Chapter II: Literature Review
Levamisole Hydrochloride is an anthelmintic synthesized drug was also found to have immunomodulatory activity. The use of herbal medication is limited due to lack of scientific evidence, variability in activity according to season and sources, etc. Levamisole Hydrochloride was selected for the present research work and proposed formulation was nanoparticles.

2.1 Patents:

US patent no. 4153678 gives information for Levamisole effervescent tablets, which have shown increased solubility, providing crystal clear solution in water, with good storage stability and ease in use. The tablets are designed for animal usage via oral delivery. (37)

US patents like 4439439, 3980791, 4395407, 4278684 provides novel pour on formulation of Levamisole, which are non-irritating, non-toxic in nature. The formulation may contain some surfactant, carboxylic acid, organophosphates and adhesion promoters, which improves anthelmintic property for animal use. (38; 39; 40; 41)

US patents like 4287176 and 4543358 provides information for anthelmintic gels which will be stable between temperature ranges of -27°C to +60°C. The gel may contain anti-foaming agent, coloring agent and preservatives which increase helminthic property. (42; 43)

US patent 3937825 provides information related to anthelmintic composition comprising Levamisole and 0, 0- dimethyl (2, 2, 2-trichloro-1-hydroxyethyl)-phosphonate for control of gastrointestinal, cutaneous and lung-infesting parasites in equine species. (44)

US patent 4096271 gives information for the slow release injectable formulation of tetramisole derivatives in benzyl benzoate which will be safer, less toxic and cause milder side effects when administered in larger quantity than recommended to homothermic farm and companion animals. (45)

US patent no. 2006/0128641 A1 gives information for potent solvent comprising Levamisole and avermechin/milbemycin against cattle parasites with specific target to tapeworm and round worm, with stability at different pH. (46)

US patent 2004/0033271 A1 gives information related to potentiation of anticancer activity of 5-FC in combination with Levamisole. The dosage regimen is given in such a way that the tumor tissues are coming in exposure to both the drug with increased level. (47)
US patent 5332577 provides information related to transdermal preparations, intended for any of anthelmintic, anti-parasitic, reproduction modulating agent, animal growth promoting agents, antibiotics, anti-allergic, cardiovascular drug, anti-inflammatory agents, bronchodilating agents and micronutrient delivery to both animals and humans.\(^{(48)}\)

US patent 5543158 gives information regarding biodegradable injectable nanoparticles. The prepared particles are not cleared rapidly from blood stream by macrophages of RES, and are modified to achieve targeted delivery with controlled release. The particles are having composition of biologically active material and poly alkelene glycol moiety. The surface can be modified by attaching biodegradable polymers to same structure.\(^{(49)}\)

US patent 7081450 B2 provides information for water soluble nanoparticles of hydrophilic and hydrophobic active materials and an apparatus and method for their production. The prepared nanoparticles are soluble in nature with even particles having core of water insoluble lipophilic compound and amphiphilic polymers. The lipophilic compound may consist of pharmaceutical compound, cosmetics, food additive compound, agricultural product and veterinary product. The invention also provides a chemical reactor for manufacturing purpose.\(^{(50)}\)

US patent 7265090 B2 gives information for nanoparticles for paracellular drug delivery. The nanoparticles were having composition of Chitosan/poly-\(\gamma\)-glutamic acid with positive charge and they have shown enhanced permeability to caco-2 cells.\(^{(51)}\)

Thus, a patent non-infringement approach was used for the present research work, which involves nanoparticles of Levamisole Hydrochloride loaded on Chitosan-STPP matrix for immunomodulatory purpose via targeting through Peyer’s patch.

2.2: Drug profile \(^{(52; 53; 54; 55; 56; 57)}\)

2.2.1: Physicochemical properties: (Levamisole Hydrochloride)

Synonym: L-Tetramisole, Phenyl imidothiazole, dl-Tetramisol, dl-Tetramisole

Brand names: Ergamisol, Ascandil, Decaris, Ergamisole, Ketrax, Solaskil, levasole, tramisol, ripercol, Dewormis, Dicaris, Dicaris, Jetomisol-P, Jetomisol-P, Levomol, L-Vin, Vermisol,
LEVOMYSOL, Lepuron, Levamisol, Nilverm base, Tetramisol, Tetramisole, Tramisol, Vermisol 150, Wormicid, Levam, Meglum, Decarns, vizole, askamex, sitraks, paraks. \(^{(58; 59; 60)}\)

Structure:

Chemical Name: \((6S)-6\text{-Phenyl}-2,3,5,6\text{-tetrahydroimidazo[2,1-b]thiazole Hydrochloride}\)

Molecular formula: \(C_{11}H_{12}N_{2}S\cdot HCl\)

Molecular Weight: 240.8 gm/mole

CAS- 16595-80-5

Content: 98.5% to 101%

Appearance: A white or almost white crystalline powder

Solubility: Freely soluble in water, soluble in alcohol, slightly soluble in methylene chloride, practically insoluble in ether.

pH: 3-4.5 (5% solution)

Specific optical rotation: -121° to -128° (2.5 gm powder dissolved in 50 ml carbon dioxide free water)

Melting point: 227°C-229°C

Dissociation constant: pKa 8.0

Partition coefficient: \(\log P\) (octanol/water) 1.8

Specific gravity: 0.697
2.2.2 Pharmacology:

Levamisole is a synthetic imidazothiazole derivative that has been widely used in treatment of worm infestations in both humans and animals. As an anthelmintic, it probably works by targeting the nematode nicotinergic acetylcholine receptor. As an immunomodulator, it appears that Levamisole is an immunostimulant which has been shown to increase NK cells and activated T-cells in patients receiving this adjuvantly along with 5FU for Stage III colon cancer. \(^{(58)}\)

2.2.3 Mechanism of action:

The mechanism of action of levamisole as immunostimulant is as follow: \(^{(62; 63; 64; 65; 66; 67; 68)}\)
Levamisole stimulates cGMP production, which helps many anticancer drugs. It is also having “thymomimetic” effect, which increases the percentage of T lymphocytes in the circulation. \(^{(62)}\) It protect many cells against necrosis by acting as a free radical scavenger. \(^{(64)}\) It was first considered to inhibit T-suppressor cell activity, increase interferon induction and lymphokine induced macrophage phagocytosis. \(^{(63)}\) It increases biodisponibility of cytostatins by reducing their catabolism. It also enhances NK cells, IL2 receptors and CD4/CD8 ratio along with cytokine production. \(^{(63; 65)}\) They also enhance IL-1 production in macrophage cell line and shifts Th1 cytokine productions. In in vivo, it may enhance the serum levels of thymic hormone like factor. Thus it can stimulate formation of antigens to various antibodies \(^{(66)}\), enhances T cell activation and proliferation, potentiate phagocytosis and chemotaxis and increases neutrophil mobility with their adherence. \(^{(58)}\)

The mechanism of action of Levamisole as an antiparasitic agent appears to be tied to its agonistic activity towards the L-subtype nicotinic acetylcholine receptors in nematode muscles. This agonistic action reduces the capacity of the males to control their reproductive muscles and limits their ability to copulate. \(^{(58)}\)

2.2.4 Pharmacokinetics and Metabolism:

Levamisole is rapidly absorbed from the gastrointestinal tract. Maximum plasma concentrations are attained within 1.5 to 2 hours. Parent drug represented 40% of total reactivity. It is extensively metabolized in the liver. The plasma half-life for Levamisole is 3 to 4 hours and for the metabolites is 16 hours. It is excreted mainly in the urine as metabolites and a small
proportion of 3-5% in the feces. About 70% of a dose is excreted in the urine over 3 days, with about 5% as unchanged Levamisole.\(^{(56)}\)

### 2.2.5 Biotransformation:

About 50 metabolites were determined suggesting four different processes, but other pathways can also be responsible because nearly 20% metabolites were remaining unidentified. The extensive metabolism was observed in hepatic. Nearly 20-25% protein binding was also observed. The most important quantitatively is (1) the oxidative introduction of a double bond into the imidazole ring, accompanied by or followed by oxidation of the sulfur to sulfoxide and introduction of a hydroxy group in the para position of the phenyl ring. The second most important pathway (2) is the hydrolysis of the thiazolidine ring to an oxoimidazole metabolite. The third (3) pathway is formation of p-hydroxytetramisole and its subsequent conjugation with glucuronic acid. The least important pathway is (4) the hydrolysis of the thiazole ring to yield the mercaptoethyl intermediate and subsequent oxidation to sulphoxide and sulphone. The metabolic scheme is depicted in Figure 2.1.\(^{(69)}\)

### 2.2.6 Interactions:

It is advised that Levamisole should be taken on empty stomach.

