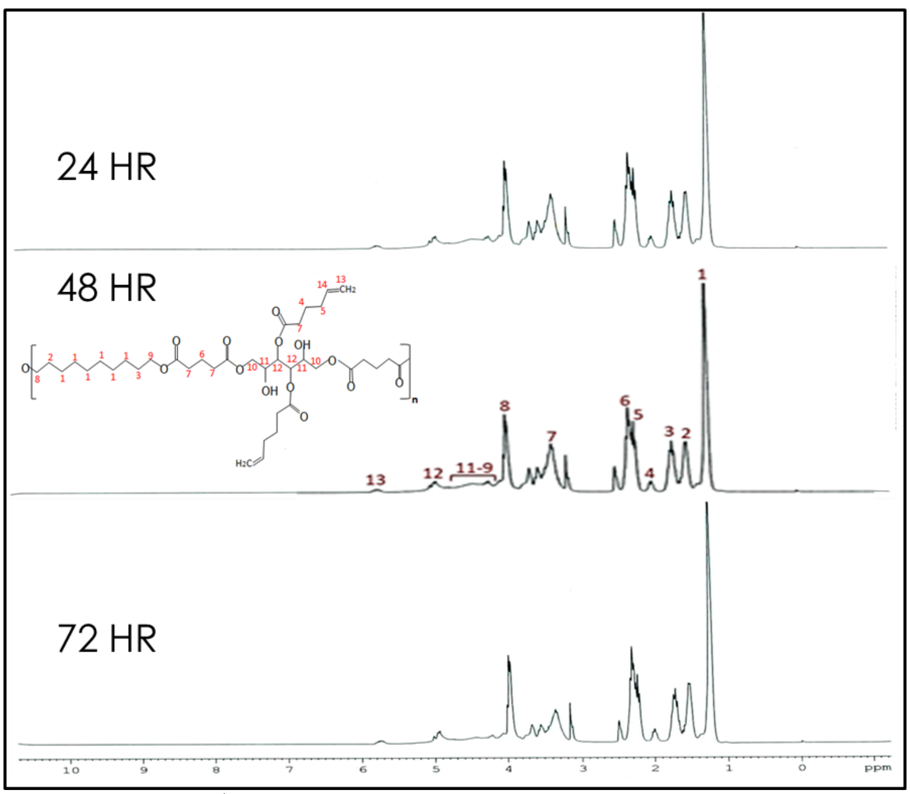

Figure 8. ^{1}H NMR Spectra of **polymer-2** time dependent study

II. ^{13}C NMR Spectroscopy:

The carbon-13 NMR spectra for the four biodegradable monomers and polymer samples are shown in **Figures 7-8**. The solvent peak for DMSO-d_6 manifests as a strong multiple at 40 ppm in each of the spectra.

There are four main types of C, all of them recognized in the NMR spectra, and we depend on Spectral Database for organic compounds (SDBS) and ChemDraw software to promote our read of ^{13}C NMR Spectra polymers. The most obvious peaks are ester carbonyl (C=O) that are represented at 172 ppm and that indicate to glutaric and hexenoic acid sites. The second type of C has observed at 138 ppm and 114 ppm and both of them represented C=C group in hexenoic acid monomer in both polymer samples. The third type is (C-X) where X here represent O between 64 to 76 ppm in these peaks CH_2 attach with O. In addition, at about 65 ppm there is CH_2 attached with (OH) hydroxyl group that indicate to the sorbitol. The last sort of C we can identify is methylene group that observed between (20-34 ppm) and these CH_2 aliphatic group indicate to hexanediol and decanediol. In both polyester polymers, we can notice that the number of peaks more than that in the first polymer due to the large number of CH_2 in decanediol than hexanediol.

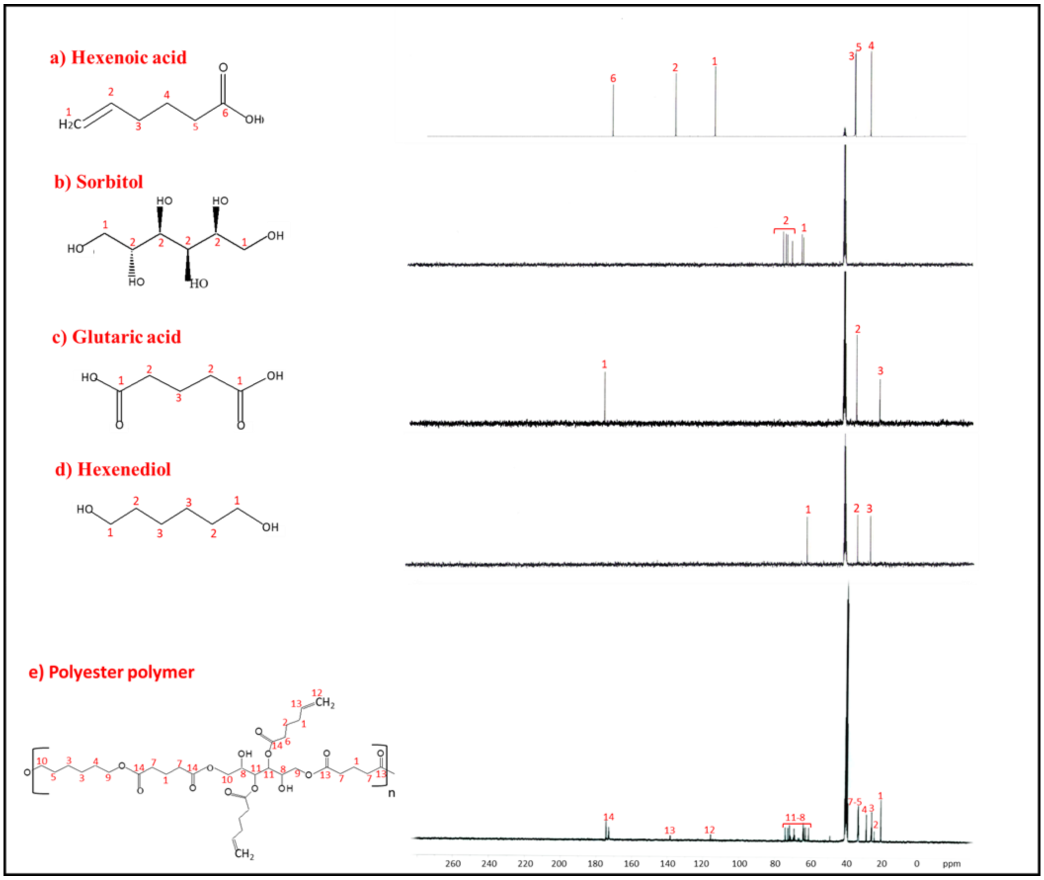

Figure 9. ^{13}C NMR spectra of the polyester **polymer-1** and the monomers.

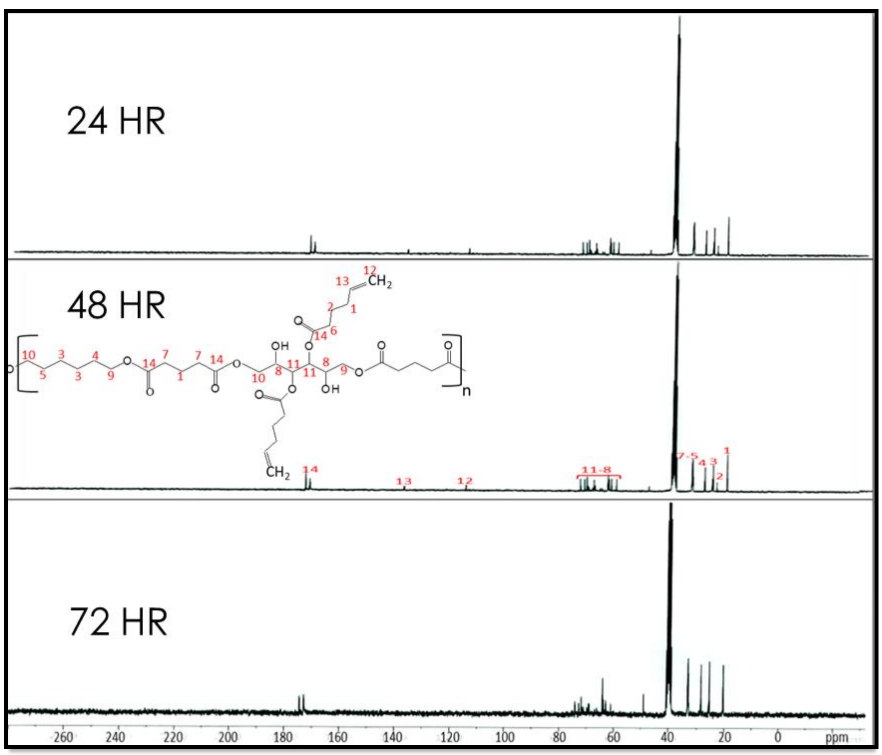

Figure 10. ^{13}C NMR spectra of the polyester **polymer-1** time dependent study

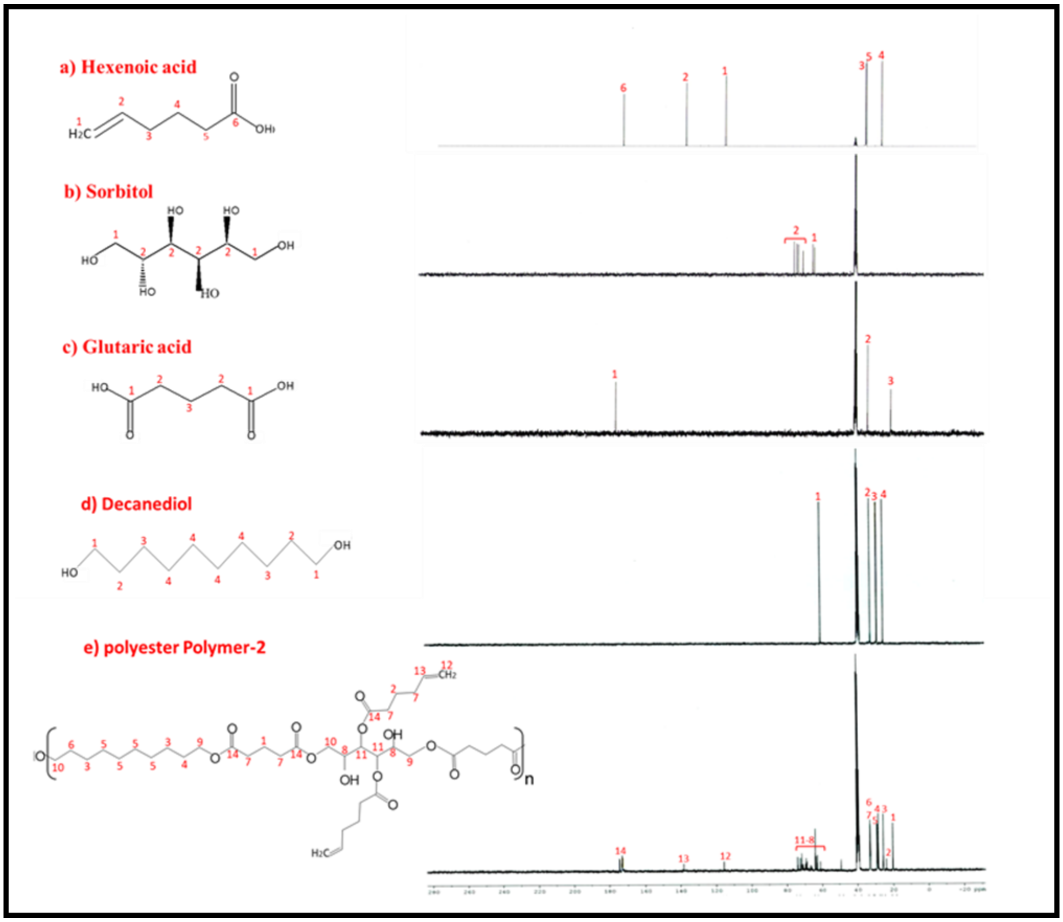

Figure 11. ^{13}C NMR spectra of the polyester **polymer-2** and the monomers.

