PHYSICO-CHEMICAL CHARACTERIZATION AND EVALUATION OF THERAPEUTIC EFFICACY OF THIOTEPA LOADED NANOPARTICLES

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY PHARMACOLOGY AND TOXICOLOGY
(Minor Subject: Animal Biotechnology)

By

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CERTIFICATE – I

This is to certify that the thesis entitled **"Physico-Chemical Characterization and Evaluation of Therapeutic Efficacy of Thiotepa Loaded Nanoparticles"** submitted for the degree of **M.V.Sc.** in the subject of **Veterinary Pharmacology and Toxicology** (Minor subject: **Animal Biotechnology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Manmeet Malpotra (L-2019-V-86-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged

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ABSTRACT

The present study included the process optimization of different method for preparation of Thiotepa loaded nanoparticles. Ionic gelation method of chitosan nanoparticle preparation was standardized and the physico-chemical characterization was done. Effects of parameters such as chitosan concentration, Chitosan to sodium tripolyphosphate ration and pH of the chitosan solution on the particle size distribution were investigated. The characterization parameter involved estimation of particle size which was 200.37±5.40nm and PDI 0.33±0.01 for the drug loaded chitosan nanoparticles. TEM images depict smooth surface and spherical shape of the nanoparticles. The optimum condition to obtain the smallest nanoparticle were found to be 0.1% chitosan concentration, 4.6 pH of the chitosan solution and 3:1 ratio of chitosan to sodium tripolyphosphate. Model fitting revealed that thiotepa loaded chitosan nanoparticles followed the Korsmeyer-Peppas Model of kinetics. Pharmacodynamic studies were also performed for thiotepa loaded chitosan nanoparticles and standard thiotepa formulation using L1210 cancer cell line which indicated ED_{50} values for thiotepa 96.62 µg while 78.36 µg for the thiotepa loaded chitosan. It was concluded that thiotepa loaded chitosan nanoparticles are suitable nano preparations. Also, the pharmacodynamic studies indicated a decrease in the drug dose thus increasing the anti-neoplastic efficacy. Therefore, nano-formulation can be further used *in vivo* studies.

Keywords: Thiotepa, nanoparticles, chitosan, TEM, drug release, encapsulation efficacy, ED_{50}, PDI.

Signature of Major Advisor **Signature of the Student**

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LIST OF ABBREVIATIONS

ANOVA	:	Analysis of variance
%	:	Percent
^{o}C	:	Degree Celsius
amp	:	Amplitude
µm	:	Micrometer
µl	:	Microlitre
CNPs	:	chitosan loaded nanoparticles
nm	:	Nanometer
DDS	:	Drug delivery system
DLS	:	Dynamic light scattering
DW	:	Distilled water
et al	:	And associates
EE	:	Entrapment efficiency
Fig.	:	Figure
FTIR	:	Fourier Transform Infrared
GADVASU	:	Guru Angad Dev Veterinary and animal Sciences
gm	:	Grams
hr	:	Hour
i.e.	:	That is
In vitro	:	Outside the living body and in an artificial environment
In vivo	:	Inside the living body
l	:	Litre(s)
log	:	Logarithm
ml	:	Millilitre
mg	:	Milligram
min	:	Minutes
mm	:	Millimeter
pH	:	Hydrogen ion concentration
PBS	:	Phosphate Buffer saline
Rpm	:	Rotations per minute
R^2	:	Correlation coefficient
TEM	:	Transmission electron microscopy
TPP	:	Tripolyphosphate

CHAPTER I

INTRODUCTION

The scourge of cancer has afflicted mankind since the dawn of time. Every year, an estimated 7.6 million people died due to cancer every year in which cancer accounting for over 70% of deaths in poor countries. Cancer occurs as a result of uncontrolled cell division or as a result of modifications that cause normal cells to acquire abnormal functions. Except for a few cancer forms that require hormone treatment or immunotherapy, cytotoxic medicines remain the most common method of cancer chemotherapy (Strauss et al., 2004). Cytotoxic medicines are a varied group of chemicals that are selectively toxic to rapidly dividing and developing cells because they experience fast growth and multiplication (Wong et al., 2007). The objective of cancer treatment is to destroy malignant cells while leaving healthy cells alone. It's a lot simpler to say than it is to accomplish.

Surgery, radiation, chemotherapy, and immunotherapy are the most commonly used neoplastic therapeutic measures around the world. However, to combat the high mortality and morbidity rates, effective new treatments are urgently needed. High toxicity and an insufficient bio-distribution and pharmacokinetic profile of cytostatic medicines are two drawbacks of traditional chemotherapy (Luque-michel et al., 2016). Neoplastic treatment includes the use of alkylating drugs. Alkylating drugs are cell cycle phase non-specific cytotoxic drugs which destroy rapidly dividing cells. They form highly reactive carbonium ions on reaction with biomolecules like nucleic acids, proteins etc under physiological conditions (at 37°C and pH 7.4)(Agents, 2010). They are more active during the late G1 and S phases of the cell cycle, as time for repair is less before the DNA synthesis occurs (Fu et al., 2012). The indiscriminate activity of cytotoxic medicines makes it difficult to treat both cancerous and healthy cells. Any method that allows cytotoxicity to be directed selectively to cancerous regions of the body would be extremely beneficial (Gregoriadis et al., 1974).

Traditional drug applications are characterized by limited efficacy, poor bio-distribution, and lack of selectivity. Drug targeting to particular organ and tissue has emerged as one of the century's most significant initiatives, as the use of free medicines in conventional dosage forms frequently includes difficulties in attaining the target area at the right concentration after or during the suitable time period. As a

result, one of the frontier research field is innovative drug delivery technique and modes of action (Martinho et al., 2011).

The majority of chemotherapeutic drugs have poor pharmacokinetic profiles as well as non-specific distribution in human tissues and organs, resulting in severe side effects and systemic toxicity. Improvement in the therapeutic efficiency while minimizing adverse effects is the heart of anticancer drug development. On a microscopic level, both of these objectives impose that identification of therapeutic molecule that binds the target with the best possible selectivity. Thiotepa (IUPAC name: 1,1',1"-phosphorothioyltriaziridine) is an anti-cancer medication that was developed first almost 60 years ago and is currently used in medicine and physiology (Nikolaienko et al., 2014).

N, N′, N″-triethylenethiophosphoramide (Thiotepa) is a classic chemotherapeutic trifunctional alkylating agent with a broad antitumor range. It is used in high-dose combination regimens for breast, ovarian, bladder, and other solid tumour malignancies (Torabifard & Fattahi, 2012). There are two mechanisms by which Thiotepa and its metabolite act. First is by protonation of the aziridine that initiates the ring opening reaction, which subsequently becomes the primary target of nucleophilic action by the N7 Guanine of DNA. The direct nucleophilic ring opening of aziridinyl groups is the second mechanism. The presence of electron withdrawing substituents (–P0S and –P0O substituents in Thiotepa and Tepa) leads the aziridinyl group to react with nucleophiles to form ring–opened products (Agents, 2010).

The primary constraints with cancer treatment are that the high doses of chemotherapeutics administered systematically could eradicate all neoplastic cells. However, several adverse effects in individuals may also occur (Alexiou et al., 2011). Early detection of cancer improves patient survival substantially. Despite this, existing diagnostic techniques (biopsies, imaging procedures, and marker detection) are frequently invasive, have limited sensitivity, or only identify cancer in its later stages, which is the primary cause of the high death rate. Although novel biomarkers are being studied, so more specific and sensitive diagnostic tools, as well as new therapeutic techniques are still required (Luque-michel et al., 2016).

To enhance cancer management, two primary research paths are currently being followed. The first entails using genomics and proteomics research to identify

particular targets in order to manufacture therapeutically beneficial medicines with minimal adverse effects ("targeted pharmaceuticals"). The second is nanomaterial design for transporting and delivering biomedical chemicals via biological systems for cancer therapy, diagnostics, and theranostics (by combining diagnostic and therapeutic molecules into multifunctional nanoplatforms) (Luk & Zhang, 2014). The use of nanotechnology in the development of these systems has been well established in pharmaceutical research and clinical practise over the last decade. Nanosystems have programmable size, shape, and surface properties, and they may target malignant tissue in two ways: passive and aggressive. The passive accumulation of nanocarriers in solid tumours is based on the so-called enhanced permeability and retention effect, which involves the retention of nanocarriers due to increased neovascularization, leakiness and decreased lymphatic outflow in tumour tissues. The surface of the nanocarriers can be functionalized with biological targeting moieties to enable active targeting (ligands). These biomolecules allow for the selective targeting of cancer-specific receptors as well as tumour endothelial cells (Luque-michel et al., 2016). As a result, significant progress has been made in the field of nanotechnology to address these issues and provide a potential and effective alternative for cancer treatment (Egusquiaguirre et al., 2012).

The main goal is to deliver medicines to the appropriate anatomical region, at the right concentration, and for the right amount of time, regardless of the method of administration (Lager & Peppas, 1981). This can be achieved by the use of particulate systems like nanoparticles as drug carriers. Attaching medicines to specially engineered carriers allow for cell-specific targeting. For a targeted therapy, the method of conjugating the medication to the nanocarrier, as well as the strategy for targeting it, are critical. A medicine can be adsorbed or covalently bonded to the surface of the nanocarrier, or it can be encapsulated inside it. Covalent linking offers an advantage over other methods of attachment in that it allows for exact control of the number of drug molecules linked to the nanocarrier, i.e., the amount of therapeutic chemical supplied (Wilczewska et al., 2012).

Nanoparticles to be used for parenteral administration, should be reasonable, cheap, biodegradable and easy to prepare. They should have small size, high loading efficacy. Properties like prolonged circulation and specifically accumulation at a

required site in the body would favour over the standard chemotherapeutic agent. Sailaja and Vineela (2014) stated that nanoparticles can directly assist their payload to specific cells or intercellular compartments due to their tailoring capabilities, which allow them to bypass physiological obstacles. With this, adverse effects can be reduced and a drug's therapeutic benefits are improved. Polymeric nanocarriers can be designed to be triggered by changes in the pH, chemical stimuli, or temperature, depending on the polymer properties.

Nanostructured and nanocrystalline nanomaterials are the two primary types of nanomaterials. Polymer-based, non-polymeric, and lipid-based nanoparticles are the three types of nanostructured materials. Dendrimers, nanoparticles, micelles, nanogels, protein nanoparticles, and drug conjugates are all polymer-based nanoparticles. Carbon nanotubes, nanodiamonds, metallic nanoparticles, quantum dots, and silica-based nanoparticles are examples of non-polymeric nanoparticles. Liposomes and solid lipid nanoparticles are two types of lipid-based nanoparticles. Polymer- or lipid-based nanoparticles make up the bulk of clinically approved therapeutic nanoparticles so far. Targeted therapy is a disease treatment strategy that involves delivering suitable quantities of therapeutic agent to the afflicted area of the body over an extended length of time. Another important factor in controlling the circulation and biodistribution of therapeutic nanoparticles is their size. Because nanoparticles are internalised by the targeted cells, their form is also important for biodistribution. The surface charge of therapeutic nanoparticles affects their clearance and targeted administration. Furthermore, nanoparticles having a surface potential of -10 to +10 mV are less vulnerable to phagocytosis and non-specific interaction (Yetisgin et al., 2020).

Although several nanoparticle-based drug delivery and drug targeting systems are being developed, no work has been done on nanoparticles loaded with thiotepa. Keeping this background in mind, the current research was designed with the following objectives in mind:

1. Optimization for synthesis of nanoparticles loaded with ThioTEPA.
2. To measure the therapeutic efficacy of ThioTEPA nanoparticles against cancer cell line.

CHAPTER II

REVIEW OF LITERATURE

2.1 Thiotepa

Aziridines are cyclic amines with three members (azacyclopropanes). These molecules are extremely reactive due to the ring-strain associated with them. Several compounds with an aziridine ring have been shown to have biological action.

Thiotepa is a multifunctional organophosphorus alkylating compound that inhibits DNA replication as well as RNA transcription. Thiotepa is stable at physiological pH in aqueous biological systems, it penetrates quickly into cells, and then releases its metabolite TEPA to impair DNA synthesis (Betcher & Burnham, 1991). It is an antineoplastic drug approved for the treatment of breast, ovarian, and bladder cancers. The discovery that the dose of thiotepa may have been increased substantially when bone marrow toxicity is not dose-limiting, as in the case of bone marrow transplantation, had reignited interest in the compound (Van Maanen et al., 2000). Although thiotepa has been in clinical use now for almost 70 years, pharmacological data on the drug are still incomplete. Questions regarding the metabolic profile, mechanism of action and pharmacokinetic parameters, particularly in high dose regimens still exist.

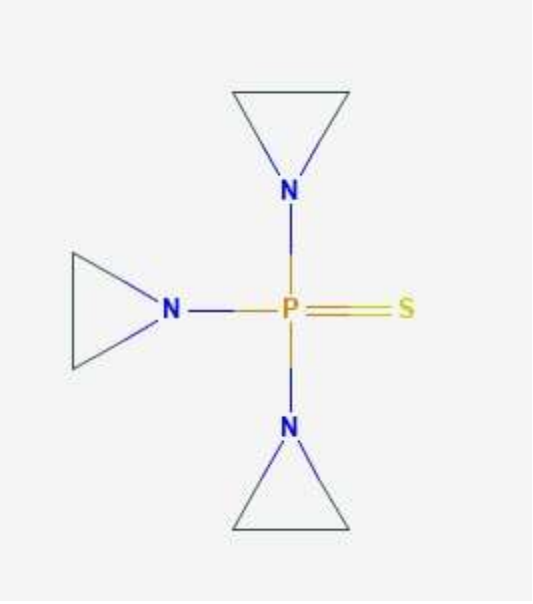

Fig 1: Chemical structure of Thiotepa

2.1.1 Structure

Aziridine, 1,1'1"-phosphinothioylidynetris-, or Tris (1-aziridinyl) phosphine sulphide is the chemical name for thiotepa. It has a white hue and a crystalline look. It has a melting point of 52 - 57 °C. Thiotepa has the molecular weight of 189.22g/mol and the empirical formula $C_6H_{12}N_3PS$. The resultant solution has a pH of roughly 5.5 to 7.5 when reconstituted with sterilized water for injection. Thiotepa is stable in an alkaline environment, but unstable in an acidic one. Thiotepa is transformed to highly reactive ethylenimine groups after dosing, which covalently attach to nucleophilic groups in DNA and favour the N7 position of guanine bases. This causes alkylated guanine bases in double-stranded DNA to crosslink, interfering with DNA replication and cell division, as well as inducing apoptosis and inhibiting cell development (Van Maanen et al., 2000).

2.2 Drug Targeting

Targeted drug administration is a means of administering medication to a patient in such a way that the medication concentration in some regions of the body is higher than in others. Targeted drug delivery aims to concentrate medicine in certain tissues of interest while lowering its relative concentration in the remainder of the body. This increases the drug's effectiveness while lowering its adverse effects. It is extremely difficult for a medication molecule to reach its goal in an organism's intricate cellular network. The goal of targeted drug delivery, as the name implies, is to help the drug molecule reach the chosen place as quickly as possible (Gupta and Sharma, 2011).

There are three criteria for a therapeutic medication delivery to be effective: getting the appropriate quantity in the right form to the right location (pharmacokinetics), and taking action once it reaches its target (pharmacodynamics). The use of nanoparticles can transform the delivery process from free-flowing circulation to targeted release in a regulated setting. Drug conjugates with antibodies and other macromolecules; microencapsulation, and nanoparticles can all be used to produce targeted and timed drug release (Tibbalis, 2017)

The objective of directing medicines to particular locations in the body where the desired pharmacological impact is sought while sparing other tissues has been

explored for many years. The notion of Ehrlich's "magic bullets" has now evolved into "magic wands" in the form of tailored medication delivery devices. The magic is due to particular targeting ligands that direct drug carriers to their molecular targets, whether they are on the cell surface or in nuclear membranes. Nano-systems are the emerging magic wands because they can target medicines to particular locations and maintain pharmacologically relevant drug levels at the site for the duration necessary for intended therapeutic effect (Vasir et al., 2005).

Focused on nanoparticle-interceded drug conveyance might be utilized to guide the particles to explicit tissues (limiting harmfulness), further develop oral bio-accessibility, support drug/quality impact in the objective tissue, solubilize drugs for intravascular conveyance, and additionally work on the solidness of remedial specialists against enzymatic debasement(Chenthamara et al., 2019).

Targeted nanoparticle-mediated drug delivery can be used to deliver therapeutic agents to specific tissues (while minimizing toxicity), improve oral bioavailability, maintain drug/gene effect in the target tissue, solubilize drugs for intravascular delivery, and/or improve therapeutic agent stability against enzymatic degradation (Xu et al., 2007).

2.3 Drug delivery and nanotechnology

In the treatment of many diseases, getting medicinal compounds to the target site is a serious issue. A traditional drug application is marked by limited efficacy, poor biodistribution, and a lack of selectivity. Controlling drug delivery can help to overcome these limits and drawbacks. The medicine is carried to the site of action in controlled drug delivery systems (DDS), minimising its impact on important tissues and undesired side effects. DDS also protects the medicine against contamination. Medication breakdown or clearance is accelerated, which improves drug efficacy. As a result of the higher drug concentration in target tissues, smaller medication doses are required. This is a new type of therapy. When there is a disparity, this is extremely crucial between a drug's dosage or concentration and its effectiveness. Whether it's the therapeutic outcomes or the side effects, there's something for everyone (Wilczewska et al., 2012).

