

Synthesis and characterization of Alendronate functionalized Poly (l-lactide) polymers for engineering bone tumor targeting nanoparticles

by

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Abstract

Nanomedicine-based therapeutics have exhibited clear benefits when compared to unmodified drugs, which include improved pharmacokinetics, drug retention, targeting efficiency, and minimizes toxicity. Every year thousands of bone cancer cases are diagnosed in the United States. Moreover, development of bone metastasis occurs in over 80% to 90% of various cancers that metastasize and signals the entry of the disease into an incurable phase. Cancer in bones can cause pain, fractures, hypercalcemia, and compression of the spinal cord, due to deposits that can erode into the bone using bone-absorbing cells. Bisphosphonates are drugs that reduce the activity of bone-absorbing cells and targets overexpressed calcium. They are characterized pharmacologically to inhibit bone resorption, skeletal distribution, and renal elimination. In addition, they can target bone microenvironment and bind strongly with calcium. The goal of this thesis is to engineer targeted nanomedicine drug with the ability to spatiotemporally control therapeutics delivery to the bone.

Herein we synthesized biopolymers with functional end group moieties as alendronate (a molecular member of bisphosphate), which can target overexpressed calcium ions at the vicinity of the bone lesion where bone resorption takes place. In order to achieve our goal, a ring opening polymerization of cyclic L-lactide initiated by ALE in the presence of catalytic amount of stannous octoate was conducted in an inert environment. Thus, formed polymers are characterized for their chemistry and physicochemical properties using various analytical tools. These polymers were characterized by nuclear magnetic resonance ($^1\text{H-NMR}$) and Fourier Transfer Infrared Spectrometer (FT-IR), which shows monomer conversion and the presence of amide and phosphate moiety.

Thereafter we engineered bone-homing polymeric nanoparticles of 80nm diameter by nanoprecipitation for controlled delivery of Dox, a first line anticancer drug used in clinics. The in-vitro results show that the nanoparticles have the ability to accumulate and internalized into the bone cancer cells, deliver drugs efficiently, and are least toxic. Therefore, innovative and efficient bisphosphonate functionalized Poly-l-lactide polymers were synthesized to target bone microenvironment.

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Chapter 1 - Introduction

1.1 Overview

1.1.1 Bone:

Bone is made up of composite material with inorganic mineral calcium phosphate to which bisphosphonates (BPs) have high affinity through Calcium ion-mediated coordination. Calcium in the form of hydroxyapatite (HAp), Figure 1, is a bivalent metal complex, which is one of the major mineral components of the skeletal system. The chemistry of HAp is like the inorganic component of bone matrix with a general formula of $Ca_{10}(OH)_2(PO_4)_6$.¹

Formation of bone in a biological environment is a complex process, which is regulated by different biomolecules of the extracellular matrix (ECM) such as

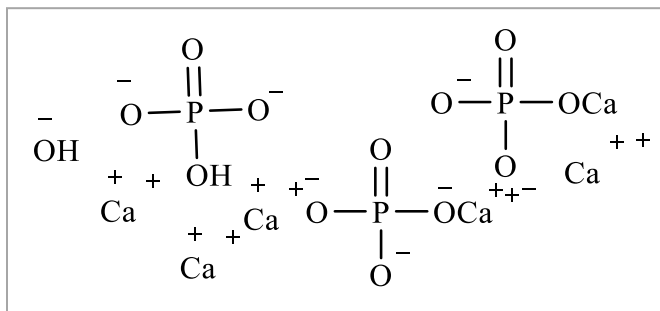


Figure 1 . Structure of Hydroxyapatite (Bone)

collagen, chitin, and proteins. ECM is secreted by cells and performs diverse cellular activities, including sending or receiving instructional information between cells for regulating their growth and terminal cell differentiation, which are highly dependent on the type of cells and the origin of tissue.^{1,2} One special role of ECM is to provide a template for the mineral to deposit in a specific orientation and organization to shape target structure.^{3,4} Formation of bone starts during the development of the fetus and continues throughout childhood and adolescence as the skeleton grows. Bone remodeling meanwhile is a life-long process, consisting of *resorption* (the breaking down of old bone) and *ossification* (formation of new bone), and is key to shaping the skeleton

and to the repair of bone fractures.⁵ There are three types of cell present in bone that are of interest, which are – osteoblasts, osteocytes, and osteoclasts⁶, Figure 2, and are respectively responsible for the production, maintenance and resorption of bone⁷.

- ***Osteoblasts***

Mononucleated “bone-forming” cells found near the surface of bones. They are responsible for making *osteoid*, which consists mainly of collagen. The osteoblasts then secrete alkaline phosphatase to create sites for calcium and phosphate deposition, which allows crystals of bone mineral to grow at these sites. The osteoid becomes mineralized, thus forming bone.

- ***Osteocytes***

These are osteoblasts that are no longer on the surface of the bone but are instead found in lacunae between the lamellae in bone. Their main role is homeostasis – maintaining the correct oxygen and mineral levels in the bone.

- ***Osteoclasts***

Multinucleated cells responsible for bone resorption. They travel to specific sites on the surface of the bone and secrete acid phosphatase, which unfixes the calcium in mineralized bone to break it down

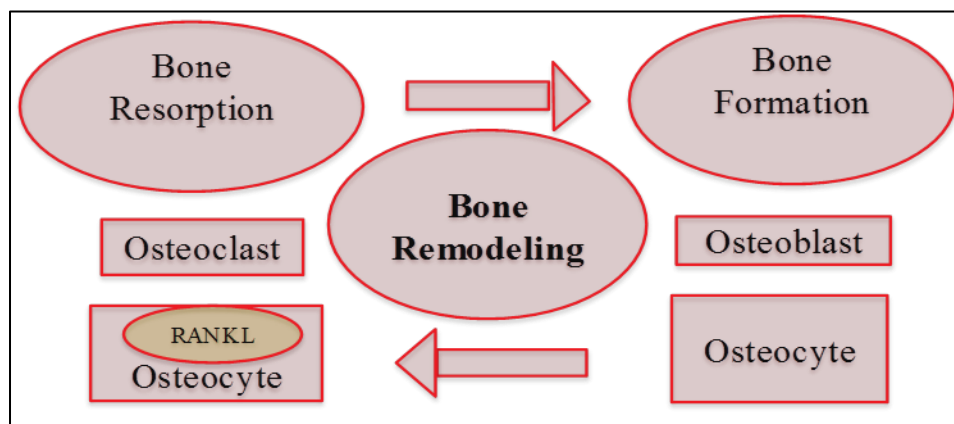


Figure 2. The Scheme of Bone Remodeling

1.1.2 Bone Diseases and Current Standard of Cure Using Bisphosphonates:

The bone tissue is one of the most affected organ that is susceptible to many diseases. Most of them can decrease the bone density thus weaken the tissue. The low bone density and osteoporosis can cause bone fractures. One of the diseases, Osteogenesis imperfecta can make bones brittle. Paget's disease can make them weak and thus can cause them to break⁸. Cancers, infections, and poor nutrition affect the rate of bone growth or rebuilding, thus increase their chances to break.⁹ Although, Bone cancer accounts for about 0.2% of all cancers, it is one of the dreadful diseases in the United States. In adults, over 40% of primary bone cancers are Chondrosarcomas. This is followed by Osteosarcomas (28%) and the others account for the remaining 32%. However, in children and teen, Osteosarcoma is more common than the other forms of cancer that lead to bone degradation.^{10,11} In addition to primary bone cancer, bone is one of the highly susceptible sites for distant cancer cells to reside thereby growing a metastatic bone cancer, which is the major problem in the management of bone related cancers. Breast, prostate, lung, thyroid, and kidney cancers are more likely to spread to the bone. In people with breast and prostate cancer, the bone is often the first distant site of cancer spread.¹² More than 2 out of 3 breast and prostate cancers that spread to other parts of the body spread to the bones. Of lung, thyroid, and kidney cancers that spread to other parts of the body, about 1 out of 3 will spread to the bones¹³. This, in turn, increase the bone cancer-related cases enormously.

Bone metastases are the most common outcome of advanced cancer, especially prevalent in breast and prostate cancer. The symptoms involve severe pain, bone fractures etc. Bone metastases is explained as mostly incurable phases of the disease. The other most common bone

disease is Osteoporosis, a disease which makes bones fragile and thereby develop fractures. It is one of the most common side effects of bone cancer.¹⁴ A fracture in the bone may be developed at the later stage of the tumor.¹⁵ Bone diseases such as osteoporosis and bone cancer are closely associated with the aging process and abnormal cellular growth. Osteoporosis is a debilitating disease of bone mass and microarchitecture which causes nearly 1.3 million fractures each year.¹⁶ On the other hand, many cancers are Osteoporotic and either metastasize to the skeleton or grow primarily within the bone marrow, where this growth frequently leads to hypercalcemia, severe bone pain, skeletal destruction, and pathologic fractures.^{17,18} Cancer in bone induce a sequence of changes in the microenvironment such as secreting cytokines to increase the activity of osteoclasts via the parathyroid hormone-related protein (PTHrP), receptor activator of nuclear factor- κ B ligand (RANKL), and interleukin-6 (IL-6), resulting in increased bone resorption and secretion of growth factors from the bone matrix¹⁹. This creates a “vicious cycle”, Figure 3, of bone cancer, where bone marrow becomes packed with cancer cells that develop resistance to conventional chemotherapy, and leads to devastating consequences of bone fractures, pain, hypercalcemia, and spinal cord and nerve compression syndromes.²⁰

Thus, the complex interactions between cancer cells and the bone microenvironment underlie the homing of cancer to bone and the subsequent progression of

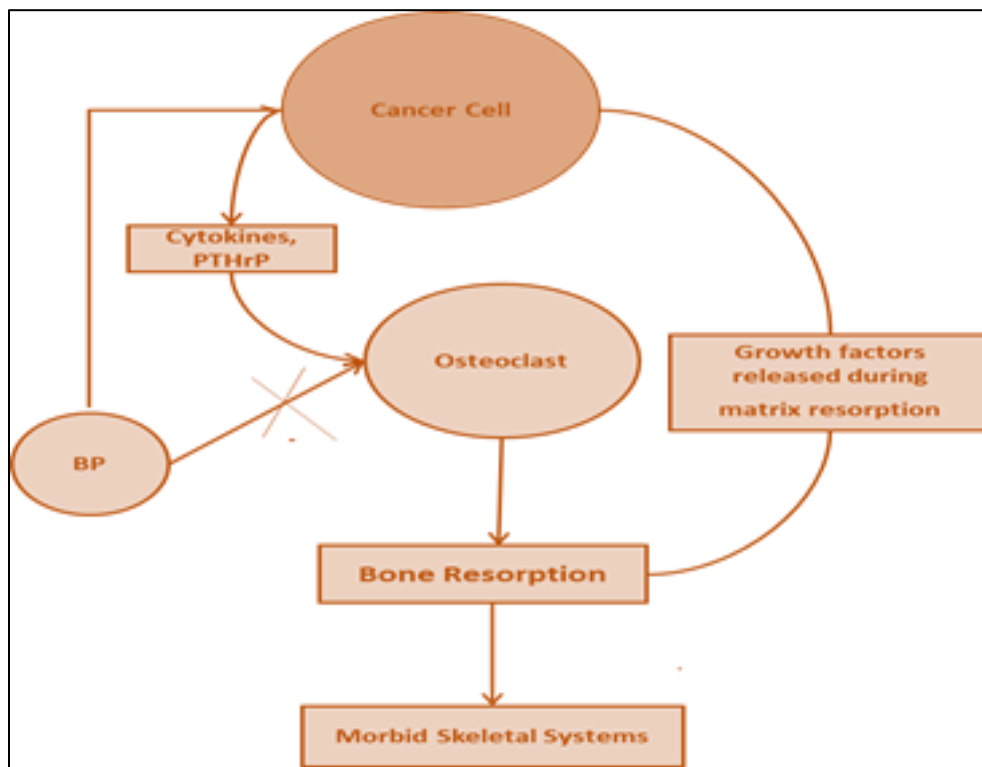


Figure 3. Action of Bisphosphonates Against Bone Resorption

osteolytic lesions. Indeed, the skeleton is the most common site of metastatic disease, and 90% or more of patients with advanced cancer develop skeletal lesions²¹. Therefore, there is an urgent need of development of a drug delivery system that specifically targets bone. Our research is focused on the synthesis of Bisphosphonate functionalized polymers that can target bone microenvironment.

1.1.3 Bisphosphonates:

The most common drugs used in bone-related disorder are bisphosphonates which can inhibit bone resorption for the treatment of osteoporosis and also in inducing apoptosis in osteoclast and tumor cells. The bisphosphonates are drugs that reduce the activity of bone-absorbing cells and can target over-expressed calcium in the sites of bone tumor.²² Structurally, bisphosphonates are chemically stable derivatives of inorganic pyrophosphate (PPi), a naturally occurring compound in which two phosphate groups are linked by esterification. It can act as a physiological modulator of calcium metabolism and have been developed clinically for the treatment of bone metabolic diseases, hypercalcaemia of malignancy, bone metastasis, and osteoporosis²³. Reduced morbidity from skeletal metastases in breast cancer patients during long-term bisphosphonate, Aminohydroxy propylidene bisphosphonate (APD) treatment.

Bone organ-specific drug delivery systems can be devised based on the attachment of BPs to drug molecules, imaging agents, therapeutic proteins or conjugation of these ligands to a common polymer carrier. Current therapeutic agents, however, do not usually display a preferential affinity to bones and non-specifically distribute throughout the body after administration. Attempts to design bone-specific agents have relied on engineering a desired therapeutic agent with bone-seeking molecules so that the latter delivers the therapeutic agents specifically to bones. Bisphosphonates are primary agents in the current pharmacological arena against osteoclast-mediated bone loss due to osteoporosis, Paget disease of bone, malignancies metastatic to bone, multiple myelomas, and hypercalcemia of malignancy²⁴. BP's are now used to treat such varied conditions as heritable skeletal disorders in children, postmenopausal and glucocorticoid-induced osteoporosis (GIO), and bone metastases in patients with malignancies. Bisphosphonates can offer substantial clinical benefit in conditions in which an imbalance

between osteoblast-mediated bone formation and osteoclast-mediated bone resorption underlies disease pathology.²⁵

1.14 Bisphosphonates and their Derivatives:

Bisphosphonates are the analogs of pyrophosphates characterized by the phosphorus-carbon-phosphorus backbone, which makes them resistant to hydrolysis. The structural differences are compared in the following Figure 4.

Substituting the central oxygen atom for carbon renders the bisphosphonate structure more stable and resistant to thermal, chemical, and enzymatic degradation. The distinct chemical properties of bisphosphonates are due to different substitutions at the carbon atom of the backbone²⁶

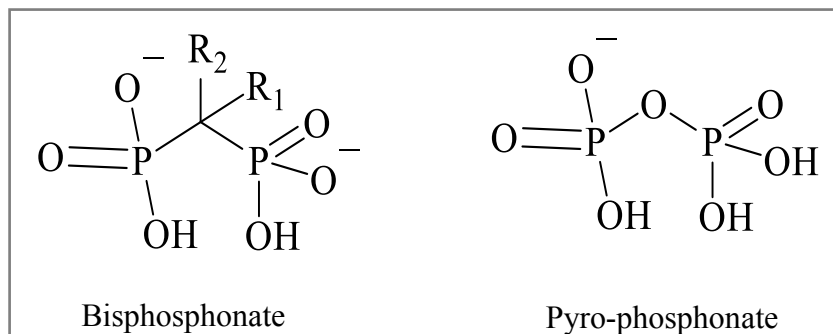


Figure 4. Comparison of Structures between Bisphosphonate and Pyro-Phosphonate

1.15 Classification of Bisphosphonates:

Bisphosphonates are classified based on the substituent at the bridge C-atom. The examples include (Table 1, Table 2, Figure 5): Alendronate, Clodronate, Etidronate, Pamidronate, Tiludronate, Ibandronate, Zoledronate, Clodronate, Residronate and Neridronate.²⁷ The side-chain substitutions (R₁ and R₂) of the carbon atom with halogen, sulfur, nitrogen,

hydroxyl, or other groups, result in a wide range of structures with varying anti-resorptive potencies as mentioned earlier.

Bisphosphonates are very effective inhibitors of bone resorption. Their activity is classified into two levels. At bone tissue, a level they combine to the bone mineral and thus increase the bone density, by avoiding dissolution. At, cellular level act on Osteoclasts and lead to their apoptosis. The Osteoclasts are bone-resorbing cells. These Bisphosphonates after attaching to the mineral surface, get internalized into the Osteoclasts, accumulate there and finally cause their apoptosis.

They may not act solely through direct actions on osteoclasts. They can stimulate the activity and proliferation of osteoblasts, which are important inhibitors of Osteoclast formation and activity. One of the possible mechanisms of bisphosphonate action is to stimulate the osteoblast to produce inhibitor(s) of osteoclast formation.

The molecular mechanism of bisphosphonates (like Etidronate and Clodronate) may include their incorporation into ATP. Then may result in compounds that are resistant to hydrolysis, subsequently kill osteoclasts. The nitrogen-containing bisphosphonates can inhibit enzymes in the mevalonate pathway, through which the post-translational modification of small GTP ases take place. These small GTP ases are the proteins that regulate an array of cell processes, which are required for osteoclast function.