During treatment avoid drinking alcohol because it may generate disulfiram like reaction.

It increases anticoagulant activities of acenocoumarol, anisindione, dicumaril and warfarin.

It increases free concentration of phenytoin concentration, when given along with 5-FU.

Do not take Levamisole during any vaccination like smallpox vaccine, measles virus vaccine, mumps-rubella virus vaccine, influenza virus vaccine, polio virus vaccines, rotavirus vaccine, typhoid vaccine, BCG vaccine, varicella virus vaccine, yellow fiver vaccine, zoster vaccine, etc.

It is also having interactions with adalimumab (TNF blocker), leflunomide, certolizumab, clozapine, etanercept, golimumab, infliximab, natazilumab and samarium sm 153 laxidronam, etc.\(^{(56;70)}\)

Nicotine like compounds (pyrentel, morantel) and cholinesterase inhibitors can enhance toxic effects of Levamisole.
Figure 2.1: Biotransformation of Levamisole Hydrochloride (69)

2.2.7 Precaution: (56)

The Levamisole should be used with precaution in patients having pre-existed blood disorders mainly bleeding/blood clotting disorder, liver diseases, seizure disorder and patient having poor bone marrow function.
2.2.7.1 Rheumatoid arthritis: The presence of HLA B27 in seropositive rheumatoid arthritis is an important predisposing factor to the development of agranulocytosis with Levamisole; it is recommended that the use of Levamisole in this group should be avoided.

2.2.7.2 Sjogren’s syndrome: The appearance of adverse effects in 9 of 10 patients with rheumatoid arthritis and Sjögren’s syndrome while being treated with Levamisole led to abandonment of the study. Levamisole should be given with caution, if at all, to patients with Sjogren’s syndrome.

Don’t take Levamisole in pregnancy (category C according to FDA). The information about lactation is unavailable.

Levamisole poisoning causes a syndrome of lip leaking, salivation, head shaking, tremor, excitement and increased frequency of urination and defecation.\(^\text{(71)}\)

It may cause vaginal dryness.

2.2.8 Adverse Effects:

Generally adverse effects are limited to vomiting, nausea, diarrhea, abdominal pain, headache, and dizziness, change in smell and taste, skin rash, fatigue, muscle ache, when given for single dose. Even lip leaking, salivation, head shaking, tremor, excitement and increased frequency of urination and defecation may be seen.\(^\text{(71)}\)

Adverse effects also includes hypersensitivity reaction such as fever, flu like syndrome, arthralgia, skin rashes, muscle pain, cutaneous vasculitis, convulsion, hematological abnormalities like leucopenia, agranulocytosis, thrombocytopenia and gastric disturbances like abnormal taste in mouth, etc. when given for prolonged time.

Patient receiving Levamisole along with 5-fluorouracil, may develop abnormal antidiuretic hormone syndrome and inflammatory leukencephalopathy.

It may cause liver toxicity by increasing aspartate aminotransferase concentration in patient receiving treatment for nephrotic syndrome.

Allergic reaction happens including difficulty in breathing, closing of throat, swelling of tongue, lips, face, hives. It may decrease bone marrow function. Nervous system problem like confusion, extreme fatigue, numbness/tingling, speech disturbances, etc. may be seen.\(^\text{(72; 73; 74)}\)
2.2.9 Indication:

Levamisole Hydrochloride is levo-isomer of tetramisole Hydrochloride is used as anthelmintic and as an adjuvant in malignant disease. It is also used in many conditions where its immunostimulant effect is also useful.

Levamisole is active against small intestinal nematode worms, abomasal nematodes, large intestinal nematodes, lungworms, hook worms, round worms, etc. It is also used in ascariasis.

It modulated cell mediated immune responses, restore depressed T cell functions. It has been tried in viral infections, bacterial infections, rheumatic disorder. It is also used in melanoma, head-neck cancer. It has been used in combination therapy for influenza due to its nature for inducing interferon.

Adjuvant treatment with 5-fluorouracil and Levamisole is used in adenocarcinoma of the Dukes’ stage C colon with regional lymph node involved. It is also used in malignant melanoma, lung cancer, breast cancer and squamous head/neck cancer. It has been used with mebendazole to treat *mansonella* infection.

It has also been used in mouth ulceration. It has been used in glomerular kidney disorders including steroid dependent nephrotic syndrome. It has also been used in vitiligo.

It is protecting liver cell necrosis from NADPH ascorbate and X-irradiation induced lipid peroxidation which was proved by using carbon tetrachloride (CCl₄) as model hepatotoxic substance.

It is reducing HBV DNA levels in patients with chronic hepatitis B infection and also enhance immune response to hepatitis B vaccination in chronic hemodialysis patients.\(^{(65)}\)

It has been used in combination with certain drugs for various dermatological diseases like lichen planus, erythema multiforme, aphthous ulcers of mouth, recalcitrant warts, leprosy, collagen vascular diseases and inflammatory skin diseases.\(^{(75)}\)

2.3: Nanoparticulate Drug delivery system

The success ratio for finding the new chemical entity (NCE) is decreasing day by day and the cost involved is increasing. It is assumed that out of about 5000 compound in preclinical trials, only 5-10 compounds make it human testing of which only one reaches to human clinical trial costing about $ 800 million with a total time consumption of 10-15 years.\(^{(76)}\) Investors want to spend less money and want high profit in short life cycle, which is totally opposite condition in
By 2011 many blockbuster drug will go off patent, giving rise in competition of generic market. It has been observed that top pharma companies have doubled their investment cost in R&D in last seven years; but their sale is decreasing due to competition, self-withdrawal from market, etc. requiring dramatic improvement in R&D product and methodology. Therefore industry is now focusing on nanotechnology which can meet the basic criteria for reducing side effects, toxicity and targeting to a specific site.

2.3.1 Principle method of nanoparticle preparation

As the size will be decreased, the surface area will be increased, which will cause increased solubility of the drug. Even there will be a change in magnetic and optical properties which can be used for diagnosis and targeting purposes. The two general principals used for preparation of nanoparticles are (a) Breaking down process and (b) building up process.

2.3.1.1 Breaking down process/top-down manufacturing:

In this process, stress is applied on material resulting in breakage of particles. The obtained particles are having cracks causing fracture of particles, but as the particles become smaller, it becomes difficult to grind them. Thus is very essential to know material’s brittleness and hardness before any process of milling on it. Ball mill is the only tradition mill used for this purpose using the principle of erosion. High pressure homogenization is also used, which are either using piston-gap principle or jet stream principle. Nanocrystal compounds are also formed by spray drying technique, but require contact of drug with water, which may cause degradation of drug. In hot melt matrices, aqueous contact can be avoided to get nanocrystal by high pressure homogenization.

2.3.1.2 Building up process/bottom-up manufacturing:

In this process, drug is dissolved in to a solvent, and then the precipitate of nanoparticles are obtained by either removing solvent rapidly or by adding non solvent to solution, reducing its solubilizing strength.

Supercritical fluid technology is also used for manufacturing of nanomaterial by using supercritical fluids like carbon dioxide in which the solubility of drug can be regulated by
regulating pressure. By sudden reduction in pressure the solubility changes resulting in formation of nanoparticles.\(^{(17;21)}\)

A new technique known as high gravity reactive precipitation technique, suppresses crystal growth, produces nanoparticles in range of about 10 nm.\(^{(21)}\)

It is found that generally, nanoparticles are made of drug and some excipients/polymers, in which the drug is either entrapped, dissolved or adsorbed to the surface. Two common preparation method includes polymerization and by preformed polymers.