Nanoparticles utilized as drug delivery vehicles typically have a diameter of less than 100 nanometers in at least one dimension and are made of biodegradable

materials such as natural or manufactured polymers, lipids, or metals. Because nanoparticles are more readily absorbed by cells than bulkier micro molecules, they might be designed as effective transport and delivery systems (Suri et al., 2007). According to Jahangirian et al. (2017) nanomedicines have gained popularity in recent years as a result of their ability to encapsulate medications or bind therapeutic chemicals to nanostructures and transport them to target tissues more accurately and with a controlled release.

Nanotechnology is the creation and usage of materials, gadgets and frameworks through the control of issue on the nanometer scale. Given the innate nanoscale elements of the natural parts of living cells, it was inescapable that nanotechnology would be applied to the existence sciences. Such applications bring about the term nanobiotechnology (Jain, 2005). As per the definition from NNI (National Nanotechnology Initiative), nanoparticles are structures of sizes going from 1 to 100 nm in no less than one measurement. Be that as it may, the prefix "nano" is usually utilized for particles that are up to a few hundred nanometers in size (Wilczewska et al., 2012).

These nanoparticles (NPs) are appealing for medical applications because of their crucial and distinctive characteristics, such as their huge surface to mass ratio compared to other particles, quantum properties, and capacity to adsorb and carry other chemicals. NPs have a large (functional) surface area that can bind, adsorb, and transport other molecules like medicines, probes, and proteins (Jong & Borm, 2008).

Saxena et al. (2004) used the biodegradable polymer poly(dl-lactic-co-glycolic acid) to create indocyanine green (ICG)-loaded biodegradable nanoparticles (PLGA). The mean diameter of all PLGA NP formulations was found to be between 300 and 410 nm, with PDI ranging from 0.01 to 0.06. ICG-loaded PLGA nanoparticles were synthesised and the formulation was adjusted. ICG entrapment increases as the amount of polymer in the formulation increases. The NPs that were generated were spherical with porous surfaces and showed the typical release pattern of a monolithic matrix-based system.

Encapsulation of hydrophobic photosensitizers (PS) into polymeric nanoparticles (NP) has shown to be a viable alternative to organic solvents for their formulation according to (Vargas et al., 2009). An emulsification-diffusion approach

was used to encapsulate a hydrophobic PS, the meso-tetra (p-hydroxyphenyl) porphyrin, inside biodegradable NP composed of poly (D, L-lactide-co-glycolide) 50:50. The chorio-allantoic membrane (CAM) model was used to examine the efficacy of the encapsulated medication. The results revealed that the medication was encapsulated in nanoparticles, which stayed in the vascular compartment for longer period, and it was inferred that the NP size regulates the photodynamic activity of the encapsulated PS.

Liposomes appear to meet many of the requirements for an ideal medication carrier. They are organic, suitable for storing a therapeutic agent in isolation from the environment, and can deliver agents to target cells through specific surface manipulation, as proposed in 1974, leading to several breakthrough discoveries using nanoparticles as drug carriers (Gregoriadis et al., 1974).

Although a lot of drug delivery systems have been successfully implemented in recent years, there are still some issues to be addressed and sophisticated technologies to be created in order to successfully transport medications to their target sites. Nanomedicines have gained popularity in recent years as a result of their ability to encapsulate pharmaceuticals or bind therapeutic chemicals to nanostructures and transport them to target tissues more precisely and with a controlled release (Patra et al., 2018).

Conventional chemotherapeutic drugs are dispersed in the body in an ad hoc manner, affecting both malignant and non-cancerous normal cells, reducing the amount that can be delivered within the tumour, resulting in unsatisfactory treatment and severe toxicities. In order for anticancer medications to be effective in cancer treatment, they must first be able to penetrate barriers in the body and reach the desired tumour tissues with little loss of volume or activity in the blood circulation after delivery. Second, with a regulated release mechanism of the active form, medications should be able to selectively destroy tumour cells without damaging normal cells once they reach the tumour tissue. Boosting the intracellular concentration of medications while concurrently lowering dose-limiting toxicities, these two basic techniques have been linked to gains in patient survival and quality of life. Nanoparticles have been created for optimal size and surface features to optimise cancer medicine biodistribution and increase their circulation time in the bloodstream. Nanoparticles can increase the intracellular concentration of medications in cancer

cells while avoiding harm on normal cells utilising passive and active targeting techniques. Nanoparticles have the potential to concentrate in cells without being identified by P-glycoprotein, one of the key mediators of multidrug resistance, resulting in higher drug concentrations in the intracellular space (Cho et al., 2008).

Nano-sized colloidal drug carriers can be considered a breakthrough in pharmacotherapy. The promise of tailored, site-specific medicine delivery is the most exciting application of nanomaterials. The possibility of removing a tumorous expansion without causing collateral harm by using nanomaterial-based drug delivery has piqued curiosity, because nanoparticles are the foundation for bio-nano-materials (Mirza & Siddiqui, 2014). According to Gao et al. (2012), nanomedicine makes a significant contribution to cancer treatment by directing drugs to tumours and lowering multidrug resistance. Nanotechnology offers a promising alternative to traditional chemotherapeutics, avoiding multidrug resistance (MDR) by encapsulating, attaching, and conjugating medications or therapeutic biological products to nanocarriers, as well as by specifically targeting tumour cells.

Abraxane, which is Nanometer-Albumin-Bound (NAB) paclitaxel for head and neck cancer, metastatic breast cancer, non-small cell lung cancer, and anal cancer, was the first nanoparticle medicine formulation to earn US-FDA approval in 2005. Doxorubicin is a widely used anti-cancer medicine that works by preventing cancer cells from synthesising nucleic acids. Despite its high anticancer efficacy, doxorubicin's nonspecific action generates substantial adverse effects in patients, which has been a big challenge to overcome. As a result, doxorubicin was conjugated to PLGA via an ester bond, which was more easily cleavable under physiological conditions. The *in-vitro* anti-cancer activity of doxorubicin nanoparticles was assessed using a HepG2 cell line after nanoparticles containing doxorubicin PLGA conjugates were synthesized (Yoo & Park, 2000).

Due to the presence of two anatomical and biochemical dynamic barrier; the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier, the majority of medicines and biotechnological agents do not readily permeate into brain parenchyma. As a result, the availability of effective brain targeting technology is one of the most serious issues facing CNS medication development. Nanotechnology advancements have presented promising solutions to this problem. For the delivery of

CNS therapies, nanocarriers ranging from the more established systems have been investigated. A drug with poor brain distribution can be loaded on a nano-carrier system with or without targeting moieties (ligands) that interacts with endothelial microvessel cells at the BBB and results in greater drug concentrations in the brain parenchyma. The use of nano-carriers can also improve brain delivery of anti-retroviral drugs (ARVs) by improving ARV availability in the CNS-compartment, allowing for lower doses and shorter treatment times (Wong et al., 2012).

2.4 Physico-Chemical Properties

Nanoparticles have features that are very different from tiny molecules, and their chemistry and manufacturing require treating them more like complicated mixtures rather than small molecules (Christian et al., 2008).

The manifestations of nanoparticles and engineered nanomaterials are influenced by physicochemical parameters such as size, shape, chemical composition, physiochemical stability, crystal structure, surface area, surface energy, and surface roughness. In the cellular absorption and biological processes of nanoparticles, minor physicochemical variations have profound biological significance (Alexis et al., 2008).

2.4.1 Size of particle

He et al. (2010) observed that the cellular uptake of NPs was influenced by changes in particle size and surface charge, as well as distinct cell lines, and several processes were engaged in the process. *In vivo* biodistribution showed that NPs with a little negative charge and a particle size of 150 nm accumulated more efficiently in tumors. These findings might be used as a guideline for rationally designing drug nanocarriers with maximum therapeutic efficacy and predicted *in vivo* features, in which particle size and surface charge were important factors. Redhead et al., 2001 concluded that particle size has an impact on drug release. Because smaller particles have a bigger surface area, so majority of the drug associated with them will be at or near the particle surface, resulting in rapid drug release. Larger particles, on the other hand, have larger cores, allowing more medication to be encapsulated and slowly diffuse out.

Nanoparticles with a diameter of 100–200 nm have the finest qualities for cellular uptake. Not only does this dispel the myth that the smaller the particle, the

better the cellular absorption, but it also highlights the reality that there is an optimal particle size range (Win & Feng, 2005). To optimise delivery of these drugs, a better understanding of the functional size and physiological modulation of transvascular routes is required. The ideal size of a nanoparticle is also determined by the location and kind of tissue being targeted (Hobbs et al., 1998).

According to Hoshyar et al., 2016, the size of nanoparticles has a big impact on their therapeutic and diagnostic applications. Nanoparticle size influences therapeutic delivery characteristics such as blood circulation half-life, cellular absorption, and tumour penetration. To get pass the renal filtration barrier, nanoparticles must be larger than 10 nm. A diameter of more than 200 nm activates the complement system, which removes it from the bloodstream fast, collecting in the liver and spleen. In thermodynamic models and most experimental research, a diameter of roughly 50 nm demonstrates the maximum cellular absorption within this range.

Nanoparticles (particles with a diameter of 1–100 nm) are developing as a new class of cancer therapies. Because of qualities including better specific localisation in tumours and active cellular uptake, early clinical studies suggest that nanoparticle therapies can improve efficacy while minimising side effects (Davis et al., 2010). Systemically delivered nanoparticles (through intravenous injection) move through the bloodstream and reach tissues with large blood vessels that are not target organs. Designing nanoparticles that can bypass the clearance processes of the lungs, liver, spleen, and kidney and reach target tissues is a plausible goal. Nonetheless, nanoparticles that are too tiny are susceptible to renal excretion and clearance from target organs. According to a study that used *in vivo* fluorescence imaging after intravenous injection of fluorescent quantum dots, hydrodynamic diameter is a major determinant of renal excretion (Choi et al., 2007).

2.4.2 Shape of particle

The influence of particle shape on several aspects of drug delivery, including cellular uptake, biodistribution, anticancer effectiveness, and nanoparticle toxicity, has been investigated. Non-spherical forms, as opposed to spheres, have a higher specific surface area, which allows for more surface modification with different ligands for targeted drug delivery. Non-spherical particles had lower cell uptake in the

majority of cell lines, improved cytotoxicity, a different biodistribution profile, better *in vivo* antitumor effectiveness, and better immunization potential (Jindal, 2017).

Recent developments in nanoparticle technology have permitted the production of nanoparticle classes with distinct sizes, shapes, and materials, paving the way for substantial advances in nanomedicine. It also aims to show how a nanoparticle's form may influence its *in vivo* trip and destination, influencing biodistribution, intravascular and transvascular transport, and eventually, cancer site targeting. According to studies, the oblate form of particles helps them circulate in the bloodstream by reducing macrophage absorption. In addition to influencing macrophage clearance, the form of nanoparticles appears to influence normal and cancer cell endocytosis. Furthermore, shape has a role in targeting nanoparticles via receptor–ligand systems (Toy et al., 2014). Given that metastatic disease is responsible for the vast majority of cancer deaths, designing nanomedicines capable of highly selective delivery to micro metastases via vascular targeting of biomarkers on the endothelium associated with metastatic disease may be the most effective strategy for reaching micro metastases. As a result, ‘shaping' nanomedicine can open up new avenues for addressing critical and unmet therapeutic needs.

2.4.3 Charge of particle

When it comes to using nanoparticles as a drug delivery method, the surface charge of the particles is crucial. The net charge of particles has an impact on circulation half-life, tissue retention, and/or cell entrance capabilities, therefore it is important to understand in order to enhance particle delivery. To determine cellular uptake, several researchers looked at the charge on the particle's surface. With the exception of some macrophages, the majority of researches agree that positively charged particles are better absorbed into most cell types (Gratton et al., 2008).

Surface charge and other physicochemical characteristics of nanoparticles are thought to play a key role in cellular uptake and particle–cell interactions. The amount of carboxymethyl groups included in the dextran chains determine the surface charge of carboxymethyl-substituted dextran-coated nanoparticles which ranges from -50 to 5 mV, as measured by zeta potential tests. Nanoparticles with a higher negative charge was shown to have higher absorption rates. Internalization patterns imply that

non-specific interactions are involved in the absorption of the most negatively charged particles (Ayala et al., 2013)

The surface charge or zeta potential, has a significant impact on particle stability in suspension due to electrical repulsion between particles. It can also affect how nanoparticles interact with bacteriae cell membrane, which is generally negatively charged. The zeta potential of Chitosan nanoparticles (CSNPs) was found to be 50.3 mV. The fact that CSNPs had a greater zeta potential implies that they were rather stable. The lengthy amino groups appear to prevent aggregation by inhibiting anion adsorption and maintaining a high value of electrical double layer thickness (Agarwal et al., 2018).

Mucosa is a semipermeable membrane that acts as a barrier to the delivery of nanoparticles via the oral route. To estimate the surface charge of particles, the zeta-potential was measured using a dynamic light scattering method. The impact of surface charge on nanoparticle penetration over the mucus layer was studied using particles with various surface functional groups, including sulfate/carboxylate, amino, and neutral. When compared to amino surface nanoparticles, neutral surface nanoparticles penetrate the mucosa barrier to a larger extent. In conclusion, the particle size and surface charge that are most desired for nanoparticle mucosal penetration are 50 nm and neutral, respectively (Bandi et al., 2020).

After exposure in a human keratinocyte cell line, we investigated whether modulating the surface charge of 1.5 nm gold nanoparticles (Au NPs) caused changes in cellular morphology, mitochondrial function, mitochondrial membrane potential (MMP), intracellular calcium levels, DNA damage-related gene expression, and p53 and caspase-3 expression levels (HaCaT). The study of three distinct Au NPs (positively charged, neutral, and negatively charged) revealed that all three NPs have different cell shape and that their toxicity was dose-dependent; the charged Au NPs exhibited toxicity at 10 μg ml^{-1} and the neutral at 25 μg ml^{-1}. In conclusion, these findings show that surface charge is a key determinant of how Au NPs affect cellular processes, with charged NPs promoting apoptosis and neutral NPs causing necrosis.

After opsonization in fresh mouse serum, RAW 264.7 murine macrophages took up NPs with a high surface charge, whether positive or negative. Several different endocytic mechanisms (e.g., clathrin-mediated endocytosis, caveolae-

mediated endocytosis, and macropinocytosis) were implicated in the cellular absorption of NPs, according to mechanistic investigations. Positively charged NPs had dose-dependent hemolytic and cytotoxic effects on RAW 264.7 cells, but negatively charged NPs didn't have any significant hemolytic or cytotoxic effects. When the surface charge of NPs was slightly negative, liver absorption was very low while for tumor uptake it was very high. Based on these findings, we may deduce that a minor negative charge on the NPs surface could minimise unwanted clearance by the reticuloendothelial system (RES), such as the liver, enhance blood compatibility, and therefore transport anti-cancer medicines more efficiently to tumour locations (Xiao et al., 2011).

2.5 Entrapment efficiency

The direct or indirect approach may be used to calculate the entrapment effectiveness of nanocarriers. In one study after centrifugation, the unentrapped or free quantity was calculated in the supernatant using the indirect technique. The difference between the initial drug amount and the free or unentrapped quantity of drug in the supernatant in relation to the total quantity included in the nanocarrier preparation was used to calculate the entrapment efficiency or percentage of the content (Gaikwad & Bhatia, 2013).

Encapsulation allows for the preservation and regulated release of bioactive chemicals at the appropriate time and in the right place. Nanoencapsulation continues to be one of the most promising methods for encapsulating bioactive substances. Nanoencapsulation of bioactive substances provides a number of benefits, including targeted site-specific delivery and effective cell absorption (Ezhilarasi et al., 2013).

The effectiveness of encapsulation of bovine serum albumin (BSA) improved considerably as the pH value increased. The effect of the crosslinker (TPP) solution pH was also investigated, with results revealing that chitosan nanoparticles made with a basic TPP solution (pH 9.5) had greater size, yield, and BSA encapsulation efficiency than those made with an acidic TPP solution (pH 5.5). To summarise, an acidic TPP solution should be used to crosslink CS in order to generate monodispersed CSNPs, whilst a high pH is advised for high protein encapsulation efficiency (Mattu et al., 2013).

The amount of drug bound per mass of polymer (typically moles of drug per mg polymer) is referred to as drug loading of nanoparticles. It can also be expressed as a percentage dependent on the polymer. Yang and Hon (2010) observed when the degree of deacetylation of chitosan was 90% and the quantities of chitosan and TPP were both 0.5 mg/mL, the drug loading increased. The prolonged release of 5-FU from chitosan nanoparticles with a 90 percent degree of deacetylation was seen *in vitro*, indicating that these nanoparticles have the potential to be used as drug delivery agents.