Drug	Generation	Relative potency Anti-Resorptive Potencies
Etidronate	First	1
Clodronate		10
Tiludronate	Second	100
Alendronate		100-1000
Pamidronate		100-1000
Risedronate	Third	1000-10,000
Ibandronate		1000-10,000
Zoledronate		10,000+

Table 1 . Relative Potencies of the existing Drugs and their classification per bisphosphonate generations

Bisphosphonate	R₁ side chain	R₂ side chain
Etidronate	OH	CH ₃
Clodronate	Cl	Cl
Pamidronate	OH	CH ₂ CH ₂ NH ₂
Alendronate	OH	CH ₂ -3-pyridine
Risedronate	OH	CH ₂ -S-Phenyl-Cl
Tiludronate	H	CH ₂ -CH ₂ N(CH ₃) (pentyl)
Ibandronate	OH	CH ₂ -imidazole
Zoledronate	OH	CH ₂ -2-imazo-pyridinyl

Table 2 . The structural classification of BP (Bisphosphonates) based on the R₁ and R₂ side chain substituents.

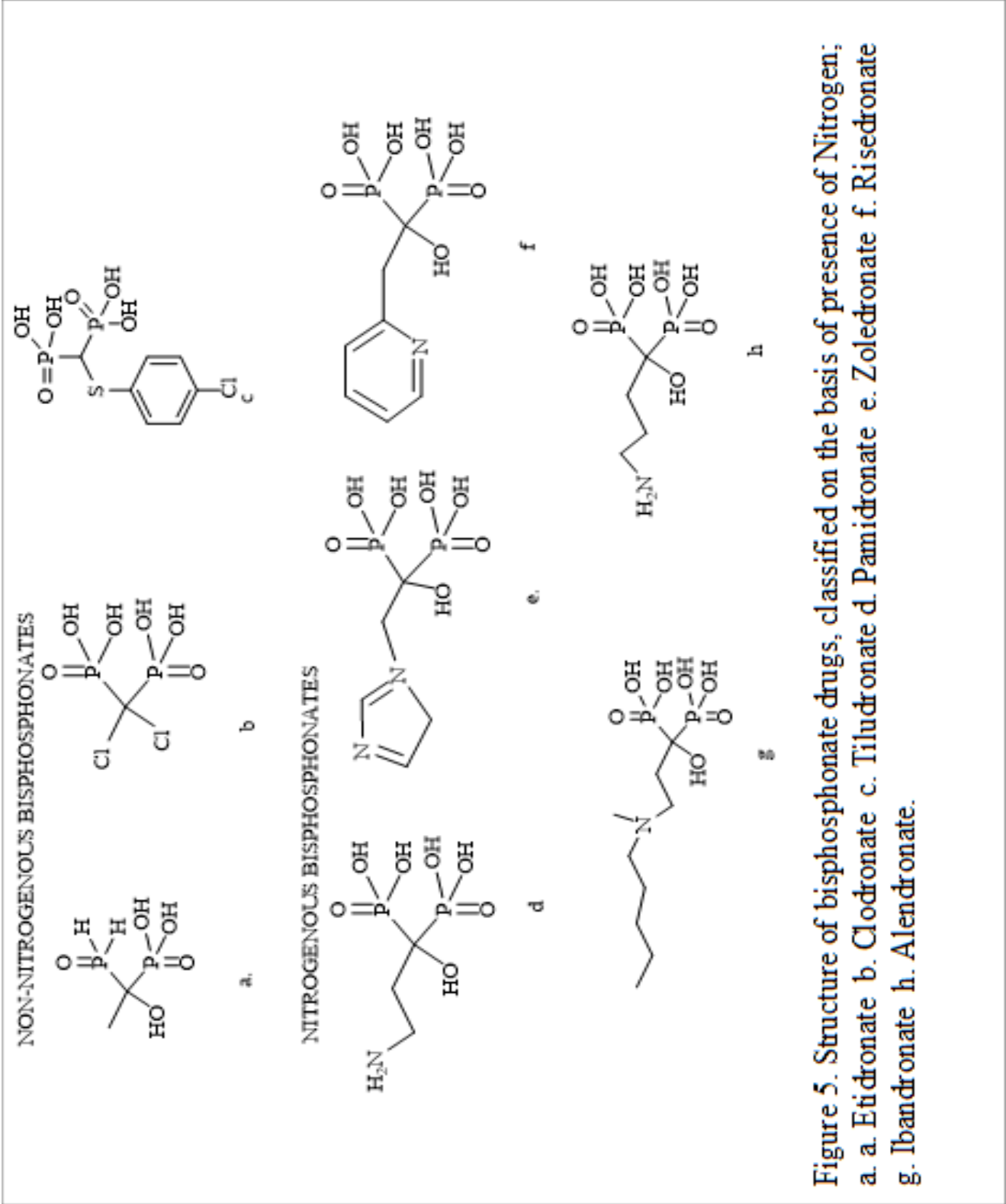


Figure 5. Structure of Bisphosphonate Drugs

1.16 Affinity of Bisphosphonate towards Bone:

Bisphosphonates have the excellent binding ability to the bone because of formation of coordination bonds with calcium ions.

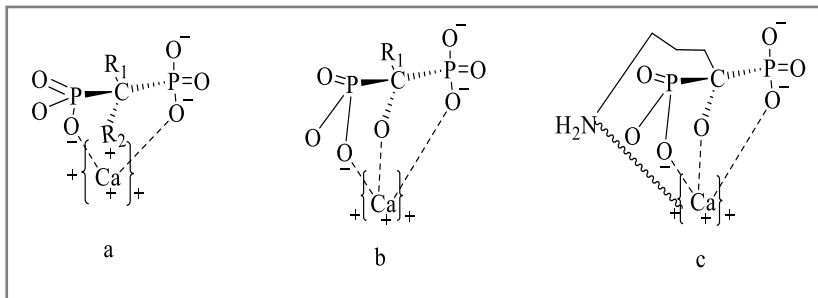


Figure 6. Explaining the chemistry between calcium and bisphosphonate interactions.

The ability of bisphosphonate to bind to the bone depends on upon the interaction between the hydroxyl group and calcium ions. The hydroxyl group in the (*gem*) geminal carbon involves in binding process and increases the bisphosphonate's ability to bind. Its binding property is inversely proportional to a number of ester groups. The location of the ester groups also affects the bisphosphonate's binding ability. Bisphosphonates have the ability to bind very strongly to the hydroxyapatite of bone tissues.²⁸

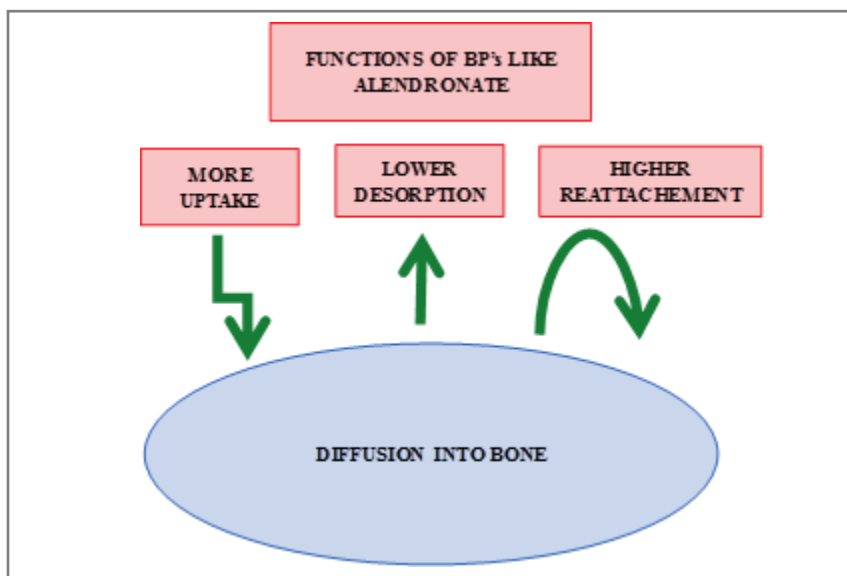


Figure 7 . Schematic representation of relative affinities of bisphosphonates.

(a) The Bidentate binding involving the two phosphonates Figure 6, (b) tridentate binding from the R_1 side group (OH), and (c) additional interactions from a nitrogen-containing R_2 side group (e.g., alendronate).²⁸ This binding affinity can be further explained by the structure of bisphosphonate. The P-C-P group is essential for binding to hydroxyapatite biological activity. When R_1 is a hydroxyl group, the binding activity increases. R_2 chain determines the potency and their relative efficacies as inhibitors of bone resorption, Figure 7. The most potent bisphosphonates to date, Risedronate and Zoledronate, contain a nitrogen atom within a heterocyclic ring. They are up to 10,000 times more potent than Etidronate in some experimental systems.²⁹

1.17 Bisphosphonates in Targeted Medicine:

The skeleton is the most common organ and it is one of the sites that has higher morbidity rate as it occupies the larger surface area in the body.³⁰ Thus safe, effective, and targeted (specific) drugs are needed for treating bone diseases such as; bone metastatic cancers and Osteoporosis. Although there are many skeletal diseases, viz.; osteoporosis, bone metastasis, Paget's disease, hypercalcemia, and osteoarthritis herein focus is towards treatments and increased selectivity to the bone tumors. In a similar manner, there are many therapeutic drugs, but there is no universal drug to deal with all these diseases. Every disease requires a specific or combination of drugs. Further, governed by several factors such as the type and amount of drug, mode of administration, and time frame of treatment.³¹

However, the main concern regarding the treatment of any disease is selecting the most appropriate type of drug administration that circumvents unwanted absorption of the drug at non-desired sites. Because of the uptake of drugs at non-desired sites of the body, treatments

sometimes involve higher drug dosages than required, which may lead to a toxic side effect.³² Therefore, most of the skeletal diseases require a targeted delivery of therapeutic agents to bone tissue or diseased tissue.³³ Tumor metastasis is a fatal cause and bone tissue is one of the prime sites for cancer to metastasize especially from the bladder, breast, Melanoma, Prostate, Thyroid, and Uterus cancer.³⁴

Thus, treatment is very important to enhance the lifetime of the patients. The most interesting ligand for targeting Bone are Bisphosphonates. The specific affinity of bisphosphonates to bone makes them deliver nanoparticles to the tissue. Although they are known for their therapeutic uses in the treatment of Osteoporosis, a lot of studies are being conducted to use them for Bone metastasis treatment. The Clinical data supports that bisphosphonates can limit the progression of breast cancer both in bone as well as other tissues.³⁵ The bisphosphonate-modified nanoparticles have high specificity and studies have also shown that these also strengthen the bone.³⁶

Generation	Drug	Drug Delivery System bound	Anti-Cancer Agents	Effects
I	Clodronate	Liposomes	Clodronate	Decreased Metastasis
II	Zolendronic Acid	PLGA	Docetaxel	Increased Cellular Uptake
III	Risendronate	PLL-CD	Cyclodextrin	Prevention of Bone Metastasis

Table 3 . Generation of Bisphosphonates and their Impacts while conjugation

Bisphosphonates have undergone three generations (as listed in Table 3). From the clinical data, prevention of bone metastases by the early-generation BP, Clodronate, have yielded fruitful results in patients with breast cancer, and trials have been undertaken to assess its efficacy³⁷. The second-generation Bisphosphonates (zoledronic acid) can inhibit angiogenesis, invasion, and adhesion of tumor cells, overall tumor progression, and the substantial amount of evidence suggests that the use of these agents may hinder the development of bone metastases.

A bone tumor is characterized by higher growth factors when compared to the normal cells. They are abundant in calcium which is the logic behind the attack of bisphosphonates and their selectivity³⁸. Calcium, which is abundant in the bone matrix, has a great effect on tumor cells. Breast and prostate cancer cells use the calcium-sensing receptor (CASR) and respond to ionized calcium, therefore have greater chance to localize around bone microenvironment.

Calcium stimulation of these cells leads to an inhibition of apoptosis and a stimulation of proliferation. Further, ionized calcium leads to increased PTHRP (Parathyroid Hormone-Related Protein) secretion by tumor cells and hence induces further resorption and calcium release. The Ionized calcium can be a potent chemo-attractant to breast cancer cells and could support bone localization in addition to tumor cell proliferation. The increased amount of calcium ions, when compared to the normal bone cells, is the chemistry that explains to attack the tumor by bisphosphonates³⁹. The various drug conjugates, nanoparticles can be modified with bisphosphonates which we can attack the tumor environment selectively.

1.18 Targeted Drug Delivery:

In general, specific targeted delivery of therapeutic agents to the site of interest is an ideal approach for the treatment of any disease. It has immense benefits and, thus has been very well explored. It can deliver the drug at the desired site with better drug action. Targeted drug delivery reduces the drug dosage and gives more uniform drug action compared to conventional drug delivery methods. It reduces the side effects of the drug and gives a localized drug effect at the diseased site. But, it requires a drug delivery vehicle which could direct the drug at the desired site.^{40,41,42}

In the case of skeletal diseases, it requires a vehicle that could direct and/or deliver the drug molecules to bone tissue. This can be achieved by using molecules that have a natural attraction or affinity to bone. Although there are several bone-seeking molecules such as; BPs, D-aspartic acid Octapeptide, Polymalonic acid, Monoclonal Antibodies, strontium, rhenium, lead, and fluoride,⁴³ except BPs, other molecules have disadvantages like high toxicity, heterogeneous distribution on the skeleton, sex dependency, inability to be conjugated to drugs, and poor affinity to bones. Among the above bone-seeking molecules, BPs are the most studied agents because of their bone affinity and anti-resorptive properties, studied widely for drug delivery.

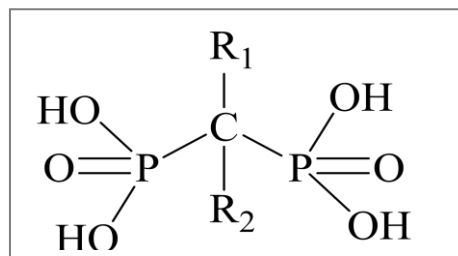


Figure 8. The P-C-P group is essential for binding to hydroxyapatite biological activity.

As the chemical structure of BP has two substituents (R_1 and R_2), Figure 8, which can be modified to introduce a selective functional group for the attachment of a drug molecule, a variety of strategies of BP-drug conjugation has been explored.⁴⁴ In addition, there are various linkers commercially available which are being used for BP and drug conjugation.

Overall, the formation of the BP-drug conjugate has been a subject of prime importance for targeted drug delivery. The Bisphosphonates can be linked to the drug through a linker that helps in degradation and release of the drug. The primary purpose being invasion of the bone tumor.

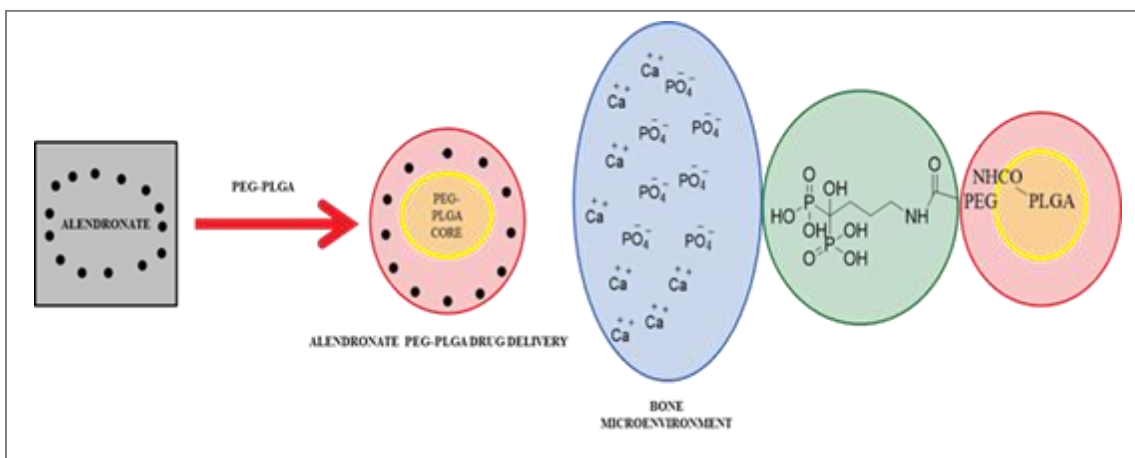


Figure 9. An Example of Alendronate(BP) based targeting Drug Delivery system for Bone Microenvironment

1.19 Chemistry of Bisphosphonate Drug delivery systems:

A library of conjugates with various bonds has been reported to link BPs with therapeutic agents⁴⁵. The conjugates can be synthesized to be labile or non-labile in nature thus can be classified into two:

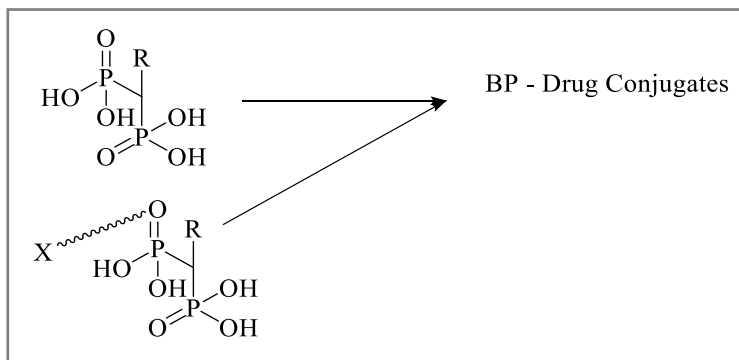


Figure 10. Explaining the linking of Bisphosphonates to enhance selectivity to tumor.

Stable conjugates and Unstable conjugates. Amide, ester, thioester, thioether, disulfide, and hydrazone bonds have been commonly used as linkages for bisphosphonate conjugation. In stable conjugates, BPs are conjugated to drugs via stable linkages, such as C-C, C-N, amide, shown in Figure 10, etc.⁴⁶ The stable conjugation is crucial for applications such as bone imaging and radiotherapy of skeletal sites.⁴⁷ In addition, stable linkages are more valuable in the case of drugs that remain active in their conjugated form. For other applications, a link between BP and a therapeutic agent could be designed to be cleavable under certain conditions, such as temperature, pH, specific proteases, etc. Some drugs remain active in their BP conjugated form and, thus, there is no need to release them from BPs.⁴⁸

However, most drugs are not fully active in their conjugated forms (more like prodrugs) and need to be cleaved from BP to maximize their activity. For delivery of such drugs, a cleavable linkage between Bisphosphonate and the drug needs to be designed in a way so that it gets cleaved at the required site in the body; this should take place with a specific cleavage rate to deliver the required amount of drug. With this understanding of Bisphosphonate chemistry,

multi- functional drug conjugate, delivery systems, can be designed. Most of them may have an anti- cancer drug, an imaging agent in a biodegradable polymer carrier, attached to bisphosphonate⁴⁹. The bisphosphonate which are used in osteoporosis can thus be used in enhanced drug delivery systems as it preferentially goes to the bone.