During nanoparticles preparation by polymerization, the monomers and catalytic agents are solubilized in aqueous system having emulsified lipophilic droplet or micelles. The nanoparticles are formed due to reaction at interface of aqueous and non-aqueous phase which can be controlled by control of temperature, pH, concentration of reactants and monomer supply. But the limitations include toxicity due to polymerization medium, the process is limited to vinyl addition, the molecular weight of polymer is not controlled, etc.

In preparation of nanoparticles by preformed polymers, the polymer is dissolved in organic water miscible or immiscible solvent, which is then evaporated/desolvated. Thus, it is divided into following.\(^{(21)}\)

Emulsion-solvent evaporation: the polymer along with drug is dissolved in volatile organic solvent, which is then emulsified in aqueous medium having stabilizer. The continuous emulsification at high speed allows solvent evaporation, forming nanoparticles in dispersed condition. Even double emulsification technique is used for water soluble drugs.\(^{(21)}\)

Solvent displacement technique/ Nanoprecipitation: In this technique, drug, polymer and stabilizer in semi-polar solvent miscible with water is injected into anti-solvent with moderate stirring, and the organic solvent is removed under reduced pressure.\(^{(21)}\)

Salting out technique: In this technique, drug and polymer in water miscible solvent are added to aqueous solution having stabilizer with a salting out agent, producing nano sized ranged particle by reducing solubility of polymer and drug. Later on salting out agent is removed.\(^{(21)}\)
2.3.2: Types of nanoparticles

A nano particulate drug delivery system can be categorized mainly into two categories: phospholipid type and polymeric type nanoparticles.

2.3.2.1 Phospholipid based nanoparticle system:

Phospholipids are part of cell. Liposomes are prepared using phospholipids by thin layer lipid hydration method in which, the solvent containing lipid in a flask is rotated under low pressure to remove organic solvent and form thin film of lipid, which is later on hydrated. The size can be reduced by ultrasonic wave method, extruder method. *(80)*

Lipid nanocapsules are particles made of lipid surrounded by semisolid/solid shell. If the lipid is solid in nature, it is known as solid lipid nanoparticles (SLN). SLN have lots of advantages like controlled release, increased bioavailability of poor soluble drug, protecting drug from light and environment. Disadvantages are polymorphism, particle growth, unpredicted gelation tendency, etc. *(79; 81)*

2.3.2.2 Polymeric nanoparticle system:

Size control of polymeric nanoparticle is difficult, which are prepared by monomers or polymers. The polymers used can be either biodegradable or non-biodegradable in nature. They can be form in such a way that they will control the release rate at specific site. *(79; 82; 83)*

Hydrogel nanoparticles: they are polymeric 3-D configuration capable of imbibing high amount of water with a size in nano range, have gained potential biomedical and pharmaceutical application. The water retention property depends upon the presence of hydrophilic group of polymers used, which also affects rheological property of nanoparticles. But still they swell instead of being dissolved when come in contact to the biological fluid, which is due to cross linking of polymers. Thus the drug is released by passive diffusion mechanism. Chitosan, alginate, poly vinyl alcohol, poly vinyl pyrrolidone, poly-N-isopropylacrylamide vinylpyrrolidone, acrylic acid, methylene bis acrylamide, etc. are the common polymers used in hydrogel nanoparticles. *(84)*

Dendrimer: they are three dimensional tree like branched macromolecules developed from a monomer having symmetrical structure with a narrow molecular weight distribution. They are
basic building blocks for large scale nanostructure. They can be used for delivery of photosensitizers, which can be used for cancer treatment. They will be having lot of application as they allow precise atom by atom control of nanostructure synthesis.\textsuperscript{(79; 85)} The common method for preparation are either divergent method (molecule is assembled from core to periphery) or convergent method (synthesized beginning from outside and terminated at core).\textsuperscript{(86)} PAMAM, PAMAMOS, PPI, tecto etc. are few type of dendrimer mainly used in pharmaceutical application.\textsuperscript{(87)} They are also having application in gene delivery.\textsuperscript{(88; 89)}

\textit{Carbon nanotubes (CNT)}: CNT, composed of graphite sheets wound around them in one or more layer, are crystalline form of pure carbon. They can be single walled, multi walled, torus etc. are few types of CNTs. Drug is attached by activation of carbon nanotubes. As the diameter is very less and the end part is very sharp, it can go to any cell and thus toxicity is a major issue for the use of CNT in pharmaceutical industry.\textsuperscript{(85; 90)}

\textit{Fullerenes}: they are crystalline form of carbon with 28-100 atoms, forms hollow sphere/tube/ellipsoid shape. Chemical groups can be attached to carbon atoms, which will be having impact on their property and toxicity.\textsuperscript{(85; 91)} It is proposed that they will be having applications in treatment of cancer and AIDS.\textsuperscript{(92)}

\textit{Protamine based nanoparticles}: Protamine is cationic, nonantigenic, nontoxic peptide; having application in DNA delivery. They have advantages like provides stability to particles and increases uptake to cells with disadvantages of precipitation when come in contact with salts.\textsuperscript{(79)}

\textit{Albumin and gelatin nanosphere}: Albumin nanosphere are having no toxicity and are non-antigenic with good shelf life, stability and high loading capacity of hydrophilic drug giving control over release property of drug. Gelatin nanospheres are used for carrier of antibody for targeting lymphocytes.\textsuperscript{(79)}

\textit{Polystyrene nanospheres}: It is having application in cell biology, immunodiagnostic assay, as size standard for calibration of many equipment. They are able to prevent viral transmission.\textsuperscript{(79)}

\textit{Magnetic nanoparticles}: Magnetic nanoparticles are used by manipulating magnetic field for the use in magnetic resonance imaging, magnetic immunoassay, magnetic particle imaging and biomedical field. They are used in cancer treatment by using external high gradient magnetic field to target at specific site. This not only reduces side effects of cytotoxic drug, but also
reduces the dose of drug. This technique can be used in sarcoma tumor and brain tumor, which is extending its application in gene delivery. Even hyperthermia phenomenon is used in cancer treatment. (79; 93; 94; 95; 96; 97; 98)

*Gold nanoparticle:* Gold nanoparticle are used in tumor cell detection, with some limited use as carrier for hydrophobic drug delivery. They are having better stability at room temperature compare to when stored at refrigerated temperature. They have also been used in rheumatoid arthritis and Alzheimer’s disease. (79; 98; 99; 100)

### 2.4: Chitosan nanoparticles

Natural polymers are having advantages like biodegradability and biocompatibility, which makes them favorable for most of the drug delivery system. (21) Nanoparticle drug delivery system is used because of its high bioavailability, reduced dose and toxicity, target specific delivery, etc. Chitosan (CS) is one of the commonly used naturally obtained polymer for various therapeutic use. Chitosan ((1→4)-2-amino-2-deoxy-β-D-glucan), a linear polyamine with a high ratio of glucosamine to acetyl-glucosamine units is natural mucoadhesive cationic polymer which is obtained by partial deacetylation of chitin. (101) Chitosan (pKa= 6.5) is solubilized in acidic medium. (102) The primary amino groups lead to special properties that render Chitosan very interesting for pharmaceutical applications like development of controlled release drug delivery systems like Chitosan gels, tablets, capsules, microspheres, microcapsules and nanoparticles for parenteral, nasal, ophthalmic, transdermal, and implantable delivery of drugs, proteins, peptides, and gene materials. (103) The free amino functional group in Chitosan makes it possible to form nanoparticles by cross-linking, emulsion cross linking, spray drying, desolvation with cationic salts, ionic complexation/coacervation or ionic gelation method by interacting with various other reactive groups such as alginates, dextran sulphate, sodium tri poly phosphate (STPP), polyethylene glycol (PEG), different ligands, antibodies, DNA and pH sensitive moieties, etc. (104) Chitosan can enhance the transmucosal absorption by increasing the paracellular permeability of intestinal epithelia. Chitosan nanoparticles are having good potential for the ocular drug delivery system because of its mucoadhesive nature. (105) It has been extensively used in nasal drug and vaccine delivery. (106) Main advantage of Chitosan nanoparticle is it can be used for hydrophilic drug entrapment. The hydrophilic nanoparticle remains in circulation for long time by avoiding reticulo endothelial system (RES) without PEGlyation. The other advantage of using Chitosan...
nanoparticles is that they don’t require any organic solvent or any other extreme conditions mainly in ionotropic gelation or complex coacervation technique which results in more stabilization of proteins and peptides.