2.6 Release of drug

"Drug release" in polymeric systems usually refers to how a drug molecule is carried from a starting point in a polymeric matrix to the it's outer surface and then released into the surrounding environment. Drug diffusion through water-filled pores, diffusion through the polymer matrix, osmotic pumping, and erosion are the four-primary controlled-release methods. For drug diffusion through water-filled pores, the drug release rate in degradable polymeric systems is regulated by diffusion via a network of pores, which evolves as the polymer matrix degrades. Water is absorbed by polymeric NPs almost instantly, making it a quicker mechanism than medication release. In 'diffusion through the polymer matrix', diffusion is the major driving force for drug release in nondegradable drug delivery reservoirs or depots, with the rate of release being constant and unaffected by concentration gradients, but rather by the characteristics of the polymeric membrane. For third release method, convection-driven drug transport through water-filled pores is another option. Osmotic pressure causes the entry of water into a no swelling system, and drug transfer as a result of this force is known as osmotic pumping. When the rate of erosion is larger than the rate of water penetration in the bulk polymer, surface erosion occurs. It happens when polymers deterioration starts at the matrix/scaffold surface, steadily diminishing the size of the matrix/scaffold from the exterior to the inside. (Kamaly et al., 2016).

The dialysis tube containing doxorubicin (DOX) conjugated Au NPs was transferred to a beaker containing 50 mL of phosphate buffer solution, maintained at pH 5.3, immediately after the purification of DOX conjugated Au NPs. The drug release experiment was carried out at 37 °C with constant stirring at 100 revolutions per minute. To determine the drug release content, samples (1 mL) were withdrawn

and replaced with an equal volume of phosphate buffer solution on a regular basis. A spectrophotometer set to 485 nm was used to measure the quantity of DOX emitted. The DOX release rate from DOX loaded Au NPs in an acid solution (pH 5.3) was found to be much greater than in physiological circumstances (i.e., pH 7.4) (Aryal et al., 2009).

The extracellular environment of solid tumors is acidic so the pH gradient between cell compartments is changed. The corona and a core make up the nanoparticles, and they respond to pH by changing their soluble/insoluble or charge state in the solution. For cellular targeting and internalization, nanoparticles with positively charged or soluble coronas have been created, making their targeting groups available for binding at the tumor pH. For rapid release of medication into the cytosol or the extracellular fluid, nanoparticles with soluble cores or changing architectures that release the medicines carried at the tumour extracellular pH should be created. Compared to their pH-insensitive counterparts, these pH-responsive nanoparticles offer therapeutic benefits (Shen et al., 2008).

Siepmann (2001) investigated the effect of tablet size on drug release patterns and found that 99.8% of the medication was released from tiny tablets after 24 hours, 83.1 percent from medium-sized tablets, and just 50.9 percent from big tablets. The cause of this occurrence is self-evident. Smaller tablets have a greater relative surface area (volume) than larger tablets. This supports the drug release from the drug loaded nanoparticles.

2.7 Characterisation of nanoparticles

2.7.1 Type of Morphology

Nanoparticles are studied using advanced microscopic methods such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy to determine their size, shape, and surface charge. The TEM can provide information on nanoparticles' particle size, size distribution, and shape. The physical stability and *in vivo* dispersion of nanoparticles are influenced by their average particle diameter, size distribution, and charge. The overall morphology of polymeric nanoparticles, which may affect their toxicity, may be determined using electron microscopy methods (Akbari et al., 2011).

2.7.2 Size and Surface area of particle

The size of the NPs may be estimated using a variety of methods. SEM, TEM, X-ray powder diffraction (XRD), atomic force microscope (AFM), and dynamic light scattering are examples of these techniques (DLS). Although SEM, TEM, XRD, and AFM can provide a better understanding of particle size (Kestens et al., 2016), the zeta potential size analyzer/DLS can be utilised to determine NP size at extremely low levels.

2.7.3 Structural characterization

The composition and nature of bonding materials in nanoparticles are primarily studied using structural properties. It gives a wealth of information on the subject material's bulk characteristics. The typical techniques used to examine structural characteristics of nanoparticles include XRD, energy dispersive X-ray (EDX), X-ray photoelectron microscopy (XPS), Fourier transform infrared spectroscopy (FTIR), and the Zeta size analyzer.

The Zetasizer series of instruments uses the Dynamic Light Scattering method to measure the particle size of distributed systems. Electrophoretic Light Scattering (ELS) and Static Light Scattering (SLS) techniques are also used to assess particle mobility and charge (Zeta potential) and the molecular weight of particles in solution utilising Zetasizer devices (SLS). The infrared spectrum of absorption, emission, and photoconductivity is obtained using the Fourier transform infrared spectroscopy (FTIR) method. Between 4000 and 400 cm, FTIR spectrum is obtained. A nanoparticle polymer was dissolved in chloroform and placed over a NaCl crystal for FTIR analysis, and the polymer film was exposed to FTIR after the chloroform was evaporated (Sindhu et al., 2015).

2.8 Chitosan as Drug Carrier

Chitosan is obtained from chitin $(C_8H_{13}O_5N)_n$, a characteristic biopolymer generally bountiful in exoskeletons of crustaceans and insect cuticles and cell walls of fungi and shells. Chitosan is a polymer of deacetyl α-(1, 4) glucosamine $(C6H11O4N)_n$ units that can ordinarily be gotten by deacetylation of chitin with NaOH after demineralization and deproteinization of the exoskeletons. The level of deacetylation of chitin goes from 60 to 100 % and sub-atomic load of economically viable chitosan goes from 3800 to 20,000 daltons. It is a multipurpose material

because of its nontoxicity, biocompatibility, biodegradability, and adsorptive conduct. In this way, it may be utilized very well for designated drug conveyance with more productivity and less incidental effects. It has been found to have antineoplastic effect with negligible harmfulness on noncancer cells and such action against various malignant growth cell lines essentially relies on atomic weight and level of deacetylation. Solvent type of chitosan oligosaccharide with low atomic weight has been accounted to show amazing biological activities and concealment of tumorous growth (Adhikari & Yadav, 2018).

The antineoplastic medications can be encapsulated in the chitosan NPs showing potential as medication transporter and target explicit delivery bringing about the killing of malignant growth cells without influencing or hurting the typical cells of the patient. Malignancy medications can be stacked over chitosan NPs utilizing epitome or by direct connection. The medications are blended completely with chitosan arrangement and take into consideration self-gathering. The construction of chitosan nanosphere comprises of a strong center for hydrophobic medications. The chitosan nanosphere are steadier with uniform size and controlled medication focusing on limit. Sometimes, chitosan NPs are made delicate and particular by greater refinement. It tends to be done by adding PEG, focusing on ligands and pH touchy or hypothermic polymer forms. During the disease treatment utilizing chitosan NPs, chitosan focuses on the cancer instigated cells by focusing on metabolism, cell development lastly prompts apoptosis of the growth cells. A report on chitosan NPs has demonstrated that cancer cells have been instigated by low sub-atomic weight chitosan and chito-oligosaccharides. The chitosan NPs are recognised by macrophage and delivered through body flow. The plasma proteins are adsorbed by the surface charge of the chitosan NPs, this aide's simple acknowledgment by the macrophages. This system helps in the simple entrance or entering of medications inside the cancer tissue. Chitosan NPs with high surface potential, high extremity and amphipatic or hydrophilic property are effectively overwhelmed and remain longer inside the tissue (Shanmuganathan et al., 2019).

Qi et al. (2004) synthesized and characterized chitosan nanoparticles and copper-loaded nanoparticles. The nanoparticles created had a tiny particle size and a positive surface charge, which rendered them stable in biological cations and

increased antibacterial activity owing to interactions with negatively charged biological membranes and site-specific targeting *in vivo*, according to the researchers.

Because of the longer drug interaction with the mucosal layer and the high surface-to-volume ratio of nanoparticles that may further increase this impact, the mucoadhesive property of chitosan renders the produced particles capable of improving both drug absorption and bioavailability. Mucoadhesion, in which chitosan adheres to particular mucosal surfaces in the body such as the buccal, nasal, and vaginal cavities, can also be used to produce drug release at a specific place and over a longer length of time (Chowdary & Rao, 2003).

2.8.1 Preparation of Chitosan Loaded Nanoparticles

2.8.1.1 Ionic gelation

Ionotropic gelation (IG) is a method that uses electrostatic interactions between two ionic species to produce nanoparticles and microparticles under specific circumstances. A polymer must be present in at least one of the species. When a drug or bioactive molecule is introduced to the process, it can become caught between the polymeric chains, resulting in the drug or bioactive molecule being trapped inside the nanoparticle or microparticle structure (Pedroso-Santana and Fleitas-Salazar, 2020). Chitosan's positively charged cations are obtained by dissolving it in an aqueous acidic solution. After that, under continual magnetic stirring, this solution is added dropwise to a negatively charged anionic sodium tripolyphosphate solution. Chitosan undergoes ionic gelation as a result of electrostatic forces forming a complex between polyanion and cation, resulting in spherical particles (Naskar et al., 2019).

The ratio of chitosan (CS) to tripolyphosphate (TPP) solution determines the size of the chitosan nanoparticles. As the ratio of chitosan and tripolyphosphate rises up to a certain concentration, the size of nanoparticles increases; beyond that, aggregation occurs. Individual chitosan and tripolyphosphate concentrations also play a role (Vaezifar et al., 2013).

The efflux mechanism of P-glycoprotein on the blood–brain barrier limits methotrexate (MTX) therapy for brain tumours. The size and zeta potential of MTX NPs were measured after they were produced using the ionic gelation method. Cytotoxicity tests against the C6 glioma cell line and transport over the MDCKII-MDR1 monolayer were also carried out. The particles were shown to be cytotoxic to

C6 cells and capable of breaking through the MDCKII-MDR1 cell barrier. The findings show that even a modest quantity of Tween 80 is adequate to improve MTX transport through MDCKII-MDR1 cells from NPs. The nanocarriers appear to be a viable approach for delivering MTX to brain tumours, and they need to be studied further *in vivo* (Trapani et al., 2011).

2.8.1.2 Emulsion cross-linking or Microemulsion method

To make a water-in-oil (W/O) emulsion, an aqueous chitosan solution is first emulsified in the oil phase, and then aqueous droplets are stabilised using a suitable surfactant. The stable emulsion is then cross-linked using glutaraldehyde, one of the most versatile cross-linking agents. Cross-linking occurred between the amino groups of chitosan and the aldehyde groups of glutaraldehyde, resulting in the formation of particles (Vaezifar et al., 2013).

To make a homogenous polymer solution, sodium alginate and the muco-adhesive polymer were dissolved in filtered water. Glipizide, the active ingredient, was added to the polymer solution and vigorously stirred with a mixer to create a viscous dispersion. The resultant dispersion was then manually dropped into a 10% wt/vol calcium chloride solution using a syringe. To complete the curing process and generate spherical hard microcapsules, the additional droplets were kept in the calcium chloride solution for 15 minutes. Decantation was used to collect the microcapsules, and the resulting product was rinsed several times with water before being dried at 45°C for 12 hours (Avadi et al., 2010).

2.8.1.3 Emulsion droplet coalescence method

Two stable emulsions are created first in this approach. In liquid paraffin oil, a stable emulsion including an aqueous solution of chitosan and the medication is first created. Then, in liquid paraffin oil, another stable emulsion comprising chitosan aqueous solution of NaOH is created. The two emulsions are then blended together at high speed. When these two emulsions are combined, the droplets from each emulsion collide at random and coalesce, causing the CS droplet to precipitate and form tiny particles (Naskar et al., 2019).

2.8.1.4 Spray drying method

A nanospray drier is used to make CSNPs in this approach. The technique relies on atomized droplets drying in a heated air stream. To begin, a CS solution is made by dissolving pure CS powder in an acetic acid solution and keeping it in the refrigerator overnight. Atomization results in the formation of small droplets. The solvent is instantly evaporated from tiny droplets, resulting in the production of NPs. Using mannitol as an aerosol carrier, spray dried powders of salmon calcitonin (sCT) loaded chitosan nanoparticles (sCT-CS-NPs) were created. Ionic gelation was used to make CS/TPP nanoparticles, which were then loaded with sCT. Co-spray drying of sCT-CS-NPs and mannitol aqueous solutions resulted in micro-sized particles appropriate for inhalation (Sinsuebpol et al., 2013).

2.8.1.5 Reverse micellar technique

Water-in-oil (W/O) droplets are known as reverse micelles in the reverse micellisation process. To begin, a W/O emulsion is created by dissolving a lipophilic surfactant (sodium bis (ethylhexyl) sulfosuccinate or cetyl trimethyl ammonium bromide) in an organic solvent such as n-hexane. To avoid turbidity, an aqueous CS solution, medication, and glutaraldehyde are then added to the organic phase while continuously stirring. Finally, the NPs are extracted (Naskar et al., 2019).

2.9.2 Application of chitosan loaded nanoparticles

Mucosal delivery- For its mucoadhesive properties, CS has been widely utilised as a mucosal medication delivery carrier. Baltzley et al. (2014) looked at the possibility of using CSNPs as an intranasal delivery method to boost olanzapine systemic bioavailability. They used an ionotropic gelation technique to make these NPs. Particle size, drug loading, and in vitro release were all investigated. Al-Ghananeem et al. (2010) investigated if CSNPs might be used as an intranasal delivery method to boost didanosine systemic and brain targeting efficiency. As a result, both the intranasal mode of administration and the formulation of didanosine in CSNPs improved didanosine transport to CSF and the brain.

Cancer drug delivery- CSNPs are an emerging topic in cancer medication delivery due to the compatibility and biodegradability of CS. CSNPs have been widely utilised to deliver anti-cancer medicines such as methotrexate (Yang et al., 2008) and paclitaxel (Li et al., 2009), with the goal of increasing anti-tumor

effectiveness, controlling drug release, and directing drugs to the tumour while decreasing toxicity.

Ocular delivery- CS, a natural hydrophilic biodegradable polymer, increases stability, precorneal retention, and eye mucosa contact. Furthermore, nontoxic, minimal eye irritation, prolonged release, mucoadhesive, *in situ* gelling, transfection, and permeation boosting characteristics distinguish CS as the polymer of choice for ocular drug administration (Kapanigowda et al., 2015).

Pattani et al. (2009) observed IL-6 gene expression was not stimulated at any of the dosages tested; however, at doses more than 68.18 g/mL comparable to chitosan, a statistically significant dose-dependent increase in NO generation was found. Furthermore, as compared to the control, CSNPs demonstrated statistically significant and dose-dependent lymphocyte proliferation ($P<0.05$). When measured using differential scanning calorimetry, it was discovered that blank CSNPs caused significant membrane disruption. A wound healing model was used to assess the in vivo effects of the blank chitosan nanoparticles. In comparison to the control, blank chitosan nanoparticles produced considerably more NO in vivo. Overall, the findings show that chitosan nanoparticles are immunoactive and have a high membrane interaction potential. Bivas-Benita et al. (2004) investigated that the dendritic cell (DCs) maturation was induced by chitosan-DNA formulation, but not by chitosan solution alone, showing that the DNA was liberated from the nanoparticles and could activate DCs. When compared to pulmonary delivery of plasmid in solution or the more often utilised intramuscular vaccination method, pulmonary injection of the DNA plasmid encapsulated in CNPs was demonstrated to cause higher levels of IFN-secretion. These findings suggest that pulmonary administration of DNA vaccines against TB may be more favourable than intramuscular vaccination, and that delivery of DNA encapsulated in CNPs might result in higher immunogenicity.

2.10 *In-vitro* studies

In latin, the term *in vitro* means "inside the glass." Various *in-vitro* experimental models have been developed as an alternative to elucidate the mechanisms of biochemical modifications caused by xenobiotics and to explain the mechanisms by which these chemicals cause injury to the host tissue studied ‘outside

the animal body' due to ethical issues and the hassle involved in maintaining animal models.

The interaction of the antineoplastic drugs N, N', N"-triethylenethiophosphorainide (thioTEPA) and N, N', N"-triethylenephosphoramide (TEPA) with the DNA of LI210 cells was investigated. There were no single-strand breaks identified when L1210 cells were treated with thioTEPA alone or with thioTEPA in the presence of microsomes and NADPH. Incubation of L1210 cells for 2 hours with thioTEPA at concentrations of ≥100 μM induced a dose-dependent rise in interstrand cross-linking that peaked 2 hours later. Lymphoblastoid cell lines obtained from Fanconi's anaemia patients were significantly more sensitive to thioTEPA and mechlorethamine hydrochloride than lymphoblastoid cell lines derived from healthy people, but not to TEPA or bleomycin. This is in line with the known hypersensitivity of patient cells. The findings suggest that thioTEPA causes interstrand crosslinks, but that TEPA, the main metabolite of thioTEPA, causes alkali labile DNA damages (Ross et al., 1991).

Under varying oxygenation settings, the cytotoxicity of N,N',N"'-triethylenethiophosphoramide (thiotepa) was investigated *in vitro* in the MCF-7 human breast cancer cell line and *in vivo* in the EMT6 mouse mammary tumour model. The presence of oxygen throughout the duration of drug exposure had a significant impact on the cytotoxicity of thiotepa toward exponentially developing MCF-7 cells, with 3 log higher cell death at 500μ M thiotepa observed when the cells were regularly oxygenated compared to hypoxic cells. One or more microsomal cytochrome P-450 enzymes present in the S-9 fraction catalysed a NADPH and O2-dependent process. Triethylene phosphoramide, a thiotepa metabolite that hydrolyzes much quicker than thiotepa, was substantially less lethal to MCF-7 cells than thiotepa, indicating that the S-9 metabolite is unlikely to be responsible for the observed increase in drug cytotoxicity. Furthermore, triethylene phosphoramide cytotoxicity was only partially O2-dependent and was significantly unaffected by incubation in the presence of the S-9 preparation, indicating a different mode of action than thiotepa. These findings show that thiotepa's cytotoxic effects are oxygen-dependent and may entail metabolic processes mediated by cytochrome P-450 enzymes, at least in part (Teicher et al., 1989).

