FORMULATION DEVELOPMENT AND CHARACTERIZATION OF BSA-LOADED CHITOSAN NANOPARTICLES FOR PROTEIN DRUG DELIVERY

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of his Master of Pharmaceutical Technology studies. All pieces of information in this document have been obtained and presented per academic rules and ethical conduct. I also declare that as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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Dedicated to My Parents and Family Members

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ABBREVIATIONS

BSA- Bovine Serum Albumin

CS- Chitosan

CS-NP- Chitosan Nanoparticle

FTIR- Fourier-transform infrared

GIT- Gastrointestinal tract

MW – Molecular weight

NPs-Nanoparticles

PBS- Phosphate Buffer Solution

SEM- Scanning Electron Microscope

TPP- Tripolyphosphate

XRD- X-ray diffraction

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CHAPTER-I

INTRODUCTION

1.1. Drug delivery

Drug delivery is the method or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals. To obtain a given therapeutic response, a suitable amount of the active drug must be absorbed and transported to the site of action at the right time and the rate of input can then be adjusted to produce the concentrations required to maintain the level of the effect for as long as required. For the treatment of human diseases, nasal and pulmonary routes of drug delivery are gaining increasing importance. These routes provide promising alternatives to parenteral drug delivery, particularly for peptide and protein therapeutics. For this purpose, several drug delivery systems have been formulated and are being investigated for nasal and pulmonary delivery [1]. These include liposomes, proliposomes, microspheres, gels, prodrugs, cyclodextrins, among others. Nanoparticles composed of biodegradable polymers show assurance in fulfilling the stringent requirements placed on these delivery systems, such as the ability to be transferred into an aerosol, stability against forces generated during aerosolization, biocompatibility, targeting of specific sites or cell populations in the lung, the release of the drug in a predetermined manner, and degradation within an acceptable period. Intravenous (IV), intramuscular (IM), intranasal (IN), intradermal (ID)/transdermal and oral administration are the main drug delivery routes. Other routes, such as ocular delivery, have also been developed for localized, site-specific drug administration without unwanted systemic side effects. Each administration method faces specific barriers against the delivery of the drugs. In addition, drugs can be incorporated into delivery devices, which considerably contribute to the preservation of the drug, targeting and therapeutic efficacy [2].

1.2. Routes of drug delivery

Among the various drug delivery routes, the oral pathway has attracted the most attention due to its unique advantages, including sustained and controllable delivery, ease of administration, feasibility for solid formulations, patient compliance and an intensified immune response in the case of vaccines. However, the oral drug delivery system faces the harsh physiological and physicochemical environment of the gastrointestinal tract, which limits the bioavailability and targeted design of the oral drug delivery system. The different anatomical characteristics, such

as limited surface in the oral cavity, gastric mucin–bicarbonate barrier, and enteral enzymes could also be obstacles to drug absorption. Since oral delivery has very limited oral bioavailability, nanoparticles have presented great potential in drug delivery. Numerous nanocarriers including nanoparticles, liposomes, emulsions etc., have been applied for oral drugs. Most nanocarriers showed advantages in protecting drugs from harsh conditions in the GIT, increasing the absorption into the circulatory system from GIT, targeting specific sites and guaranteeing a controlled release. The limitations of oral drug delivery can be summarized by anatomy factors, biochemistry factors, and physiology factors in GIT. Anatomically, the GIT consists of the oral cavity, oesophagus, stomach, small intestine, and colon, each part having different factors that affect drug delivery. The different anatomical characteristics of GIT show varying effects on drug absorption. Different pH environments and digestive enzymes were regarded as the main biochemical barriers for oral drug delivery systems. The pH varies distinctly in different parts of the GIT, it rises gradually from the stomach to the colon in the range from 1 to 8. The variation from acidic to alkaline environment affects the drugs' activities and bioavailability. The GIT exerts a low permeability to the bloodstream and extraneous substances, which restricts the bioavailability and absorption of drugs. The physiological barriers mainly consist of the epithelium cellular barrier and the mucus barrier [1-3].