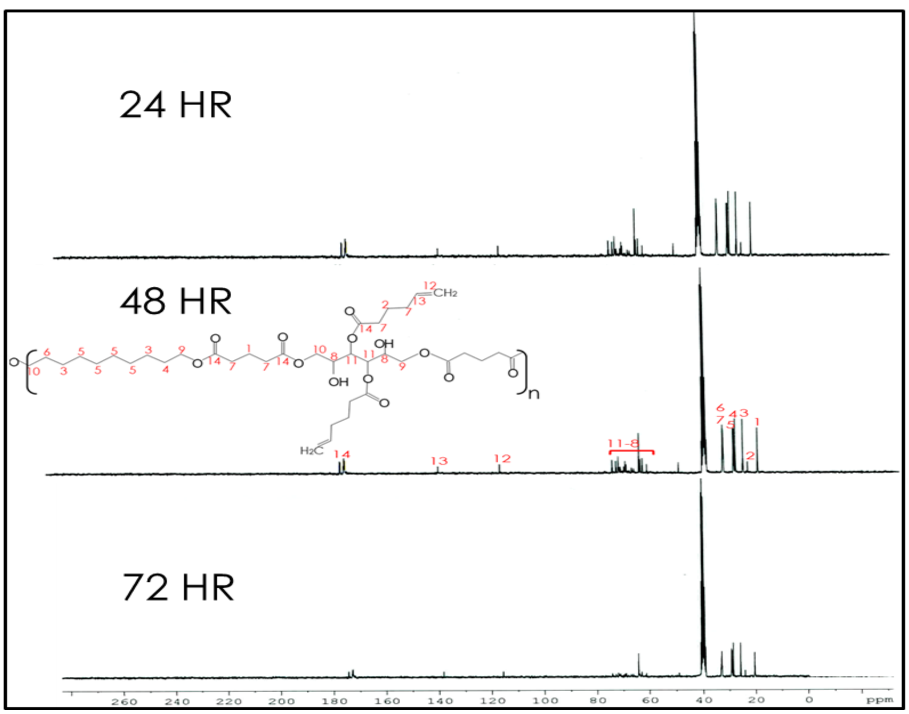

Figure 12. ^{13}C NMR spectra of the polyester **polymer-2** time dependent study

1.2.2 Fourier transform infrared spectroscopy (FT-IR):

In the FTIR spectrum of both polyester polymer samples, we will see some different types of bands. The most obvious band is OH that can be recognized by its strong and very broad wavenumbers at (3400 cm^{-1}), and that indicates to OH group of sorbitol. Next zone can be recognize (C-H) aliphatic stretch band at 2950 cm^{-1}, also there is another one at 2800 $cm^{-1.}$ The carbon-oxygen double bond C=O which is ester carbonyl can be found at 1720 cm^{-1}. Alkene stretching (C=C) was observed at 1400 cm^{-1}. However, between (1000-1300 cm^{-1}) there are two main peaks that we cannot emphasize which one of them is (C-O) band and the other one is (C-H) band because they are in a close range or nearby band stretches (**Figure 13, 15**). To carify that there are two seprat study for each polymer 1&2 (**Figure 14, 16**).

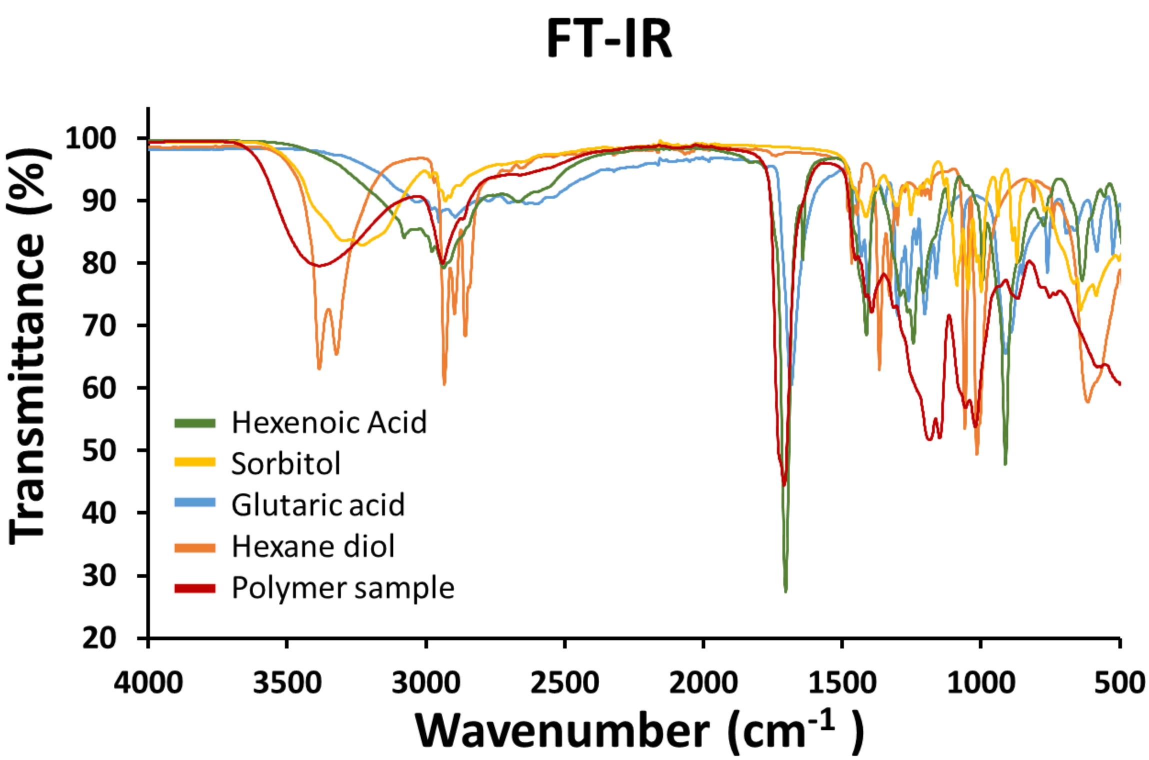

Figure 13. FT-IR Spectra of the polyester **polymer-1** and biodegradable monomers

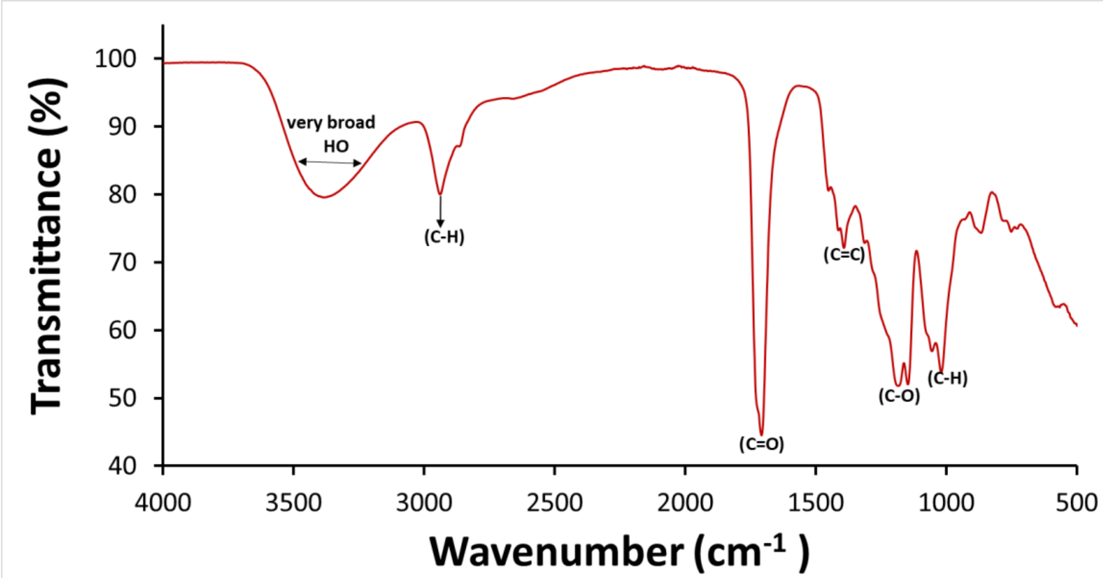

Figure 14. FT-IR Spectra of the polyester **polymer-1**

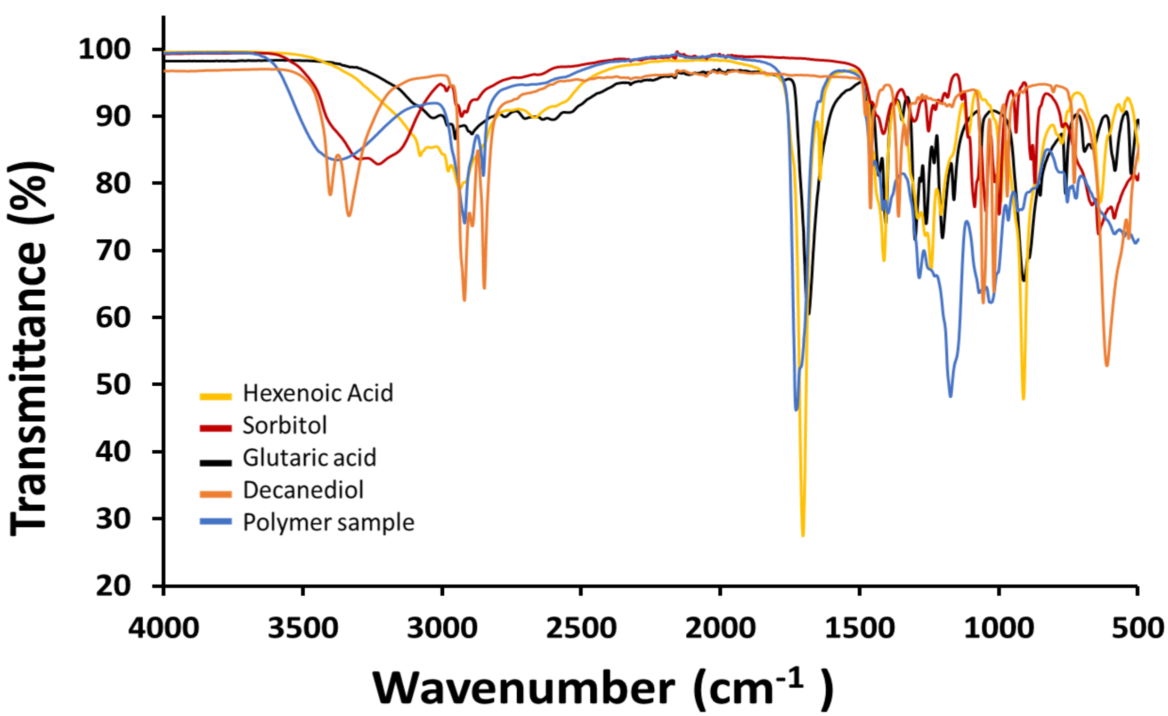

Figure 15. FT-IR Spectra of the polyester **polymer-2** and biodegradable monomers