In vitro cytotoxic effects were investigated to see if the DLNPs had any cytotoxic or anti-proliferative properties. Cellular damage produced by DLNPs against anti-cancer medicines was utilized to assess toxicity. The percent survival following treatment with medicines and DLNPs was investigated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test. The MTT test is a colorimetric method for determining cell metabolism. Under certain situations, NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of live cells present. The tetrazolium dye MTT may be reduced by these enzymes to its insoluble formazan, which has a purple hue.

In live cells, MTT, a yellow tetrazole, is converted to purple formazan. To dissolve the insoluble purple formazan result into a coloured solution, a solubilization solution (typically dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulphate in diluted hydrochloric acid) is added. A spectrophotometer may be used to measure the absorbance of this coloured solution at a certain wavelength (generally between 500 and 600 nm).

In hepatic metastasis-bearing mice, free doxorubcin and doxorubicin coupled with polyisohexlycyanoacrylate were evaluated for therapeutic efficacy. The M 5076 reticulum cell sarcoma was the source of the metastases. The decrease in the number of métastases was considerably greater with the doxorubicin-loaded nanoparticles than with free doxorubicin, regardless of dosage or delivery schedule. Histological testing definitely verified this. Although pharmacological and pharmacokinetic results showed that the nanoparticles were well captured by the hepatic metastasis, the mechanism of nanoparticle therapeutic efficacy remains unknown (Chiannilkulchai et al., 1989). Soma et al. (2000) observed that in mice with hepatic metastases of the M5076 tumor, doxorubicin-loaded polyalkylcyanoacrylate nanoparticles were shown to be more effective than the free drug.

CHAPTER III

MATERIALS AND METHODS

The nanoparticles were synthesized using many ways, and their standardization was carried out using the available techniques. The size, surface shape, zeta potential, encapsulation efficiency, and release kinetics of thiotepa loaded chitosan nanoparticles in the proposed system were all characterized physiochemically. Routine pH, viscosity, and zeta size average were also analyzed to assess the stability of the created nano solutions. The experiment was carried out on a mouse lymphoblast leukemia (L1210) cell line which was obtained from the National Centre for Cell Science in Pune,

3.1. Chemicals and reagents

Thiotepa and Sodium tri-polyphosphate (TPP) were obtained from Sigma Aldrich Co. LLC, New Delhi. Low molecular weight chitosan powder, acetic acid was purchased from Merck (Mumbai, India).

Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), Dulbecco's Phosphate Buffered Saline and Antibiotic–Antimycotic solutions were acquired from HiMedia Laboratories Pvt. Ltd. (India), trypan blue and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were secured from Sigma Chemical Co. (St Louis, MO, USA). Dimethyl sulphoxide (DMSO), ethanol (96%) and glacial acetic acid (100%) were procured from Merck (Darmstadt, Germany). Sterile cell culture flasks and culture plates were purchased from Nest Biotech Co. Ltd, China. All other chemicals utilized were of expository evaluation and bought from manufacturers. Buffers and reagents were prepared in autoclaved Milli-Q water and filtered through membrane filters (0.22 μm) which were bought from Millipore (Molsheim, France).

3.2 Preparation of nanoparticles

Chitosan nanoparticles/ sodium tripolyphosphate nanoparticles

Low molecular weight chitosan was dissolved in acetic acid at a concentration of 1 % (w/v). By adding the necessary amount of NaOH, the pH of the solutions was raised to 4.6–4.8. Dissolving sodium tripolyphosphate in distilled water yielded aqueous sodium tripolyphosphate solution. The tripolyphosphate solution was then

dropped into a chitosan solution while being agitated with a magnetic stirrer. The solutions were taken in various ratios, such as 2:1, 3:1, 4:1 and 5:1, with the former being the chitosan solution and the latter being TPP solution. Drug was added in a 2:1 ratio to the TPP solution, this solution was vortexed so as to make a uniform solution. Drug-TPP solution was subsequently added dropwise into the chitosan solution kept for stirring on the magnetic stirring at 700 rpm for 4-6 hours. The solution was then given an ultrasonication bath.

0.1% of chitosan in 1% acetic acid solution and 0.1% of TPP in conductivity water were formulated separately.

↓

Thiotepa was then added to the TPP solution. This solution was then added dropwise to the chitosan solution.

↓

Different ratios of Chitosan: TPP i.e., 1:1, 2:1,3:1, 4:1and 5:1 was taken.

↓

Ultrasonication bath

3.3 Physico-chemical characterization of nanoparticles

3.3.1 Particle size and zeta potential:

The size, size distribution and zeta potential of the nanoparticles were analyzed by Dynamic light scattering (DLS). DLS measurements were performed using a dynamic light scattering instrument model Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) having 632.8nm red laser at 25^0C at an angle of detection of 173^0 with incubation time of 60 seconds in the round aperture glass cell. It was done at Central Institute of Post-Harvest Engineering and Technology, Ludhiana.

The nano samples were made by diluting the nano-suspension 1:50 times and afterward assessing the zeta size and zeta potential of nanoparticles. The investigation of size was done in a ZS 90 Malvern cuvette which was put in the analysis chamber to get different peaks and next to detect its average zeta size. For zeta potential analysis a disposable folding capillary cuvette was used. Suitably, the results of z-average diameter, polydispersity index (i.e., relative width of the particle size distribution) and zeta potential of the nano-dispersion were recorded.

3.3.2 Particle Shape

Electron microscopy (EM) is used to determine the morphology of nanosuspensions particles. These techniques play an important role in validating the

reliability of other routine particle sizing techniques such as Dynamic light scattering. Transmission electron microscopy (TEM) provide two-dimensional pictures with nano- and microscopic detail of nanoparticles. Internal composition features like crystallinity and lattice structure were determined via TEM. It displays particle information in both qualitative and quantitative terms. The transmission electron microscope (TEM) uses electrons instead of light as a "light source," and their significantly shorter wave length allows for a thousand times greater visual resolution than a light microscope. The morphology of the nanoparticles in the suspension can be seen using TEM. The sample was imaged using a Hitachi H-7650 with a 40-120 kV accelerating voltage; 0.2 nm resolution; magnification range 200x-200000x in high contrast (HC) mode; 4000x-600000x in high resolution mode (HR); tungsten and LaB6Filaments; and a 1024x1024 pixels digital camera. It was carried out in the Punjab Agriculture University's Ludhiana Electron Microscope Lab. The TEM sample was made by centrifuging 1 ml of the solution at high speed and then resuspending the pellet in deionized water. This was then applied on the support grids, dried, and then examined.

3.3.3 Encapsulation Efficiency:

The difference between the total amount of drug added to the preparation at the start and the amount of free drug remaining in the solution was used to calculate the nanoparticle's encapsulation efficiency. 1 mL of the sample was placed in an ultracentrifuge tube and centrifuged for 30 minutes at a high speed of 20000 g using micro ultracentrifuge. The amount of free drug in the supernatant was measured by UV spectrophotometer at 227 nm. The samples were analyzed in triplicates. The encapsulation efficiency (EE) was calculated by the equation. (Manimekalai et al., 2017)

$$EE\% = \frac{(T-F)}{T} \times 100$$

Where F represents free drug in the supernatant and T is the total amount of drug added into nanosuspension.

3.4 Drug release from the nanoparticles:

The quantity of drug that will be released in a biological system is determined by drug release tests of the nano-suspension. For *in-vitro* drug release estimate, the

dialysis bag diffusion method was utilized (Venkateswarlu and Manjunath 2004). Washing under running water and overnight incubation in phosphate buffer pH 7.0 at 37°C regenerated the dialyzing bag (4Å pore size), which was 10cm long. 3 mL of nano-suspension was put within a pre-washed dialysis tube, which was then hermetically sealed before being dialyzed against 30 mL of 0.2M phosphate buffer pH 7.0. This system was kept at 37°C in a water bath shaker with light agitation. 1ml of the sample was taken from the system at predefined intervals and tested for drug estimation. The samples were taken at intervals of 0, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 10 hours, 12 hours, 24 hours. To achieve a cumulative release pattern, the whole buffer solution was replaced every time using fresh buffer.

3.5 Standard preparation

3.5.1 Stock and working standards

Stock solution of thiotepa (standard drug), drug loaded nanoparticles, blank nanoparticles were prepared in stock media and further working standards were prepared in DMEM.

3.5.2 Composition of phosphate buffer Saline (PBS)

Sodium chloride (NaCl)	8.0 g
Potassium chloride (KCl)	0.20g
Disodium hydrogen orthophosphate (Na_2HPO_4)	1.44g
Potassium di-hydrogen phosphate (KH_2PO_4)	0.27g

The above given components were mixed in 800ml of milli-Q water. Then pH was adjusted to 7.4 with the help of 0.1M NaOH and 0.1M HCl. Final volume was made up to 1000 ml. PBS solution was autoclaved and used for the experiment.

3.6 Culture medium and supplements

3.6.1 Stock medium

To 500 ml Dulbecco's Minimum Essential Medium- High Glucose (DMEM-HG) medium, 3.7 g of NaHCO3 was added. This solution was sterilized via filter assembly sterilization method by passing through 0.22 μm filter membrane. An aliquot from the filtered stock solution was incubated at 37°C for 48 hours to check the contamination and rest of the stock solution was stored at refrigerated temperature till further use.

3.6.2 Growth medium (GM)

For 100 ml cell culture growth medium following compositions were used:

Components	**Amount**
Stock DMEM	70 ml
Vitamins	1.25 ml
Triple distilled water (TDW)	13.75 ml
Fetal Bovine Serum (FBS)	15 ml
Amoxicillin	60 µl
Gentamycin	250 µl

The pH of the GM was adjusted to 7.2. It was then sterilized with a 0.2 µm syringe filter, a small quantity of the GM was incubated at 37°C to ensure sterility, and the rest was kept at 4°C.

3.7 Collection of cancer cell lines

L1210 cancer cell samples were obtained from National Centre for Cell Science (NCCS), Pune.

3.7.1 Processing and sub culturing of cell lines

Contamination free cancer cell suspension was cultured in 25 cm^2 cell culture flasks. Flasks were incubated for 48-72 hours at 37 °C. Flasks were observed under inverted microscope to check the morphology of dividing cells. GM was replaced after every 2-3 days or according to requirement and cell cycle. When the confluency of flask was around 70-80% with cells, it was sub-cultured to another 25 cm^2 flask.

For subculturing, cells along with media was centrifuged at 800 rpm for 8-10 minutes, cell pellet obtained was washed with PBS (pH- 7.2) twice (to remove any debris) and finally resuspended in GM. Gentle pipetting (to prevent bursting of cells) was done for the formation of uniform suspension. Fresh sterile 25 cm^2 flasks were seeded with the cells at a split ratio of 1:2. To the newly seeded flasks 5ml of GM was added and incubated at 37 °C in growth chamber having controlled humidity and CO_2 level for proper multiplication and growth of cells. Microbial contamination was routinely examined (every 12 hours) in the flask, and the cell development pattern was seen under an inverted microscope. When there was a change in the pH of the medium, it was replaced.

3.7.3 Cryopreservation of cells

For future use cells showing exponential growth were cryopreserved using the freezing mixture.

3.7.3.1 Freezing mixture

Components	Amount (%)
Growth media (GM)	60
Fetal Bovine Serum (FBS)	35
Dimethyl sulfoxide (DMSO)	5

For appropriate mixing, the freezing mixture was gently vortexed and filtered through a 0.22 μm sterile syringe filter membrane before being kept at 4°C.

3.7.3.2 Freezing of cells

Harvested cells were centrifuged at 800 rpm for 10 minutes. The supernatant containing media was discarded, and the obtained cell pellet was resuspended in the freezing solution. This cell suspension was poured as 1ml aliquot into sterile cryovials, they were labelled and the lids were firmly closed. To avoid temperature shock, vials were gradually cooled before being stored in liquid nitrogen, then placed in a deep freezer (-80°C) overnight, and lastly moved into a liquid nitrogen container.

3.8 Cell Viability

Cells were centrifuged at 800 rpm for a period of 10 minutes, the supernatant was discarded, cell pellet obtained was diluted in 1 ml of the GM. Cell suspension of 20 μl was taken in a 1.5 ml microcentrifuge tube and to this 180 μl of filtered 0.1% trypan blue dye solution, made in PBS, was added. Pipetting of the solution followed by immediately charging of the hemocytometer was done. It was left to stand for a minute. The living (bright) and dead (blue) cells in 64 secondary squares were counted under a light microscope at 10x magnification (4 large corners squares). The following calculations were then made.

- Average number of viable cells = (Total cell count in the large corner squares) /4
- Total viable cell count/ml = (Average number of viable cells) $\times 10^4 \times 10$ (Dilution Factor)

$1x10^5$ cells/mL of culture media were being used as cell concentration. Cells were cultured in culture flasks with GM at 37 ^{0}C in a humidified environment with

5% CO_2. Cells were sub cultured when the cells reached more than 80% confluency. The trypan blue dye exclusion technique was used to evaluate cell number and viability. In the studies, cell suspensions with a vitality of greater than 95% were utilized.

3.9 Experimental protocols

3.9.1 Inhibitory concentration (IC_{50}) determination

Working solution of thiotepa and drug loaded nanoparticles ranging between 10-500 µM were prepared in maintenance medium. L1210 cells were exposed to these concentrations for 72hrs time period. After completion of exposure period cell viability assay/MTT assay was performed to calculate the IC_{50} concentration. A log dose response curve was plotted for the determination of IC_{50}.

3.9.2 MTT cytotoxicity assay

The most extensively used methods to measure cellular viability are tetrazolium-based assays. Tetrazolium salts are redox sensors that can be reduced into formazan derivatives by metabolically active cells, which may then be dissolved for spectrophotometric analysis.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) testing involves metabolically active cells carrying NAD(P)H-dependent oxidoreductase enzymes converting the water-soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to insoluble formazan crystals. Indirectly the assay can also determine the cytotoxicity and growth rate. The MTT assay is based on the protocol as described by (Ezhilarasi et al., 2013).This assay was standardized for the L1210 cancer cell line used in this study.

The viability test was done on 96-well cell culture plates. Cells were seeded at a density of 2 $x10^4$ cells/mL (5,000 cells/well) and grown for 24 hours before being exposed to thiotepa-loaded nanoparticles (10-500 g/ml). At the end of the exposure period, an MTT experiment was carried out (48hrs). Each well received 20 µl of 5 mg/ml MTT dye, with one set of wells receiving MTT but no cells (control). The experiment was carried out in an aseptic manner. Under the culture hood, the plates were incubated for 3.5 hours at 37°C in the dark. After that, using a plate centrifuge, the medium was extracted without disturbing the cells. 150 µl MTT solvent (DMSO) was used to extract formazan. Tinfoil was used to cover the microplate, and

the cells were shaken on an orbital shaker. The absorbance was measured at 570 nm after 30 minutes. Each group was tested three times in three different wells. The absorbance levels and findings were expressed as a percentage of the control values after analysis. The toxicity of thiotepa and drug loaded nanoparticles or the percentage of cell inhibition was expressed using the following relationship:

$$\% \text{ Of cell inhibition} = \frac{\text{Absorbance (570 nm) tested compound}}{\text{Absorbance (570 nm) control}} \times 100$$

On the basis of these results, IC_{50} was calculated to be 96.27 µM for thiotepa and 76.29 µM for drug loaded nanoparticles. Further IC_{25}, $IC_{12.5}$ and $IC_{6.25}$ values were calculated.

3.10 Cell Morphology

Thiotepa loaded chitosan NPs-induced morphological alterations in MDBK cells were examined under an inverted phase contrast microscope (Nikon ECLIPSE-Ti, Japan). Varying degree of morphological changes such as cellular swelling, rounding of cells, chromatin condensation, cell shrinkage, formation of blebs, nuclear degradation, plasma membrane disintegration and loss of cytoplasmic content were examined.

3.11 Controlled release kinetic models

Drug release kinetics from nanoparticles was carried out using conventional and our novel models with the aim of finding a general model applicable to multi mechanistic release.

3.11.1 Zero Order Kinetic Model

Dissolution of the drug from pharmaceutical dosage forms that do not disaggregate and release the drug slowly and they don't depend on the concentration of the drug can be represented by the following equation (Lokhandwala et al., 2013): The equation is

$$C = C_0 - K_0 t$$

Where, C = Total amount of drug release or dissolved (supposing that drug release occur immediately after the drug is dissolved), C_0 = Amount of drug in solution at 0-hour, K_0= Zero order rate constant, t = total time taken to study release kinetics

The graph is plotted between the amount of cumulative drug released verses time.

3.11.2 First Order Kinetic Model

The application of this model to drug dissolution studies was first proposed by Gibaldi and Feldman. (1967). Although it is difficult to conceptualise this process on a theoretical basis, this model has been utilised to describe the absorption and/or elimination of various medications. It is dependent on the drug concentration (Lokhandwala et al., 2013). The drug release which follows the first order kinetic can be expressed by the equation

$$\text{Log } C = \text{Log } C_0 - K_t/2.303$$

Where, C_0= Concentration of drug at 0-hour, K=First order rate constant, t=time

The data obtained are plotted as log of cumulative percentage of drug remaining verses time, which gives a straight line with slop=K/2.303.