1.3. Protein drug delivery

Protein drug delivery refers to the administration of therapeutic proteins to target sites within the body for the treatment of various diseases and medical conditions. Unlike conventional small molecule drugs, which are often orally administered, protein drugs typically require specialized delivery systems due to their large size, susceptibility to degradation, and potential immunogenicity. The introduction of protein drugs has revolutionized the field of medicine, offering targeted and potent therapies for diseases ranging from cancer and autoimmune disorders to metabolic diseases and infectious diseases [4]. Therapeutic proteins and peptides represent an appreciable part of pharmaceuticals contributing to various treatment protocols like cancer therapy.

However, permeability and stability are the main disadvantages of these therapeutics. To address these limitations, the parenteral route is the most effective route for the delivery of these drugs [5]. However, novel formulations are being explored by researchers other than

parenteral due to their ease of administration. Macromolecular drugs such as therapeutic proteins and peptides have become needful in the treatment of cancer as well as metabolic disease. Pharmaceutical biotechnology has a good impact on developing such proteins and peptides [6]. However pharmacokinetic properties of these therapeutics are the main drawback in the delivery aspect. However, the research on routes of administration other than parenteral continues. Successful delivery of an unchanged form of protein or peptide to the targeted site is not feasible for major factors like high proteolytic activity and acidic condition of the gastrointestinal tract. To achieve successful protein formulation, stability and the storage conditions of the formulation must be carefully considered and optimized [7].

1.4. Carriers for drug delivery

Without drug carriers administration of drugs is not possible, Drug carriers are biocompatible tools for the transport of molecules for pharmaceuticals. The term drug carrier was used, for the first time, to define a system that has the capability of incorporating a fixed number of molecules to enhance their selectivity, bioavailability, and efficiency. The effectiveness of a drug carrier during its delivery stands in the necessity of having a valuable protective barrier. This barrier can add an important resistance to mass flow and diffusion from the inner core to the external bulk form [8].

Drug carriers can be classified according to their shape, geometry, and production methods.

- **Hydrogels**

  Hydrogels are characterized by a network of hydrophilic polymers that can hold large amounts of water, saving the backbone of the structure. The key consists of using a polymer able to form a three-dimensional network by cross-linking its chains, exploiting weak forces, such as covalent bonds, hydrogen bonds, or van der Waals forces. Hydrogels can protect loaded drugs from the external environment, such as the acidic pH of the stomach during oral administration. Similar to hydrogels, lipogels are similar preparations based on vegetables or animal oils used as gelification agents. Aerogels are low-density solids with high porosity, with the ability to keep their structure intact after exchanging their liquid pores with gas. They are employed in several biomedical applications, such as diagnostic agents [9].

- **Liposomes**

  Liposomes consist of one or more double layers of phospholipids. These systems can be used to load hydrophilic drugs in the inner core and/or lipophilic drugs in the double layer of phospholipids. Examples of drugs that are generally loaded into liposomes and transported to target cells are amphotericin and daunorubicin, especially for their anti-carcinogenic properties. The main problem of liposomes is linked to their production; several methods have been developed, but industries prefer to use batch-mode methods, which are characterized by low repeatability. Moreover, the raw materials employed are particularly expensive [10].

- **Foams**

  Foams are generally defined as two-phase systems in which a gas is dispersed into a liquid continuous phase. Bubbly foam is formed when the amount of gas is low enough to create spherical-shaped bubbles. In the absence of a stabilizing agent, disproportionation occurs very quickly, but this is the largest cause of instability [10].

- **Niosomes**

  Niosomes are novel drug delivery systems characterized by layers of non-ionic surfactant active agents. Their main difference with liposomes is that they use non-ionic surfactants in place of phospholipids. For this reason, they are substantially similar to liposomes in terms of shape and geometry but provide several advantages, due to their reduced tendency to aggregation and enhanced stability [11].

- **Microparticles**

  Microparticle sizes range from 1 to 500 μm and the well-known matrix or reservoir they exist in various structures. Beyond the excipients used, the structure and the shape determine the function as well. Microspheres can be characterized as matrix systems in which the drug is homogeneously dispersed, either dissolved or homogenously suspended. Microcapsules are heterogeneous particles where a membrane shell is surrounding the core forming a reservoir. A classical microsphere structure contains solid or liquid API dispersed or dissolved in a matrix. Microcapsules are reservoirs of microscopic size surrounded by a wall that can control the release from the reservoir [12].