1.20 Polymers in Drug Delivery:

Around a century, various procedures such as compression, spray, and dip coating, and encapsulation have been used in the therapeutics to incorporate bioactive agents with polymers⁵⁰. Most of these biopolymers include cellulose derivatives, poly (ethylene glycol) PEG, and poly (N-vinyl pyrrolidone). The classification of biopolymers in drug delivery are diffusion-controlled (monolithic devices), solvent-activated (swelling- or osmotically-controlled devices, chemically controlled (biodegradable), or externally-triggered systems (e.g., pH, temperature).⁵¹ These Polymer-mediated drug delivery systems have evolved over five decades as explained in Figure 11.

These biopolymers are very crucial in drug delivery. These polymers undergo degradation rather than just a cleavage like a covalent bond. They absorb water from the surrounding environments and thus undergo degradation through gradient concentration, pH, temperature even Hydrogen Bonding⁵². These erosion and degradation are surface or bulk phenomenon. In surface degradation, the polymer matrix is completely removed from the surface, but the polymer volume fraction remains mostly unchanged. On the other hand, in bulk degradation, not much change occurs in the physical size of the polymer carrier until it is completely degraded or eroded, but the fraction of polymer remaining in the carrier comes down over time. The leading process is determined by the relative rates of solvent getting into the

polymer, diffusion of the degradation product, and degradation or dissolution of the macromolecular structure⁵³.

The aim of a drug delivery system is controlled release of therapeutics at the desired disease site and to maintain the drug concentration within a therapeutic band for a required duration.

Irrespective of administration of drug like oral, parental or transdermal, bioavailability in the bloodstream allows for distribution to all organs in the human body⁵⁴. In the blood, these therapeutics disseminate to all or most organs by crossing endothelial barriers or by draining through endothelial gaps in tissues with the leaky vasculature. Moreover, active targeting mechanisms may be employed by the polymer carrier with an agent ALE, a polymer-drug conjugate, or the drug itself to disproportionally partition itself into the organ of interest.

Because of this interesting chemistry and attributes the polymer mediated drug delivery systems with drugs are very part of the Pharmaceutical products and companies across the globe.

Some of the drug delivery systems and their purpose are discussed in Table 4⁵⁵.

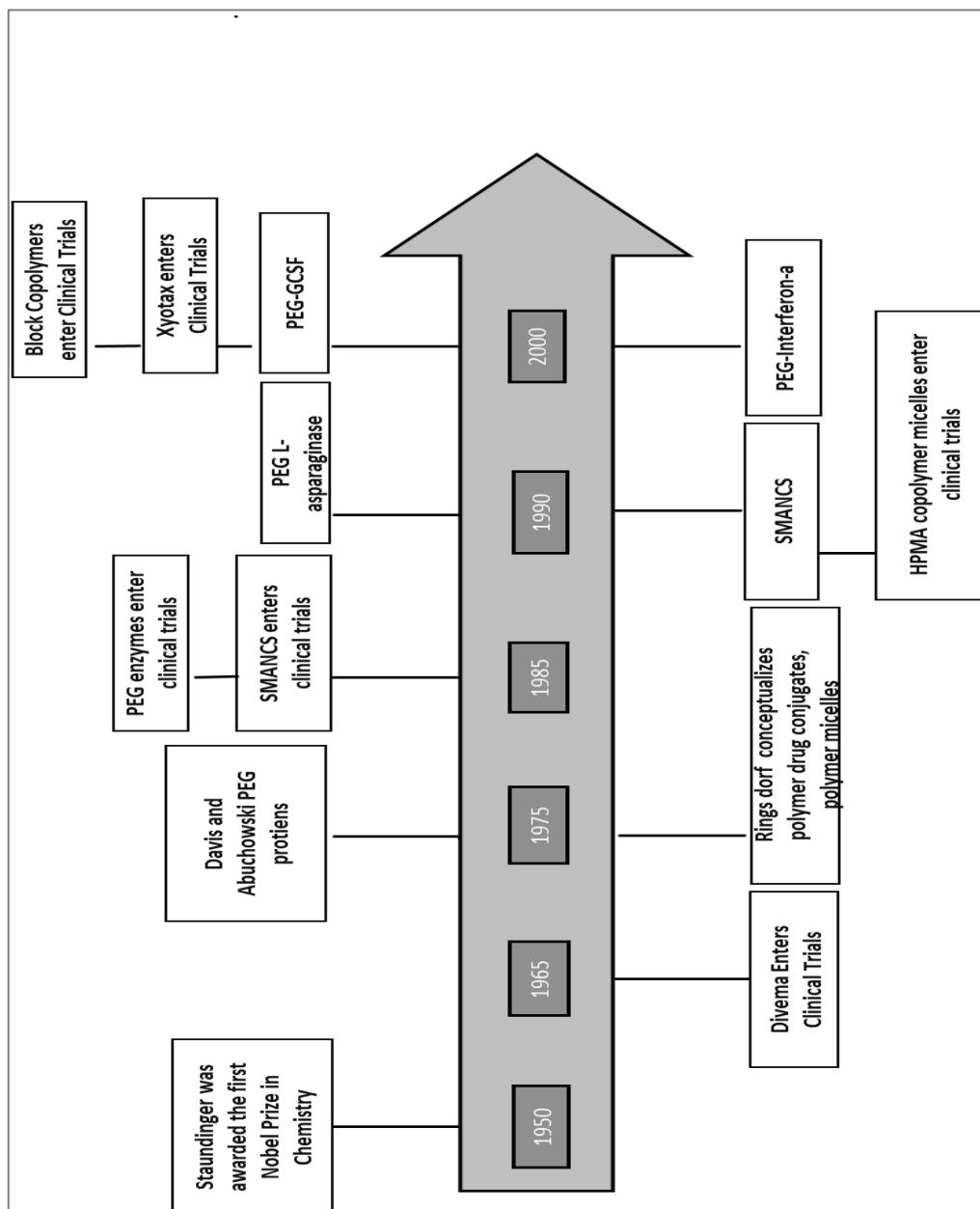


Figure 11. Polymer-Mediated Drug Delivery Evolution in Five Decades

Trade Name	Primary Objective	Incorporated drug	Progress	Company
NK-911	Targeting	Doxorubicin	Phase II	Nippon Kayaku Co., Japan
NK-105	Targeting	Paclitaxel	Phase II	Nippon Kayaku Co., Japan
NK-6004	Targeting	Cisplatin	Phase I	Nano Carrier Kayaku Co., Japan
NK-012	Targeting	SN-38	Phase I	Nippon Kayaku Co., Japan
GENEROL-PM	Solubilization	Paclitaxel	Phase II	Samyang Corp., Korea
SP-1049C	Anti-MDR effect	Doxorubicin	Phase II	Supratek Pharma Inc., Canada

NK-911: PEO-b-P(Asp)-doxorubicin; NK-105: PEO-b-Poly(4-phenyl-1-butanoate)-L-aspartamide; NK-012, NK-6004: PEO-b-Pglu; Genexol-PM: PEO-b-poly(D,L-lactide); SP-1049C: PEO-b-PPO-b-PEO

Table 4 . The trade name of various polymer mediated drug delivery systems and the incorporated drugs.

Although, the most commonly used Polymers in the drug delivery are PEG (polyethylene glycol) with an array of commercial products in the market more than half of the population has developed resistance to these drugs⁵⁶. Thus, a need for alternative biopolymers is in demand

1.21 Poly (L- lactide) Based Drug Delivery:

Poly(l-lactide) (PLA) is one of the most desirable polymer owing to its biocompatibility, biodegradable, and ease of synthesis with various end functionality. It is widely used in various Biomedical applications like Tissue Engineering, biomedical device fabrication, and Drug delivery⁵⁷. This material can be used in the synthesis of various block co-polymers like PLGA, PLA-PEG, PLA-PCL-PEG etc. which can degrade in short times when compared to the traditional homopolymers. The PLA can further be used in the synthesis of nanoparticles by using Nano emulsification technique. These particles can be incorporated with bioagents and

therapeutics for screening and treatment of diseases.⁵⁸ The common route of preparation includes Condensation and Ring-Opening Polymerization (ROP) with later being a better synthetic route as the water removal process is difficult in the condensation process.

Three reaction mechanisms have been proposed so far for ROP of lactide: They are anionic, cationic and coordination mechanisms. In the anionic polymerization, damaging reactions such as racemization, back-biting reaction, and other side reactions are often caused by the highly active anionic reactants that hinder the chain propagation⁵⁹. In the cationic polymerization, side reactions and racemization likely occur because of the nucleophilic attacks on the activated monomers and the propagating species causing a loss in molecular weight and purity of the compound, further decrease the crystallinity and mechanical strength of the obtained products.⁶⁰ On the other hand, coordination polymerization with metal catalysts can give a large molecular weight with the high optical purity maintained as mentioned in Figure 12.

In Ring-Opening Polymerization (ROP) the PLA polymers are synthesized by using Toluene as solvent and Tin(II) Octate as catalyst owing to higher molecular weight and it is Food and Drug Administration-approved to use in drug manufacturing process.⁴⁵

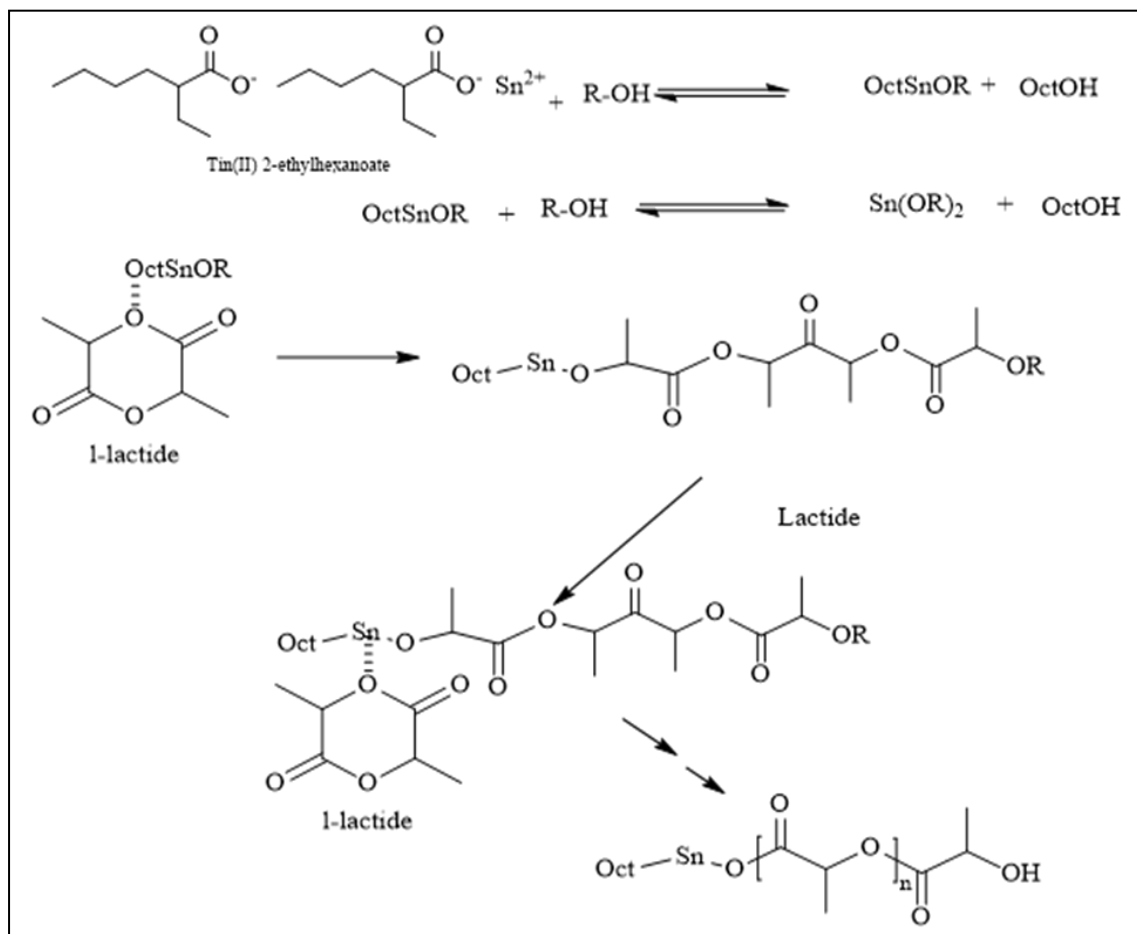


Figure 12. Schematics of Ring Opening Polymerization explaining synthesis of PLA

The synthesized PLA are used for nanofabrication of particles to delivery with nanoprecipitation being one of the best methods of synthesis⁶¹. It was first described by *Fessi et al.*, which is based on the interfacial deposition of polymers following the displacement of a semi-polar solvent miscible with water from lipophilic solutions.⁶² An easy and widely used technique especially in the preparation of PLA- and PLGA based nanoparticles. The advantages of this method include-

- (a) Huge amounts of toxic solvents can be avoided- Green Chemistry (b) Excellent particles with high payload capacity and good polydispersity are obtained (c) Simple and Cost-effective.^{63,64,65} The process involves PLA which is amphiphilic in nature is dissolved in

organic solvent and precipitated in aqueous solution. The particles of nanoscopic range are formed with hydrophilic layer out and a hydrophobic core. The hydrophobic core can carry drugs and other bioagents for targeted drug delivery⁶⁶.

1.2 Objective

This project is focused on the controlled synthesis of ALE functionalized Poly(l- lactide) polymers which are innovative and economical for targeting bone tumor and delivering therapeutics. The polymers are used to engineer nanoparticles using nanoprecipitation technique. These particles are further used to synthesize Drug conjugates. The whole idea of Polymer Mediated drug delivery is since PLA polymers are biocompatible and biodegradable.

The PLA was synthesized from l-lactide monomer as they are better than lactic acid molecules for dehydration process is not needed and high molecular weight polymers can be synthesized. For this process, Ring Opening Polymerization technique is implied in presence of metal alkoxide, Tin(II) octoate, as a catalyst which is approved by FDA for the synthesis of least toxic biomaterials. ALE protected with Calcium is the initiator to initiate nucleophilic ring opening of L-lactide monomer. Toluene is used as a solvent and in an inert, nitrogen, atmosphere, 180 degrees' Celsius temperature and for 24 hours' time.

The ALE functionalized Poly(l-lactide) polymers are synthesized with three differ molar ratios of monomer to the initiator to understand and control the synthesis of polymers. The so synthesized polymers are characterized with ¹H-NMR, FT-IR for the functional groups confirming the presence of Amide and phosphate moieties. Later MALDI-TOF is used to analyze and elude out the controlled synthesis of polymers.

The highest molecular weight ALE functionalized PLA (PLA-ALE) is selected to engineer polymeric nanoparticles with high targeting efficient and excellent payload capacity.

The goal of phosphate moiety is to attach to excess calcium in the tumor vicinity. The hydrophobic core part holds therapeutics to selectively and efficiently release the drug to the tumor cells. This is explained by Dynamic Light Scattering (DLS) and negative zeta potential values. In addition to that, the spherical nanoparticles (SEM) are to be less toxic

The Nanotoxicology studies are conducted to elude out that the material is biocompatible and can be used to synthesis drug conjugates. The targeting efficiency (FACS) and cell internalizations (Confocal Microscopy) are the important assumptions towards the Bone Osteosarcoma cell line completing the first objective of this study.

The second objective is the loading of enormous DOX into PLA-ALE particles. The reason being DOX an important frontline drug in chemotherapy of Bone and Breast Cancer. The particles loaded with DOX have a similar diameter (DLS) and still have negative zeta potential explaining our important objective that the phosphate moiety is on the surface which will direct these drug conjugates to the bone tumor. Then the toxicology studies have to show that they are comparable in killing tumor cells, to the free DOX concentrations. The load capacity is another important factor which is well satisfied. In addition to this, the release studies show promising results efficiently releasing the drug in vitro.

To conclude, the overall goal of the project is to control synthesize alendronate functionalized polymeric nanoparticles which are biocompatible, biodegradable and have excellent targeting and payload capacity to the bone tumor. This also opens a new paradigm in the field of cancer nanomedicine.