3.11.3 Korsemeyer-Peppas Model (The Power law)

Korsemeyer et al. (1983) derived a simple relationship which describes the release of drug from a polymeric system. To illustrate the mechanism of drug release, first 60% of drug release data was fitted in Korsemeyer-Peppas model.

$$Ct/C\infty = kt\ n$$

Where, $Ct/C\infty$=fraction of drug release at time t. k=rate constant n=release exponent. A modified form of this equation was developed to adjust the lag time (l) in the beginning of release of drug from the pharmaceutical dosage form.

$C(t-1)/C\infty = a(t-l)_n$ where there is possibility of a burst effect, b this equation becomes

$$Ct/C\infty = at_n + b$$

In the absence of lag time or burst effect l and b values would be zero and only at_n is used. This mathematical model, also known as the "Power Law", has been used very frequently, to describe the drug release from several different Pharmaceutical modified release dosage forms.

CHAPTER IV

RESULTS AND DISCUSSIONS

Cancer is a prominent cause of death in the globe. Despite improvements and intensive research on novel therapies like as immunotherapy and gene therapy, surgery, radiation, chemotherapy, and immunotherapy remain the most common treatments now available (Zhu & Liao, 2015). These medicines are unquestionably effective in the elimination of cancer cells, but they come at the cost of an ever-increasing number of side effects (Bisht & Rayamajhi, 2016). Except for a few cancer types (e.g., breast cancer), which are treated with hormone treatment or immunotherapy, cytotoxic medicines are still the most common kind of cancer chemotherapy.

Generally, cytotoxic medications are given as an intravenous bolus or infusion, usually in the form of free drug solutions. Thiotepa (TH, triethylene thiophosphoramide) is a cytotoxic ethylene amide that was created by Lederle Laboratories in 1952 and has mechlorethamine-like alkylating action. It's most commonly used as a second-line therapy for breast cancer. Intrathecally for carcinomatous meningitis and intravesically for bladder carcinoma, TH has been used. TH has shown to be one of the most effective medications in bone marrow transplantation (Abdul-Hai et al., 2007)

Traditionally given cytotoxic drugs frequently attach to bodily tissues and serum protein in an unpredictably large and indiscriminate manner. Only a tiny percentage of the drugs make it to the tumour. This might lower therapeutic effectiveness while also increasing systemic medication toxicity. Furthermore, while cytotoxic medications are designed to kill cancer cells solely, they are also hazardous to non-cancerous cells, particularly rapidly proliferating cells such as bone marrow cells and gastrointestinal tract cells. Even when typical therapeutic dosages of anticancer medicines are provided, normal tissue toxicities occur.

The non-specific effect of cytotoxic chemicals can irritate or harm different organs or tissues, depending on the medicines used. While practically all cytotoxic medicines have side effects such as nausea, vomiting, exhaustion, and hair loss, other side effects are drug-specific. Anthracyclines, for example, can cause cardiotoxicity

(Wong et al., 2007), as well as a local toxic impact leading in thiotepa hyperpigmentation (Horn et al., 1989)

The use of nanoparticles to deliver small compounds in anticancer treatment is a fast - growing field of study (Chen, 2010). Nanomaterials have the following distinguishing characteristics, as a result of which they have lately become a heavily debated research issue and a viable alternative to traditional cancer treatment methods. 1) Biomolecules, which are similar in size to NPs, play a key function in regulating the body's numerous cellular cycles and preserving cellular homoeostasis. NPs may be targeted in any system in the body and imitate the function of biomolecules with correct engineering, thereby hacking the body's system biology for human advantage. 2) Because of their tiny size, NPs are extremely soluble, and their solubility can be further enhanced by suitable surface modification. 3) Because NPs have a large surface area to volume ratio, they have plenty of room to encapsulate medicines and other materials, resulting in a greater therapeutic payload. 4) Because of their selective targeting, NPs can deliver a therapeutic payload to the target while minimizing negative effects in normal cells. Aside from cancer, nanomedicine is increasingly being used in personalized medicine as well as the detection and treatment of cardiovascular disorders (Bisht & Rayamajhi, 2016).

The present study aimed at developing thiotepa loaded nanoparticles using biodegradable nanocarrier and then comparing this system to the standard drug preparation to determine the best suitable. Dimensionality, surface charge, surface modification, and hydrophobicity are all factors that influence the reactivity, physical, and chemical characteristics of nanoparticles. Nanocarriers with optimum physical, chemical, and biological qualities can enter cells where bigger materials would be rejected or destroyed. Nanocarriers of smaller diameters (50–300 nm) can interact with biomolecules in the cell or on the cell surface, and hence can influence biological responses (Bronze-Uhle et al., 2017). The nanoparticles consisting of biodegradable polymers and drug encapsulation are appropriate for long-term intracellular drug delivery, especially for drugs with cytoplasmic targets.

Slight changes in the physio-chemical characteristics of the nanoparticle can have significant biological ramifications in the cellular absorption and biological processes of the nanoparticle (Alexis et al., 2008). As a result, multiple

formulations were created, and their physio-chemical characteristics as well as the system's release kinetics were compared. Furthermore, the system was also put through pharmacodynamics experiment, which included comparing it to a commercially available drug on a cancer cell line to see what the minimal inhibitory concentration was.

4.1 Thiotepa loaded chitosan nanoparticles

4.1.1 Overview

Deacetylation of chitin, a polysaccharide found abundantly in nature, is a convenient way to get chitosan (e.g., crustaceans, insects and certain fungi). Chitosan is a linear copolymer composed of -(1-4)-linked d-glucosamine and N-acetyl-d-glucosamine units. Chitosan has been investigated for pharmaceutical formulation and drug delivery applications, with its absorption-enhancing, controlled-release, and bio adhesive characteristics being of particular interest. This polymer has been demonstrated to be both biocompatible and biodegradable after being synthesized from a naturally occurring source (Dodane & Vilivalam, 1998). Chitosan becomes a polyelectrolyte, positively charged in acidic environments due to the protonation of the $–NH_2$ groups (Rinaudo et al., 1999). The CS-STPP nanoparticles are prepared using sodium tripolyphosphate (STPP) as a cross-linker. In nature, it is non-toxic and biodegradable.CS nanoparticles are frequently utilized in colon and mucosal delivery, cancer treatment, and target medication delivery because they are positively charged and have mucoadhesive characteristics. The electrostatic interaction between the protonated amine group of chitosan and the negatively charged group of a polyanion (such as TPP) or an anionic polymer results in the production of chitosan NPs (Kouchak & Azarpanah, 2015).

4.1.2 Analysis of Particle size and zeta potential

Ionic cross-linking method was used to make chitosan nanoparticles with slight modifications. Sodium tripolyphosphate was the most typical cross-linking agent. Two components with opposing charges united to generate nanoparticles when it was constantly added to a water solution of chitosan with steady stirring at optimum condition (Amidi et al., 2010). In the present study, various ratios of CS: TPP were taken and their particle size, zeta potential and polydispersity were analyzed.

Table 1: Particle size and PDI of thiotepa loaded nanoparticles for varying concentration at 5% level of significance

S. No.	Chitosan Concentration	Z- Average	PDI
1	1	1472.0^{a}±51.06	0.7630^{a}±0.07
2	0.5	543.4^{b}±28.64	0.6347^{b}±0.05
3	0.25	465.6^{b}±25.35	0.5977^{b}±0.04
4	0.1	212.1^{c}±3.05	0.3690^{c}±0.06

There is no significant difference between 0.5 percent and 0.25 percent concentrations, according to Critical Value for Comparison (83.484) of Z-size avg. and Critical Value for Comparison (0.1539) of PDI. Although there is a substantial variation between the concentration levels of 1 and 0.1 percent. According to the findings, chitosan with a concentration of 0.1 % has the lowest particle size of 212.1nm and a mean polydispersity index of 0.3690. The results obtained were found to be similar with the study conducted by Amanjot. (2019).

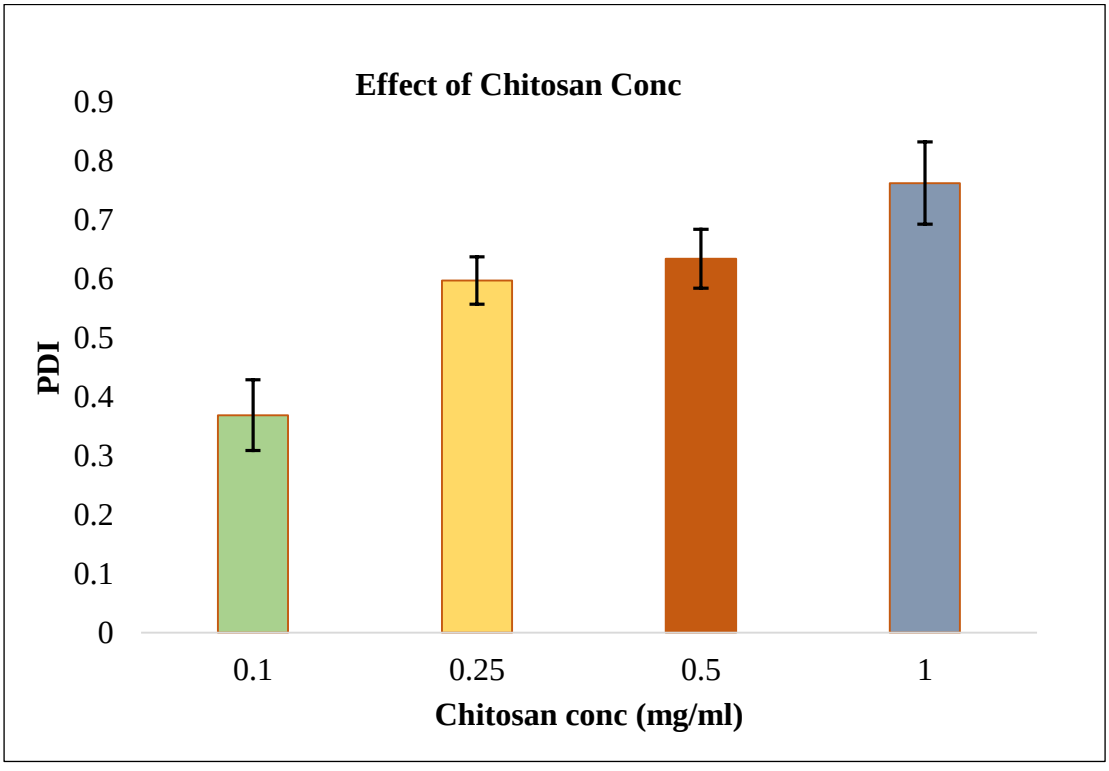

Fig. 2: Comparison between varying concentration of thiotepa loaded chitosan nanoparticles for PDI

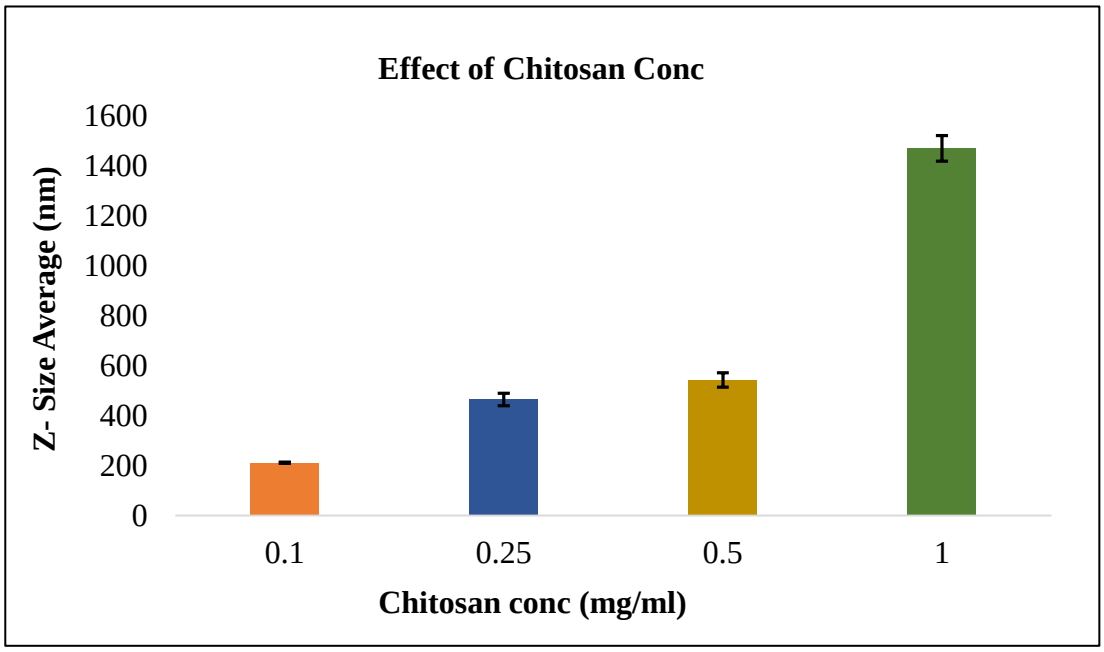

Fig. 3: Comparison between varying concentration of thiotepa loaded chitosan nanoparticles for particle size

Results

			Size (d.nm):	% Intensity:	St Dev (d.n..
Z-Average (d.nm):	213.5	Peak 1:	266.8	96.0	188.3
PdI:	0.373	Peak 2:	4953	4.0	636.8
Intercept:	0.947	Peak 3:	0.000	0.0	0.000
Result quality :	Good	D(10): 91.6		D(50): 267	D(90): 642

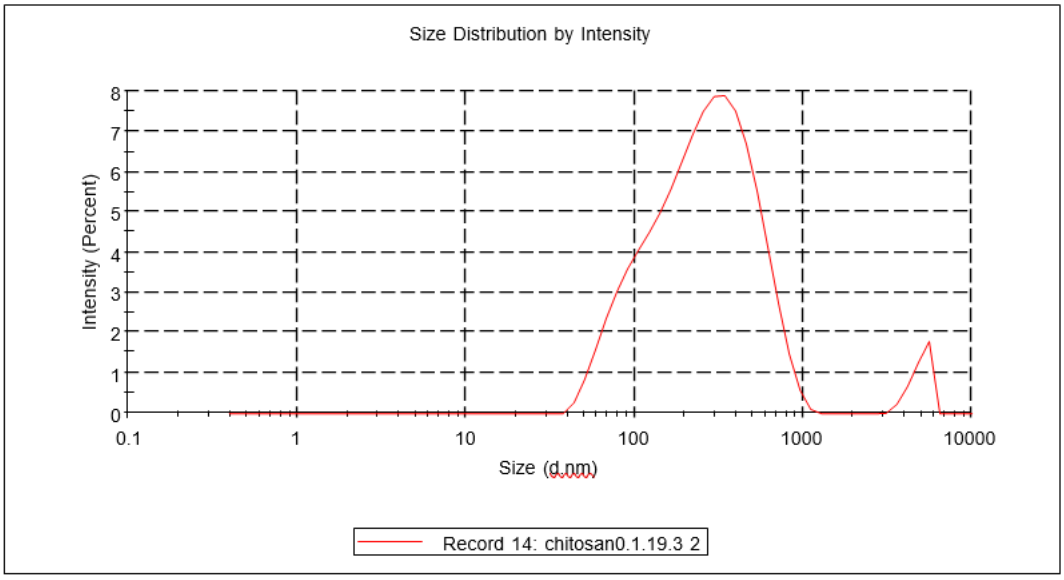

Fig. 4: Particle size at 0.1% chitosan concentration for Thiotepa loaded chitosan nanoparticles

Table 2: Particle size and PDI of thiotepa loaded nanoparticles for varying formulation at 5% level of significance

S. No.	Formulations	Z- Average	PDI
1	(5:1) F1	1032.3^{a}±150.70	0.9877^{a}±0.01
2	(4:1) F2	944.0^{a}±48.84	0.7887^{b}±0.03
3	(3:1) F3	212.1^{b}±3.05	0.3690^{c}±0.06
4	(2:1) F4	307.3^{b}±34.44	0.4853^{d}±0.01

Various formulations including specific and moderate concentrations of chitosan and tripolyphosphate have been developed earlier. In this investigation, four formulations were created using different chitosan ratios: particle size and polydispersity of chitosan to tripolyphosphate ratios of 2:1, 3:1, 4:1, and 5:1 were determined. F1 formulations had a 2:1 chitosan:TPP ratio, F2 formulations had a 3:1 chitosan:TPP ratio, F3 formulations had a 4:1 chitosan:TPP ratio, and F4 formulations had a 5:1 chitosan:TPP ratio. Table no. 2 and fig. 4 shows the particle sizes and polydispersity index of chitosan nanoparticles in various ratios of chitosan and TPP (tripolyphosphate) solutions.