- **Nanoparticles**

  Nanoparticles are characterized by several applications, such as cancer therapy, gene therapy, viral treatment, and radiotherapy, and they are largely employed in the delivery of proteins, antibiotics, vitamins, and vaccines. According to the great improvement of nanotechnology, these drug carriers can find all of the solutions necessary to improve the delivery of drugs to overcome disadvantages such as poor bioavailability, low tissue absorption, and loss of drugs during transport to the target cells. Nano-carriers are the choice of delivery of protein because of their resistance towards the gastric environment [13].

1.5. Advantages of Nanoparticles as Protein and Peptide Drug Carriers [15]

- ❖ Reduces the enzymolysis and aggregation of protein and peptide drugs in the gastrointestinal environment and increases the transmembrane absorption of the small intestinal epithelium.
- ❖ Changes the distribution of the drug in the body
- ❖ Both preparation material and preparation process are simple
- ❖ Achieves the therapeutic effect of controlled release and target to treat diseases
- ❖ Be targeted by the modified target ligand and prolong the retention time at a specific absorption site.

1.6. Polymeric nanoparticles

Polymeric nanoparticles are colloidal particles composed of synthetic or natural polymers, typically ranging from 1 to 500 nm. These nanoparticles are designed to encapsulate therapeutic agents such as drugs, proteins, or nucleic acids and deliver them to specific target sites within the body for therapeutic purposes. These carriers are usually categorized as either nanospheres or nanocapsules. In nanospheres, the drug is dispersed in a polymeric matrix whereas nanocapsules are reservoir systems in which the drug is confined within a polymeric shell. Both polymeric nanospheres and nanocapsules have been explored for the delivery of protein and peptide therapeutics. The properties of polymeric NPs are significantly affected by the nature

of polymers either natural or synthetic and the method of preparation. A few examples of commonly employed natural polymers include chitosan (CS), gelatin and alginate. These polymers are abundantly present in nature and have been extensively applied in oral proteins and peptide delivery. Among natural polymers, CS has shown the most interesting potential, which is attributed to its better solubility at the intestinal pH, improving mucoadhesiveness and permeation enhancement. In the small intestine, CS NPs can adhere to and infiltrate into the mucus layer and open the tight junctions between contiguous epithelial cells. Proteins can be encapsulated, adsorbed or chemically linked to the surface of polymeric NPs. Chitosan-based nanoparticles have emerged as a promising platform in pharmaceuticals, and biotechnology fields. These nanoparticles are prepared from chitosan, a naturally occurring biopolymer obtained from chitin, which is found in the exoskeletons of crustaceans like shrimp and crabs, as well as in fungal cell walls. The unique properties of chitosan, such as biocompatibility, biodegradability, low toxicity, and mucoadhesive nature, make it an attractive material for nanoparticle formulation. One of the most significant advantages of chitosan-based nanoparticles is their ability to encapsulate and deliver a wide range of bioactive compounds, including drugs, proteins, DNA, and vaccines. The encapsulation protects these bioactive agents from degradation and enhances their stability, bioavailability, and targeted delivery to specific cells or tissues. Moreover, chitosan nanoparticles can be surface-modified or functionalized with targeting ligands, such as antibodies, peptides, or aptamers, to achieve site-specific delivery and enhanced therapeutic efficacy. This targeted delivery minimizes off-target effects and reduces the required dosage of drugs, thereby lowering the risk of side effects. In addition to drug delivery, chitosan nanoparticles have applications in tissue engineering, wound healing, gene therapy, imaging, and diagnostics. Their versatile nature and tunable properties make them a versatile platform for developing innovative biomedical technologies [4].

Protein incorporation in polymeric NPs can be achieved by various methods. Natural polymers are generally more sensitive to processing conditions. Therefore, NPs with natural polymers are generated using mild techniques including ionic gelation, polyelectrolyte complexation and coacervation. NPs composed of synthetic polymers are normally prepared by more extensive techniques such as interfacial polymerization, emulsification–polymerization, emulsification-solvent evaporation, nanoprecipitation, salting out, supercritical fluids and emulsification solvent diffusion [14].