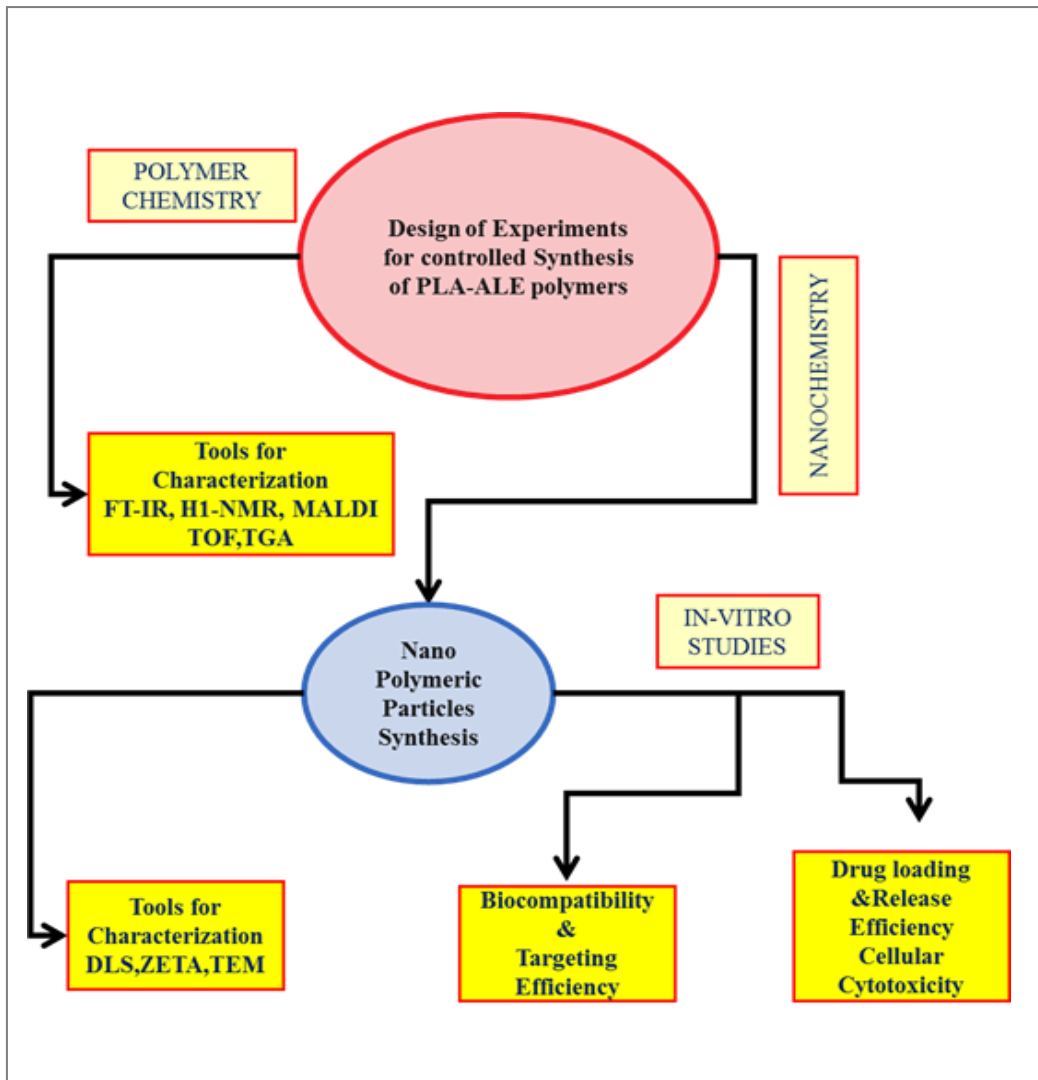


Figure 13 . Flow chart explaining the Objective and Overview of the project.

Abbreviations

Alendronic Acid - ALE

Calcium Alendronate – Ca-ALE

Doxorubicin – DOX

Dynamic Light Scattering- DLS

Fourier Transformation Infrared Red spectroscopy- FT-IR

Poly(l-lactide) Alendronate – PLA-ALE

Poly(l-lactide) – PLA

Poly(ethylene-glycol) – PEG

Poly (lactide-co-glycolic acid) – PLGA

Nanoparticles – Nps

Nuclear Magnetic Resonance Spectroscopy- ¹HNMR

Ring Opening Polymerization – ROP

Chapter 2 - Synthesis of Alendronate Functionalized Poly(L-lactide) polymers

Abstract

Poly (l-lactide) polymers have tremendous applications in drug delivery. They have been in use for effective intravaneous delivery systems which have ability to deliver drugs to specific sites. ALE with a hydroxyl or an amine group at its R1 position facilitates tridentate binding to bone and hydroxyapatite (HA). The overall nature of the R2 substituent also contributes toward enhancing the bone-seeking ability and pharmacological properties of BPs. They are used in clinical practice to inhibit bone resorption in bone metastases, osteoporosis, and Paget's disease. This chapter deals with the synthesis of ALE functionalized poly(l-lactide) polymer. The synthesis was carried in various ratio of ALE and l-lactide monomer. The resulting polymer was characterized for their molecular weight and structural integrity.

Introduction

The Poly (l-lactide) (PLA) polymer is one of the most important and highly commercialized polymer owing to its biocompatible and biodegradable nature. It is approved by United States Food and Drug Administration (FDA) to use in various applications like Tissue Engineering and Drug delivery systems⁶⁷. The PLA and its derivatives like PLGA block-co-polymer, PLA-PEG etc. have been continuously used in medical field as a device for various biomedical application. The PLA can further be used in nanoparticle synthesis by using emulsification techniques thereby forming spherical delivery vehicles⁶⁸. These particles can be incorporated with bioagents and therapeutics for screening and treatment of diseases.⁶⁹ The

common route of preparation includes condensation and ROP with later being a better synthetic route because of better control over polymer molecular weight and homogeneity.

The reaction mechanisms have been proposed so far for ROP of l-lactide are anionic, cationic, and coordination mechanisms. In the anionic polymerization, damaging reactions such as racemization, back-biting reaction, and other side reactions are often caused by the highly active anionic reactants that hinder the chain propagation⁷⁰. In the cationic polymerization, side reactions and racemization likely occur because of the nucleophilic attacks on the activated monomers and the propagating species causing a loss in molecular weight and purity of the compound, further decrease the crystallinity and mechanical strength of the obtained products.⁷¹ On the other hand, coordination polymerization with metal catalysts can give a large molecular weight with the high optical purity maintained as mentioned in Figure 12.

In ROP the PLA polymers are synthesized by using Toluene as solvent and Tin(II) Octate as catalyst which results in the formation of higher molecular weight with well controlled polydispersity.⁷²

Therefore, ROP was implemented in our study using Calcium Alendronate as initiator which follows a nucleophilic mechanism to form a long chain polymer in presence of Tin (II) catalys, PLA-ALE. Thus formed PLA-ALE polymers was characterized for the physicochemical properties and Chemistry using the techniques like FT-IR and ¹H-NMR. FT-IR gives information regarding the functional group moieties like amides, ester, phosphates, alkyl etc. and H¹-NMR confirms the structure of the polymers. Later the molecular weight was determined by MALDI-TOF technique, which is most popular tool for understanding the fragmentation pattern of long chain bio-polymers.

2.1 Chemicals and Reagents:

98% L-Lactide and 99% Dichloromethane were purchased from ACROS ORGANICS. ALE was purchased from TCI Chemicals. 92.5-100% Tin (II) 2-ethylhexanoate, 99.9% Methanol and 99.9% Dimethyl Sulfoxide were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Anhydrous Calcium Chloride, 99.9% Toluene were purchased from Fischer Chemicals. PBS Buffer(1X), pH=7.4, for dialysis, from Fischer. Dox stock solution was prepared from Dox Hydro Chloride salt from Fisher Bioreagents. Acetate Buffer pH= 4.7 was prepared from Acetic Acid and Sodium Citrate Dihydrate from Sigma-Aldrich.

Osteosarcoma Cell line K7M2 was purchased from ATCC and maintained per the manufacturer's recommendation. All other chemicals and solvents were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used as received.

2.2 Synthesis Alendronate PLA:

The Calcium Alendronate was synthesized using a ratio of ALE

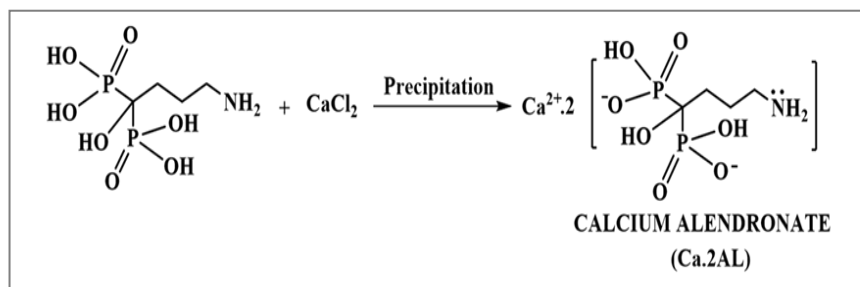


Figure 14. Synthesis of polymer initiator calcium alendronate.

acid vs Calcium chloride of 1:2 in the water at 60°C, heating and stirring around 1200 rpm, leading

to the precipitation
of Calcium
Alendronate. Thus
formed calcium
alendronate was
washed several

SNO	POLYMER TYPE	MOLAR RATIO INITIATOR: MONOMER	MOLAR RATIO TIN(II) OCTATE:INITIATOR	TIME	TEMPERATURE
1	PLA CAL 300	1:300	1:100	24 hours	180 °C
2	PLA CAL 200	1:200	1:100	24 hours	180 °C
3	PLA CAL 100	1:100	1:100	24 hours	180 °C

Table 5 . Controlled synthesis of PLA -ALE polymers

times using Milli-Q water in order to remove unreacted calcium and ALE.

Herein, 10 mg, stoichiometric amount, of purified ALE was used as an initiator with 1/100th ratio of Tin(II) octoate, in a catalytic amount, to initial ROP of l-lactide , three different ratios with respect to Alendronate:l-Lactide Viz.; 1:100, 1:200, 1:300 by moles was used .⁷³The ALE end-functionalized polymer, PLA-ALE, was synthesized in a solution phase using toluene as a solvent at 180°C, for 24 hours, under nitrogen inert atmosphere.⁷⁴ Thus formed polymer was dissolved in 5ml of Dichloromethane. Then it was precipitated in 1:2 ratios of Diethyl ether and Methanol. This process of dissolution and precipitation was done three times to remove the unreacted monomer units.

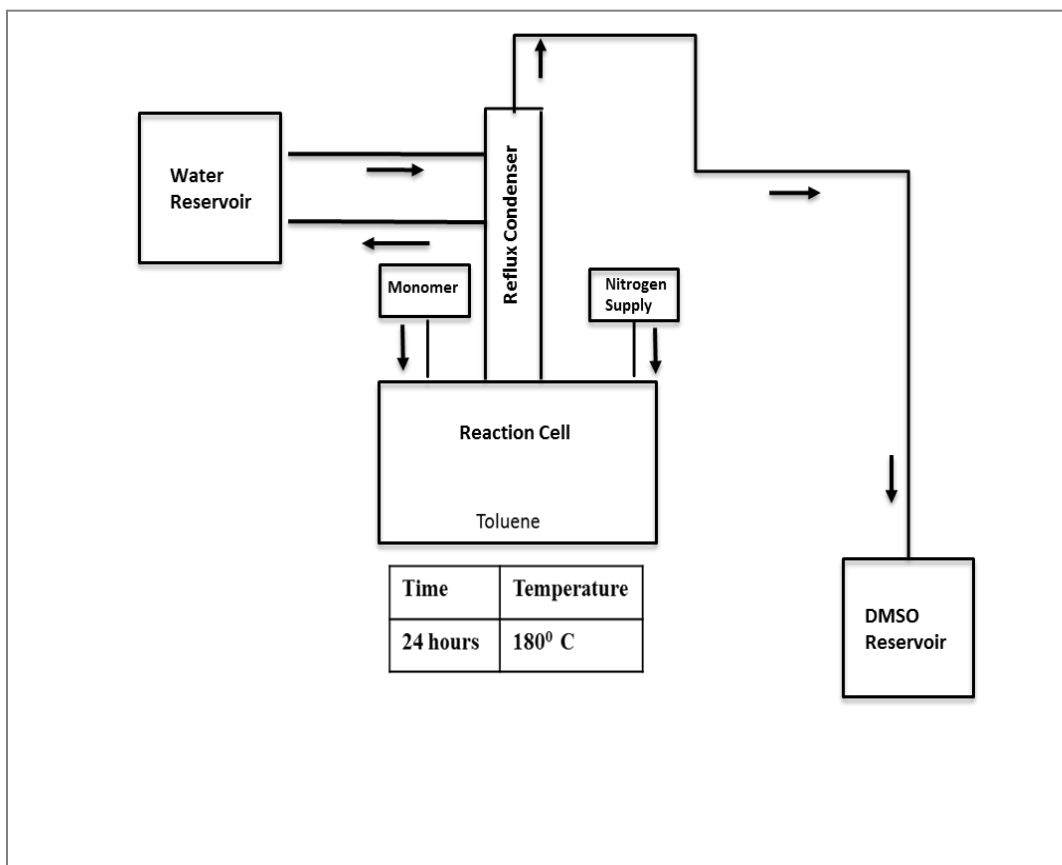


Figure 15. Schematic representation of polymer reaction and reaction conditions

Thus, ring opening polymerization of the monomer, l-lactide, leads to the formation of calcium alendronate conjugated polylactide, which was a bisphosphonate functionalized biocompatible polymer with high targeting efficiency.

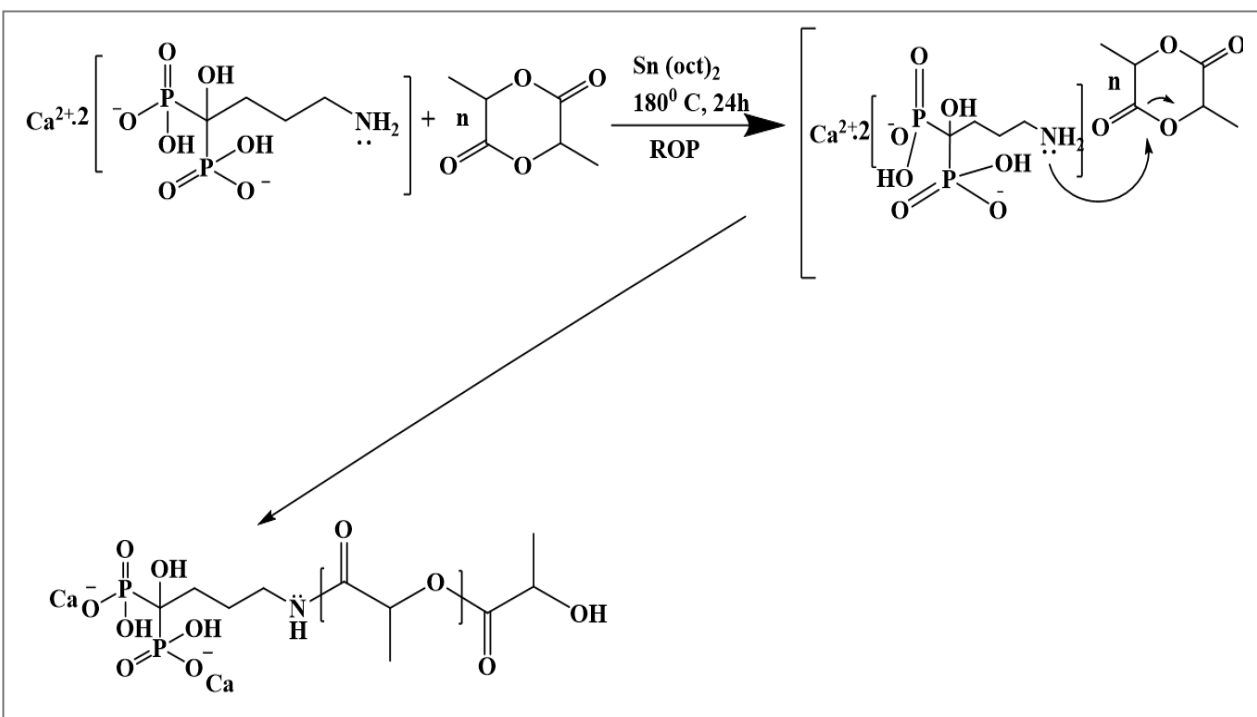


Figure 16. Chain Initiation for Polymer synthesis.

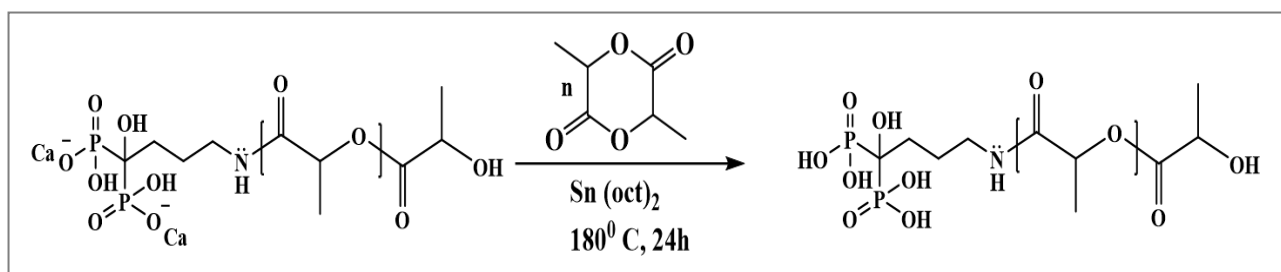


Figure 17. Chain Propagation and completion of polymer reaction.

2.3 Characterization of PLA-ALE Polymers:

The Synthesized PLA-ALE polymers in three different ratios were characterized using Fourier Transform Infrared Spectroscopy (FT-IR) and Proton Nuclear Magnetic Resonance Spectroscopy $^1\text{H-NMR}$.⁷⁵ The FT-IR analysis involves identification of functional groups, which confirm the polymerization and bond formation. The ALE functionalized polymers will give signature peaks at range of wavenumbers.

$^1\text{H-NMR}$, Varian 400MHz NMR, instrument explains the structure prediction and further corroborates the results from FT-IR, Cary 630 FT-IR. The chemical environment of the protons attached will give different shifts in ppm which we observe and analyze the structure.

Then the molecular weight assay was done using Matrix Assisted laser Desorption/Ionization-Time of Flight, MALDI-TOF, Finnigan LaserMAT 2000, which gives peak profile explaining the units present in polymer.⁷⁰ The profile is very helpful in eluding out the polydispersity of the macromolecules. Thermogravimetric analysis (TGA) was done to understand the amount of calcium in the polymer due to calcium alendronate conjugation. The disintegration profile with respect to the temperature explained weight ratio of constituents present in the sample.^{76,77}

2.4 Results and Discussions:

The synthesis of Calcium Alendronate Poly-lactide (PLA-ALE) polymer was initiated by Calcium Alendronate via ROP. The chemical structure of synthesized PLA-ALE was confirmed by FT-IR and $^1\text{H-NMR}$.