Ionic gelation and complex coacervation are almost same except that in ionic gelation uses electrolyte such as tripolyphosphate (TPP), whereas in complex coacervation oppositely charged ionic polymer such as alginate are used. Ionic gelation method using TPP is more common for entrapment of hydrophilic drugs, proteins and plasmids. By ionotropic gelation method, we can obtain size from 100-1000 nm and zeta potential between +20mv to +60 mv by varying the ratio of Chitosan/STPP. (107)

Size, surface charge, release characteristics and percentage drug entrapment efficiency of these Chitosan/STPP nanoparticles can be modulated by (i) using different molecular weight Chitosan, (ii) by incorporating additional polymers such as poloxamer, hyaluronic acid, cyclodextrin etc. or (iii) by using chemically modified Chitosan derivatives, such as N-trimethyl Chitosan. (106)

Figure 2.2: Structure of A- Chitosan and B- Sodium tri poly phosphate

A common pie chart can be given to explain the use of Chitosan in various applications as biomedical material: (108)
2.4.1 Methods of Chitosan nanoparticle preparation:

Chitosan based nanoparticles can be prepared by following methods.

1. Ionic gelation
2. Covalent crosslinks
3. Complex coacervation
4. Desolvation method
5. Emulsion-droplet coalescence method
6. Reverse micellar method

We will mainly discuss ionotropic gelation method in detail. Other methods are discussed in brief.

2.4.1.1 Ionic cross linking (Ionic gelation)

This method is the most common technique to prepare Chitosan nanoparticles. In this method only aqueous phases are used and no organic solvent is used, and thus the chances of degradation of peptides are decreased. Chitosan is dissolved in one phase, and in other phase negative charge generating polyanion like STPP is dissolved. Both the phases are mixed under magnetic stirring at room temperature which generates gel like nanoparticles. The nanoparticles are formed due to intra and inter molecular linkage between Chitosan and STPP. pH of both the phases can play important role on particle size and percentage entrapment efficiency. Generally the method doesn’t require any stabilizer or surfactants and the nanoparticles generated are stable in nature. (109)
To obtain high yield of Chitosan-TPP nanoparticles, the Chitosan: TPP weight ratio should be between 3:1 to 6:1. The nanoparticles can also be made by introduction of other hydrophilic polymer. Even it may increase versatility for association and delivery of proteins. In nanoparticles of Chitosan and diblock copolymer of ethylene oxide and propylene oxide, PEO-PPO is attached to surface of particle which was confirmed by transmission electron microscopy (TEM). In general, protein loading can be obtained as high as 50%. Highest loading efficiency can be obtained when pH of solution is above isoelectric point of protein, so that it has negative charge. In addition to ionically crosslinking with TPP, other mechanisms may also work for association of macromolecules, e.g., insulin nanoparticles prepared at pH where insulin is positively charged, showed 30% entrapment efficiency. These mechanisms could be hydrogen bonding, hydrophobic interaction and other physicochemical forces. Even the drug release profile of insulin varies with of pH medium. Cross linking induced by incorporation of TPP will make the nanoparticles compact and resistant to freeze drying.\(^\text{(110)}\) The cross linking density, crystallinity, and hydrophilicity of cross-linked Chitosan can allow modulation of drug release. Even the swelling behavior of cross linked Chitosan depends on pH of TPP. Cross linked Chitosan shows higher swelling ability.\(^\text{(111)}\)

There were some studies in which the effects of cationic surfactant like cetyltrimethylammonium bromide were studied. The addition of cationic surfactant might reduce the size, while increasing the zeta potential. The decreased size may improve permeability through extracellular membrane. The increased surface charge density may facilitate absorption efficiency and may prevent nanoparticles from enzymatic degradation.\(^\text{(112)}\)

![Schematic representation of Chitosan- STPP nanoparticle](image)

Figure 2.4: Schematic representation of Chitosan- STPP nanoparticle
2.4.1.2 Covalent crosslinks

The process involves the precipitation of the polymer followed by chemical crosslinking by oppositely charged polymer. Precipitation can be done by sodium sulfate followed by chemical crosslinking using formaldehyde, glutaraldehyde or even using a natural crosslinking agent such as genipin. Later on the solvent is removed by rotary evaporator and dry nanoparticles are obtained. In this method, the drug is immobilized on nanoparticles instead than encapsulation.\(^{(84)}\)

2.4.1.3 Complex coacervation

The process is spontaneous phase separation which occurs upon mixing of oppositely charged polyelectrolyte in aqueous medium. This method is almost similar to ionic gelation method, but here opposite charge generating polymer like alginate or dextran sulfate is used. The mechanical strength is comparatively high than that of ionic gelation method.\(^{(103)}\)

2.4.1.4 Desolvation method

In this method, a more water soluble polymer or water miscible nonsolvent for Chitosan is added drop wise under stirring and then liquid particles are hardened by chemical crosslinking of glutaraldehyde. These nanoparticles can be prepared by either o/w or w/o/w emulsion.\(^{(18)}\)

2.4.1.5 Emulsion-droplet coalescence method

As Chitosan is having solubility in acidic medium (below its pKa= 6.5), it will precipitate when it will come in contact with other alkali medium. In this method, two emulsions are prepared. In one emulsion, only Chitosan is dissolved along with the drug while in other emulsion, the Chitosan is dissolved along with little quantity of sodium hydroxide. Finally both the emulsions are mixed at high speed resulting in formation of small solid Chitosan nanoparticles due to collision between the two emulsions.\(^{(113)}\)

2.4.1.6 Reverse micellar method

In this method, Chitosan and drug solution is added to organic solvent having surfactant. The transparency of the solution should be maintained by adding water additionally if required. Cross linking agent is added to above system and is stirred continuously to evaporate the organic solvent to obtain transparent dry mass. Then, the obtained transparent material is dispersed in water and then suitable salt is added to precipitate out the surfactant which was separated by centrifugation. The obtained supernatant is having drug loaded nanoparticles. By dialyzing the solution, free drug is removed and the remaining solution is lyophilized.\(^{(114)}\)
2.4.2: Applications of Chitosan nanoparticles:

The applications of chitosan nanoparticles are described below:

2.4.2.1 Molecular imaging

Chitosan based system such as microbubble, micelles and liposomal nanoparticles are used in molecular imaging. The incorporation of hard and brittle calcium phosphate or hydroxyapatite with Chitosan yields a bioresorbable composite with favorable mechanical property for bone and cartilage tissue engineering. Particles with ferric oxide can be used in MRI for hepatocyte targeting imaging. The Chitosan coated Superparamagnetic iron oxide nanocrystals (SPION) are used as MRI contrast agent, which can have high labeling efficiency and high uptake efficiency by stem cells. Water soluble Chitosan- linoleic acid conjugate can be used for contrast agent to target hepatocytes. Gadolinium-loaded Chitosan nanoparticles displayed prolonged retention in tumor tissue after in vivo intratumoral injection. (115)

2.4.2.2 Protein delivery

2.4.2.2.1: Via oral route

2.4.2.2.1.1 As vaccination

The n-trimethyl Chitosan –TPP nanoparticles prepared by ionic gelation method for protein delivery system by oral route have shown promising results. They have reduced the transepithelial electrical resistance (TEER) or have increased the paracellular transport to the cells. They have shown good systemic immune response when immunized with urease loaded nanoparticles. (116)

The adsorption of protein on Chitosan nanoparticles is giving high loading capacity. Hydrophilic nanoparticle with negative surface charge (excess sodium alginate) can be uptaken by rat Peyer’s patches, which can be used as carrier of mucosal vaccination. (117)

Using Bovine serum albumin, the loading efficiency can be obtained as high as 68%. As the deacetylation degree increases, the loading efficiency also increases but the drug release rate decreases. As the molecular weight of Chitosan was increased, the loading efficiency was increased but the drug release rate was decreased. PEG addition can accelerate the drug release. (118)

M cell represent a potential portal for oral delivery of peptides and for mucosal vaccination because of their transcytotic capacity. Alginate modified trimethyl Chitosan nanoparticles were used for loading of urease. Nanoparticles may influence their ability to enhance drug
permeation through paracellular pathway. The systemic and mucosal immune responses were also good. \(^{(119)}\)