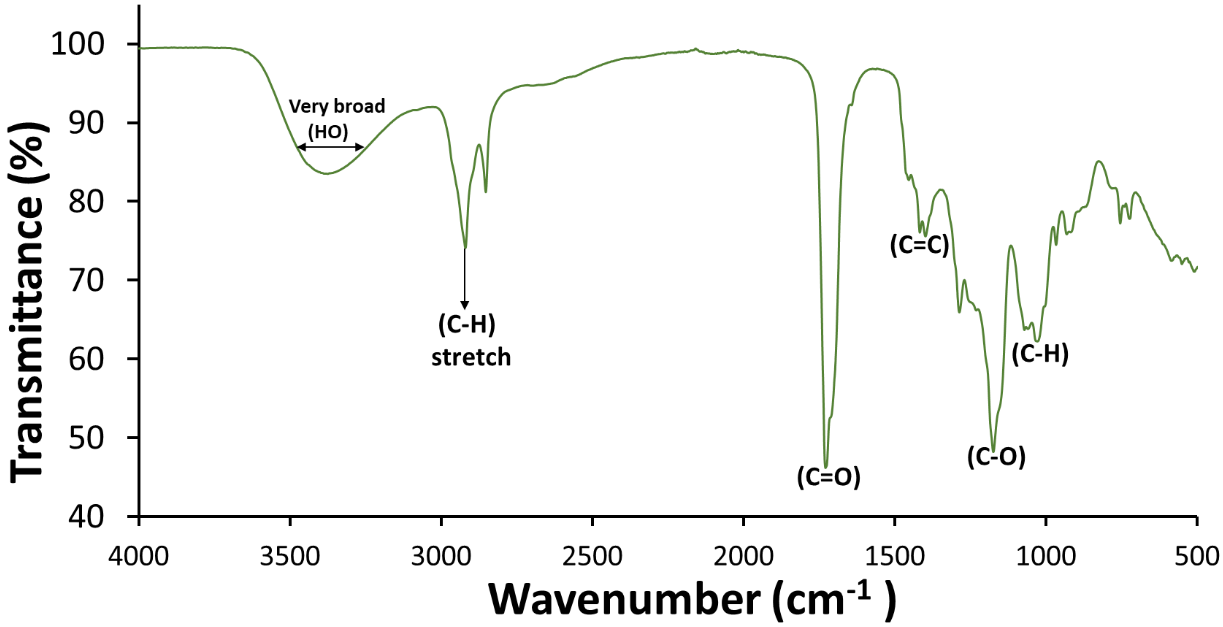

Figure 16. FT-IR Spectra of the polyester **polymer-2**

1.2.3 Gel Permeation chromatography (GPC):

The goal of getting gel permeation chromatography (GPC) spectrum is to identify the molecular weight of these two polyester polymers. We observed by using this technique that both samples have the highest molecular weight product at around 33 minutes and 31 minutes, respectively. The GPC result of both polymer samples can be seen in (**Figure 17and 19**). These results indicate that the second polymer was higher in molecular weight when subjected to equal reaction time, and the reason behind that is the long aliphatic chin in decandiol monomer. It also was determined that each sample had polydispersity index (PDI): first polymer was around 1.41while second polymer was around 1.37.

There is also in **Figure 18 and 20** compare between all monomers and both polyester polymers 1&2. At about 43 min there is a peak in all GPC figures and that peak indicate to Tetrahydrofuran (THF), is the solvent that was used to dissolve the polymer in order to get the GPC result.