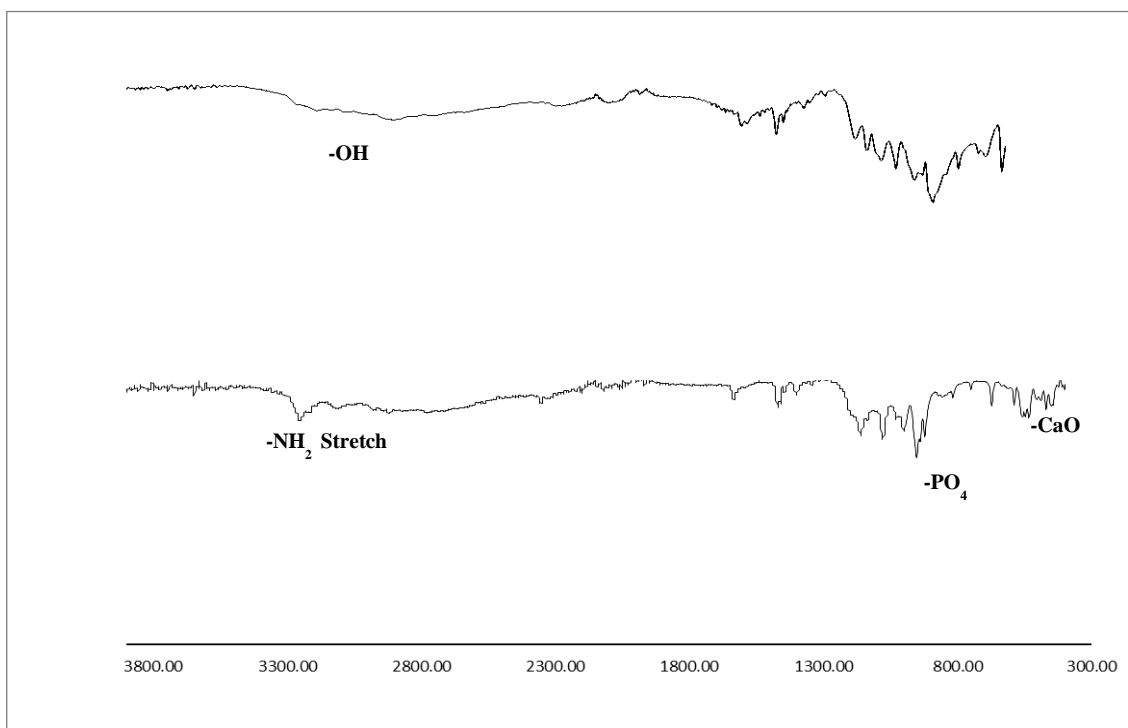


Figure 18. FT-IR Analysis of Calcium Alendronate

-OH Stretch	3200-3600
-NH ₂ Stretch	3500-3300
-PO ₄	Around 1000 -1400
-CaO	Around 400

Table 6. Signature peaks of the functional groups of initiator

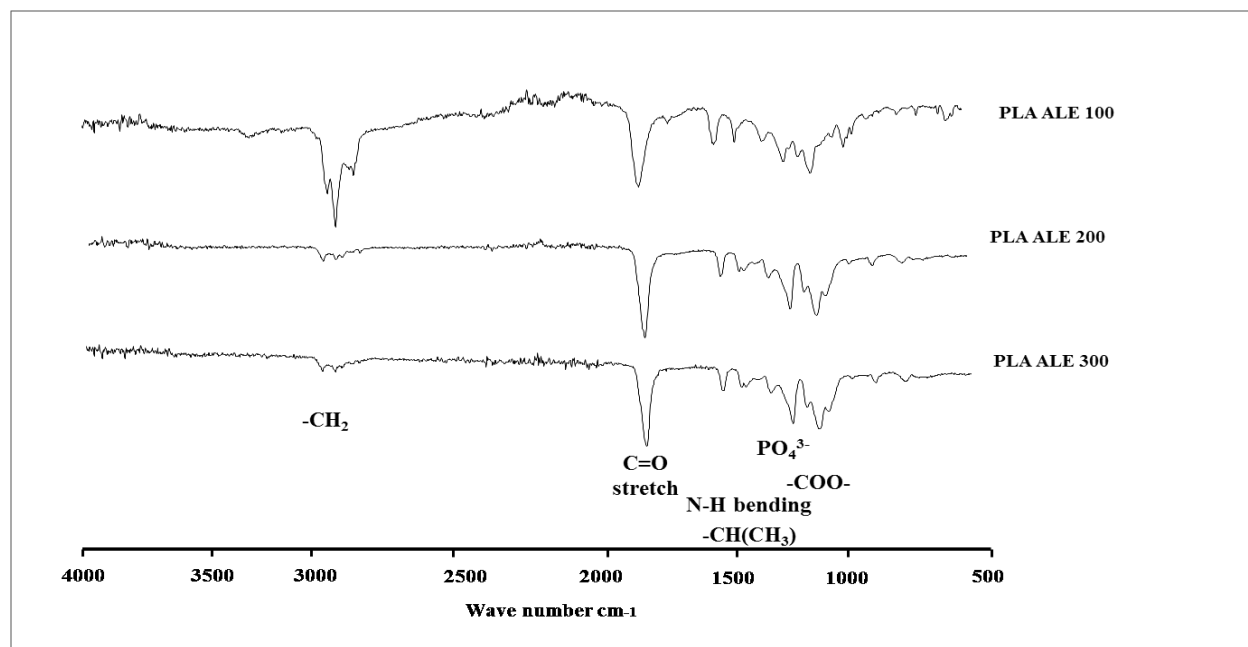


Figure 19. FT-IR spectrum of three different polymers of PLA-ALE; 300, 200,100.

-CH ₂ (Aliphatic) Stretch	Around 2994-2997
-C=O Stretch	Around 1758-1763
-COO-C	1184-1190
-CH(CH ₃)	1452-1457 1382-1389
Amide (N-H) bending	1550-1640
-PO ₄	Around 1000-1400
-CaO	Around 400

Table 7. Signature peaks of the functional groups in the three Polymeric Ratios.

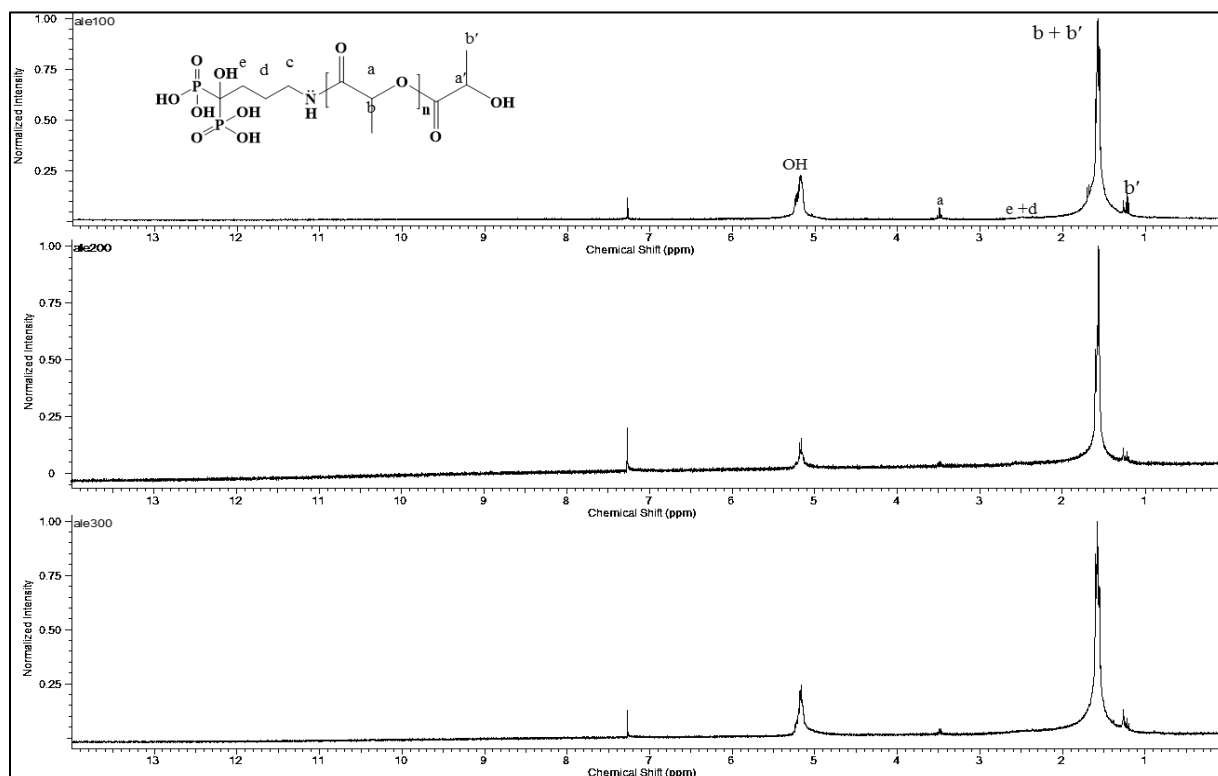


Figure 20. $^1\text{H-NMR}$ of three Polymers PLA-Ale in the increasing order of their ratios; 100,200,300

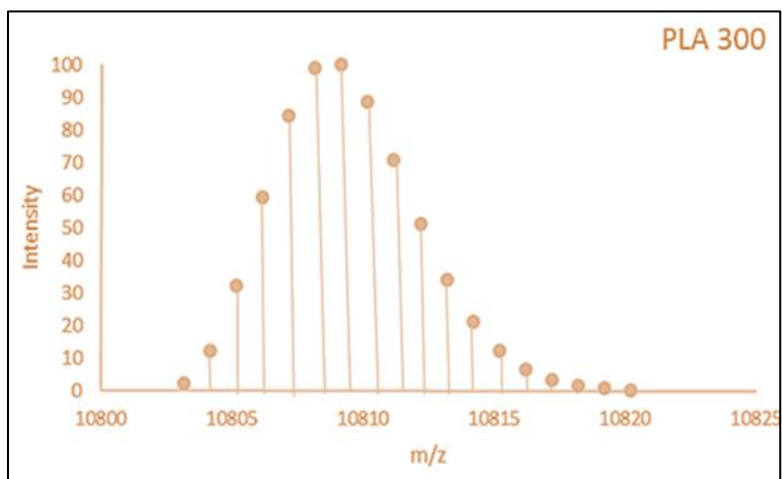
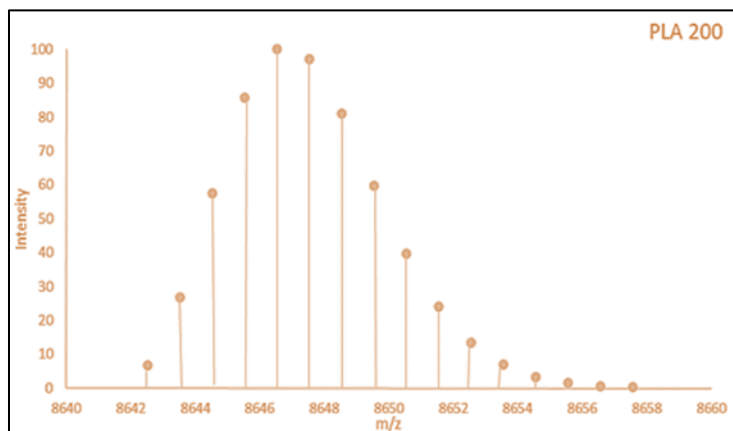
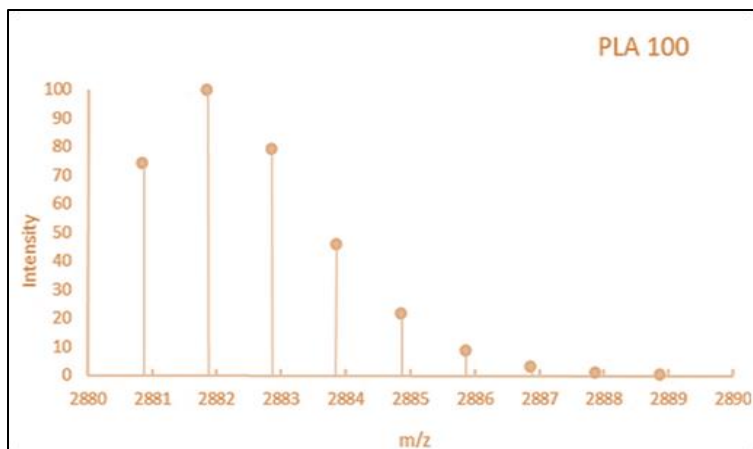


Figure 21. MALDI-TOF showing theoretical disintegration of three different Polymers

Polymer	Mw(AverageMolecular weight)	PDI
PLA ALE 300	10337.8856	1.00066
PLA ALE 200	8176.0021	1.00057
PLA ALE 100	2410.6539	1.00036

Table 8. Three Polymer, PLA-ALE showing their Average Molecular Weights and PDIs.

The FT-IR spectrums and characteristic features of the poly (L-lactide) was presented in Figure 18, 19, Table 6 and 7., The PLA spectrum shows the bands centered at 2700 to 2900 cm^{-1} from symmetric and asymmetric valence vibrations of -C-H from -CH₃, respectively. The -C=O stretch in the polymer is a broad band centered at 1600 to 1700 cm^{-1} is the indication of polyester formation due to C=O stretch. Due to the presence of amide from ALE moiety that attached to polymer backbone, we observed the band broadening. Moreover, Ca-O (metal oxide) stretch was observed around 400 cm^{-1} , indicating the presence of calcium in the in the form of salt due to the reason in is very difficult to resolve the distinct broadband of -OH functional group. In addition to Ca-O and amide, the C-N vibration was clearly observed at 1430 cm^{-1} indication the end functional ALE attachment to the polymer.

With the assurance from FT-IR, we moved forward to understand the proton environment in the polymer. As can be seen from Figure 20, the characteristic proton resonance of PLA polymer was observed, which are assigned in the structural formula of the PLA-ALE polymer. Typically, proton at α -C atom of the repeating unit of the polymer, which is near the ester functionality shows multiplete at $\delta=5.3$ ppm. In the meantime, end terminal proton at α -C atom

resonate at 3.5 ppm and shows quartet splitting due to neighboring methyl protons. On the other hand, the methyl proton of repeating unit of the monomer in the polymer exhibit high intense splitting at 1.5 ppm indicating the formation of the polymer. Most importantly, the signature peak which shows a triplet splitting due to neighboring $-\text{CH}_2$ protons and extreme upfield shift due to $-\text{NH}$ prominently observed at 1.15 ppm is the indication of the attachment of ALE at the end of the polymeric product.

Further, the MALD-TOF theoretical calculation and graphing of data of these polymers shown in Figure 21 and experimental results in Table 8, revealed that the synthesis was controlled and as the molar ratio between Monomer and Initiator increased the molecular weight also increased. The PDI (polydispersion Index) of these polymers are around 1.00057 ± 20 explaining the good distribution of molecules as units in the polymer.

2.5 Thermogravimetric analysis of Polymers:

Thermogravimetric analysis of polymers, as shown in Figure 22, explains the trend in weight loss as the temperature is increased. The three polymers were analyzed along with calcium alendronate to understand the amount of calcium bonded to polymer while conjugation. The trend explains as the molecular weight increases the amount of calcium left over in polymer decreases.

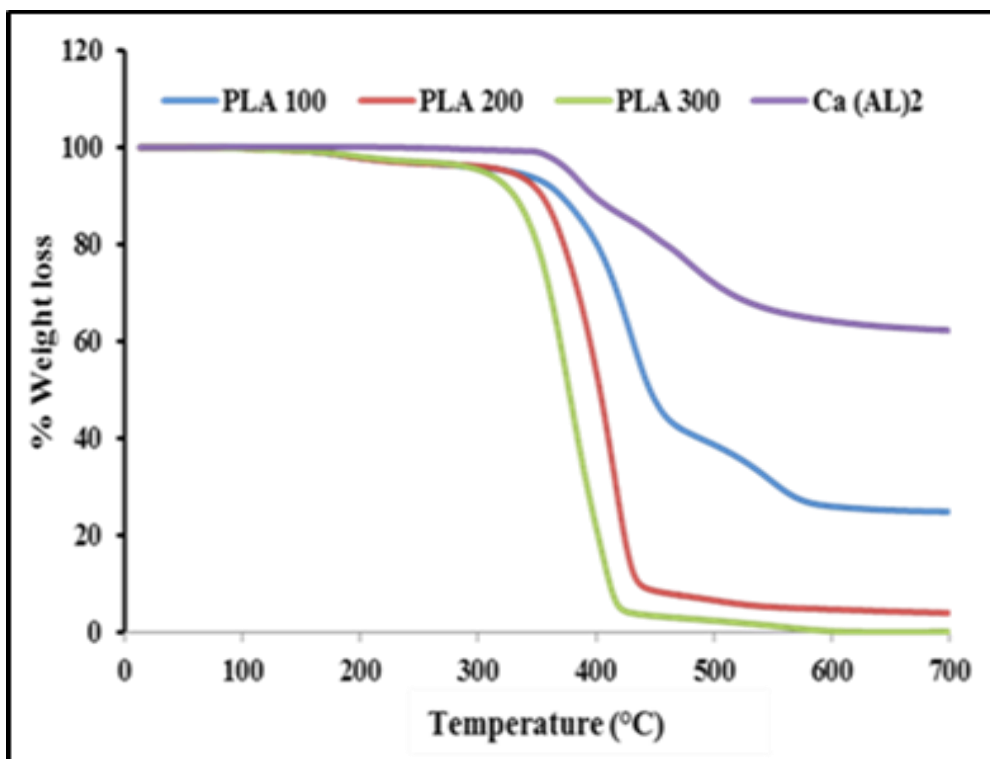


Figure 22. Thermogravimetric Analysis of Polymers PLA-ALE and the initiator.