Recombinant hepatitis B surface antigen (rHBsAg) as a model was used to prepare hepatitis B vaccine, in which Chitosan nanoparticles were prepared by Chitosan-TPP ionic gelation method. Normal vaccines prepared by using alum as adjuvant, were unstable when there was slight temperature change, and thus stable vaccine was needed. Chitosan based nanoparticles were stable, and protected the associated antigen during storage, either as an aqueous suspension under different temperature conditions (+4ºC and −20ºC), or as a dried form after freeze-drying the nanoparticles. Even the IgG level was 9-fold higher than conventional alum adsorbed vaccines. \(^{(120)}\)

### 2.4.2.2.1.2 **For hyperglycemia**

Insulin loaded Chitosan-TPP nanoparticles were prepared at controlled pH by ionotropic gelation method for oral route delivery with 63% entrapment efficiency. \(^{(121)}\)

Insulin was entrapped by alginate and calcium chloride, and then Chitosan formed polyelectrolyte complex with alginate. They protect insulin from aggressive environment of GIT, when administered orally. Insulin was released in pH dependent manner. The glucose level was decreased by more than 40% for 18 hr with 50 IU/kg. Confocal microscopy study confirms that nanoparticles adhere to intestinal epithelium. \(^{(122)}\)

### 2.4.2.2  **Via nasal route**

#### 2.4.2.2.1 **As vaccination**

Chitosan-DNA nanoparticle complexes were prepared for vaccination purpose of flu, in which haemagglutinin and Influenza A virus were used as plasmids. Even measles and respiratory syncytial virus (RSV) by nasal route are also prepared. \(^{(123)}\)

Even they are effective for targeting to nasal associated lymphoid tissues (NALT) in nasal vaccine delivery. \(^{(124)}\)

Trimethyl Chitosan nanoparticles can increase the M-cell dependent uptake and enhance the association of the antigen with dendritic cells. \(^{(125)}\)

Nanoparticles transport through M cell co-culture model is 5 fold higher than intestinal epithelial monolayer, with atleast 80% Chitosan-DNA nanoparticles uptake in 30 min. \(^{(126)}\)

Chitosan nanoparticles and Chitosan-ethylene oxide- propylene oxide polymer blocks were used for association and control release of bovine serum albumin, tetanus and diphtheria...
toxoid. Protein was released at constant rate which matches with the intensity of protein loading. Tetanus vaccine was released for atleast 15 days. (127)

2.4.2.2.2 For hyperglycemia

As Chitosan is having mucoadhesive nature, it will intensify the contact between insulin and nasal mucosa, leading to increased concentration at absorption site. The nanoparticles were prepared by ionic gelation method, which was having average size of 300-400 nm and 55% insulin loading efficiency. The molecular weight of Chitosan had no impact on drug release profile or on the level of blood glucose level, but the nanoparticles were able to reduce blood glucose level in rabbit due to increased nasal absorption. (128)

The Chitosan concentrations, osmolarity, medium and absorption enhancers in Chitosan nanoparticles have significant effect on the insulin nasal delivery. As the concentration increases, the insulin transport increases. The permeability will also increase in hypo or hyper osmotic medium compare to iso-osmotic medium. (129)

2.4.2.3 For anti-fungal delivery

The amphotericin B is the ideal candidate for many fungal infections. But it is very less soluble and its bioavailability is poor by oral route. Thus it needs to be given by parenteral route, which is causing nephrotoxicity. The nanoparticles were prepared by Chitosan- dextran sulphate coacervation method using zinc sulphate as stabilizing agent. Loading efficiency of 65% was obtained by this method. More importantly, the nephrotoxicity was reduced when checked by in vivo renal toxicity study. (130)

2.4.2.4 For gene delivery system

The Chitosan and DNA interaction is electrostatic, which is strong enough that they don’t dissociate until they have entered in to cell. Even the mucoadhesive and cationic nature of Chitosan play important role in adhesion to cell and lysosomal escape of DNA. They are divided in two categories depending on mechanism of formation. The complex can be protected from DNase to improve bioavailability of plasmid DNA.

2.4.2.4.1: Chitosan-DNA complexes:

Gentle mixing followed by incubation of Chitosan and DNA solution can generate Chitosan-DNA complexes with size from 100 to 600 nm depending on the molecular weight of Chitosan, in which proportion of Chitosan is in excess. The stability depends on positive
amino group of Chitosan to the negative phosphate group of DNA, and is also having direct effect on surface charge and particle size. Higher charge ratio can give better stability along with good transfection efficiency. \cite{131}

2.4.2.4.2: Chitosan-DNA nanosphere:

In this method, Chitosan and DNA solutions are mixed with controlled speed of mixing and temperature with addition of dilute salt in to DNA solution which works as a desolvating agent for polymer. They are having size of 200 to 500 nm with loose rodlike and toroidal structure. These nanoparticles may be entering to cell via endocytic pathway. \cite{131}

The size of Chitosan-DNA complex decreases as the molecular weight (MW) of Chitosan decreases. High MW Chitosan are superior to those of low MW Chitosan in enhancing the stability of complex, giving protection to DNA in cellular endosomal/lysosomal compartments, but on other side, it restricts release of DNA. Higher deacetylation of Chitosan will result in increase positive charge enabling a greater DNA binding capacity and cellular uptake. Chitosan in salt form have higher transfection efficiency than Chitosan base alone. pH below pKa value of Chitosan favors DNA association and dissociation. \cite{132}

Der p 2- a house dust mites dermatophagoides pteronyssinus is responsible for asthma, perennial rhinitis and atopic dermatitis. Thus allergic diseases can be characterized by sensitization of allergen specific Th2 cells and IgE production. The Chitosan pDer p 2 nanoparticles have shown 100% encapsulation efficiency with sufficient protection of plasmid. They are able to induce interferon- γ (IFN-γ) in serum, and thus prevent allergic response caused by Th2 sensitization. \cite{133}

Generally Chitosan-DNA vaccines are applied through traditional high pressure gene guns which elicit high titres of protective immunity, but will cause inevitable pain. To overcome this, a low pressure gene guns were used. The vaccine for Japanese encephalitis virus was prepared, which have produced specific antibodies, and have maintained high survival rate. \cite{134}

Chitosan lactate and Chitosan acetate have also been used as carrier of pSV β-galactosidase plasmid, have shown cell viability of more than 90%. \cite{135}

2.4.2.5: For hepatitis treatment

Glycyrrhetic acid is an aglycone and an active metabolite of glycyrrhizin which is having anti-inflammatory, anti-hepatotoxic, anti-tumorigenic activity and is used in chronic hepatitis. It is having side effect of aldosteronism. Glycerrhetic acid is metabolite of glycyrrhizin and is active...
in nature. But bioavailability of glycerrhetic is very less when given as such. Thus the nanoparticles of ammonium glycyrrhizinate were prepared by PEGlated Chitosan-TPP ionic gelation method, which was having 82% entrapment efficiency.\(^{(136)}\)

### 2.4.2.6: For anti-microbial agents

Chitosan- alginate nanoparticles were prepared for Polymixin B, a potent peptidic antibiotic having effect on gram negative bacteria. They also showed uptake by M cells. Polymixin B was earlier given by parenteral route because it was absorbed very less by oral route.\(^{(137)}\)

Amoxicillin- a broad spectrum antibacterial is having very short half-life suggesting frequent dosing. To overcome this, a controlled drug delivery system of Chitosan-TPP nanoparticle was developed.\(^{(138)}\)

Chitosan nanoparticles and copper loaded nanoparticles have shown antibacterial activity against \textit{E. coli}, \textit{S. choleraesuis}, \textit{S. typhimurium}, and \textit{S. aureus} and the results states that minimum inhibitory concentration is 0.25µg/mL, and minimum bactericidal concentration is 1 µg/mL. Exposure of \textit{S. choleraesuis} to nanoparticles disrupt the cell membrane and causes leakage of cytoplasm, which was later on confirmed by Atomic forced microscopy.\(^{(139)}\)