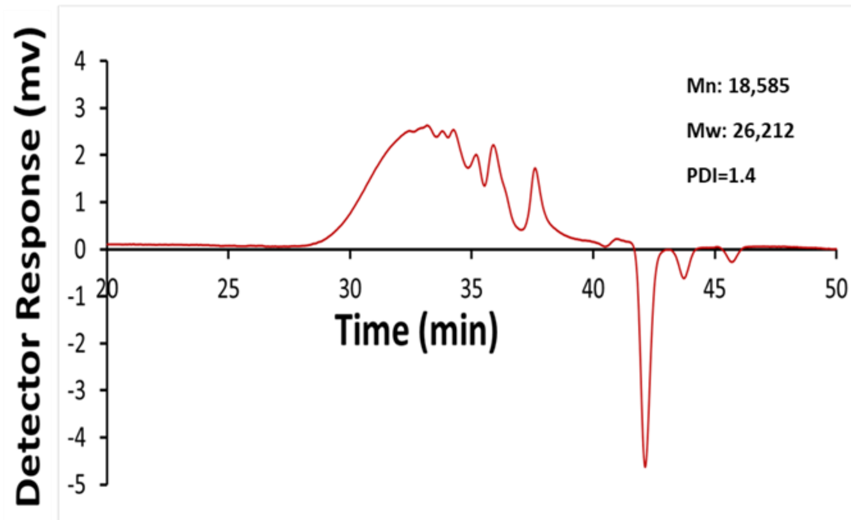

Figure 17. GPC 48 hour of polyester **polymer-1**

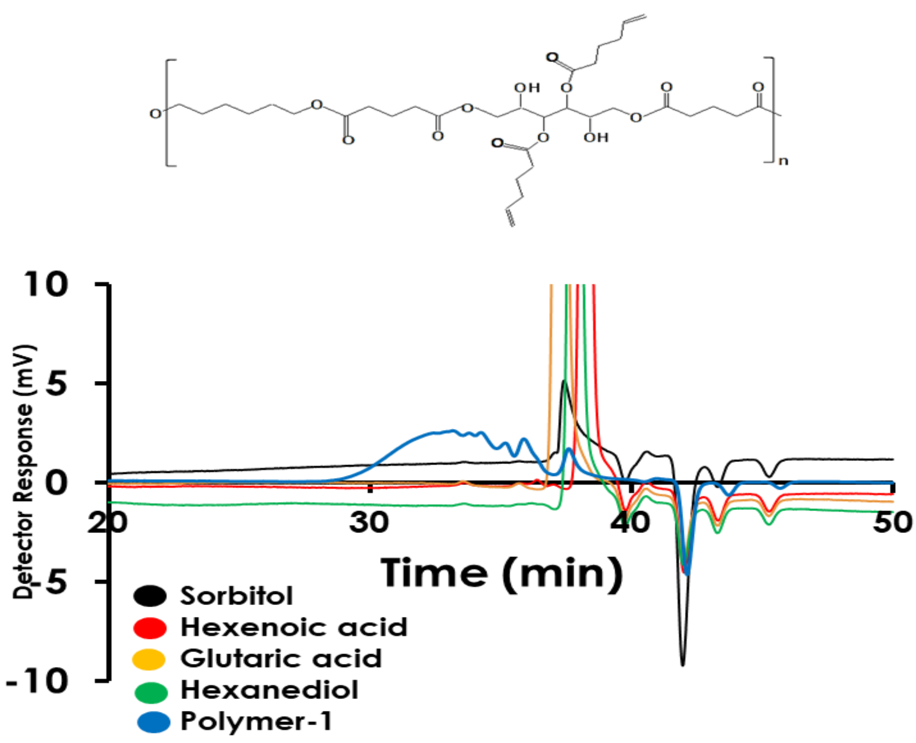

Figure 18. GPC 48 hour of polyester **polymer-1** and all bio based monomers

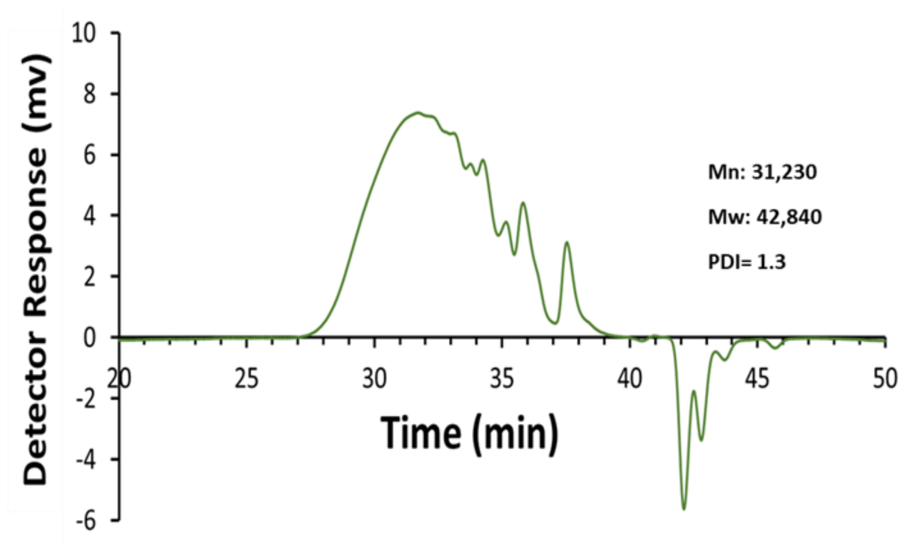

Figure 19. GPC 48 hour of polyester **polymer -2**

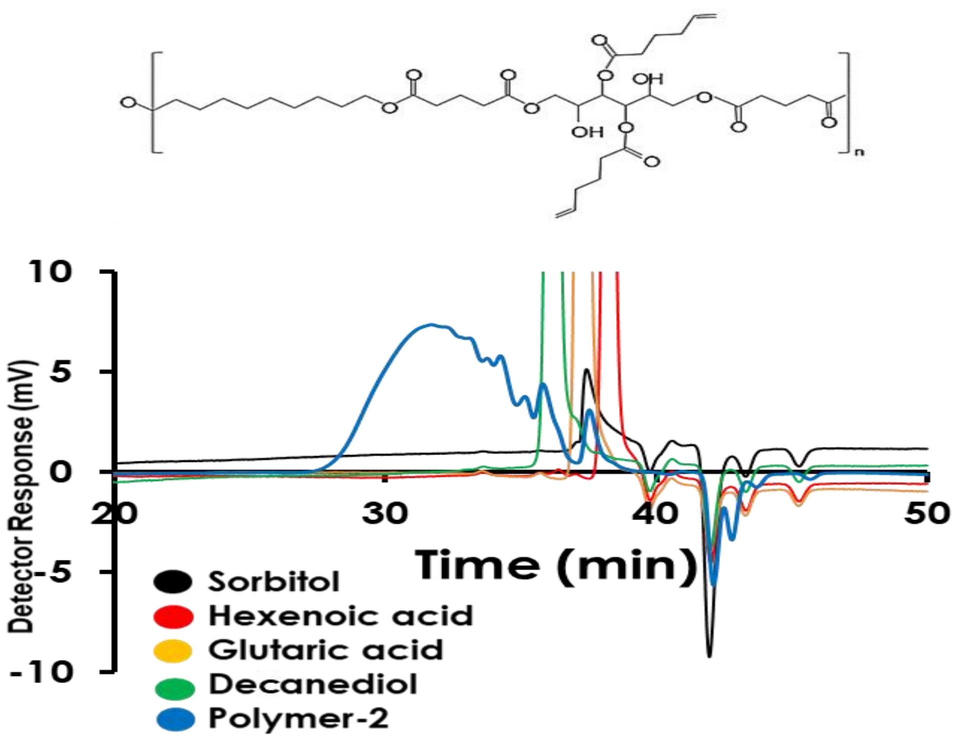

Figure 20. GPC 48 hour of polyester **polymer-1** and all bio based monomers

1.2.4 Thermal Gravimetric Analysis (TGA) Results:

Thermal gravimetric analysis (TGA) indicate the average thermal stability of the synthesized polymer (10% weight loss at around 225 °C in air) in the polymer-1 **(Figure 21)** while in the polymer-2 **(Figure 22)** (10% weight loss at around 240 °C in air) . That indicates the probability of obtaining high degradable polymers. While both polyester polymer samples started their decompositions at 37 °C, they showed degradation (10% weight loss) at around 230 °C. The typical temperature of a polyester polymer is 350 °C; this indicates to the slight difference between the typical polymer and our polymers. The high degradability were expected due to the aliphatic and non-aromatic compound in both polymers 1&2 so Tg are low. There is another characteristic that we can approve in these results both of the polymers are amorphous.

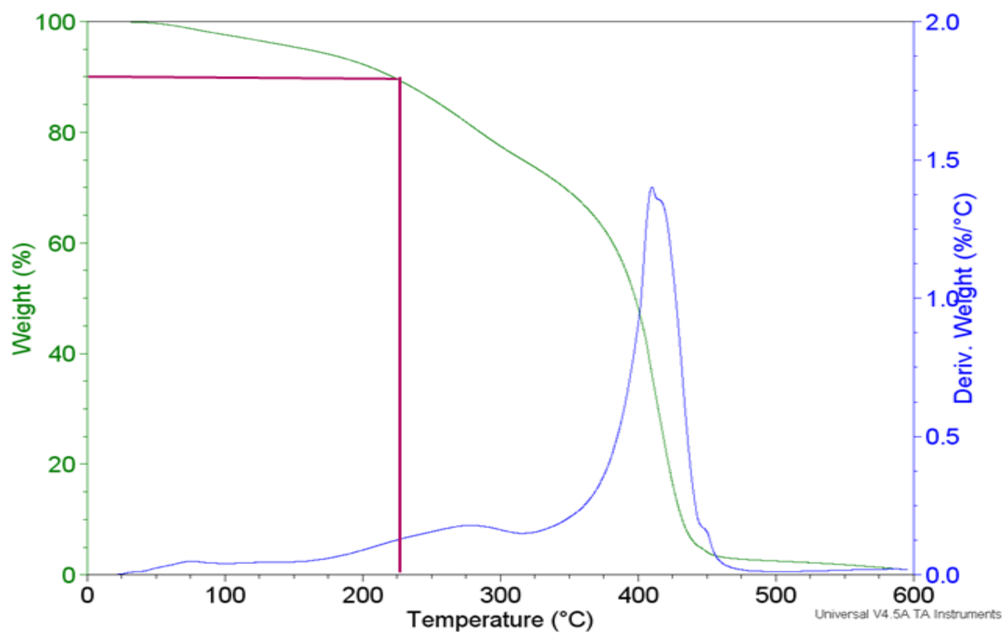

Figure 21. TGA of polyester **polymer -1**

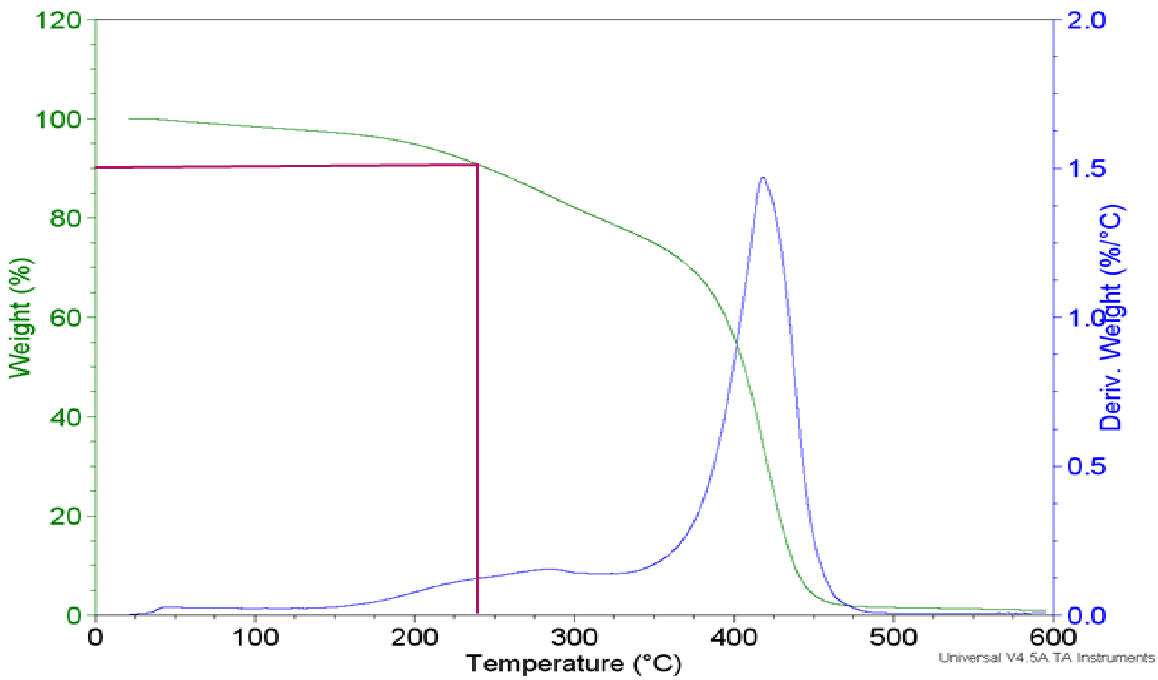

Figure 22. TGA of polyester **polymer -2**

1.2.5 Differential Scanning Calorimetry (DSC):

The **(Figure 23)** indicates to DSC curves of two different types of polyester polymers. Both of them have melting temperature Tm around 25 ºC. The rate of Tm indicates to that polyester polymer in both samples have low degree of crystallinity. In addition, there is a clear indicate to the glass transition (Tg) at around -30ºC. To clarify, due to presence of hydroxyl group (OH) and functional group (double bond) on the surface of our polymer the Tg is low.

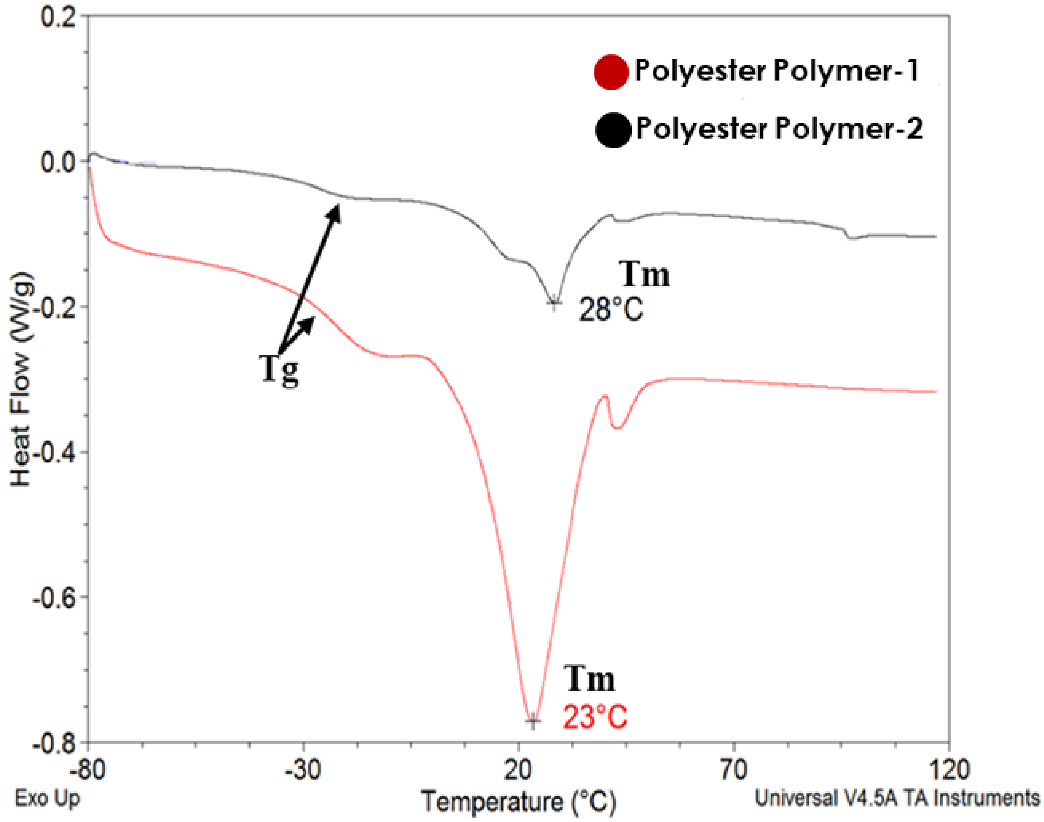

Figure 23. DSC of polyester **polymers-1 and 2**

2. Polymeric nanoparticle synthesis and Characterization:

In our study, to obtain and formulate polymeric nanoparticles (PNPs), we used an optical dye DiI (5 µg/µL) and anti-cancer drug Taxol (1 µg/µL). In addition, polyester polymer were uesed to enacpsulate various corage by uesing solvent diffusion method and two different solvet (DMSO and Dionize water). By utilization this approach, we obtained a perfect polymer mixiture because this method forace the polymer and hydrophobic cargo to interact with each other and induce the dye molecules to be encapsulated with in the hydrophobic pockets of the polymer matrix.then, to purify the PNPs, we uesed daiylisis bag method. Then, we ueded a very active approach to bioconjugate which is "click-ene" chemistry. For click-ene reaction, PNP and Fol-N_3 reacted under heat 50 °C and without uesing any catalyst only thermal. To illustrate, there are some charactirzations of polymeric nanoparticlae that can give a better vition about our these nanoparticals **(Figure 24).**