This phenomenon is probably since with the increase in the polymer chain the ratio of monomer unit of the polymer and Ca changes. Indeed with the increase in molecular weight, the overall % of calcium to that to the weight of the polymer decreases. In addition, due to the higher feeding of monomer during polymer synthesis, the unreacted lactide monomer could hydrolyzed yielding lactic acid and leached out the calcium during the purification process.

Chapter 3 - Engineering of Nano polymeric Particles

Abstract

Nanomedicine is one of the most effective way of treating diseases at cellular level. The nanoparticles have excellent targeting ability thereby reducing nonspecific cytotoxicity. Thus, polymeric nanoparticles were prepared by nanoprecipitation in our study for achieving monodispersed nanoparticles. The PLA-ALE nano particles were prepared from polymeric non-aqueous solution by reprecipitating it in aqueous medium. The particles are characterized by DLS and Zeta sizer for their physico- chemical properties and further confirmed with TEM imaging.

Introduction

Targeted delivery of therapeutic agents to the site of interest is an ideal approach for the treatment of any disease. It has immense benefits and, thus has been very well explored.⁷⁸ It can deliver the drug at the desired site with better drug action. Targeted drug delivery reduces the drug dosage and gives more uniform drug action compared to conventional drug delivery methods.⁷⁹ It reduces the side effects of the drug and gives a localized drug effect at the diseased site.⁸⁰

In the case of bone tumor, it requires a vehicle that could direct and/or deliver the drug to the bone tissue. This can be achieved by using molecules that have a natural attraction or affinity to bone. Although there are several bone-seeking molecules Bisphosphonates are the most useful bone seeking agents used for targeted drug delivery. The Alendronate (Bisphosphonate) functionalized drug delivery systems can be synthesized by nanoprecipitation technique which the most economical and efficient technique for synthesis.⁸¹⁻⁸³

In this study we have implemented nanoprecipitation technique to synthesize spherical shaped PLA-ALE particles with the synthesized PLA-Ale polymer (Chapter 2). Nanoparticle is based on the self-assembly of the amphiphilic polymer, in current design ALE is aqueous soluble moiety where as PLA is a hydrophobic moiety.

PLA is one of the most successfully used biodegradable and biocompatible copolymers in biomedical applications and after hydrolysis leads to the metabolite monomer, lactic acid [83a–d]. These monomers (l-lactic acid) is endogenous and easily metabolized by the body via the Krebs cycle, and minimal systemic toxicity is associated with the use of PLA in biomedical applications. PLA has attracted considerable attention due to its attractive properties such as: (i) biodegradability and biocompatibility, (ii) FDA and European Medicine Agency approval in drug delivery systems for parenteral administration, (iii) well described formulations and methods of production adapted to various types of drugs e.g. hydrophilic or hydrophobic small molecules or macromolecules, (iv) protection of drug from degradation, (v) possibility of sustained release, (vi) ease of surface modification to provide stealth properties and/or better interaction with biological materials, and (vii) possibility to target NP to specific organs or cells. Finally, we have loaded chemotherapeutic DOX as a model drug for the treatment of various bone cancers such as primary or metastatic cancer. These particles are characterized with DLS and Zeta sizer for their size, stability, and phosphate group presence on the surface and TEM for further diameter calculations.

3.1 Nanofabrication of Polymeric Particles:

PLA-ALE polymeric particles were prepared by nanoprecipitation technique.⁸⁴ In brief 400 μ L of PLA-ALE in acetonitrile (1mg) was added dropwise to 3 mL of Mili-Q water. The

mixture was stirred continuously for 15 minutes to facilitate the formation of nanoparticles at room temperature. These polymeric nanoparticles were further purified using Amicon Ultra-4 centrifugal filter (Millipore, MA) with a molecular weight cut-off of 10kDA and stored at 4°C for further use, as shown in Figure 23

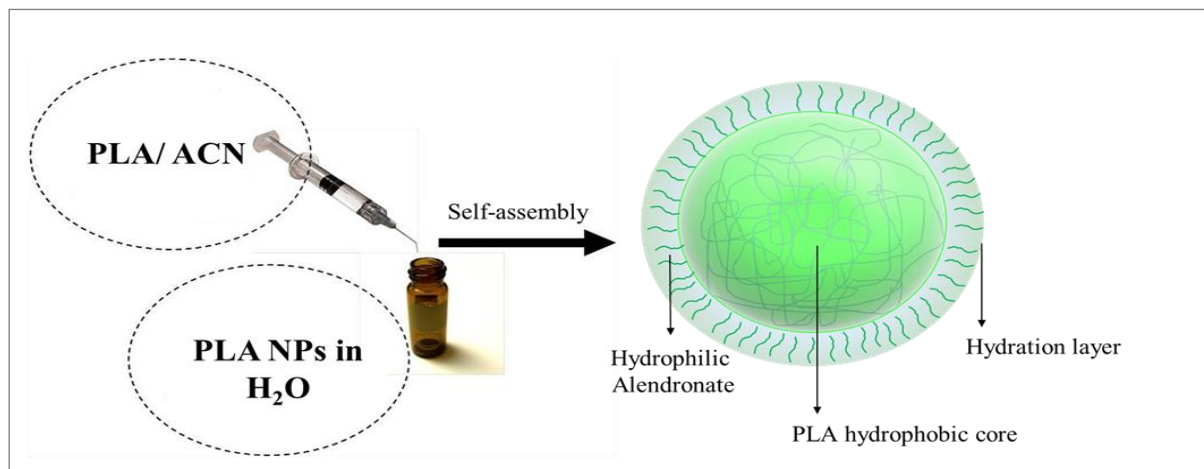


Figure 23. Schematics explaining Nanoprecipitation method to Synthesize PLA-ALE 300 Nanoparticles

Rhodamine dye labeled Nanoparticles were prepared by hydrating 20µg of L- α -Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium Salt) (Egg Liss Rhod PE) film with 200µL PLA-ALE (1mg/mL) before performing nanoprecipitation process. RhB-labeled PLA-ALE nanoparticles were also prepared by adding 400µL PLA-ALE (1mg) 20µg of L- α -Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium Salt) (Egg Liss Rhod PE) under a magnetic stirring condition at 60°C, followed by addition of 3mL Milli-Q water. The mixture was stirred continuously for additional half an hour at room temperature and purified using Amicon Ultra-4 centrifugal filter (Millipore, MA) with a molecular weight cut-off 10kDA.

3.2 Characterization of Nanoparticles

The hydrodynamic size and zeta potential measurements of the prepared PLA-ALE NPs were analyzed by Dynamic light scattering (DLS) using a Zeta-sizer Nano ZSP apparatus (Malvern, Worcestershire, UK).⁸⁵ The Smoluchowski model was used to calculate the zeta potential value. All data represents the average of triplicate measurements of samples (PLA-ALE 300) prepared in different preparations. The morphology of the prepared Nanoparticles was further analyzed using Transmission Electron Microscope (TEM, Tecnia G2, Spirit Bio TWIN). TEM samples were prepared by drop casting and evaporation technique using formvar coated copper grid (400 mesh). TEM images were analyzed by GATAN digital imaging system (GATAN, Inc.).

3.3 Results and Discussions:

PLA-ALE 300 was used for nanofabrication of polymeric nanoparticles using nanoprecipitation technique due to its optimum molecular weight for drug loading i.e; 10kDa. The physicochemical properties and morphological characterization of PLA-ALE NPs were determined using dynamic light scattering (DLS), surface charge zeta potential and transmission electron microscopy (TEM), as shown in Figure 24, 26. The hydrodynamic size of the PLA-ALE NPs showed a diameter of around 80 nm. Importantly, the DLS data showed unimodal distribution which demonstrates that the PLA-ALE NPs are highly monodispersed.

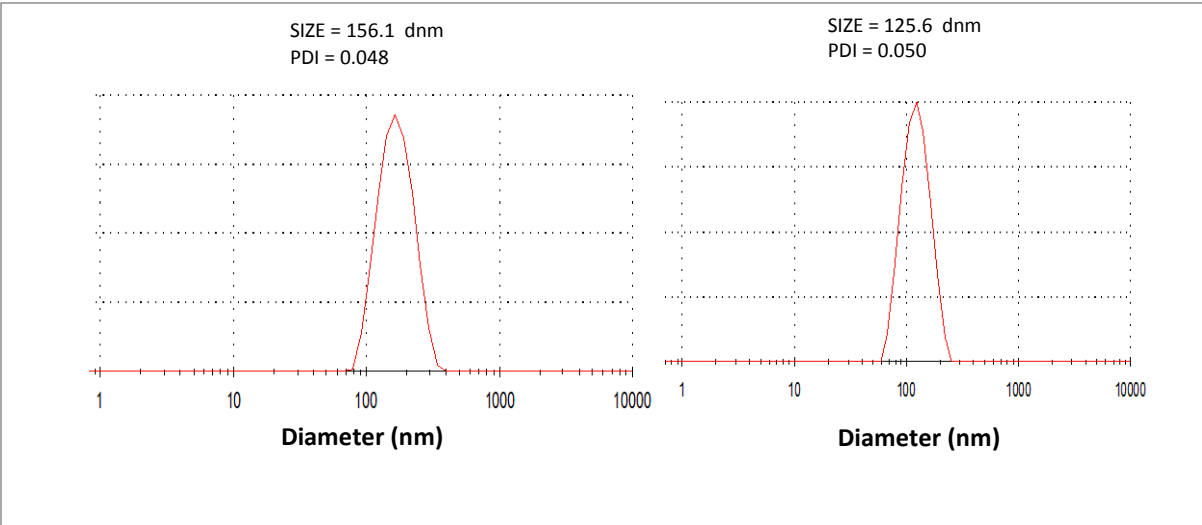


Figure 24: Dynamic light Scattering Method for PLA-ALE 100 (left) and PLA-ALE 200 (right)

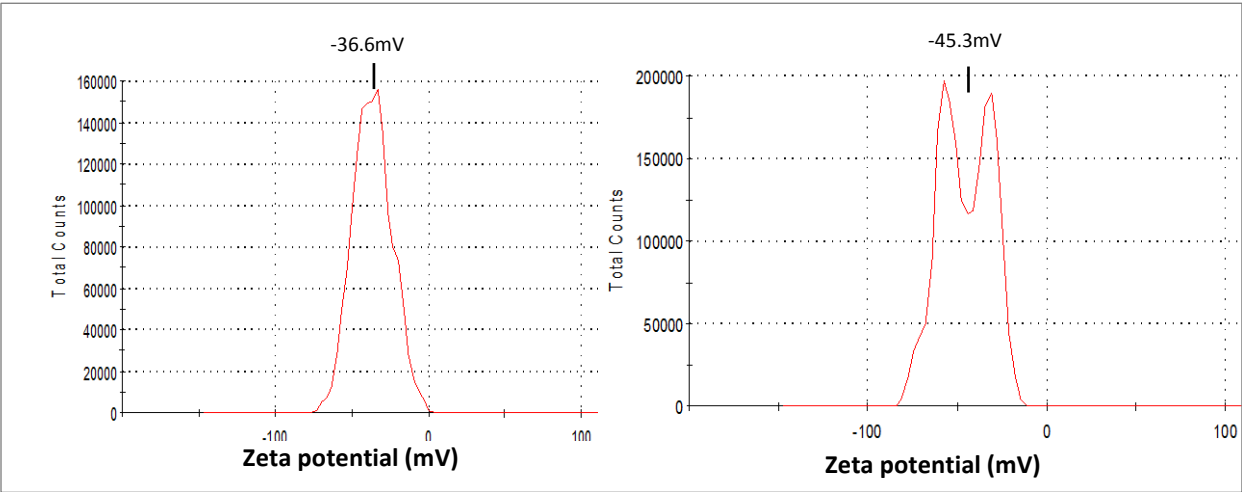


Figure 25: Zeta Potential graphs for PLA-ALE 100 (left) and PLA-ALE 200 (right)

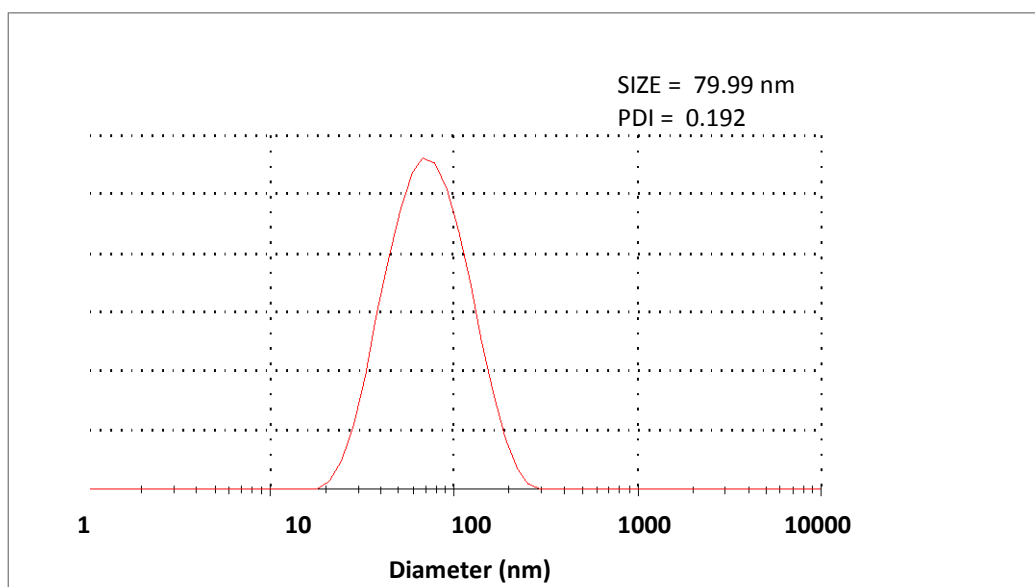


Figure 26: Dynamic Light Scattering method for PLA- ALE 300 particles

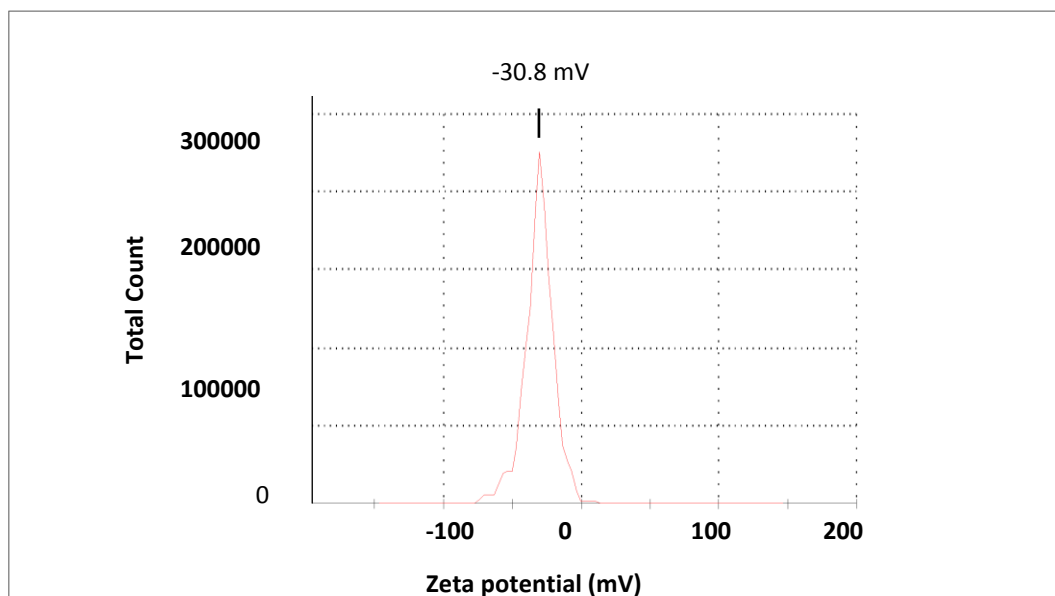


Figure 27. Zeta Potential graph for PLA-ALE 300

The TEM image of PLA-ALE clearly shows the spherical structure of nanoparticles of around 50 nm diameter, Figure 28. The measurement of the PLA-ALE NPs surface zeta

potential revealed negative values, Figure 25, 27 in agreement with the presence of phosphate moiety on the surface.