1.7. Ionic Gelation technique for preparation of polymeric nanoparticles

The ionic gelation technique is mainly employed for preparing NPs composed of natural polymers (CS, gelatin and sodium alginate). In ionic gelation, CS is dissolved in acetic acid (presence/absence of stabilizer) followed by the addition of polyanion or anionic polymer under mechanical stirring at room temperature. The preparation conditions are mild and protein can be encapsulated without the use of organic solvents or elevated temperature. However, it is difficult to achieve long-term controlled release due to the solubility of polymers. Some of the parameters affecting protein encapsulation by ionic gelation method are the molecular weight of the polymer, initial protein concentration and polymer concentration [14].

1.8. Drug Information [16]

Name: Bovine Serum Albumin is used as a model drug for protein and peptides.

Synonym: BSA, Cohn Fraction V, Fraction V

Structure:

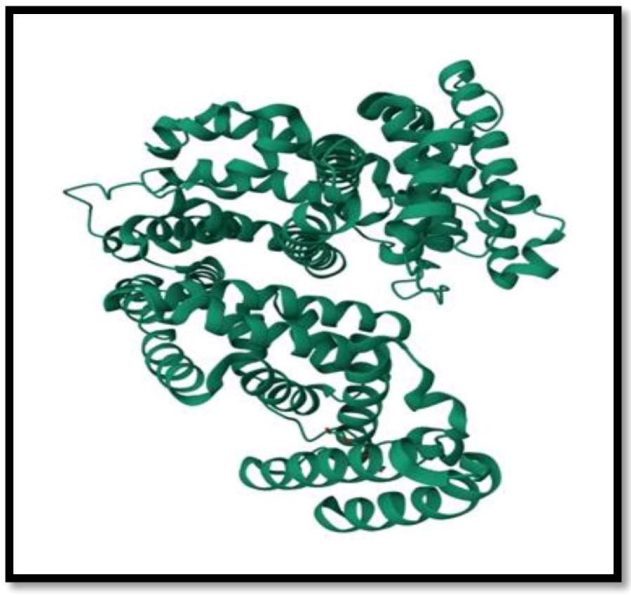

Fig.1: Three-dimensional structure of BSA

Description: Cow blood contains a monomeric protein called bovine serum albumin (BSA), which has a molecular weight of 66.5 kDa and is composed of a single chain of amino acids. Blood serum albumin (BSA) is primarily made by the liver. In labs, BSA is frequently added to biochemical and tissue culture media as a supplement. In addition to stabilizing the volume of extracellular fluid, the protein transports small molecules like thyroid hormones, fatty acids, and steroids. Moreover, BSA is employed in many industrial and scientific processes, such as food processing, protein purification, and medication development. It's not

toxic and non-antigenic, so it is not expected to cause an immune response in humans or other animals. Therefore, it is generally considered to be safe for use in research and industrial applications.

Source: Bovine Serum.

Physical Appearance: White to off-white coloured, lyophilized powder.

Purity: Greater than 97.0 %

Properties: The full-length BSA precursor protein is 607 amino acids in length. An N-terminal 18 residue signal peptide is cut off from the precursor protein upon secretion; hence the initial protein product contains 589 amino acid residues. An additional 4 amino acids are cleaved to yield the mature BSA protein that contains 585 amino acids.

Physical properties of BSA:

- Number of amino acid residues: 585
- Molecular weight: 66776 Da
- Isoelectric point in water at 25 °C: 4.7
- The molar extinction coefficient at 280nm for BSA is approximately 43,824M^{-1} cm^{-1}.

Solubility: This product is tested for solubility at 40 mg/mL in water.

Stability: If stored at 2-8°C, BSA powders and BSA solutions are stable for a minimum of 2.5 years.

Application:

- Bovine Serum Albumin has been used in the washing solution for flow cytometry analysis
- It has also been used in the resuspension buffer for flow cytometry
- Bovine serum albumin has been used as a component of PCR (polymerase chain reaction) mix.
- It is widely used for laboratory research purposes.

1.9. Polymer Information [17]

- **Name:** Chitosan
- **Synonym**: Deacetylated chitin, Poly(D-glucosamine)
- **Structure:** Chitosan is a linear copolymer polysaccharide made of β-(1-4)-linked 2-amino-2-deoxy-D-glucose (also known as D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (also referred as N-acetyl-glucosamine).