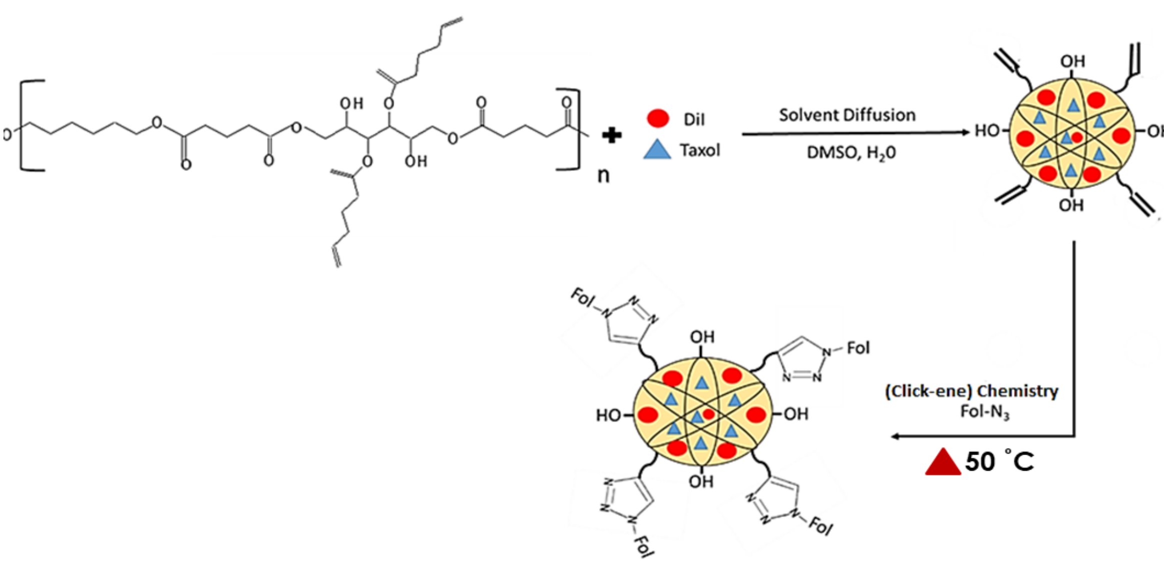

Figure 24. Conversion of polyester polymer to Nanoparticles and Surface Ligand Modification

2.1 Dynamic light scattering (DLS):

Dynamic light scattering (DLS) is a technique to measure the size of PNPs and studies of PNPs confirmed the presence of stable and monodisperse nanoparticles. The overall diameter was found at 39.40 nm and at 117.0 nm in PNP-1 (**Figure 25**). While after conjugated with folic acid, there was an obvious increase in the size about 5 nm (**Figure 26**). However, in the PNPs-2 the size presented at 121.7 nm (**Figure 27**). Then after conjugated the PNP-2 with folic acid there was also increase about 4 nm **(Figure 28)** .

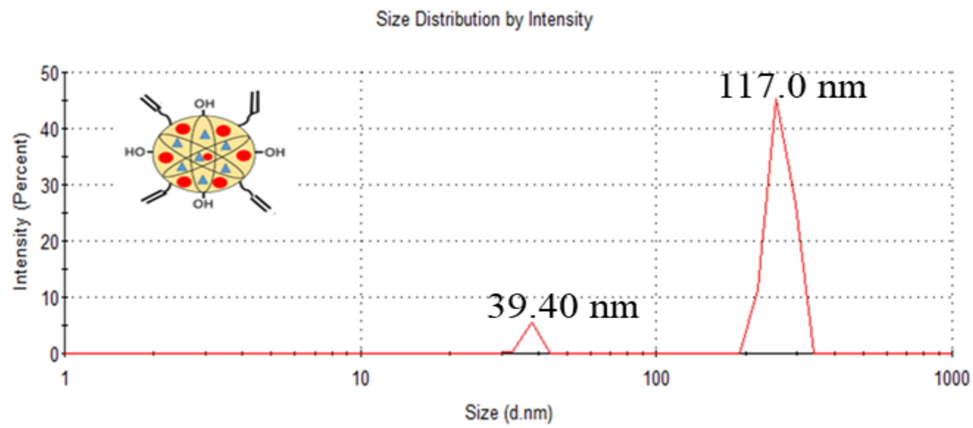

Figure 25. Dynamic light scattering of the **PNPs-1**

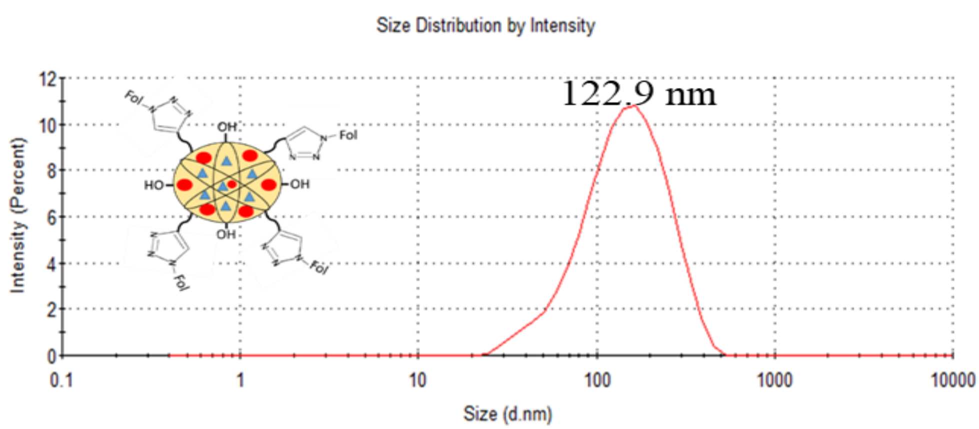

Figure 26. Dynamic light scattering of the folic acid conjugating with **PNPs-1**

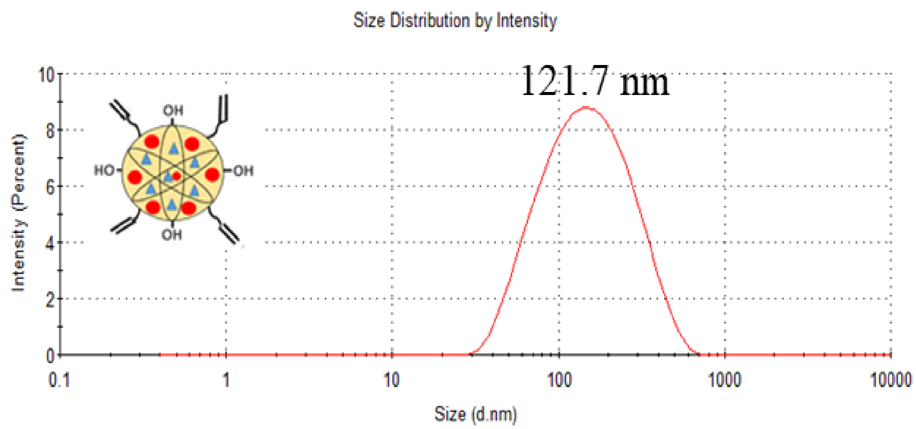

Figure 27. Dynamic light scattering of the **PNPs-2**

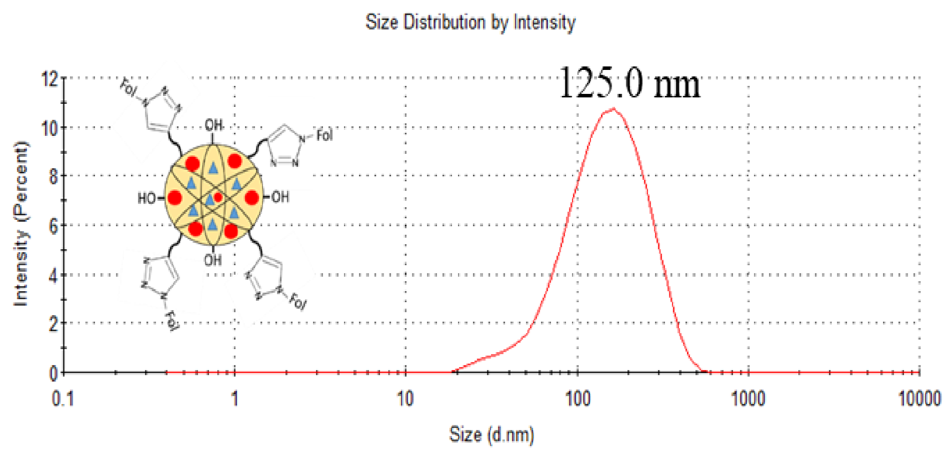

Figure 28. Dynamic light scattering of the folic acid conjugating with **PNPs-2**

2.2 Zeta Potential Determinations:

The zeta potential is a technique used to determine the surface charge of PNPs. The result of the surface zeta potential indicate that average values of -24 mV for the PNP-1 and – 22.8 mV for the PNP-2 **(Figure 29, 31)**. These values were expected due to the carbonyl oxygen (C=O) present in the hexenoic acid surface pendants and the secondary alcohols (OH) in the sorbitol component of the polymer all that will result negative charge. However, in **Figure 30** and **32**, when both PNPs 1 and 2 were conjugated with folic acid, they showed a clear decrease in there surface charge that PNP-1 was found at -22.1 mV while PNP-2 was found at -18.7 mV. Due to the positive charge in the folic acid, the PNPs presented decrease in there surface charge.