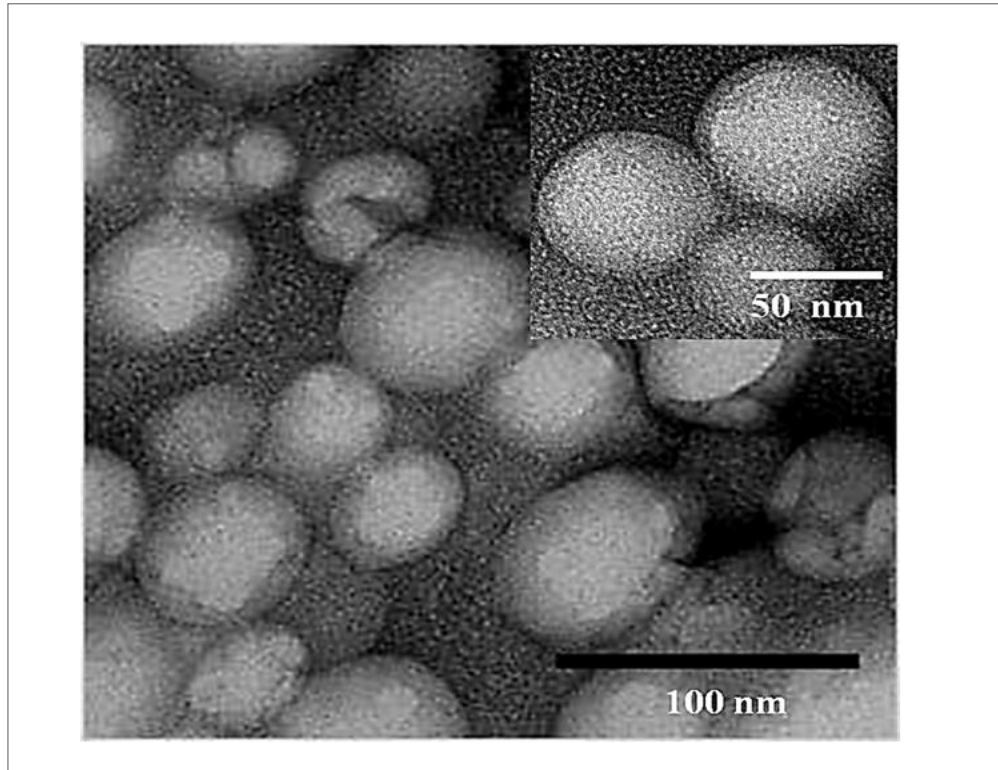


Figure 28. Transmission Electron Microscopy, TEM, image of PLA -ALE 300 Nanoparticles

Chapter 4 - Cellular Studies

Abstract

The cellular targeting ability, uptake, and biocompatibility are a few of the most crucial factors to decide the best nanoparticles in drug delivery. The targeted drug delivery increases specificity and controlled release of drugs in tumor sites. The specificity of Alendronate functionalized PLA was studied against Osteosarcoma cell line both qualitatively and quantitatively in vitro. The qualitative analysis was done using Confocal microscopy which explains the uptake of particles in to osteosarcoma cells and quantitatively with FACS to understand the targeting ability and internalization over a period. The biocompatibility of the PLA-ALE nanoparticles was studied against Osteosarcoma cell line and they were non-toxic to cells.

Introduction

The Osteosarcoma is one of the major form of bone cancers especially in Children and teenagers. Nanomedicine is an important tool in treating this disease without causing non-specific toxicity. Nano Delivery System, is gaining momentum in cancer biology due to their size and physio-chemical properties.⁸⁶ The toxicity and distribution of these systems play an important role in assigning them as drug delivery systems. In general, the drugs are susceptible to various factors like pH destabilization, non-specificity and enzymatic degradation. Nano delivery systems help in protecting drugs and carry them to the specific sites. The important feature includes nontoxic systems, effectively biocompatibility, strong targeting efficiency, good drug loading, transportation of drugs. The cytotoxicity and cellular uptake are the most important characteristics of a good drug delivery system. The cellular uptake studies are mostly done using microscopic techniques with Confocal imaging being a good tool in this analysis.⁸⁷ Then quantitative analysis is done using Fluorescence-activated Cell Sorting, flow cytometry. In this

process the particles are labelled with fluorescent dye and are compared with non-specific nanoparticles over a period to understand the internalization and accumulation in to bone tumor cells.⁸⁸ The particles then are studied for their cytotoxicity before taking decision. The polymeric particles must be compatible with tumor cell line after incubation, there by enable them to be used in nano drug delivery systems.

Thus, we have incorporated FACS and Confocal studies to understand targeting and accumulation in to osteosarcoma cell line to elucidate the targeting efficiency of our Nano Drug delivery systems. Later we conducted the cyto toxicity of the PLA-ALE particles to understand their biocompatibility.

4.1 Intracellular uptake studies:

To verify cellular uptake efficiency of PLA-ALE, RhB-labeled nanoparticles were used in Osteosarcoma bone cancer cell line (K7M2).⁸⁹ In brief, cells were seeded in Poly-D-lysine coated 8 chamber slide at a density of 50,000 cells per well and incubated for 24h. Then, the cells were treated with 50µg/mL RhB-labeled NPs suspension prepared in complete DMEM and incubated for 4h. After incubation treated cells were washed twice with 1X PBS (pH 7.4), fixed with 4% paraformaldehyde for 30 min at room temperature, stained with DAPI for additional 10 min and imaged under a Confocal Laser Scanning Microscope (Carl Ziess, LSM-700).

4.2 Fluorescence-activated cell sorting (FACs) studies:

To evaluate quantitatively the cellular internalization efficiency of PLA-ALE NPs, a comparative experiment was conducted.⁹⁰ In brief, K7M2 cells were seeded in T25 tissue culture flasks at 4×10^5 cells per flask for 24 hr. After incubation, cells were washed with 1X PBS and treated with 2 mg RhB-labeled PLA-ALE particles suspended in DMEM. Cells were then

incubated at 37°C at varying lengths of time (1 and 3 h) at 37°C. This time, point was chosen from a series of pilot experiments and should be determined for each cell line. After incubation, cells were put on ice to stop internalization, washed twice with ice-cold PBS (centrifuging at 4°C), detached with 0.08% w/v trypsin, washed twice again and analyzed on a flow cytometer. RhB-labeled PEGylated NPs were used as control particles.

4.3 In vitro Biocompatibility of PLA-ALE Nanoparticles:

The in-vitro cytotoxicity of PLA-ALE was conducted on Osteosarcoma Cells using MTT assay.⁹¹ In brief, 2×10^4 cells per well in DMEM medium were seeded in a 96-well plate and incubated for 24h. After incubation, the media were replaced with different concentration (10, 25, 50, 100, 150 and 200µg/mL) of nanoparticle and incubated for additional 24h. Control cells were also maintained without any nanoparticle treatment (n=6). After the completion of incubation, MTT was added to each well and further incubated for 3h according to the manufacturer recommendation. The insoluble formazan crystals were solubilized using DMSO and their absorbance was recorded at 570 nm using a microplate reader (BioTek, Synergy H1 hybrid reader).

4.4 Results and Discussions:

Cellular Internalization:

These studies were conducted to understand cellular internalization and targeting ability of nanoparticles to the bone microenvironment. The cellular internalization of PLA-ALE was first visualized under confocal laser scanning microscope by incubating RhB-labeled PLA-ALE NPs with K7M2 cells for 3h. Confocal micrograph revealed that a large quantity of NPs internalized into the cell and co-localized with Nuclei Figure 29. A quantitative cellular uptake

of RhB-labeled PLA-ALE NPs was then conducted using flow cytometry in compared to conventional PEGylated NPs. Our results show the cellular uptake of both PLA-ALE and PEGylated NPs is time-dependent. As the nanoparticle incubation time increased, the enhancement of cellular uptake was observed in both PLA-ALE and PEGylated group Figure 30. However, at an equal incubation time, the PLA-ALE NPs were taken up by K7M2 cells is higher than that of PEGylated NPs, suggesting that conjugation with bisphosphonate plays a significant role in targeting nanoparticles to bone cancer cell in compared to PEGylated NPs.

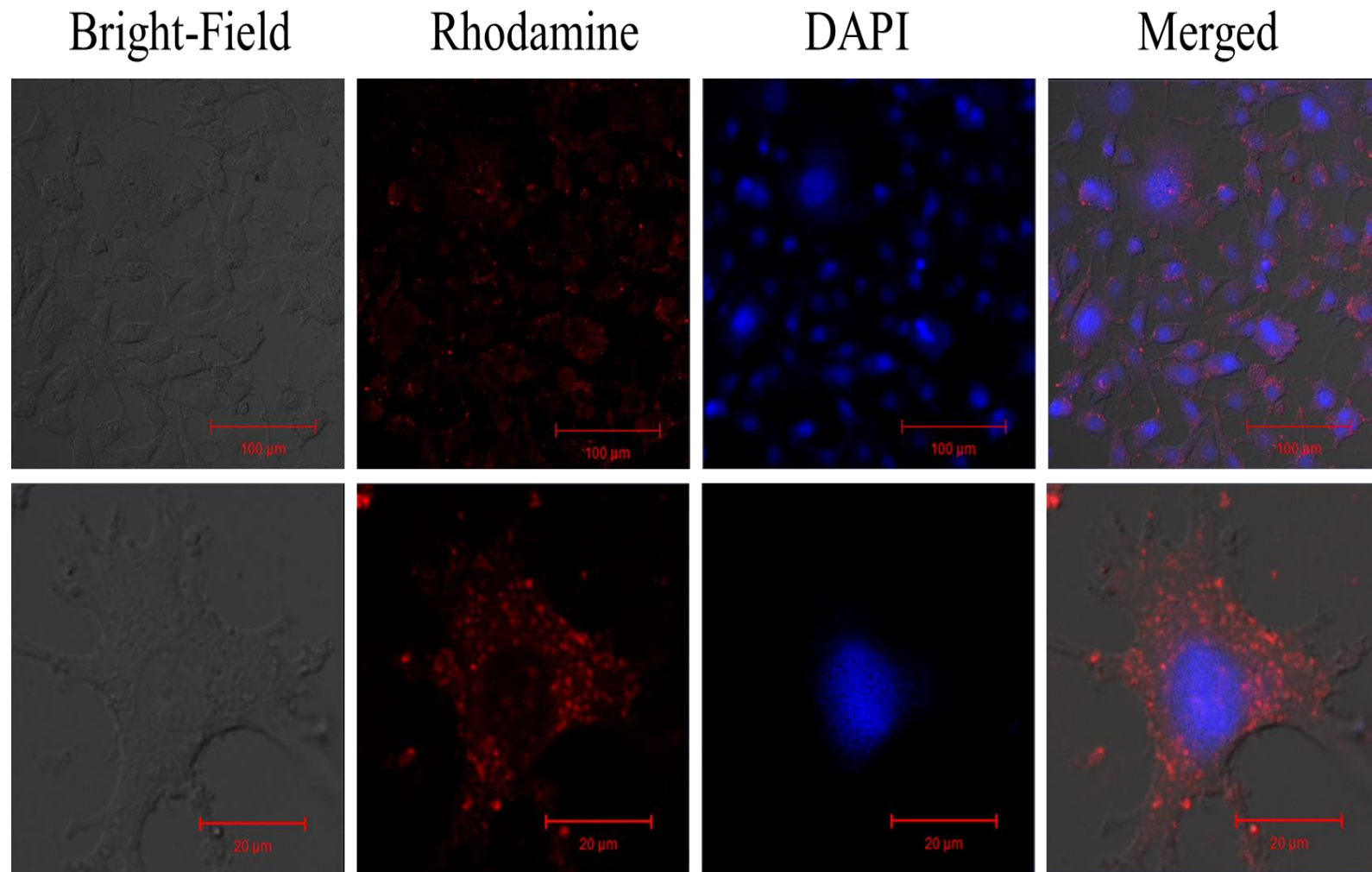


Figure 29. Confocal microscopic micrograph of Rhodamine labeled PLA-ALE

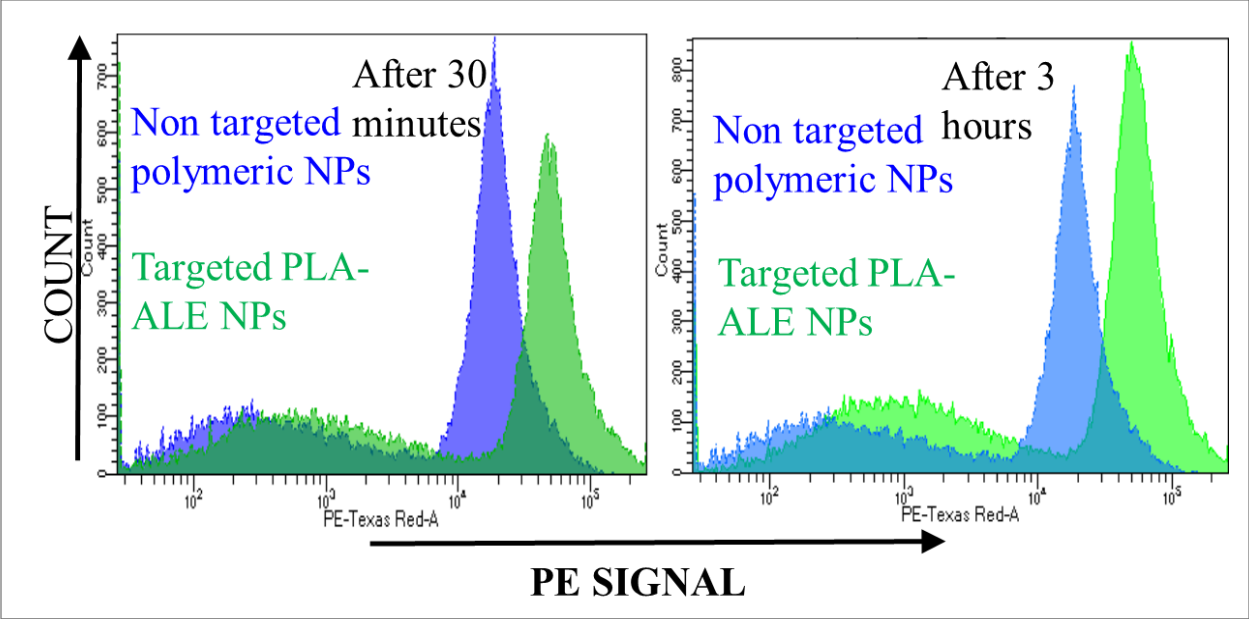


Figure 30. Fluorescence-activated cell sorting (FACS) studies showing time-dependent PLA-ALE internalization into the K7M2 cell. PEGylated NPs without bisphosphonate were used as controlled NPs. Values represent mean \pm s.d., n = 6

Bio-Compatibility of PLA-ALE Nps:

The biocompatibility of PLA-ALE Nps was studied in normal bone cells using MTT assay. As can be seen in Figure 31, over the wide range of PLA-ALE NPs concentration, no significant toxicity related to nanoparticles was observed, indicating the excellent biocompatible of PLA-ALE after nano-formulation. The PLA-ALE thus have excellent targeting capability, which are biocompatible and can internalize well into the cells.

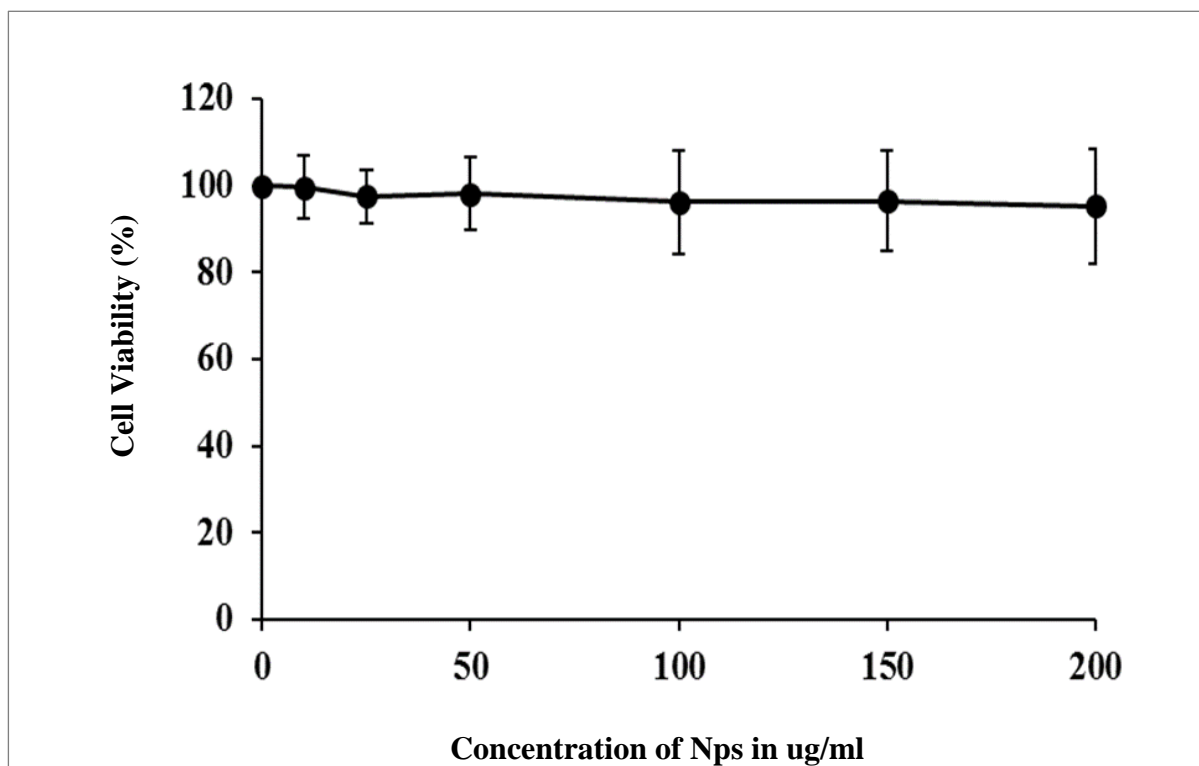


Figure 31. Cellular biocompatibility study of PLA-ALE nanoparticles with respect to Osteosarcoma Cells

Chapter 5 - Study of in-vitro therapeutic effects

Abstract

The development of target specific drug delivery systems is the most important step in cancer nanomedicine. The particles must have very good payload of drug and efficient transportation to the tumor microenvironment. Dox is the first-class drug used in the treatment of bone cancer. The Loading of doxorubicin in ug/mg into PLA-ALE nanoparticles was studied. Later the release of drug from DOX loaded PLA-ALE particles was studied in PBS buffer. The cytotoxicity of drug loaded particles was studied against Osteosarcoma cell line to understand effective nature of engineered nano delivery systems.