### 2.4.2.7: For tumor targeting

Doxorubicin is one of the potent anti-cancer agents having cardio toxicity. The nanoparticles prepared by Chitosan- dextran have shown reduction in side effects and improved efficacy in treatment of solid tumor. The size of 100±10 nm was obtained which favors the enhanced permeability and retention effect observed in solid tumors.\(^{(140)}\)

The doxorubicin was not released in cell culture, and it had entered to cell while remaining associated with nanoparticles, which was later confirmed by confocal microscopy. The positive charge carrier of nanoparticle will be useful in the treatment of solid tumor. Even the biodistribution and organ accumulation pattern will be changed when given by intravenous route.\(^{(141)}\)

5-amino salicylic acid (5-ASA), an anti-inflammatory agent used in ulcerative colitis, crohn’s disease which may provide protection against development of colorectal cancer in patient suffering from inflammatory bowel disease. It is metabolized in intestine and eliminated from there. If given orally, adverse effects like hepatitis, blood dyscrasias, pancreatitis, pleuropericarditis and intestinal nephritis can be seen. Chitosan-Ca-alginate matrix in which 5-
ASA is dispersed can be used for targeting colon because Chitosan is insoluble in pH above 6.5 and is mucoadhesive in nature. In colon Chitosan is degraded by microflora and free drug is available.\(^{(142)}\)

Gadolinium neutron capture therapy utilizes photon and electrons emitted in vivo as a result of nuclear neutron capture reaction with administered gadolinium-157 and non-radioelement. It is having highest thermal neutron capture cross section, and release of gamma rays and electron by neutron-capture reaction. Gaopentetic acid loaded Chitosan nanoparticles were prepared for gadolinium neutron capture therapy and MRI diagnosis by emulsion droplet coalescence technique. The nanoparticle have not released gadolinium in phosphate buffer and retained gadolinium in tumor for long time in vivo after intratumor injection.\(^{(143)}\)

Epirubicin is an effective anticancer agent. Chitosan was carboxymethylated and bound covalently on Fe\(_3\)O\(_4\) nanoparticles via carbodiimide activation. Thus Chitosan-magnetic nanoparticles were prepared for diagnosis and targeted therapy, it could be used in biomedicine.\(^{(144)}\)

5-flourouracil (5-FU) is mainly used in colon cancer. 5-FU loaded n-succinyl Chitosan nanoparticles were prepared by emulsification solvent diffusion method. It was having 19% loading capacity with 61% release in 24hrs. They have shown good anti-tumor activity against sarcoma 180 solid tumor with reduced toxicity.\(^{(109)}\)

Heparin- a known anticoagulant, is known to interact with diverse groups of proteins having heparin binding domain which regulates cell proliferation, differentiation and inflammation. It exhibits anti-cancer activity in process of tumor progression and metastatis. It binds with vascular endothelial growth factor (VEGF) and inhibits angiogenesis required for tumor growth. Free heparin molecules within cells interacts with transcription factors which plays important role in cell survival and growth, ultimately leading to apoptotic cell death via caspase dependent pathway. Chitosan-PEG-heparin polyelectrolyte complexes were prepared which were having higher toxicity against B16F10 cells. The cancer cells show higher internalization of complex, so higher cellular uptake of heparin resulting in dramatic cell death.\(^{(145)}\)

Colorectal cancer is having very short survival time because early detection is not excellent. 5-aminolaevullinic acid (5-ALA) loaded Chitosan-TPP nanoparticles were prepared to detect colorectal cancer at early stage. 5-ALA is zwitterionic drug, so the entrapment efficiency by this method was greatly affected by pH of solution. 5-ALA is degraded to protoporphyrin IX. The protoporphyrin IX is having different decomposition rate in cancer cell and normal cells, and is
photosensitive fluorophore, and thus it can be used for detection of colorectal cancer. Even nanoparticles were able to escape from bacterial uptake in GIT. Fluorescence microscope showed that nanoparticles are engulfed by caco-2 colon cancer cells. \(^{(102)}\)

Arginine rich hexapeptide are blocking the growth and metastasis of VEGF, which secretes human carcinoma cells. It shows significant inhibition of angiogenesis induced by VEGF in chorioallantoic membrane and rabbit cornea neovascularization. Thus it is useful in human tumor and angiogenesis dependent diseases which are related to action of VEGF. The peptide was encapsulated by Chitosan-dextran sulfate nanoparticles by coacervation process, with 75% entrapment efficiency with sustained release characteristics. \(^{(146)}\)

Tamoxifen (TMX) is used in breast cancer, is having side effect for vaginal symptoms, thrombotic events, stroke, endometrial cancer and drug resistance. TMX Chitosan nanoparticles prepared shows uptake by Peyer’s patch. The nanoparticles are transported to breast cells via lymphatic system reducing the toxicity. \(^{(145)}\)

Catechin like polyphenolic compound is having anti-oxidant property, by which heavy metals can be chelated and lipid peroxidation can be prevented, which can reduce many side effects in cancer treatment. They undergo high first pass metabolism. The catechin loaded nanoparticles were prepared by ionic gelation method, which showed entrapment efficiency of 90% along with 32% drug release in 24hr. \(^{(147)}\)

2.4.2.8: For buccal and sublingual delivery system

Chitosan and trimethyl Chitosan were even able to increase macromolecule permeation across buccal epithelium, which was later on confirmed by confocal laser microscopy, histopathology analysis and immunohistochemistry reaction. The increase in permeability was quantified by franz diffusion cell using isolated buccal epithelium. \(^{(146)}\)

2.4.2.9: For articular joint therapy

Macrophages are responsible for increased tissue permeability at inflammation site, including rheumatoid arthritis. Thus their selective elimination from such inflammatory site may be beneficial. Photodynamic is used in such therapy which involves nontoxic photoactivable dye known as photosensitizer along with harmless visible light of defined wavelength. When photosensitizers are activated, they form reactive oxygen species resulting into destruction of macrophages along with cellular death. The phototoxicity of photosensitizers entrapped in
Hyaluronate–Chitosan nanogels was decreased considerably. The nanogel encapsulated photosensitizers were retained in inflamed joint for long time compared to rapid clearance from joints of free photosensitizers. The treatment showed better result than the standard corticosteroid therapy for inflammation.\(^{(148)}\)

### 2.4.2.10: For ocular delivery system

Topical application to eye is limited due to protective physiological mechanism which exist at precorneal area resulting into drug loss, which ultimately results in to less effective concentration at site of action. The Chitosan nanoparticles were stable against lysozyme and didn’t affect the mucin viscosity. They also prolongs the retention time on eye surface. Confocal microscopy study has confirmed that nanoparticles penetrate corneal and conjunctival epithelia with 100% cell survival.\(^{(120; 149)}\)

Pilocarpine is choice of agent in open angle glaucoma. The drop causes frequent dosing schedule, while gels causes blurred vision. Picocarpine loaded Chitosan-carbopol nanoparticles were prepared which improves interaction with negatively charged biological membrane along with sustained release property.\(^{(150)}\)

Indomethacin reduces post-operative inflammation and decreases intraocular irritation cataract extraction and cystoid macular edema with side effects such as burning sensation, irritation and epithelial keratitis. Using Chitosan- TPP nanoparticles, the residence time of indomethacin was increased on cornea and thus the bioavailability was also increased. With loading efficiency of 85%, minimum 76% drug release was observed in 24h. The nanoparticles were able to treat chemical ulcer in rabbit eyes.\(^{(151)}\)

Cyclosporin A is effective in extraocular disorders like caratoconjuctivitis sicca or dry eye disease, with very slow partition rate at corneal epithelium. Nanoparticles were prepared by ionotropic method having 79% association efficiency. In vivo study on rabbit cornea suggested that therapeutic concentrations were achieved for 48h, while negligible level in inner ocular structure, blood and plasma.\(^{(105)}\)

Gatifloxin is fourth-generation fluoroquinolone antibiotic which inhibits bacterial enzyme DNA gyrase and topoisomerase IV. A Chitosan-sodium alginate nanoparticulate reservoir for ocular delivery was developed, which have average size of 205 to 572 nm and zeta potential from +17.6 mV to 47.8 mV. Nanoparticles have shown sustained release by non- Fickian diffusion process.
for ocular delivery. TEM and Atomic force microscopy (AFM) confirms that nano-particles are spherical and dense in nature.\(^{(152)}\)

### 2.4.2.11: For brain delivery

Alzheimer- a neurodegenerative disease, is becoming public health issue. Tacrine- acetylcholinesterase inhibitor, affects by reversible inhibition of cholinesterase, which increases level of acetylcholine in CNS. It is having high first pass metabolism, which result in poor oral bioavailability of 17±3 % only. Tacrine loaded Chitosan nanoparticles were prepared by spontaneous emulsification method, with good drug loading capacity. They have shown continuous slow release of drug. They have provided good result in alzhemier disease.\(^{(153)}\)

### 2.5: Characterization of Nanoparticles:

Nanoparticle’s characterization plays important role in stability, release property, site specificity, etc. Many parameters like size, shape, surface charge, drug release property, etc. play important role in characterization of nanoparticles.