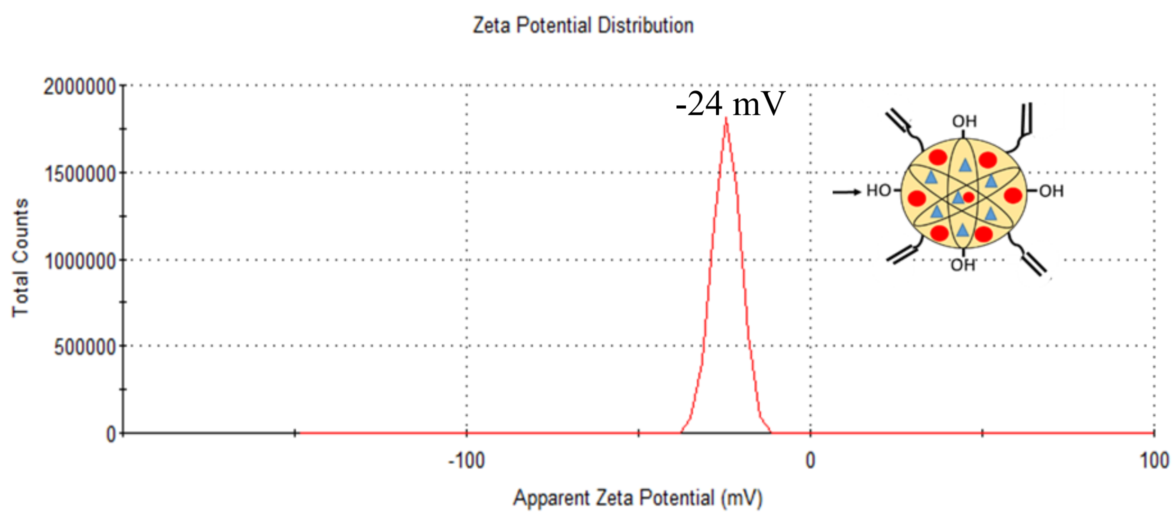

Figure 29. Zeta-potential of **PNPs-1** loaded with drugs

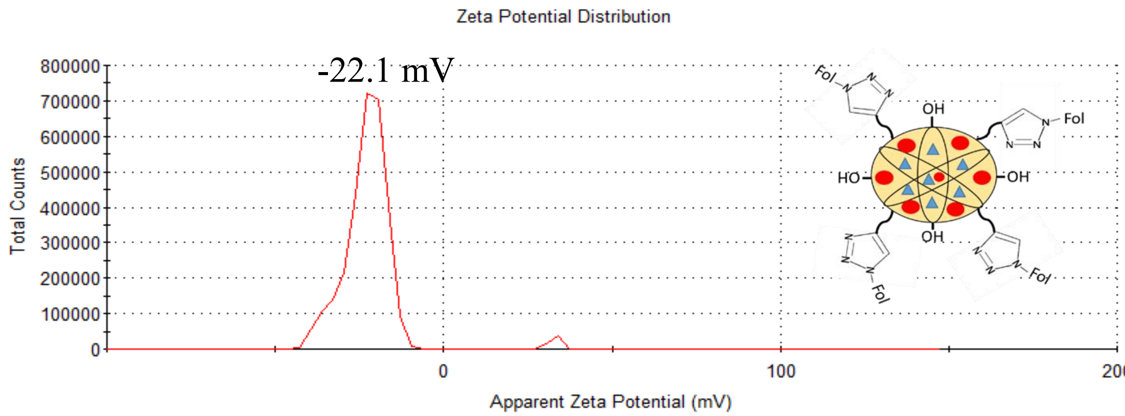

Figure 30. Zeta-potential of **folic acid** conjugated **with PNPs-1**

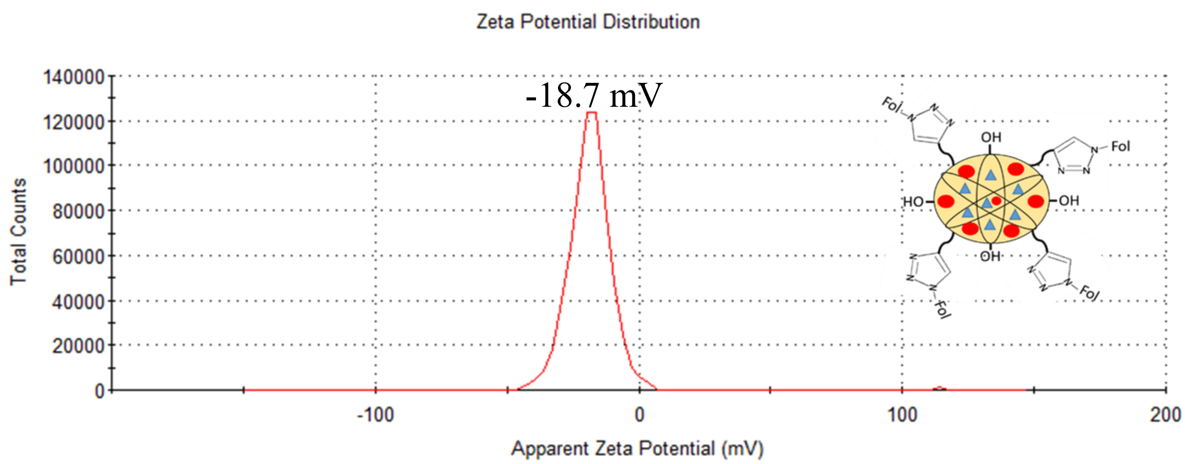

Figure 31. Zeta-potential of **PNPs-2** loaded with drugs

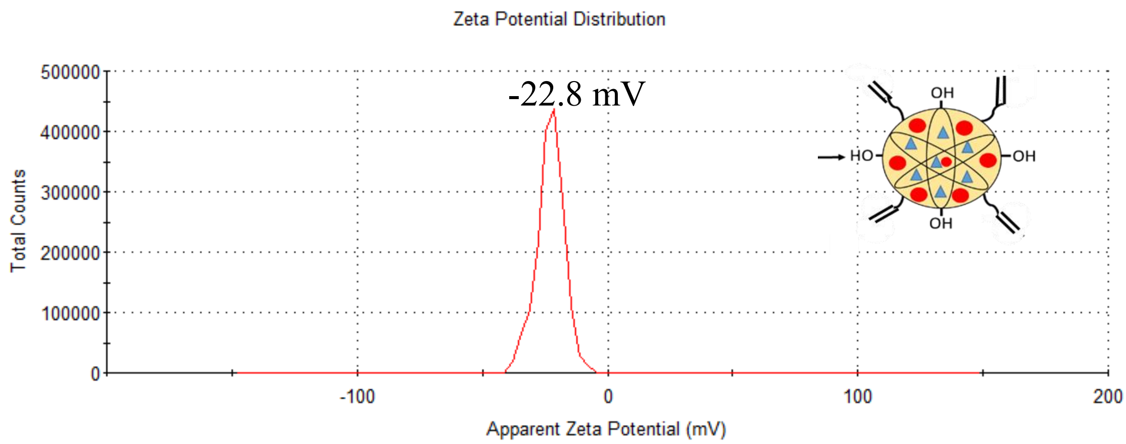

Figure 32. Zeta-potential of the folic acid conjugated with **PNPs-2** loaded with drugs

2.3 Characterization by Absorbance and Fluorescence:

In order to study and analyzes the nanoparticles, we used two different techniques to characterize them: UV/V absorbance and fluorescence spectroscopy. In addition, these methods were used to determine whether the folate and DiI dye existed or not. To illustrate, UV/V studies of folate-decorated Polyester polymer loaded with DiI were characterized by the presence of folic acid (λ_{abs} = 350 nm) and DiI dye (λ_{abs} = 565 nm) (**Figure 33**).

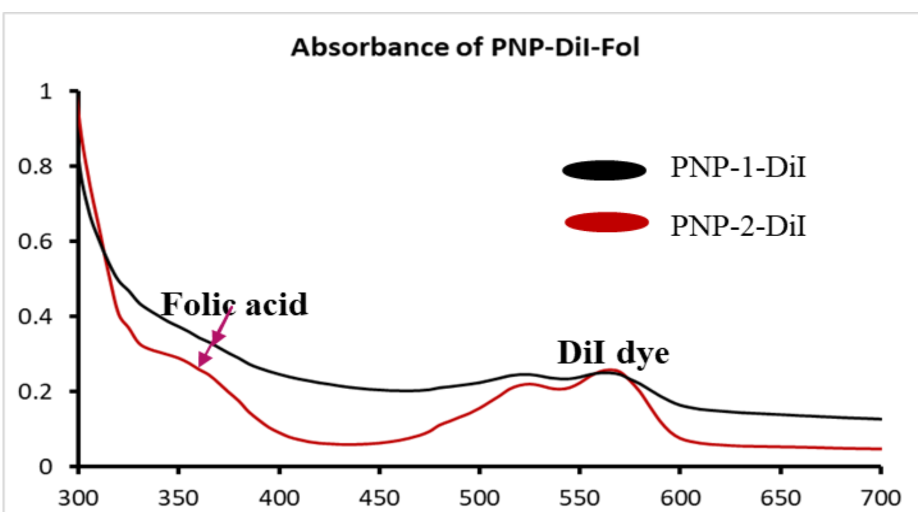

Figure 33. UV/vis spectrum indicating the presence of DiI and Folic acid of PNPs with Encapsulation.

The fluorescence emission spectra indicated to DiI optical dye in the PNPs. In our polymeric nanoparticles, there are two peaks between 550 to 650 nm represented the characteristic peak for DiI. To clarify, fluorescence emission spectra confirmed the presence of DiI in the first polyester PNP at (λ_{em} = 580 nm) and in the second polyester PNP at (λ_{em} = 570 nm) **(Figure 34)**.

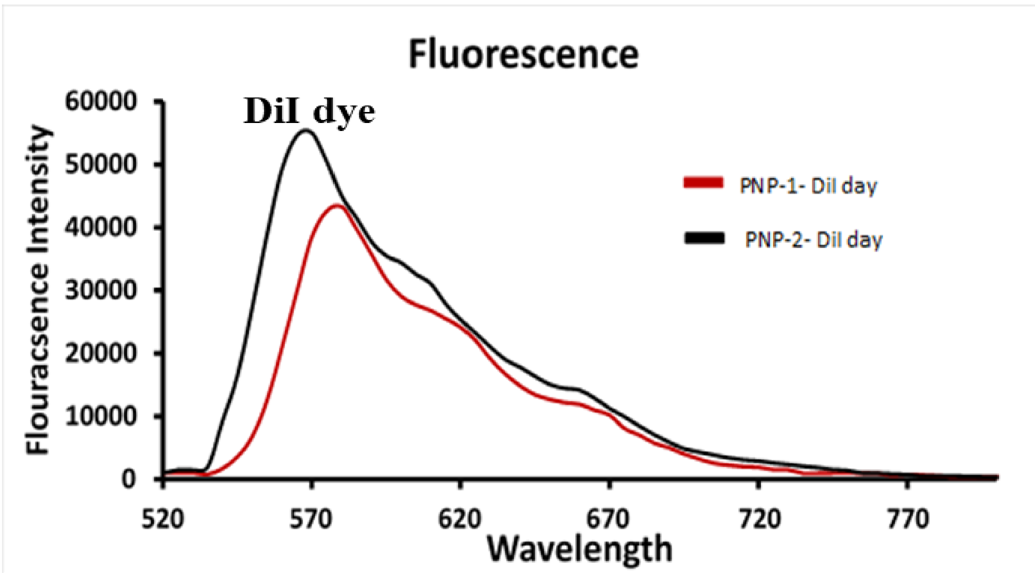

Figure 34. Fluorescence emission of PNPs with encapsulated DiI dye

3. Drug Delivery: Cell Culture and Cytotoxicity Assay:

MTT Assay is a technique to measure and determine the cellular efficiency and cytotoxicity of our Polyester polymeric nanoparticles.