Introduction

The in vitro study is one of the preliminary study based on laboratory, which helps to guide researcher about the therapeutic effectiveness of proposed medicine. In current study, DOX is loaded into the NPs capable of targeting cancer at the bone. Chemotherapy is widely accepted and used as a clinical treatment for cancer management and has a well-defined role in the prognosis of many hematological neoplasms and solid tumors. DOX is the most widely used chemotherapeutic in the treatment of breast cancer and metastatic breast cancer to bone [91a,b]. DOXIL®, a liposomal form of DOX, is currently used in clinics as one of the first lines of cancer chemotherapies for soft tissue sarcomas and lymphomas [91c-e]. This drug is also used in treatment of Osteosarcomas and Chondrosarcomas.⁹² Although this drug is widely used, it has many issues like non-specificity and dose dependent toxicity. Thus, the targeted drug delivery could enhance its specificity and transportation to the tumor microenvironment.^{92, 93}

Therefore, in our study we used DOX and loaded them into PLA-ALE polymeric nanoparticles. Then the release profile is studied in PBS buffer up to 36 hours. These Dox loaded particles were studied for their toxicity in comparison to free DOX to understand the efficiency of our drug delivery systems.

5.1 Doxorubicin Loading into Polymeric Nanoparticles:

The loading assay of DOX, a class of frontline anti-cancer drug, into PLA-ALE particles was conducted.⁸⁰ The stock solution of DOX was prepared at 1mg/ml concentration using Acetonitrile vs Milli-Q water (1:1 by volume) solution. This stock was later used for loading into PLA-ALE nanoparticles, prepared by nanoprecipitation technique. In brief, 400 μ L of PLA-ALE in acetonitrile (1mg) was added dropwise to 3 mL of Mili-Q water in vials. At the same time, the samples from DOX stock were added to these vials in increasing concentration i.e. 25ug/ml, 50ug/ml, 75ug/ml, 100ug/ml, 150ug/ml. The mixture was stirred continuously for 15 minutes to facilitate the formation of nanoparticles at room temperature. These DOX-loaded polymeric nanoparticles were further purified using Amicon Ultra-4 centrifugal filter (Millipore, MA) with a molecular weight cut-off of 10kDA and stored at 4°C for further use.

5.2 Doxorubicin Release from Nano polymeric Particles:

After successful loading of DOX into PLA-ALE nanoparticles, the sample with 150 ug/ml was used to conduct drug release studies.⁹⁴ For this 15mm snake's skin dialysis bags, of around 1cm long were used. These were first soaked in 1X PBS buffer solution of pH = 7.4 for 12 hours in order to activate these membranes.

Then 1 ml of 1mg/ml concentration DOX-loaded PLA-ALE particles were pipetted out into the activated dialysis bags and were clamped. These dialysis bags were introduced into

300ml of 1X PBS buffer, stirring up to 100 rpm speed at room temperature, to analyze the drug release from PLA-ALE particles.

A comparative study is done against free DOX release from dialysis bag. 100 ul of PBS samples were collected at the start of the experiment, 30 minutes, 3 hours, 6 hours, 12 hours, 24 hours, 36 hours and 48 hours respectively. Every time 100ul of PBS was added to the beaker, in order to maintain a constant volume. These samples were analyzed to determine the drug content using a microplate reader (BioTek, Synergy H1 hybrid reader) and absorbance were recorded at 485 nm. Experiments were repeated three time and standard deviation was calculated.

5.3 Cellular Cytotoxicity:

The comparative in-vitro cytotoxicity of DOX-loaded PLA-ALE and free DOX was conducted on Osteosarcoma Cells using MTT assay.⁹⁵ In brief, 2×10^4 cells per well in DMEM medium were seeded in a 96-well plate and incubated for 24h. After incubation, the media were replaced with different concentration (10, 25, 50, 100, 150 and 200 μ g/mL) and incubated for additional 24h. Control cells were also maintained without any nanoparticle treatment (n=6). After the completion of incubation, MTT was added to each well and further incubated for 3h according to the manufacturer recommendation. The insoluble formazan crystals were solubilized using DMSO and their absorbance was recorded at 570 nm using a microplate reader (BioTek, Synergy H1 hybrid reader).

5.4 Results and Discussions:

Doxorubicin Loading Efficiency:

The Dox encapsulation efficiency was calculated with respect to initial DOX input in ug/ml, in percentages. The efficiencies were found decreasing as we go higher concentration of drug loading from 25ug/ml to 150 ug/ml, with 150 ug /ml showing around 40 percentage of encapsulation(Figure 32). The absorbance as a function of concentration was used to calculate these concentration values from DOX standard plot obtained from DOX stock solutions.

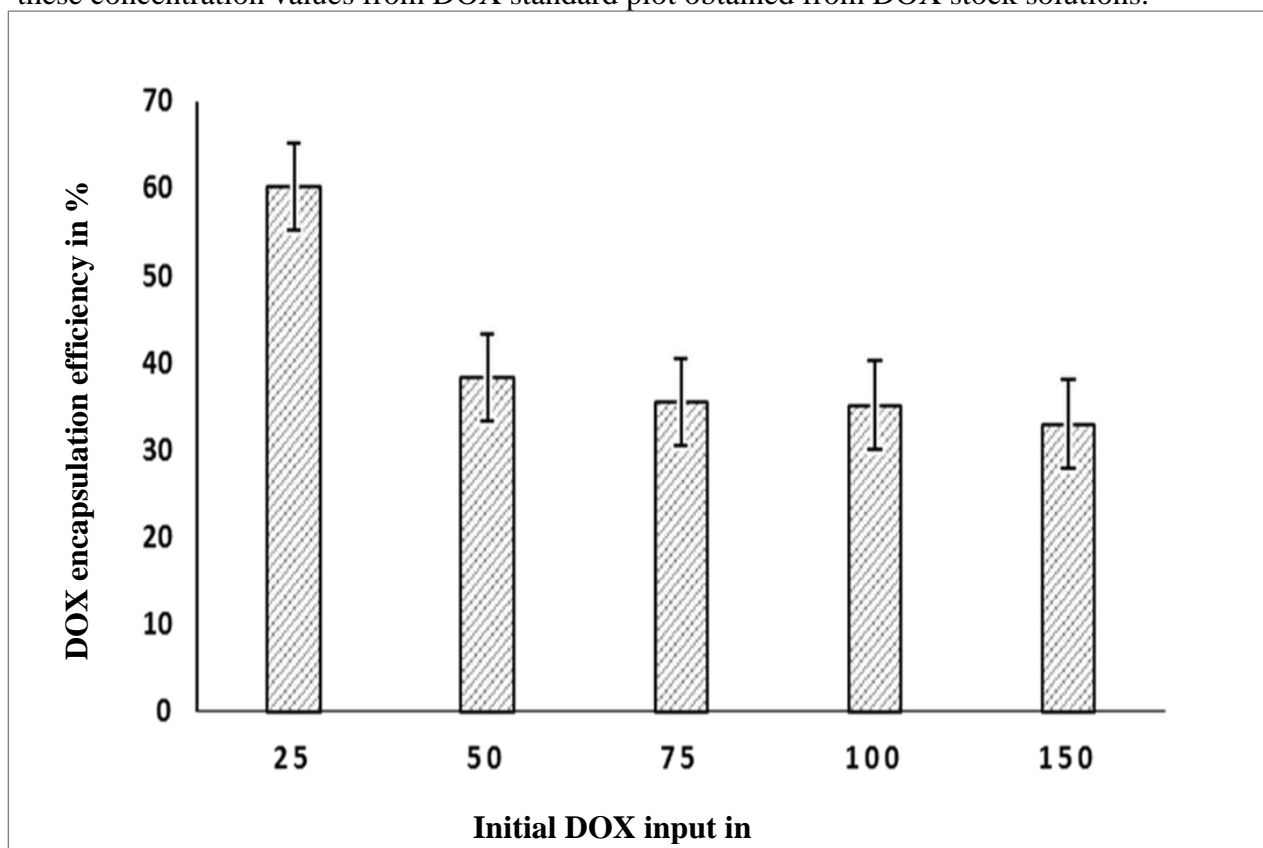


Figure 32. Dox loading efficiency in percentages into PLA-ALE particles (n = 3), mean \pm s.d

Doxorubicin Release:

The Dox release efficiency was calculated with respect to the amount of DOX in 150ug/ml initial concentration PLA-ALE nanoparticles. The release profile was plotted with respect to time of incubation and was compared with free DOX release, Figure 33. The drug release was very low at the start explaining the stability of DOX-loaded particles in the system. Then the release was happening slowing in gradient fashion over a period of time with the slope getting equal to one at 36 hours explaining the completion of experiment. The percentage release is around 40 percent at the end of the experiment.

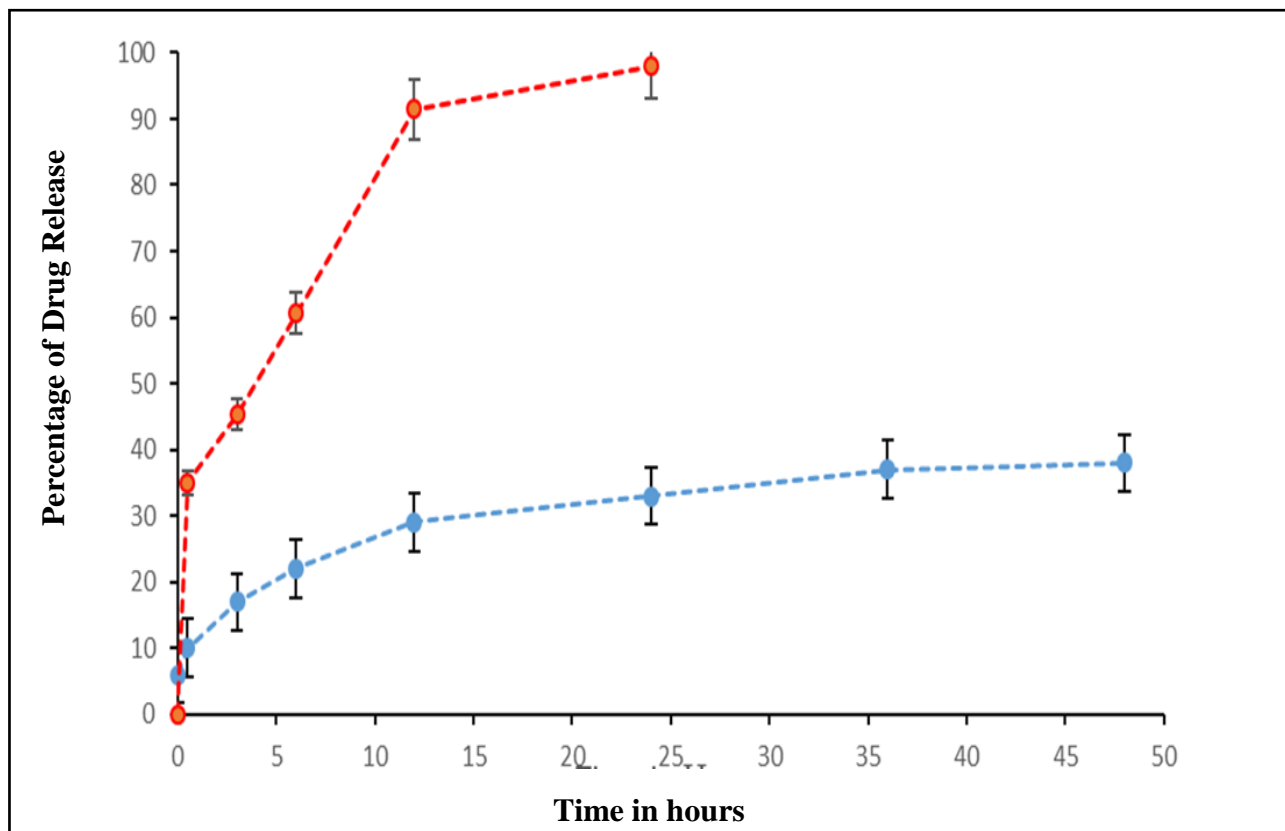


Figure 33. Dox release efficiency in percentages from PLA-ALE particles over period of time (n = 3), mean \pm s.d

Cellular Cytotoxicity:

The comparative study of cytotoxicity with respect to free Dox against Osteosarcoma cell line show that the DOX- loaded PLA-Ale have excellent tumor inhibition property, from Figure 34, and can be very effective in chemotherapy. Thus these particles have excellent release profile and therefore good cytotoxicity results can be observed from the graph.

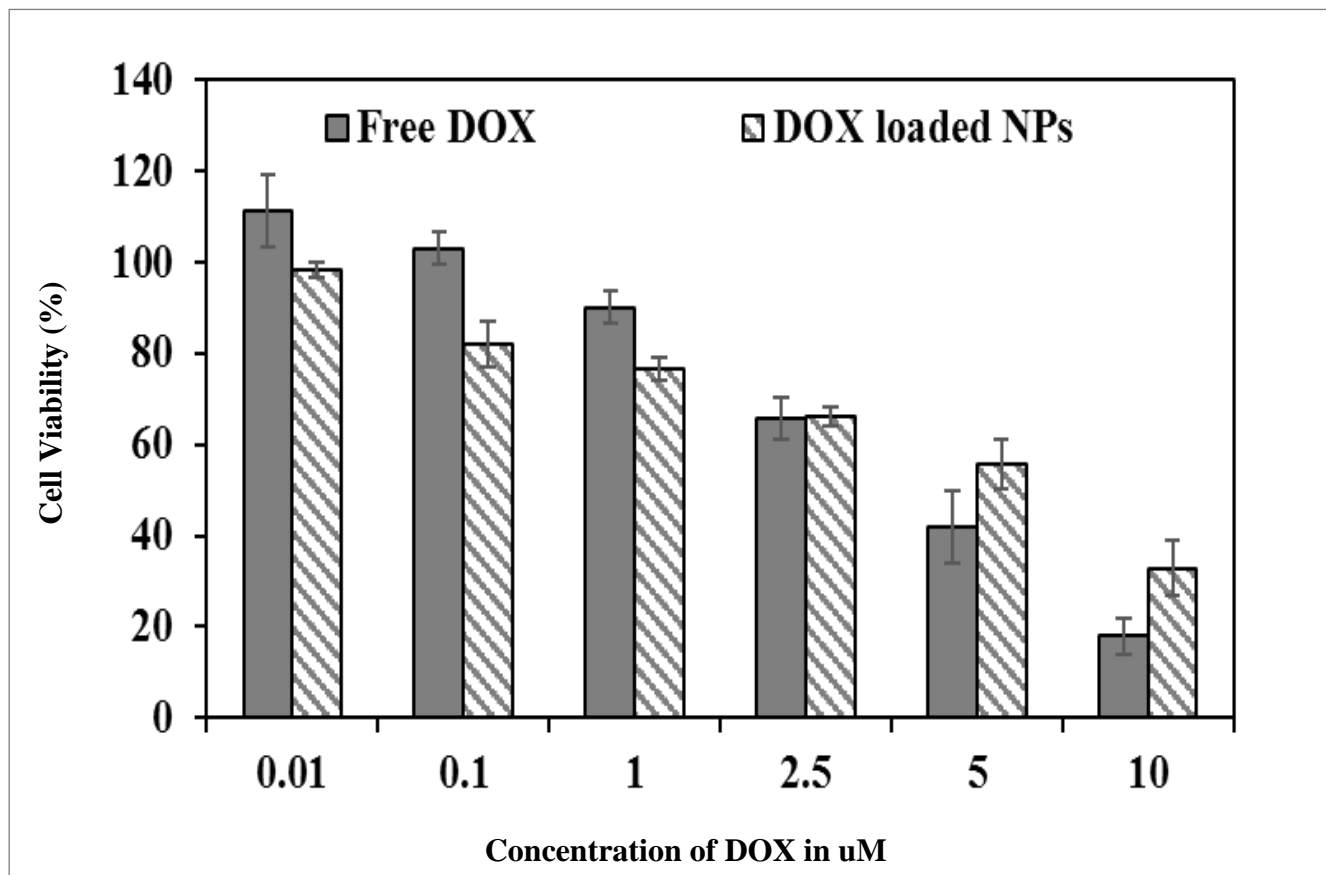


Figure 34. Comparative cytotoxicity study of free Doxorubicin Drug and Doxorubicin loaded PLA-ALE nanoparticles with respect to Osteosarcoma Cells

Chapter 6 - Conclusions and Future Work

6.1 Conclusions:

ALE functionalized poly-l-lactide was synthesized with variable chain lengths under similar conditions. The common physicochemical and spectroscopic data of the PLA-ALE were analyzed indicating the formation of the functional polymer.

As synthesized polymer was reengineered to form spherical nanoparticles of around 80 nm with a negative zeta potential of 33.2 ± 0.4 mV, which explains the presence of phosphate moiety.

The biocompatibility of the synthesized nanoparticles was assayed against osteosarcoma cells over the range of nanoparticle concentration, which suggested the excellent biocompatibility of the polymer.

The presence of ALE moiety, which is water soluble, at the surface of nanoparticles was studied using cellular internalization assay. Both nuclear co-localization and time-dependent cellular uptake of the nanoparticle was studied using confocal microscopy and FACS, which internalization and enhanced targeting ability of the nanoparticle. Therefore, bone microenvironment targeted polymeric nanoparticles was successfully engineered as an injectable nanodevice capable of delivering payload in a high degree of precision.

These particles have also shown a good ability of drug loading with respect to different concentrations of Doxorubicin. The drug release assay in two different pH ranges explains the higher efficiency for chemotherapy.

Considering the rapid cellular uptake, the polymer and its nanostructure engineered herein could open up a new paradigm in nanomedicine targeting chemotherapeutic to the bone cancer sites.

6.2 Future Work:

We have optimized the synthetic protocol for the synthesis of the polymers. Although we were successful in controlling homogeneity of the synthesized polymer (PDI ranges from 1.02 to 1.05, the practice molecular weight far from the theoretical molecular weight.

We will extend our project to understand in vivo studies using Animal models in future.

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