#### 2.5.1: Particle size

Not only particle size but its distribution also plays much important role in nanoparticle drug delivery, as it can have direct impact with uptake, biodistribution, targeting, etc. But it has been observed that results vary among instrument of same type from manufacturer to manufacturer. Thus the methods to measure light are described below.\(^{(154)}\)

**Dynamic light scattering (DLS):** It records variation in intensity of scattered light on microsecond time scale, which is due to Brownian motion of individual particles, and is quantified by compilation of autocorrelation function, which is co-related to diffusion coefficient. For this coefficient, particle size is calculated. This is the most common method used for measurement of particle size of nanoparticles with advantages like speed, sensitive and don’t require calibration along with disadvantages like requires dilution of sample, interparticle interactions, need of cleanliness in sample preparation, etc.\(^{(154)}\) But it may not give accurate results when particle size distribution is wide.\(^{(82)}\) Its application is limited to small particles which are not affected by settling forces.\(^{(155)}\)
**Static light scattering (SLS):** In this method, pattern of light scattered is collected and fitted to fundamental magnetic equations in which, size is primarily available. This method is fast, but may measure size of agglomerated particle. \(^{(154)}\) Many theoretical knowledge of Fraunhofer theory, Mie theory, Becke theory, etc. One new method is developed in which refractive index is extrapolated to 100 % solute of dissolved compound, but it may require solubility in media of wide different polarity. \(^{(154)}\)

**Brunauer-Emmett-Teller (BET):** In this method, the gas adsorbed on surface of nanoparticle is measured, from which size is evaluated assuming that they are spherical in shape. \(^{(82)}\)

**Single Particle Optical Sensing (SPOS):** It records the scattering of a beam of light that results from passage of single particles through sensor, and response is compared with calibrated result and particle size is given from it. They are able to detect large particles (with count rate of 8000 particle/sec), which is utilized for detection of any problem in production. \(^{(154)}\)

**Field flow fractionation:** It is based on separation of particles according to their size in thin channel in which eluent is flowing in perpendicular direction produced by sedimentation (according to density). It complicated method and data interpretation is hard. \(^{(154}; 156)}\)

### 2.5.2: Particle Shape and morphology

Nanoparticles can be prepared in different shapes like spherical, crystalline, tubular, toroid, disc, etc. Shape is having effect on flow in body circulation, and is even having effect on damage to the cells. \(^{(157)}\) Thus it is important to know particle shape, which can be done by microscopic images. \(^{(82)}\)

**Electron microscopy:** Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are used for knowing morphology of particle. SEM gives result via irradiating electron beam to the sample and observes the second generated beam. Conductivity can play major role in SEM. Chemical composition and drug content evaluation can be done during SEM by using energy dispersive spectroscopy (EDS). \(^{(155)}\) TEM uses electron diffraction via staining gives structural information under vacuum. \(^{(154)}\) For knowing porosity of particle, three dimensional shape analysis is required which is done by 3D-SEM (three-dimensional scanning electron microscopy) and TEM-CT (transmission electron microscopy-computer aided tomography). 3D-SEM takes two images from different angle and gives information for thickness
and surface roughness. TEM-CT takes about 120 images when sample is rotated 1 degree interval from -60 to +60 degrees, giving three dimensional shape of nanoparticle. Internal structure can be observed by cryotransmission electron microscopy at very low temperature. 

**Atomic forced microscopy (AFM):** It is a type of scanning probe microscope (SPM), which produces topological map based on forced played between surface and tip of probe. Sample with adhesive nature can be deformed when it comes in contact of probe, which may not produce accurate image. The vertical resolution is very high compare to SEM/TEM. It is used view nanoparticles to surface of glass.

Table2.1 given below gives comparison of three microscopic techniques

<table>
<thead>
<tr>
<th></th>
<th>SEM</th>
<th>TEM</th>
<th>AFM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resolution</strong></td>
<td>Several nanometer</td>
<td>Atomic resolution</td>
<td>Atomic resolution</td>
</tr>
<tr>
<td><strong>Environment need</strong></td>
<td>Vacuum</td>
<td>Vacuum</td>
<td>Air, Vacuum, Gas, Liquid</td>
</tr>
<tr>
<td><strong>Sample preparation</strong></td>
<td>Easy</td>
<td>Difficult</td>
<td>Easy</td>
</tr>
</tbody>
</table>

2.5.3: Surface characteristics

**Zeta potential:** Charge of nanoparticle surface can be measured as zeta potential. It is affected by composition of nanoparticles, ionic strength and pH of environment. It can be measure by oscillations in signal that results from light scattered by particles located in electric field. The interaction between these particles determines aggregation and repulsion. Thus by controlling zeta potential, aggregation can be prevented.

**Hydrophilicity:** It is evaluated by hydrophobic interaction chromatography, which is based on affinity chromatography. The nanoparticles will elute according to their polarity. Nanoparticles are passed through column containing hydrophobic interacting gel, thus first hydrophilic compound will be eluted and later on (gradient chromatography) hydrophobic compound will be eluted.
Chemical composition: X-ray photoelectron microscopy is used for evaluation of chemical composition of nanoparticle surface. It is used for development of surface modified nanoparticles by giving evidence of component present on the nanoparticle surface. \(^{(156)}\)

Surface adsorption capacity: The capacity to adsorb proteins on surface of nanoparticles play important role in biodistribution. 2-D polyamide electrophoresis (PAGE) is used for analysis of proteins adsorbed on to the surface of nanoparticles along with their identification. For evaluation of modification of adsorbed protein in time dependent manner, capillary electrophoresis can be used. \(^{(154; 156)}\)

2.5.4: Internal structure:

*X ray diffraction (XRD)* method is used for evaluation of its crystalline structure. It measures X-ray intensity scattered by electron of particle/crystal. It can detect change in diffraction angle from 10 to 150°. The most stable structure of material is crystalline which are having unique peaks. Thus XRD measurement is important for identification of main component. \(^{(154; 82)}\)

*Differential scanning calorimetry (DSC)* is generally used for determination of extent of interaction of drug and other component. By measuring glass transition temperature and melting point along with their enthalpy, it can be used for determination of nature of crystallinity within nanoparticles. \(^{(154)}\)

*Fourier transform infrared spectroscopy (FTIR)* is for information of chemical bonding by measuring vibrational spectra. The vibrations can be in form of twisting, stretching, rotating, etc. By very small amount of sample, in any state with minimal interference of coexisting substance, results can be obtained. It takes when molecular vibration causes bipolar molecular moments. Thus it gives details regarding bonding of drug with polymers of nanoparticles during entrapment. \(^{(158)}\)

2.5.5: Drug release behavior:

Drug release from nanoparticle is not only affected by drug solubility but also by polymer erosion/degradation, diffusion in matrix of nanoparticle and desorption from surface of nanoparticles, etc. General USP dissolution apparatus are not effective for nanoparticle dissolution testing because of reasons like difficulty in separating dissolved drug from undissolved drug, specific enzymes needed for biodegradable nanoparticles (e.g. colon targeted
delivery), specific pH and temperature requirement, problem in maintaining sink condition, etc. If the nanoparticles are dissolving too fast, i.e. within few minutes, it may not give accurate rate and mechanism of release. General method used is dialysis bag diffusion technique in which nanoparticles are placed in a dialysis bag and placed in large medium under gentle stirring, and sample is taken from medium. The reverse approach, reverse dialysis bag technique in which nanoparticles are dispersed in to medium, and sample is collected within the bag. Other techniques include ultrafiltration/centrifugal ultrafiltration, diffusion cell and agitation followed by centrifugation/ultracentrifugation. The release can be faster initially when the drug is adsorbed on nanoparticle surface, but it can be controlled by coating of nanoparticles. (160; 161)

2.6: Mechanism of nanoparticle transport (13)

With development of nanoparticles system, in vitro model has been performed for the transport studies which lack the clinical studies. There are mainly two pathway by which nanoparticle can be transported:

A. Paracellular pathway
B. Transcellular pathway

![Diagram of Particle Transport Mechanism](image)

Figure 2.5: Particle Transport Mechanism (A) Paracellular transport, (B) Enterocytes and (C) M cells
2.6.1: **Paracellular transport**

The paracellular transport can be enhanced by interaction with negative charge of cell membrane or by complexing calcium ion involved in structure tight junction. Chitosan is having effect on depolymerization of cellular F-actin and tight junction protein Zonula occludens-1 (ZO-1). It can act via activation of protein kinase C.