LNCaP and PC3 prostate cancer cells were cultured in a 96-well plate and incubated with 50 µL each of (1) PNP-DiI and (2) PNP-DiI-Fol and (3) PNP-DiI-Fol-Tax from both nanoparticle samples. A well for untreated (control) cells was also cultivated for comparative purposes. The nanoparticles were permitted 24 hour of incubation (with results assessed at 6, 12 and 24 hours) within a humidified incubator at 37 °C and 5% CO_2 atmosphere. After the incubation, the cells were treated with the MTT/ Phosphate-buffered saline (PBS) solution and incubated for an additional 4-6 hours. The apoptotic effects of the treatment are measured with respect to the absorbance intensities of the MTT compound (560 nm). The cumulative results of these experiments in **Figure 35 and 36**.

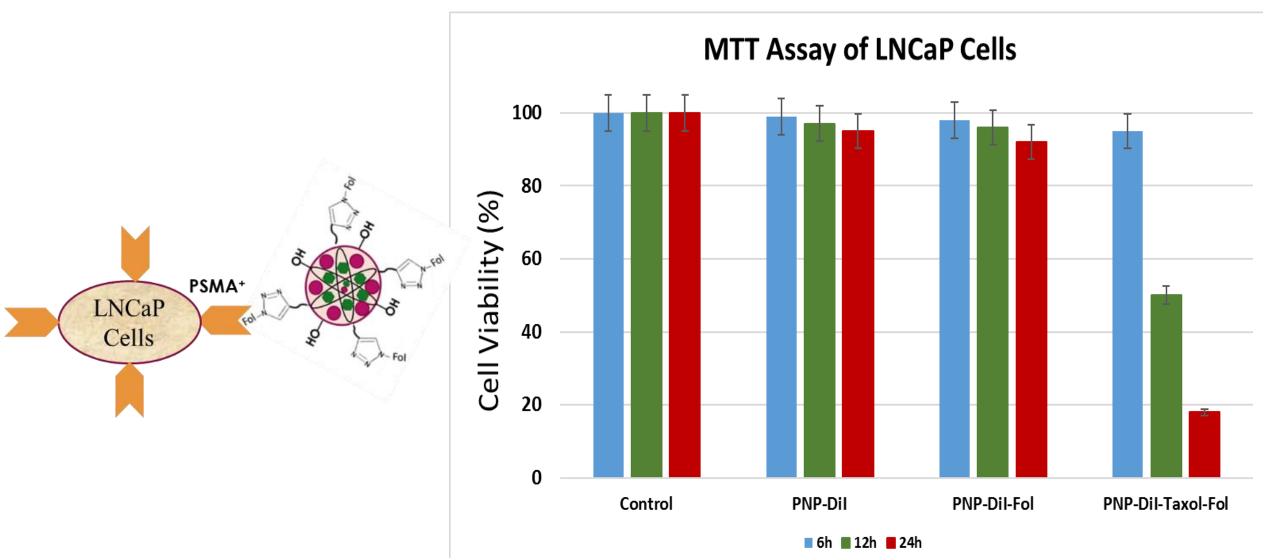

Figure 35. Evaluation of cytotoxicity of functional PNPs using MTT assay of LNCaP cells

In the MTT Assay of LNCaP prostate cancer cells, we observed the huge effect of encapsulating Taxol drug (anti-cancer) to the DiI, folic acid and PNPs. Over 12 hour, cell death occurred in about 50% of the cells, while over 24 hour there was about 80% cell death. These results indicate the efficiency of our PNPs. When we examined the DiI-Folic acid and PNPs without encapsulating the Taxol drug, we observed reduction in cell viability. These results confirm that our functionalized nanoparticles encapsulating anticancer drugs entered these cells and degraded, releasing Taxol to the cytosol and initiated apoptosis.

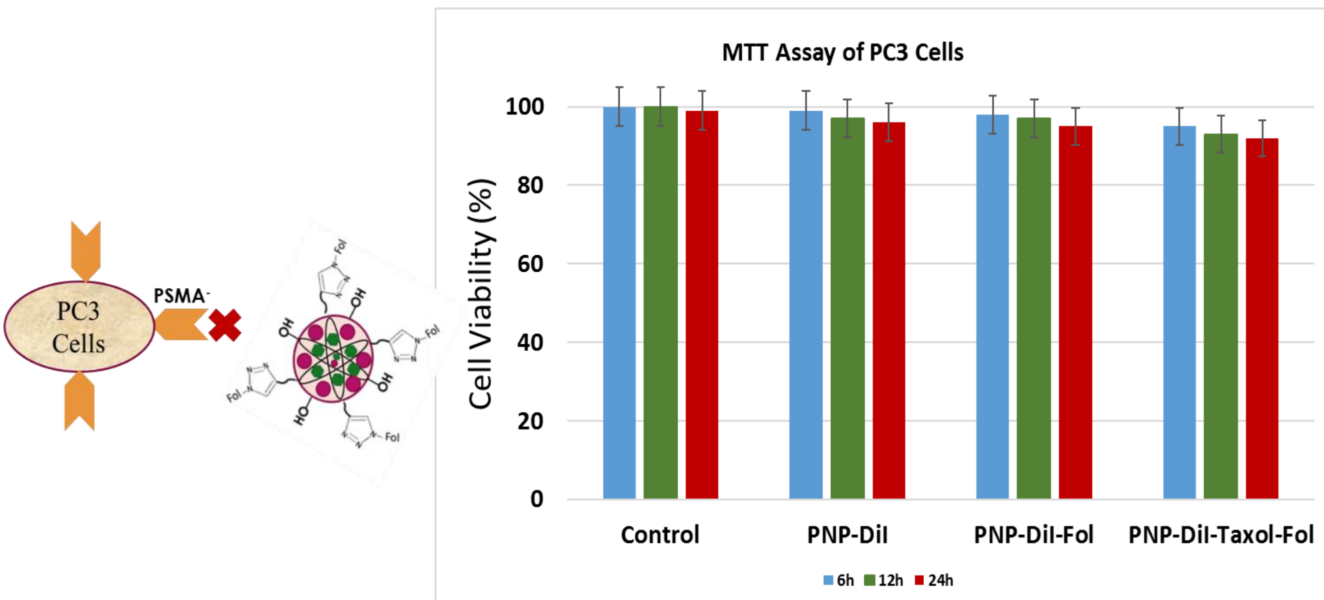

Figure 36. Evaluation of cytotoxicity of functional PNPs using MTT assay of PC3 cells

In this assay, there are normal cells and known as PC3 prostate cancer cells. In this evaluation, the cells do not have any folate receptors (PSMA); therefore, we got a successful result that indicate to the selectivity of our functionalized nanoparticles. As a result, there is no significant cell death was observed in any of the tracks. That was because of PC3 cells' lack of the PSMA receptor expressed in the LNCaP cell line, which displays a high affinity for folic acid. This provided evidence that our functionalized nanoparticles were selective for cell lines expressing the PSMA receptor, as significant cytotoxicity was only observed in the LNCaP cells. The slight reduction in PC3 cell viability observed at longer incubation times was attributed to disruption of the media (e.g. slight changes in pH) as exposure to nanoparticles increased.

CHAPTER IV

CONCLUSION

In summary, in our study, we successfully developed an efficient way to control the drug delivery for targeting cancer cells and for avoiding damage to other normal cells, organs and issues. Furthermore, two different aliphatic liner polyesters were synthesized from biodegradable and biocompatible monomers with hydrophilic functional groups OH (hexenoic acid). To characterize these two polyester polymers, NMR, FT-IR, GPC, TGA and DSC were used to demonstrate obtaining polymers successfully. With achieving our goal, polymeric nanoparticles were synthesized by using folic acid (azide group N_3) and click-ene chemistry, and by encapsulating DiI day and anticancer drug (Taxol) as well. To investigate the efficiency of the PNPs after encapsulating, there were some characterizations to demonstrate that. The size and the surface charge were observed to identify the PNPs characterization and both showed some great results. Furthermore, MTT assay was used to measure the cytotoxicity of PNPs to demonstrate the efficiency of PNPs in LNCaP cells, and the results showed significant data. About 80% 0f cancer cells were died within 24 hour by encapsulate these LNCaP cells with DiI day, folate and anti-cancer (Taxol). In the future study, drug release study will be one of the significant steps to measure the high efficiency of the PNPs in a human body. Also, reduce the PNPs size one of the main point in the future study. Finally, working more in vitro studies for performing in vivo study is a great and important step that we are looking forward to do in the future.

CHAPTER V

Experimental Methods

Materials:

Our bio-based monomers sorbitol, glutaric acid, hexanediol, decanediol, and hexenoic acid were purchased from Sigma Aldrich and used without further purification. To examine and determine the solubility of the polymers we used various solvents (methanol, dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), water (H_2O), chloroform ($CHCl_3$), and toluene) and they were purchased from Sigma-Aldrich or Acros Organics and used as received. In addition, Deuterated solvent dimethyl sulfoxide (DMSO-d_6) for use in ^{1}H NMR and ^{13}C NMR spectroscopy was purchased from Sigma-Aldrich. Near-infrared fluorescent dye 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and the chemotherapeutic drug Paclitaxel (Taxol) were purchased from Invitrogen and ThermoFisher, respectively, and 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.

Polyester Polymer Synthesis: Polymer-1 and Polymer-2

Sorbitol (1.4 g), glutaric acid (2.0 g), hexenoic acid (0.44 g) and either hexanediol (0.90 g) or 1,10 decanediol (3.29 g) were added to a 50 mL round-bottom flask containing a stir bar, then placed in an oil bath heated to 110 °C until all the compounds had melted. After melting these biodegradable monomers, the temperature was reduced to 95 °C and Novozyme-435 (400 mg), a lipase catalyst used for esterification at lower temperatures below 100 °C, was added to the melt. The flask was topped with a vacuum adapter, attached to a Schlenk line, and flushed with nitrogen gas (99.99 % purity) to create an inert atmosphere. The reaction proceeded for 12 hour under nitrogen atmosphere, after which the mixture was treated with a high vacuum ($4x10^{-4}$ mm/Hg). The vacuum exposure (applied to remove the water byproduct and drive the reaction to completion) lasted 72 hour, with two 2-3 g samples taken at 48 and 72 hours of total reaction time. To purify the polymer, each sample was dissolved in methanol and filtered through P8-grade (fine) filter paper to isolate the polymer solution from the expended catalyst. The isolated sample was placed in 50 mL round-bottom flask and subjected to rotary evaporation (low vacuum and 60 °C) to remove the methanol. If necessary, the samples were subjected to direct high vacuum to further ensure the complete removal of methanol. The purified form of the first polyester polymer was more wax-like, while the second polyester polymer was coherent. Both the 48 and 72 hour. Samples of the first and second polyester polymers were found to be soluble in dimethyl sulfoxide (DMSO), Toluene, and tetrahydrofuran (THF); the second polymer was also soluble in chloroform ($CHCl_3$).