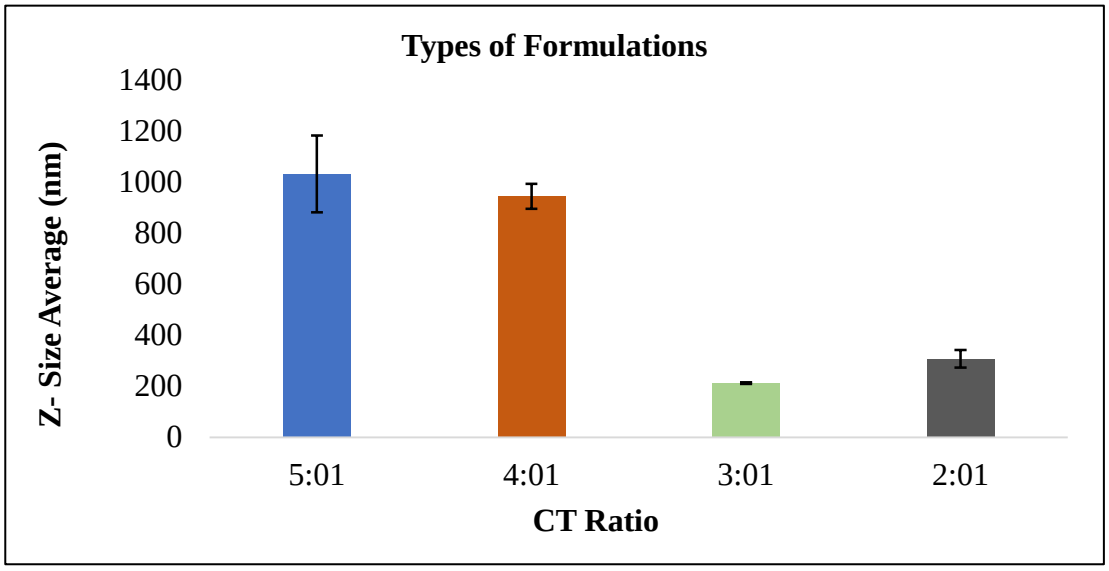

Fig. 5: Comparison between varying formulations of thiotepa loaded chitosan nanoparticles for particle size

The data was analyzed using one way analysis of variance (post hoc tukey's) which showed that the Critical Value for Comparison (215.33) and Critical Value for Comparison (0.0994) suggests that there is a significant difference among

formulations for zeta size average and PDI. F3 formulations are of appropriate size and have the lowest PDI value, so they were chosen for further studies. Results were relevant to as obtained by (Vaezifar et al., 2013).

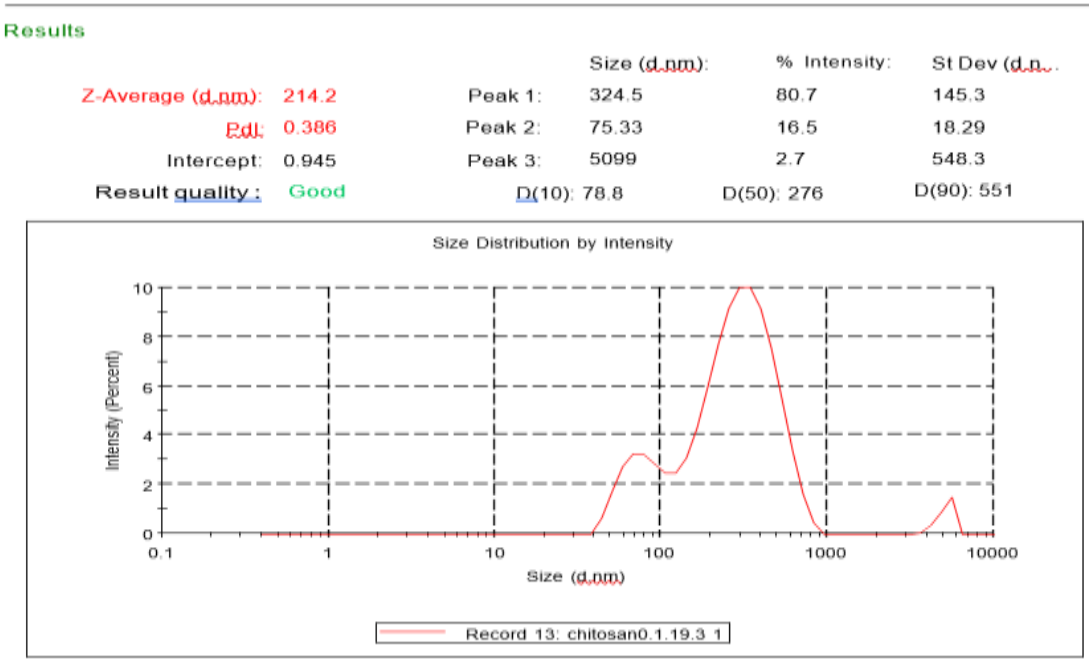

Fig. 6: Particle size at 3:1 chitosan to TPP ratio for Thiotepa loaded chitosan nanoparticles

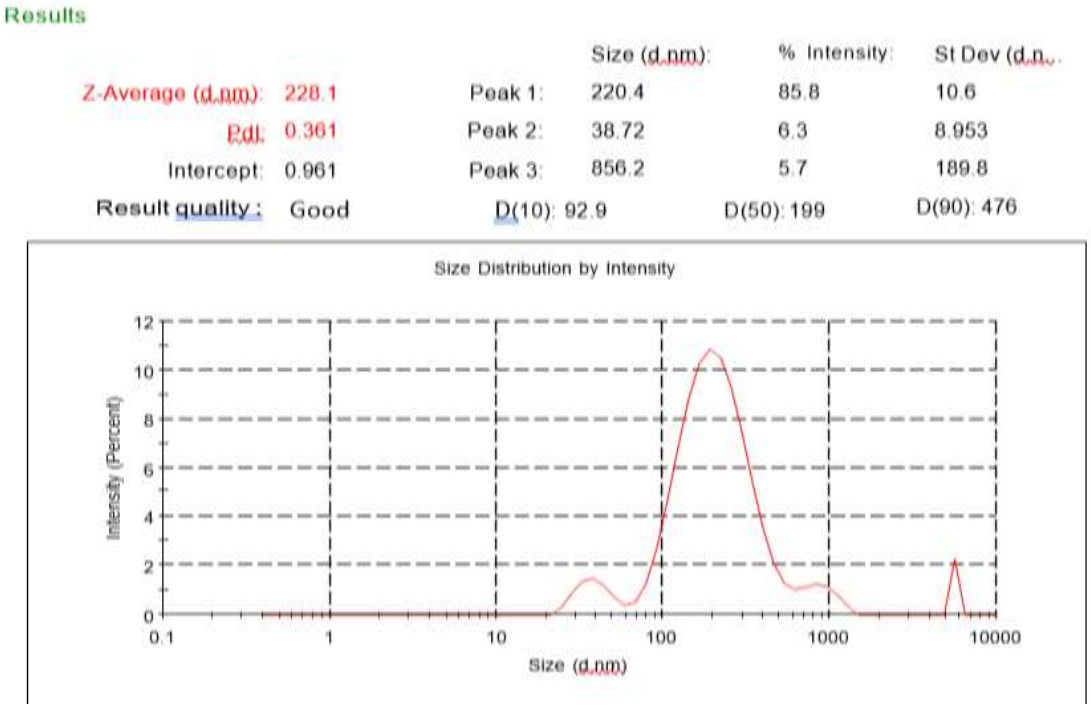

Fig. 7: Particle size at 45 min sonication time for 3:1 formulation

Table 3: Particle size and PDI of chitosan nanoparticles for different sonication timing at 5% level of significance

S. No.	Sonication time (min)	Z- Average	PDI
1	15	500.07^{b}±48.15	0.5607^{b}±0.012
2	45	249.73^{a}±15.97	0.3747^{a}±0.03

The critical value for comparison (81.40) for Z size average and the critical value for comparison (0.0631) for PDI show that the varied sonication times have a significant difference. The particle size and polydispersity index were at their lowest at 45 minutes (Table no. 3 and fig no. 6). The results were in accordance with the one obtained by (Bohrey et al., 2016) according to there findings it may be stated that when the sonication duration is increased, the applied energy rises, resulting in a reduction in nanoparticle size (from 430 to 265 nm).

Table 4: Particle size and PDI of thiotepa loaded nanoparticles for varying pH at 5% level of significance

S. No.	pH	Z- Average	PDI
1	4	469.17^{a}±54.75	0.5877^{a}±0.02
2	4.6	200.37^{b}±5.40	0.3380^{b}±0.01
3	5	787.00^{c}±28.05	0.8827^{c}±0.05

The above data was analysed statistically using one way analysis of variance (post Hoc tukey's) which indicates that there is a significant variation in pH value for the Critical Value for Comparison (89.376) of z-size avg. and the Critical Value for Comparison (0.0858) of PDI which indicates the efficiency of chitosan nanoparticles to be used as a standard and the nanoparticles having the appropriate sizes were selected for further studies. The observed mean particle size was 200.37 at 4.6-4.8 pH, and the mean polydispersity index was 0.3380, which is within the permissible range for PDI. Similar findings were reported by Gharedaghi et al. (2012), which stated that the pH of the buffer has a significant but non-linear influence on the size of chitosan nanoparticles generated using this approach; in general, raising the pH value of the buffer tends to reduce the size of nanoparticles.

Particle size and pH have the opposite relationship. High pH levels, i.e., 4.9, were shown to be the best conditions for producing nanoparticles with a minimum size of 50–200 nm.

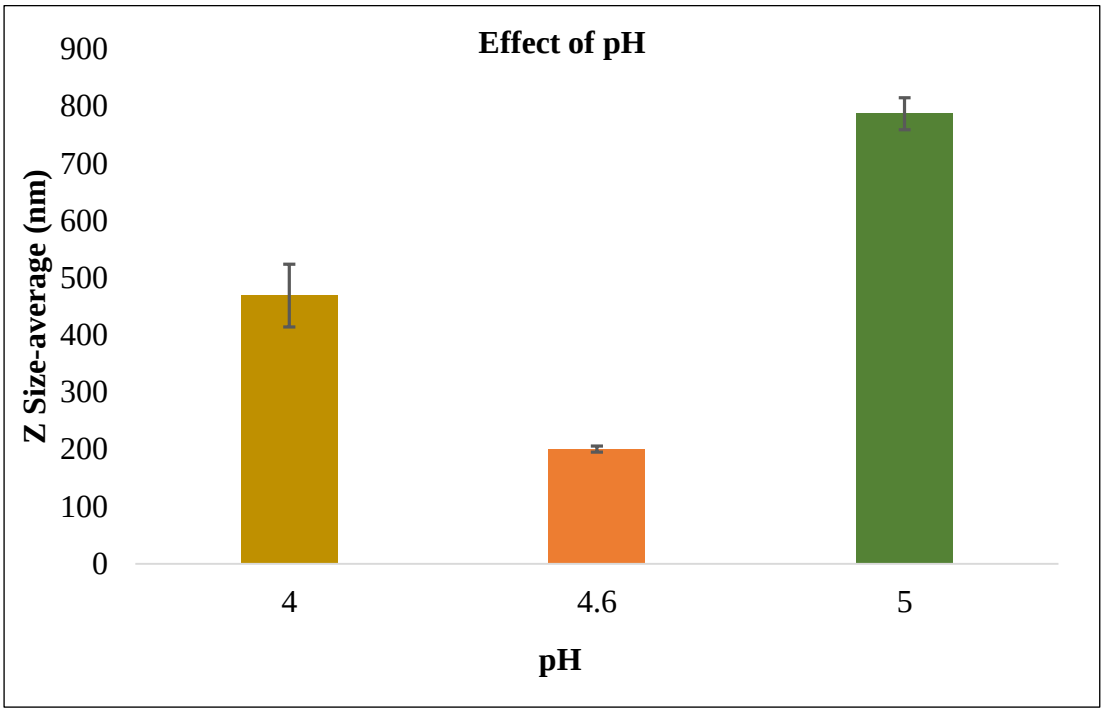

Fig. 8: Comparison between varying pH of thiotepa loaded chitosan nanoparticles for particle size

The interaction of the positively charged particle surface with the negatively charged mucosa increases the delivery system's residence time at the absorption site. Zeta potential 24.93±0.49 mV for thiotepa loaded nanoparticles.

Table 5: Z-potential of thiotepa loaded chitosan nanoparticles

S. No.	Z-potential	Z- potential avg.
1	24.7	24.93±0.49
2	25.5	
3	24.6	

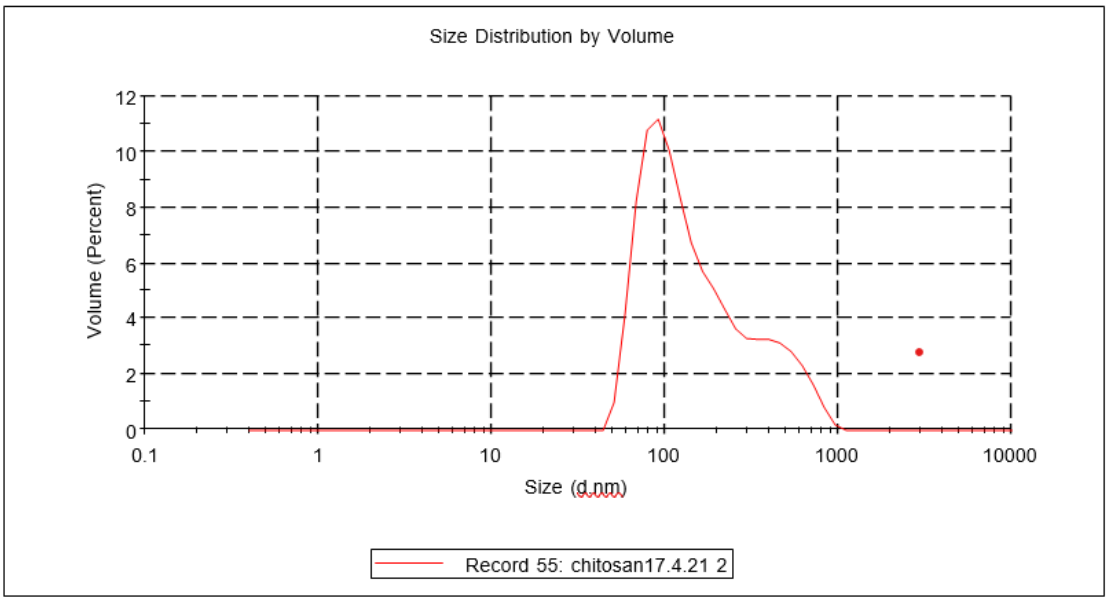

Figure 9: Z-Size Average 202.6nm and polydispersity index 0.323 for Thiotepa loaded Chitosan nanoparticles (pH- 4.6 to 4.8)

Results

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	20.9	Peak 1:	20.9	100.0	7.43
Zeta Deviation (mV):	7.43	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm):	0.153	Peak 3:	0.00	0.0	0.00
Result quality :	Good				

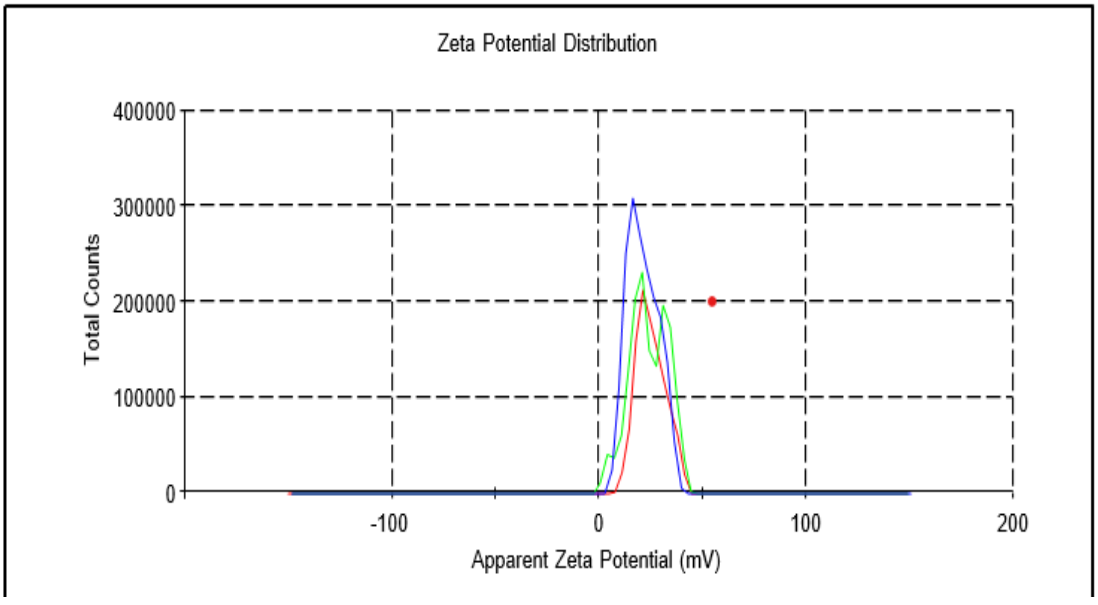

Figure 10: Zeta Potential of blank nanoparticles

Results

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	24.6	Peak 1:	27.1	76.4	4.62
Zeta Deviation (mV):	6.91	Peak 2:	14.7	23.6	3.29
Conductivity (mS/cm):	0.158	Peak 3:	0.00	0.0	0.00
Result quality :	Good				