Chitosan decrease cellular toxicity or damage because its effect on caco-2 cell line is reversible in nature.

2.6.2: **Transcellular transport**

It occurs by transcytosis- particles are taken up by cells, which begins with endocytosis and ends at the time of release at basolateral pole. Absorption occurs by mainly two kinds of cells: enterocytes and M-cells, located on payer’s patch. M-cells are able to absorb large range of materials. Some strategies for uptake of nanoparticle is given below

2.6.2.1: **Non-specific uptake of nanoparticles:**

2.6.2.1.1: By epithelial cells

Transcellualr transport can start by one of these endocytosis processes: pinocytosis, macropinocytosis and clathrin mediated endocytosis. All the activity is active transport, which requires energy. Phagocytosis and clathrin mediated endocytosis are receptor mediated, while pinocytosis is non-receptor mediated transport process.

Mucoadhesive materials improve transport by increasing residence time in contact with epithelium, thus increasing the concentration at site of absorption.

Chitosan nanoparticles may enhance oral uptake by crossing the epithelium, or that Chitosan molecules release the drug at the apical pole of epithelial cells, facilitating somehow their transcytosis. Even it can be due to interaction of Chitosan with tight junctions or adsorptive endocytosis which is saturable, energy and temperature dependent in nature. They may cause decrease in TEER value.

2.6.2.1.2: By M-cells

Particle transport by M-cells is energy dependent and transcellular which occurs by fluid phase endocytosis, adsorptive endocytosis and phagocytosis. Many factors like nanoparticle size, hydrophobicity, targeting moiety on surface, etc can play important role for nonspecific transcellular uptake. Particles below 1µm are taken up by M cells and transported to basal
membrane, while particles larger than 5 µm are taken up by M cells but they remain entrapped in Peyer’s patch.

2.6.2.2: Specific uptake of nanoparticles

2.6.2.2.1: By epithelial cells
The most popular approach is modification of nanoparticle surface. It can be done by use of lectins, wheat germ agglutinin, Concanavalin A, Tomato lactin. The level of targeting and uptake is directly proportional to the amount of targeting agent attached to particles. Targeting is a specific phenomenon, which greatly reduced in presence of free lectin or specific sugar. Chitosan nanoparticles were prepared using glucomanan, which facilitates interactions of nanoparticles with mannose receptors present in epithelial cells.

2.6.2.2.2: By M cells
The most popular approach is modification of nanoparticle surface. Most effective ligand is ulex europaeus agglutinin-1 lectin, which is highly specific for α-L-fucose, located on apical membrane of M cells. Lectins derived from Sambucus nigra and Viscum album were able to selectively label the surface of human follicle associated epithelium (FAE), and therefore could be used as ligands to human drug delivery applications.

Another strategy can be mimicking some pathogen bacteria, such as Yersinia, Salmonella, and Shigella species that are able to hijack the mucosal immune system, by using M cells to invade the intestinal mucosa. These bacteria present microbial adhesions at their surface, which are responsible for their binding and internalization by M cells. Immunoglobulins, particularly IgA, can specifically interact with M cell surface. Ganglioside GM1 could be used for targeting.

2.7: Design of experiment (DOE)/Factorial Design

Thus, it can be concluded that average size, shape, zeta potentials, percentage drug entrapment efficiency and drug release rate are important parameters to be considered for its pharmacological application, which are dependent on many factors such as drug concentration, drug solubility, polymer concentration, the evaporation rate of organic phase when using nanoprecipitation method, evaporation rate under vacuum in flask (for liposomes), concentration of surfactants, concentration of stabilizer, ratio of polymers, pH of solvent in which drug is solubilized, etc. and
may more. Thus it is becoming quite difficult to optimize nanoparticle formulation by general trial and error method, requiring a more precise method, which can provide desired outcome along with giving explanation. Design of experiment is one of the best approach used nowadays in pharmaceutical industry which is not only giving information predicting output, but also gives information for interactions between the factors. Even US FDA has also encouraged the use of factorial design.

2.7.1: Practical approach for DOE

The first step to create factorial design is to specify the no. of factors, levels and responses. It can be done by doing the following steps:

Stat> DOE> Factorial > Create factorial design, as shown in the image below:

The next image is “create factorial design” option menu.
As our Design of experiment involves three factors at three level, we have to select general full factorial design, and select the No. of factors as 3. Now select the Design option.

Now after giving input of the name of factor and their level, press Ok. Then Select the factors option.
After specifying the level values in factors, press Ok. Now select the Options menu.

After Deselecting the randomized runs and OK, Select the Results menu.

In this option, select the result in Summary table and design table and then Ok. Finally press OK in the Create factorial Design options.

It will generate a 27 run for the designed experiment. Now input the responses. i.e. Particle size (nm) and Percentage drug entrapment efficiency.
Now to analyze the factorial design follow the following steps:

Stat> DOE> Factorial> Analyze factorial Design as shown in the image below:
It will open the Analyze factorial design menu.

Select both the responses, and then select the terms menu. In this menu, select the default terms.
Now in analyze factorial design menu, select the graphs option.

After selecting the appropriate options, select results option in analyze factorial design menu.
After selecting appropriate options, select the Ok options of analyze factorial design.

It will generate the residual plots for particle size and percentage entrapment efficiency.

Regression analysis needs to be performed separately which can be performed by following way.

Stat> Regression> Regression.
Under the option of regression, select appropriate response and predictors.

After selecting appropriate options in graphs, options, results and storage, select OK.

It will generate regression equation, coefficient, P value, ANOVA table and residual plots.
Regression Analysis: PS versus X1, X2, ...

The regression equation is
PS = 1071.4 - 3780 X1 - 160 X2 - 241 X3 + 9514 X1^2 + 7.93 X2^2 + 10.8 X3^2
+ 149 X1X2 + 21.5 X2X3 + 1547 X1X3 - 154 X1X2X3

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<td>4.180</td>
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<td>7.268</td>
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<td>504.4</td>
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<td>X1X2X3</td>
<td>-153.91</td>
<td>55.81</td>
<td>-2.76</td>
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S = 5.02770  R-Sq = 90.3%  R-Sq(adj) = 84.2%

Analysis of Variance

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<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
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<td>404.44</td>
<td>25.28</td>
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<td>Total</td>
<td>26</td>
<td>4164.17</td>
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<table>
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<th>DF</th>
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<td>X2</td>
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<td>X1X2X3</td>
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<td>192.21</td>
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</table>
Now for performing Contour plots, following steps are needed to be performed. In this, we have to keep one axe constant, so our $3^3$ full factorial design will have three contour plots for each response.

Graph> contour plot
Under contour plot menu, select the appropriate options.
After selecting appropriate options, press OK. It will generate the contour plot.

Now for performing surface response plot, following steps are needed to be performed. In this, we have to keep one axe constant, so our $3^3$ full factorial design will also have three surface response plots for each response.

Graph> 3D Surface plot
After selecting 3D Surface plot, select surface or wireframe appropriately.
After selecting appropriate options in 3D Surface Plot - Wireframe, press OK. It will 3D surface plot.

2.8: Cell line study

As discussed in introduction, there are different cell lines for different tissues, which require different supplements which also differentiates their growth.