Polymeric Nanoparticle Synthesis:

Polymer (30 mg) was placed in an Eppendorf tube, and 250 mL of DMSO was added to the polymer to dissolve it. To the polymeric solution, 3 mL Taxol (drug) and 2 mL DiI dye (optical dye) were added. This mixture was vortexed for about 3-5 minutes at 1500 rpm. A 15 mL centrifuge tube was taken with 4 mL deionized water in it, and the polymeric solution having cargos (drug and dye) was encapsulation slowly into the DI water at 1700 rpm. The centrifuge tube was capped and vortexed for about 3 minutes at 2500 rpm.

By solvent diffusion method **(Figure 37)**, the dialysis bag was soaked in water for about 30 minutes and the polymeric nanoparticles encapsulated with drug and dye were placed in the dialysis bag. Dialysis was carried out for about 2 hours. The purified polymeric nanoparticles were then removed from the dialysis bag, and placed into a tube, and labelled.

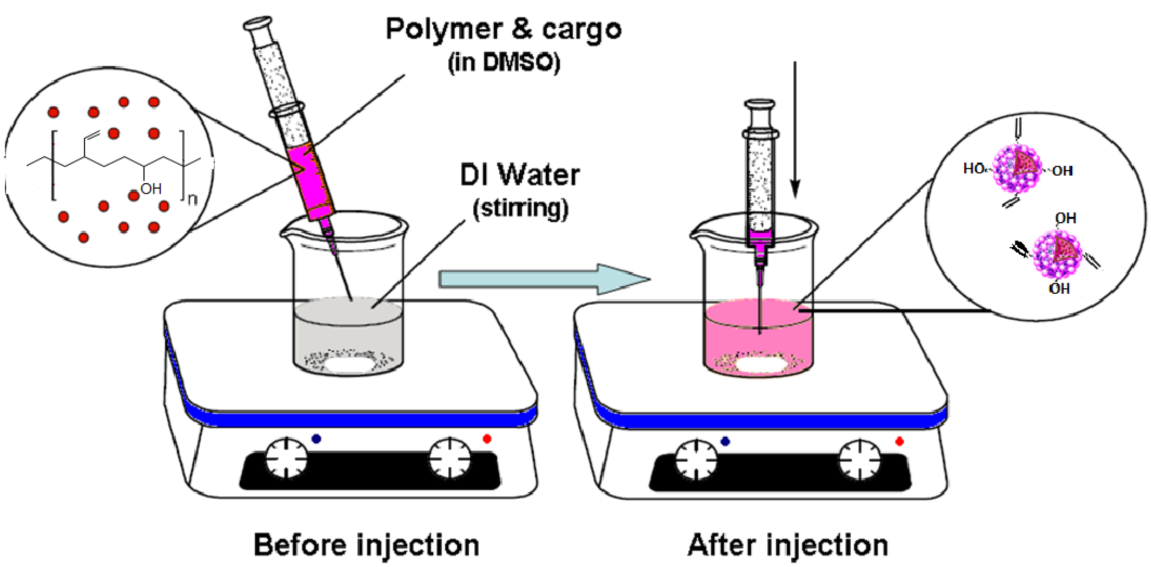

Figure 37. Solvent diffusion method[56]

Folic Acid Conjugation

It was necessary to do some modification process to the functional group on the surface of PNP for selective treatment of LNCaP cancer. Therefore, the nanoparticles were conjugated with azide functionalized folic acid (Fol-N_3) by "click-ene" chemistry, due to the presence of C=C surface functional groups. Moreover, folate ligands on the surface of cell cap cells, helps in selective uptake, which overexpress folate receptors on the surface of cell membrane. In **(Figure 38)** there is the synthesis of aminopropyl azide to modify the folic acid.

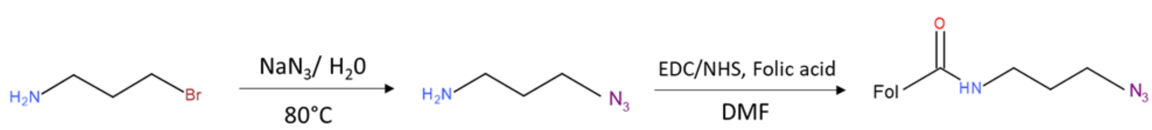

Figure 38. Synthesis of Azide-Functionalized Folic Acid

In addition, to modify the folic acid with the synthesis of aminopropyl azide that occurs by adding 3-bromopropyl amine (7 g, 0.051 mol) and of sodium azide (14.23 g, 0.219 mol) to a 100 mL round bottomed flask containing deionized water (40 mL), which is then heated to 80 °C for 20 hour. Thereafter, solvent was removed in a rotary evaporator under low vacuum, followed by the addition of potassium hydroxide (2 g, 0.036 mol) and extraction with petroleum ether.

Folic acid (0.05 g, 0.113 mmol) was dissolved in DMSO (2 mL). Two vials were taken and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.02 g, 0.129 mmol) was added in 250 μL of MES buffer (pH 5.0) in one vial. In the other vial, N-

Hydroxysuccinimide (NHS) (0.013 g, 0.113 mmol) in 250 µL of MES buffer (pH 5.0) was added.

Both of them were mixed and incubated at room temperature for 3 minutes. Within shortly incubation, aminopropyl azide (0.007 g, 0.07 mmol) was dissolved in PBS (0.025 mL), then one drop was added to the mixture, and the vials were incubated for about 3 hours. The azide-functionalized folic acid supernatant was collected by centrifuging the solution at 5000 rpm and dissolved in DMF (1 mL) until further use.

In order to complete the bonding of the nanoparticles, the nanoparticle suspension (2 mL) was mixed in a bicarbonate buffer (pH=7.4) with the azide-functionalized folic acid dissolved in DMF (0.02 mol). After which the reaction mixture was dialyzed in deionized water, and stored at 4 °C until further use.

Instrumentation of polymers and monomers characterization:

^{1}H NMR Spectroscopy: Samples of each polymer (30 mg) or monomer (5-10 mg) were dissolved in DMSO-d_6 (1 mL). The samples were processed in the Bruker DPX-300 MHz spectrometer using the TOPSPIN 1.3 program for 25 scans. Polymer samples were vacuum-dried before dissolving in the deuterated solvent.

^{13}C NMR Spectroscopy: Samples were taken of each polymer (60 mg) and monomers (20-30 mg), and they were dissolved in DMSO-d_6 (1 mL). The samples were analyzed in the Bruker DPX-300 MHz spectrometer using the TOPSPIN 1.3 program for 1000 number of scans. Polymer samples were vacuum-dried before dissolving in the deuterated solvent.

Fourier transform infrared spectroscopy (FT-IR): Polyester polymer or monomer samples (1- 5 mg) were placed in the PerkinElmer Spectrum 2 FT-IR spectrometer and scanned to obtain their wavenumber (cm^{-1}) spectra. Polymer samples were vacuum-dried and desiccated before analysis.

Gel Permeation Chromatography (**GPC**)**:** Gel permeation chromatography (GPC) was performed with a Waters 2410 DRI gel permeation chromatograph, consisting of four phenogel 5 µL columns filled with cross-linked polystyrene-divinylbenzene (PSDVB) beads. The polymer samples (5 mg) were first vacuum-dried, dissolved in THF (1 mL), then transferred to a GPC vial. The flow rate of tetrahydrofuran (THF) eluent was set to 1 mL/min at 25 °C for 50 minutes.

Thermogravimetric Analysis (**TGA**): The thermal stability of the polymer was examine on a TGA Instruments Q50 thermogravimetric analyzer. Polymer samples of about 7 mg were weighed, equilibrated, and then heated under nitrogen atmosphere using a heating ramp of 10 °C/min for 60 minutes, ranging from 25 to 600 °C.

Differential Scanning Calorimetry (DSC): The calorimetric parameters of the polymer were measured on a DSC Instruments Q100 differential scanning calorimeter. Polymer samples of about 8-10 mg were used for the test. The device was set to run three cycles ranging from -70 °C to 160 °C, with a ramp of 10 °C/min. The beginning of each cycle was precluded by a three-minute isothermal period, after which the ramping would begin again.

Instrumentation of Nanoparticle Characterization:

Dynamic Light Scattering (DLS) and Zeta Potential: The polymeric nanoparticle (10 µL) solution was added to DiI water (1 µL). This solution was then placed in a standard cuvette for DLS reading, or a specialized electrode-containing cuvette for zeta potential determination. The appropriate cuvette was placed in the Malvern ZS90 zeta sizer and the program set up (approximately 50 readings in 3 cycles) for the appropriate data acquisition.

UV/vis Absorption and Fluorescence Analysis: UV/vis spectra were recorded using a Tecan infinite M200 Pro microplate reader. Samples of polymeric nanoparticle suspension (50 µL) were placed in the wells of a 96-well plate and placed in the spectrophotometer. Absorbance scans were set to read a range of 300-700 nm, while fluorescence emission scans were set to read wavelengths of 500-800 nm. Readings were taken at intervals of 5 nm, with 10 flashes for each reading. The resulting data points were transferred to Microsoft Excel and plotted to visualize and compare the two samples.

In-vitro Cell Studies:

Cell Culturing: Both LNCaP and PC3 prostate cancer cells were grown in a specially formulated media containing, by volume, 85% RPMI-1640 media, 10% fetal bovine serum, and 5% Penicillin/Streptomycin antibiotic. These components were mixed, vacuum-filtered, and stored at 4°C until needed. The cells taken from cryo were re-suspended in this media (5 mL), transferred to a 7-mL culture flask, and incubated at 37 °C. Cells were split to new flasks with fresh media as needed to prevent overcrowding and to increase the longevity of the cells. Cell samples used for assays were taken from flasks with the most recently changed media and at least 24 hours old, or roughly 80 % confluent.

MTT Assay: LNCaP and PC3 prostate cancer cells were cultured in a 96-well plate and incubated with 50 µL dosages of the polymeric nanoparticle formulations (both with and without folic acid and Taxol) for 24 hours. Following the incubation, the media was removed, and 50 µL of 1X PBS was added to the cells for washing. The PBS was removed, and 25 µL of the MTT solution (50 mg MTT in 10 mL 1X PBS) was added to the wells and incubated for 4-6 hours. After incubation, the excess MTT solution was drained from the wells, and 30 μL of isopropanol was added. The cells then were ready to be read in the TECAN Infinite M200 PRO multi-detection microplate reader (at 560 nm absorbance) to determine the efficacy of nanoparticle treatment.

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