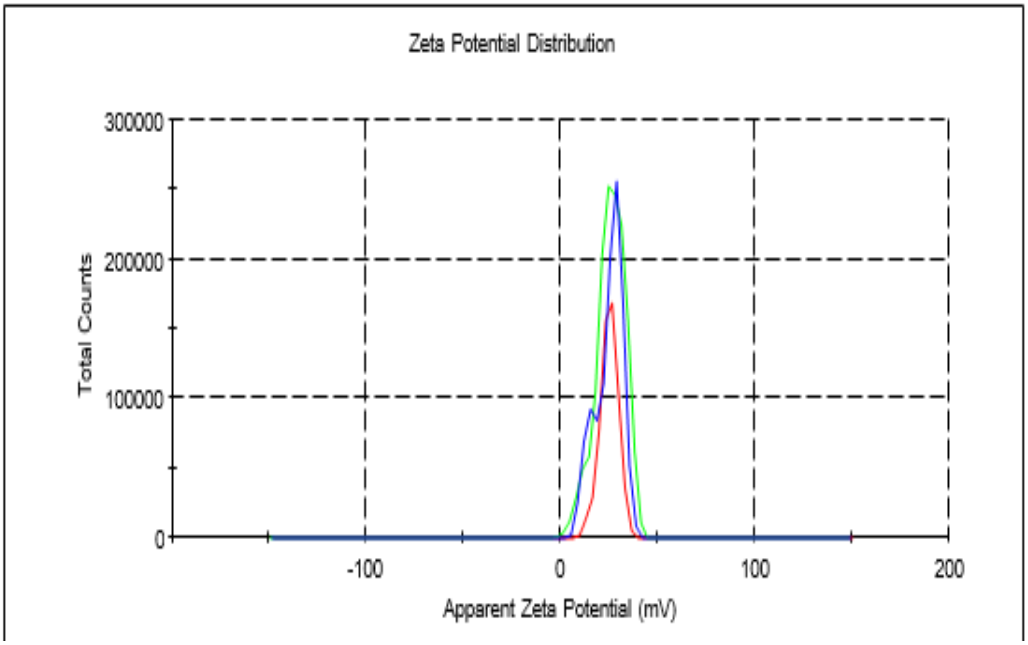

Figure 11: Zeta Potential of drug loaded nanoparticles

4.2 Encapsulation efficiency

By estimating the quantity of unentrapped drug, the amount of thiotepa entrapped within the nanoparticles was estimated by an indirect technique. As previously indicated, the nanosuspension was centrifuged using ultracentrifuge, and the clear supernatant containing the free unentrapped drug was collected, diluted with distilled water, and spectrophotometrically measured at the wavelength with the maximum thiotepa absorbance (max) 227 nm. The E.E % for thiotepa loaded chitosan NPs 56.815 ± 0.968736, which was obtained with specific ratios of drug loaded tripolyphosphate and chitosan particles, it was attributed to shorter chains of low molecular weight chitosan which are unable to entrap more of the drug content as reported by Wu et al. (2005). With increasing the amount of drug in the polymer: drug ratios, the average drug entrapment efficiency of the chitosan loaded nanoparticles

formulations was observed to improve significantly. It's possible that this is related to the increased drug that react with polymer.

4.3 Stability Studies

Stability studies for the nanoparticles were done to check if there were any changes that leads to change in size, ph. or change in the viscosity of the nanosuspensions. As the purpose of the drug loaded nanoparticle formulation is therapy, so stability of the formulation is an important part. These studies were conducted for a period of 3 months (Table no. 6). The formulations were kept at refrigeration temperature. The viscosity was measured for three months, and there was no change in viscosity throughout that period. The viscosity of nanofluids is determined by differences in the size or agglomeration of nanoparticles (Pastoriza-Gallego et al., 2009). Results indicated that there is no significant change in the pH of the formulation. The size of the formulation increased due to the increase in the pH of the solution, as the particle are formed by ionic interaction of the polymer and the polyanion. Similar results were obtained by Thandapani et al. (2017), according to their finding crosslinking agents limit the mobility of the polymer structure and increase its physio-chemical characteristics, such as mechanical qualities and dimensional stability.

Table 6: Mean values of pH, particle size and viscosity

Months	pH	Z-size average	Viscosity
March	5.38	200.37±5.40	0.86± 0.008
April	5.45	212.1±3.05	0.88±0.010
May	5.43	214.2±3.86	0.87±0.008

4.4 Study of *in-vitro* Release Kinetics

In-vitro drug release kinetics investigations are carried out to see if novel medication formulations have the potential to regulate drug release. The process through which a drug exits a drug product and undergoes absorption, distribution, metabolism, and excretion (ADME) before being accessible for pharmacological activity is known as drug release. *In-vitro* drug release studies are essential for determining the dosage forms *in-vivo* performance. The assessment of sustained and prolonged release dispersion systems is aided by drug release experiments. There are

two types of release patterns: those that release medication at a slow zero or first order rate and those that deliver an initial fast dosage followed by slow zero or first order sustained component release. Controlled release systems are designed to keep medication concentrations in the blood or target tissues at a desired level for as long as feasible. In general, controlled release systems release a portion of the dosage early in order to achieve the drug's effective therapeutic concentration quickly. Then, in order to deliver the maintenance dosage and achieve the appropriate drug concentration, drug release kinetics follows a well-defined pattern (Dash et al., 2010).

Barzegar-Jalali et al. (2008) used traditional and novel models to compare drug release kinetics from nanoparticles. Each data set's mean percent error (E), the overall mean percent error (OE), and the number of Es less than 10% were used to measure accuracy. The reciprocal powered time model was shown to be the best generic model for studying multi-mechanistic drug release from nanoparticles.

Jain et al. (2015) prepared nanoparticles utilizing polymers as drug carriers to improve the effectiveness and solubility of duloxetine hydrochloride, an antidepressant. By fitting the dissolution data into various kinetic models, it was discovered that the delayed release formulation of the drug followed Higuchi model kinetics with linearity range 0.99, and the release component (n) was obtained by the Korsemeyer Peppas model, indicating that the release mechanism was followed by an anomalous diffusion process.

Table 7: *In-vitro* drug release data for all the preparation depicting the % cumulative release

Time (hr)	Cumulative drug release %
0.5	7.704655
1	11.98502
2	18.04887
4	24.64776
8	32.31675
12	40.16408
24	48.72481

Dialysis is the separation of molecules in solution based on differences in diffusion rates via a semipermeable membrane. The release kinetics of chitosan nanoparticles were studied using the dialysis bag technique. All of the experiments were done in triplicate to ensure that the results were consistent. After 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 24 hours, and 36 hours, data was collected and computed. All of the parameters were held constant, and UV spectroscopy was used to conduct the analysis.

Chitosan loaded nanoparticles also show an initial burst release pattern with release of 18.05 % at just 2 hours. Swelling of the polymer, creating pores, or diffusion of the drug from the polymer's surface induce the first burst release from the chitosan nanoparticles. The drug release continued even after 24 hrs. since the maximum value obtain at 24 hrs. was 49.72 %

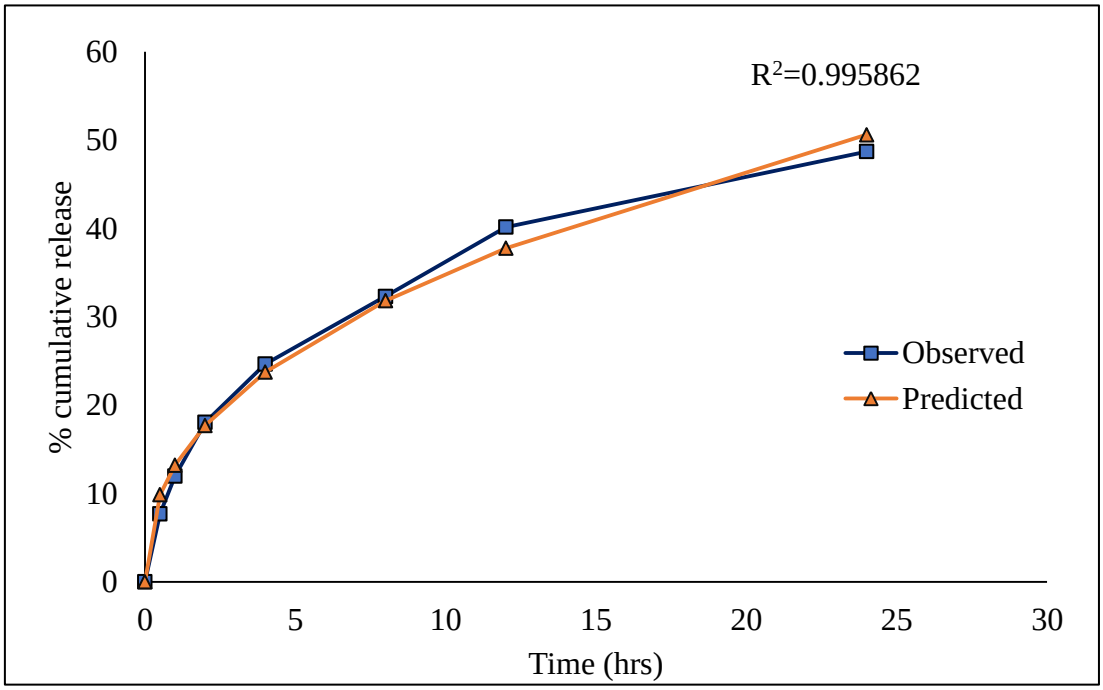

Fig. 12: Korsemeyer-Peppas kinetic model for thiotepa loaded nanoparticles

In order to understand the kinetics of drug release chitosan loaded nanoparticles data was analyzed by the three kinetic models: zero order, first order and Korsemeyer–Peppas model. The n is the value of the diffusion exponent (n), which describes how medicines are released. The value of n for a spherical system, 0.433 for Fickian release, $0.43<n<0.85$ for non-Fickian release, shows that this model is employed if the release pattern follows more than one phenomenon. When n

exceeds 0.5, the release follows a non-Fickian pattern, meaning it is regulated by both diffusion and dissolution. The release of thiotepa-loaded chitosan nanoparticles follows the Korsemeyer pattern, as seen by the R^2 value. The n value was found to be 0.42, which clearly depicts the thiotepa loaded follow fickian type of model.

Table 7: Comparison of various kinetic models for drug release kinetics

	Parameter	**CNPs**
Zero order kinetic model	R^2	0.83
First order kinetic model	R^2	0.86
Korsemeyer peppas model	R^2	0.99

4.5 Morphology of the Thiotepa loaded nanoparticles

Nanoparticle–nanoparticle interactions have lately gotten a lot of interest because they have a direct influence on nanoparticle uptake in cells. Adsorbed proteins on nanoparticle surfaces, as well as the formation of the so-called protein corona, play an important role in these interactions, as they determine the nanoparticle agglomeration state, as well as their fate and transport in biological media, mobility, and settling, among other things. The shape of the particle is just as significant as the size of the particle. The morphology of nanoparticles influences their movement inside the body, and their absorption by macrophages is also controlled to a significant extent by their shape (Champion & Mitragotri, 2006)

Chitosan nanoparticles were further analysed by using Transmission electron microscopy (TEM). It gives two-dimensional pictures of nanoparticles. TEM is one of the most powerful techniques for characterising nanomaterials at spatial scales spanning from the atomic level (from 1 to 100 nm) to the micrometre level, allowing for new applications. TEM employs more intense electron beams than SEM, allowing for more resolution and detail, such as information on a particle's crystalline structure and granularity. In the current work, TEM reveals the spherical shape of individual particles.

Chitosan can be made into a film, however in an aqueous environment and when dried, the cross-linked chitosan nanoparticles split into spherical particles. The nanosize of dried cross-linked chitosan particles was confirmed by TEM micrographs, which also revealed the dispersion of these derivatives. The dried particles ranged in

size from 60 to 280 nanometers. Smooth surface and spherical shape were observed for thiotepa loaded chitosan nanoparticles.

Results obtained by Sailaja and Vineela (2014) also were similar to the results obtained in present study. They reported similar findings of Mefenamic acid loaded nanoparticles by SEM analysis showing smooth and spherical structure of the nanoparticles. There SEM images were indicative of the agglomeration of the sample. Results of SEM are in agreement with TEM, depicting spherical shape of the nanoparticles.

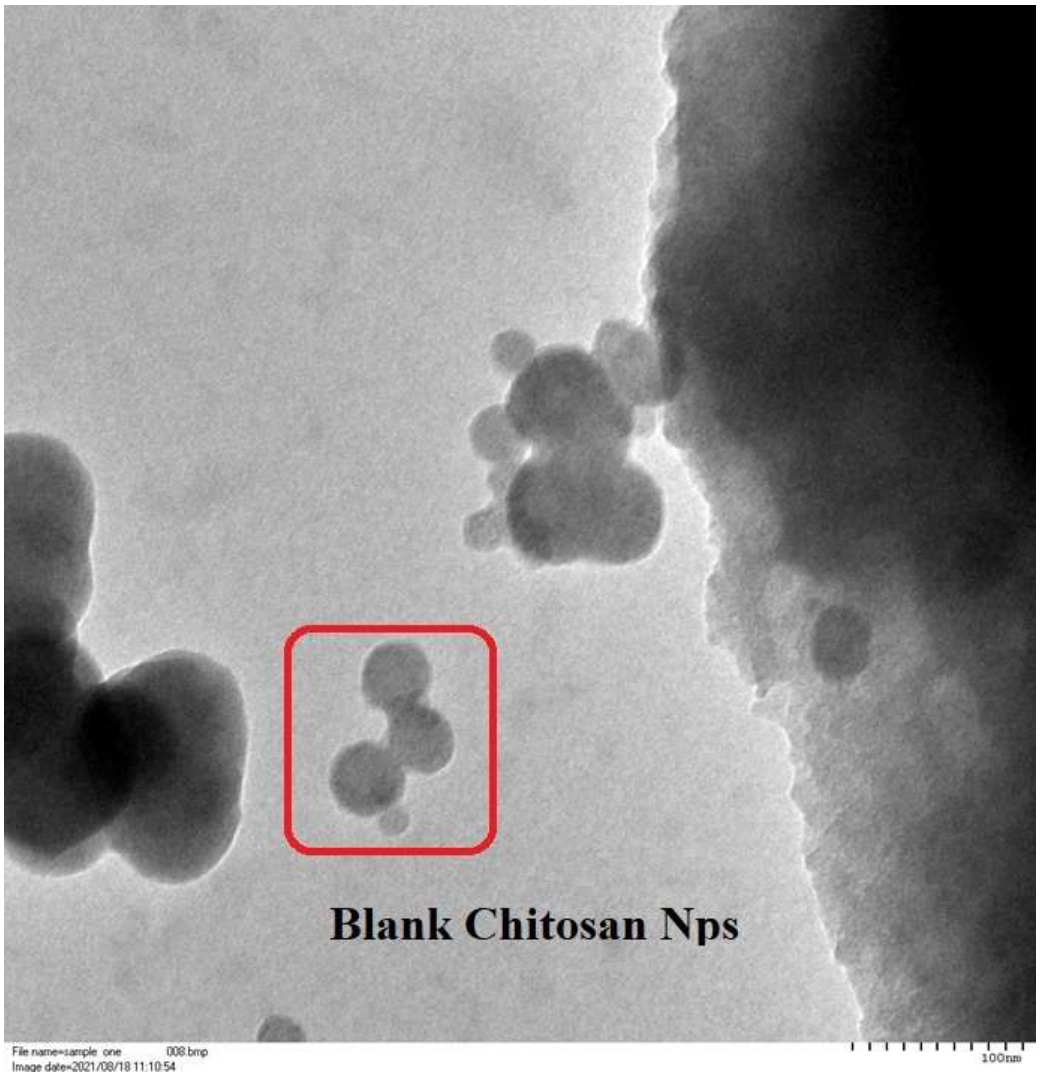

Fig. 13: TEM image of blank chitosan nanoparticles at 200nm scale

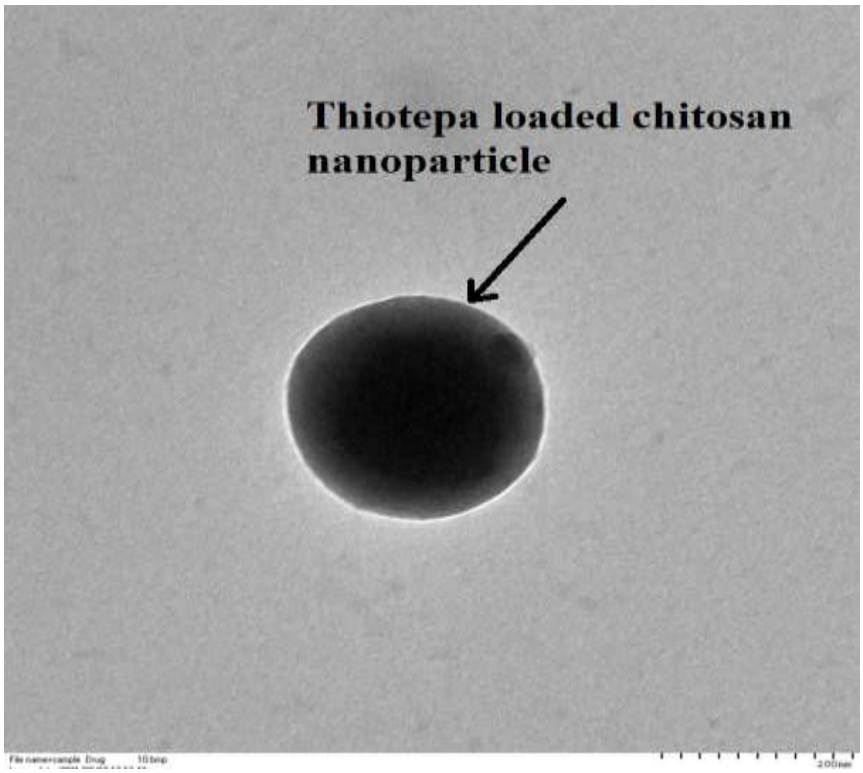

Fig. 14a: TEM image of blank chitosan nanoparticles at 200nm scale

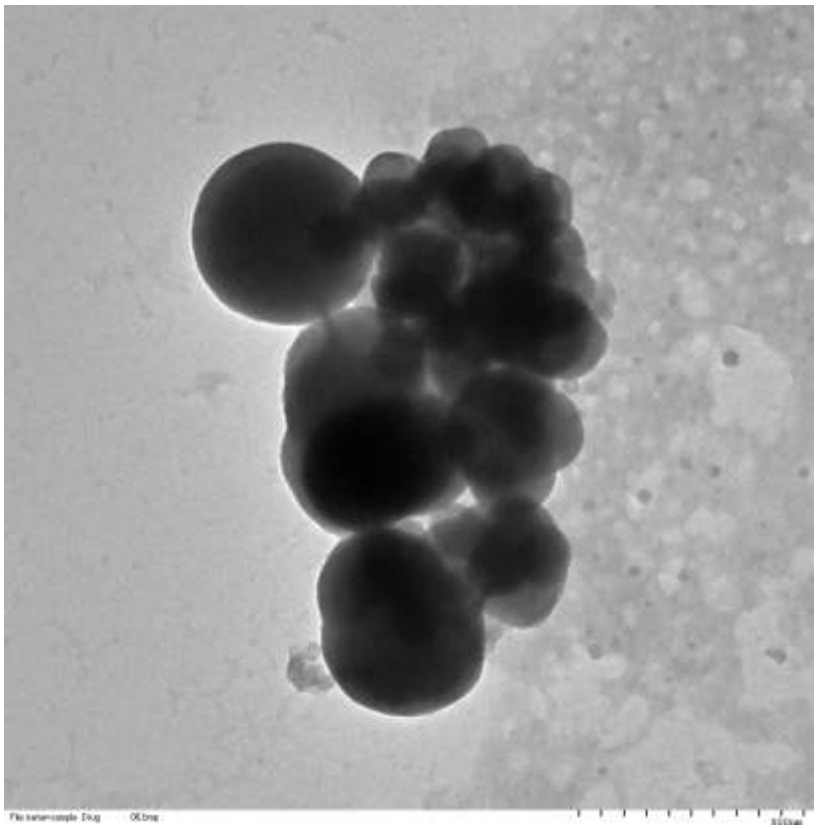

Fig. 14b: TEM image of blank chitosan nanoparticles at 200nm scale

4.6 Inhibitory concentration

Thiotepa and thiotepa loaded chitosan nanoparticles exhibited significant cytotoxic activity after 72 hr. of exposure in L1210 cells with IC_{50} value of 96.62 µM and 78.36 µM. Further, IC_{25}, $IC_{12.5}$ and $IC_{6.25}$ values were calculated to be 48.31, 24.15 and 12.07 µM and 39.18, 19.59 and 9.79 µM for thiotepa and thiotepa loaded nanoparticles, respectively.

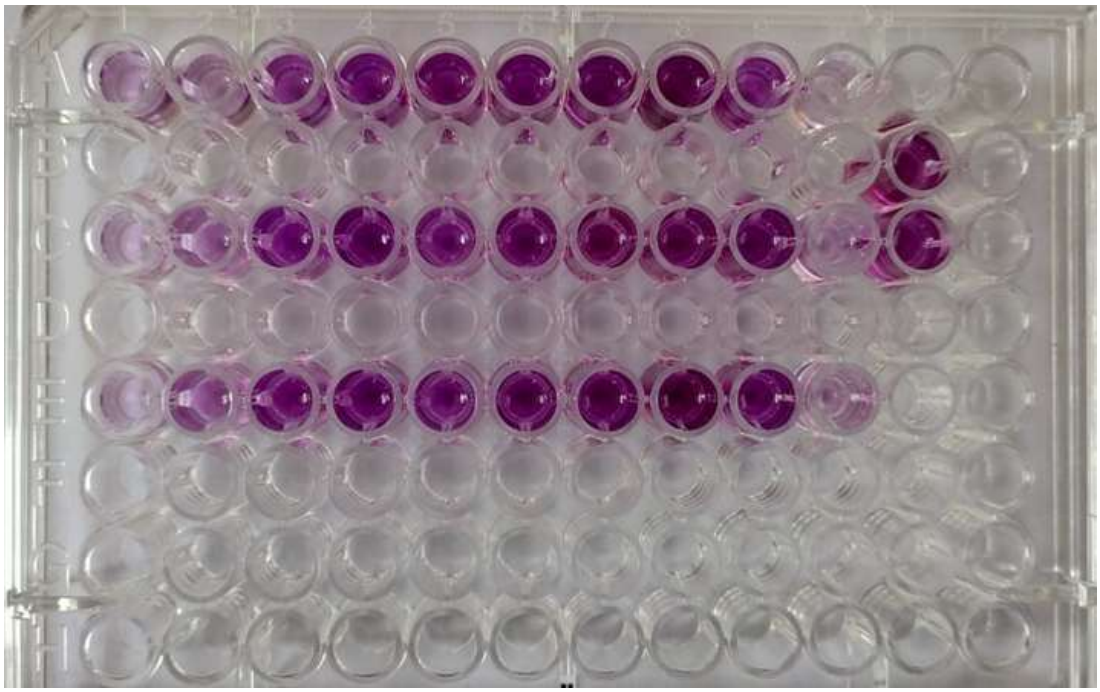

Figure 15: MTT assay performed on L1210 cells in 96 well plate

Table 8: Comparison of Standard and Nanoparticle loaded drug

S. No.	Standard drug (µM)	DLNPs (Drug loaded nanoparticles) (µM)
IC_{50}	**96.62**	**78.36**
IC_{25}	48.31	39.18
$IC_{12.5}$	24.15	19.59
$IC_{6.25}$	12.07	9.79

4.7 Cell morphology

Cytotoxic effect of thiotepa loaded chitosan nanoparticles was assessed in L1210 cell line, by observing under compound microscope and morphological changes such as cellular swelling, chromatin changes, plasma membrane blebbing, nuclear fragmentation, loss of plasma membrane integrity and release

of cytoplasmic content were examined.

Thiotepa loaded chitosan nanoparticles exposed L1210 cells showed varying degree of morphological changes such as cellular swelling, rounding of cells, chromatin condensation, cell shrinkage, formation of blebs, nuclear degradation, plasma membrane disintegration and loss of cytoplasmic content. These changes were found to be dose and time dependent. In comparison to control, these changes were severe at 72 hour. On visual observation, it was noted that the rate of cellular growth inhibition was high as compared to standard and control groups.

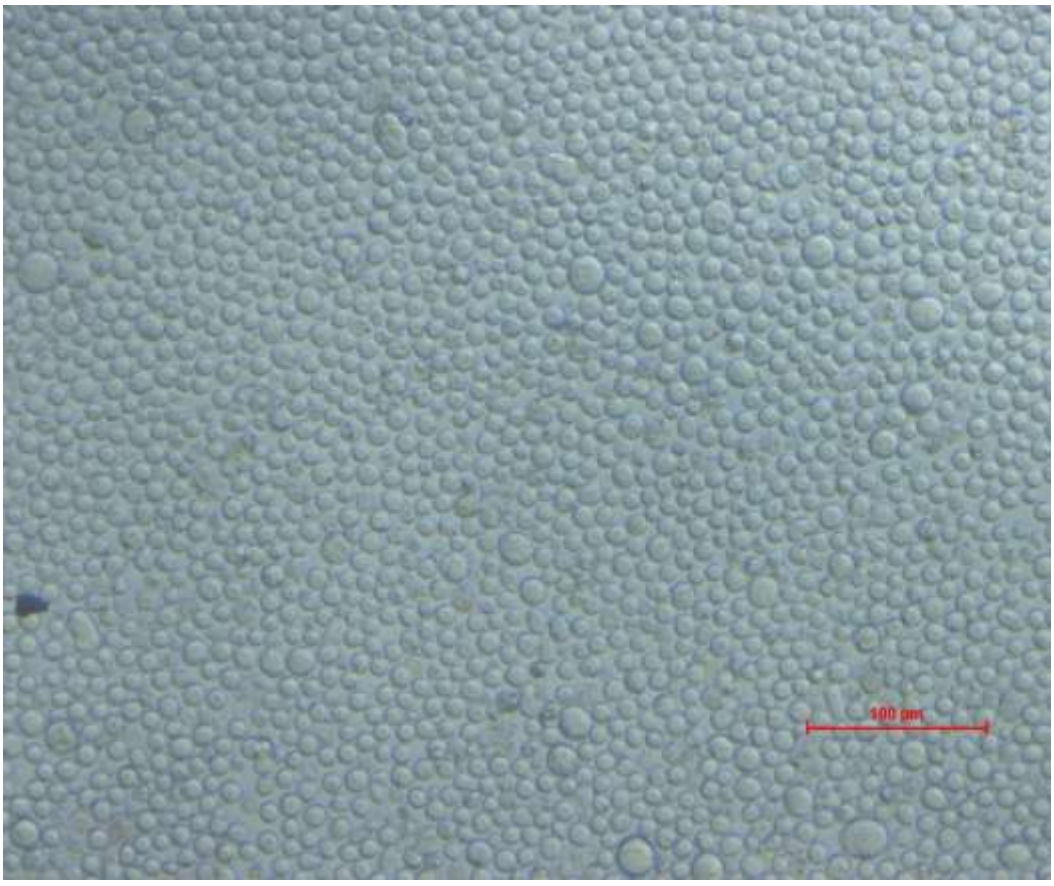

Figure 13: Control L1210 cells at (72 hour)

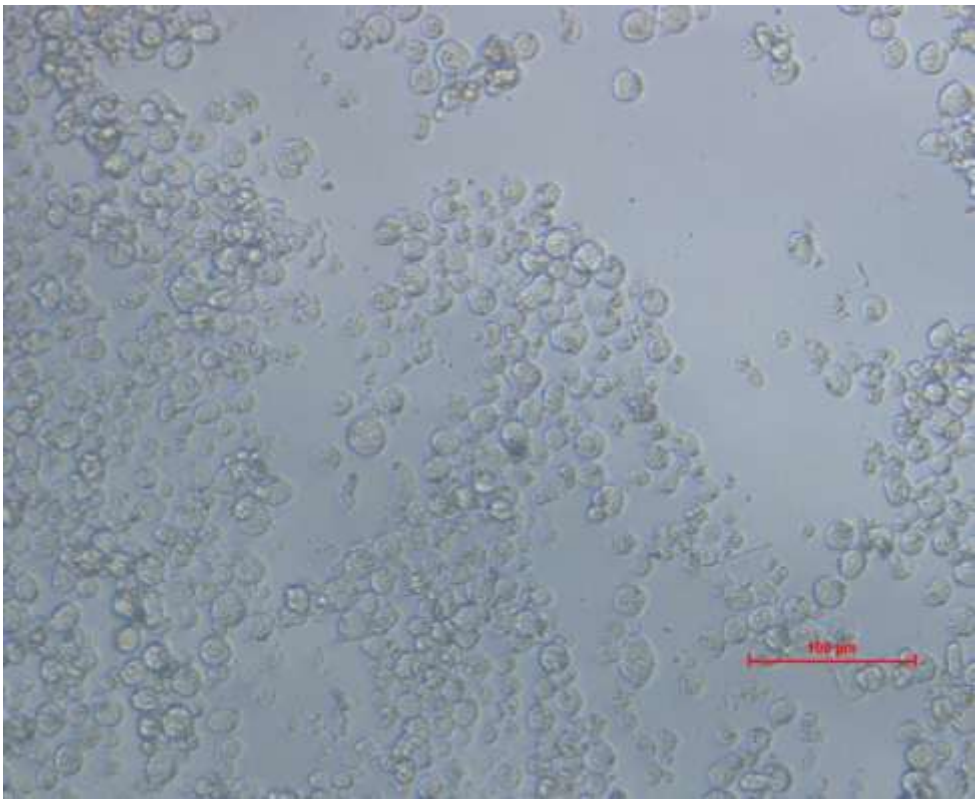

Figure 14: Thiotepa (Standard) treated L1210 cells (72 hour)

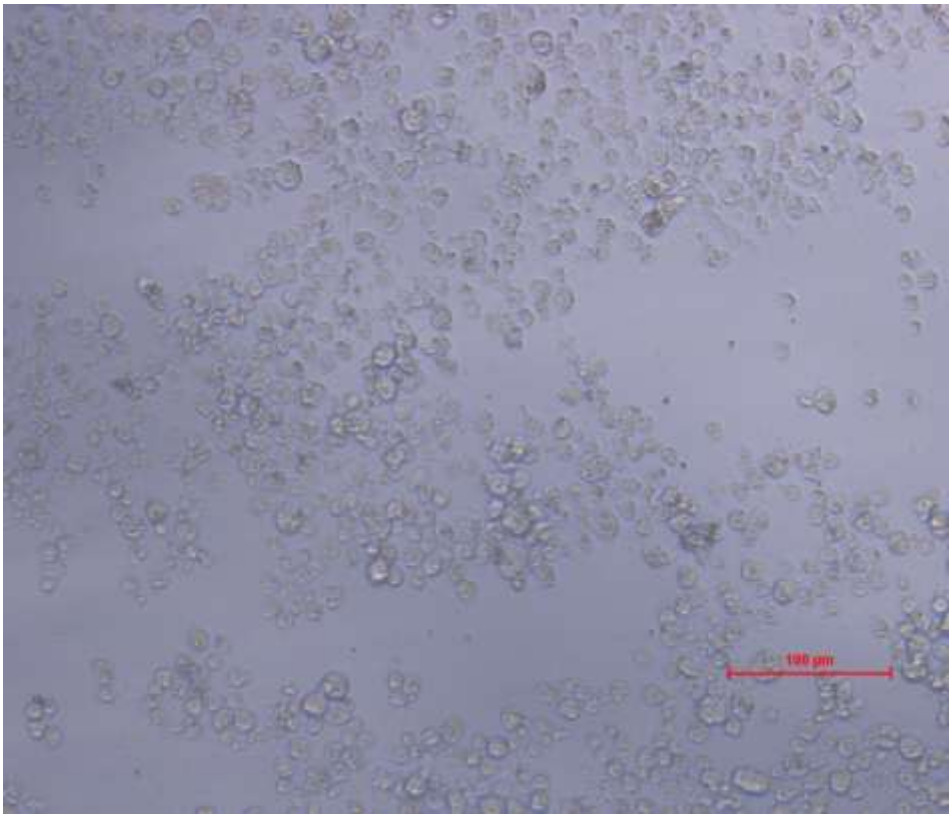

Figure 14: Thiotepa loaded nanoparticle treated L1210 cells (72 hour)

CHAPTER V

SUMMARY AND CONCLUSIONS

In the present study the process optimization of different methods for preparation of Thiotepa loaded nanoparticles was done. The method for the preparation of chitosan nanoparticles (CNPs) was standardized. Then loading of the drug was done. This method was then further tested for various characterization parameters and based on the results obtained, suitable method was chosen for further studies.

1. The characterization parameters involved estimation of particle size which was 200.37±5.40 nm and PDI of 0.3380±0.01 for Chitosan loaded nanoparticles.
2. The zeta potential value for chitosan loaded nanoparticles was 24.93±0.49 mV.
3. The entrapment efficiency was found to be 56.81 ± 0.96%.
4. The release kinetics for the formulation was also determined yielding 48.72% drug release from chitosan loaded nanoparticles. The drug release was analyzed by fitting into different mathematical kinetic models which revealed that chitosan loaded nanoparticles tend to follow Korsmeyer-Peppas model. Also, it follows fickian type of dissolution (As n-value is less than 0.5).
5. Stability studies including pH change, Z size average and viscosity studies for the three preparations was also done revealing stable nanosuspension.
6. The method of thiotepa loaded chitosan nanoparticles was chosen for further characterization, revealing a smooth surface and a spherical shape in TEM.
7. The IC_{50} value of thiotepa and thiotepa loaded chitosan nanoparticles was found to be 96.62 μM and 78.36 μM respectively, calculated by using MTT assay. It exhibited marked cytotoxic activity after 72 hours of exposure period. IC_{25}, $IC_{12.5}$ and $IC_{6.25}$ values were calculated as 48.31 μM 24.15 μM and 12.07 μM respectively for thiotepa and 39.18 μM 19.59 μM 9.79 μM, respectively for thiotepa loaded chitosan nanoparticles.
8. The present investigation confirms the hypothesis that nanodrug used in the present study causes inhibition of the cancer cells by disruption of plasma membrane integrity and cell shrinkage. This nanodrug was found to be more effective than standard thiotepa.

Conclusion

From the present study it can be concluded that Thiotepa loaded chitosan nanoparticles had optimum characteristics. They can be used for potent drug delivery. *In-vitro* studies indicated decrease in value of ED50 as compared to normal drug which might help in reducing the dose rate of drug. Therefore, chitosan loaded drug nanoparticles may be further used therapeutically. Further investigation for pharmacokinetics and cytotoxicity is required. True potential of these thiotepa loaded nanoparticles can be revealed by testing in *in-vivo* models.

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