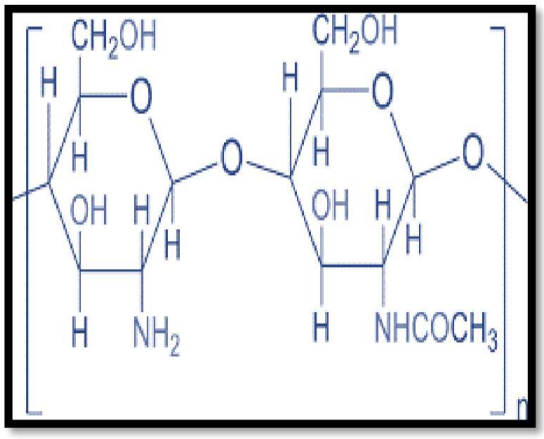

Fig.2: Structure of Chitosan

- **Source:** Shrimp and crabs are the most common sources cited in the literature as the raw material for chitosan preparation, while other species such as lobster, crayfish and oysters have also been utilized.

- **Description:** Chitosan is the second-most abundant natural polysaccharide mainly found in molluscs, insects, fungi, and yeasts. It can also be synthesized by partial removal of the acetyl group of chitin. On polymerisation through β-(1,4)-glycosidic linkage N-acetyl-glucosamine transformed to chitin while chitosan is a linear copolymer comprising of D-glucosamine and N-acetyl-D-glucosamine. The insoluble nature of chitin in water and most organic solvents used in pharmaceuticals make it unsuitable for drug delivery systems. Chitosan's positive charge is beneficial as it allows it to interact with polyanions. The solubility of chitosan in water and organic solvents is very poor but it is soluble in aqueous acidic solution. The positive charge of chitosan is due to the protonation of the amine group of glucosamine units in an acidic solution which is essential to interact with anions or polyanions. Because the chitosan molecule contains functional groups (OH and NH_2), it is possible to modify its chemical structure, resulting in the generation of derivatives with different characteristics, such as improved solubility and mucoadhesion. The extent of acetylation of high molecular weight chitosan is significant for protein delivery. Due to their significant mucoadhesive and absorption-enhancing properties, it has been extensively used to transport proteins and peptides via various administration routes for diverse therapeutic applications. It has been observed that chitosan can enhance the absorption of proteins.

❖ **Types and their properties:**

Table 1: Different types of chitosan and their properties

Parameter	**High molecular weight chitosan**	**Medium molecular weight chitosan**	**Low molecular weight chitosan**
Molecular weight	310-375 kDa	190-310 kDa	50-190 kDa
Solubility	Soluble in aq. acidic solution	Soluble in aq. acidic solution	Soluble in aq. acidic solution
Viscosity	800-2000 cP	200-800 cP	30-100 cP
Physical appearance	White to light tan powder	White to light tan powder	Coarse ground flakes and powder

1.10. Cross-linking agent information (TPP) [17]:

Name: Sodium tripolyphosphate

Synonyms: Pentasodium tripolyphosphate Anhydrous, STPP, Sodium triphosphate, Sodium triphosphate pentabasic, Sodium tripolyphosphate pentabasic.

Linear Formula: $Na_5P_3O_{10}$

Molecular Weight:367.86

Physical properties:

- **Physical state at 20 °C:** Solid
- **Colour:** White
- **Odour:** Odourless
- **pH:** 9.5-10.3 at 1g/L at 20°C
- **Melting point:**622°C
- **Solubility:** Freely soluble in water. Insoluble in ethanol.

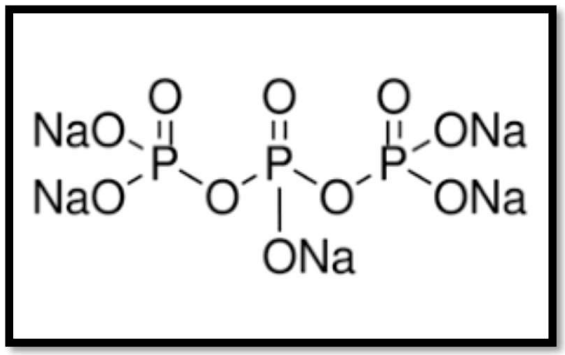

Fig.3: Structure of TPP

Structure: Pentasodium tripolyphosphate Anhydrous is sodium salt of tripolyphosphoric acid where all the five H atoms of five O-H groups are replaced with five sodium ions (Na^+)

1.11. Mechanism of cross-linking [18]

The positive charge of chitosan is due to the protonation of the amine group (NH_2) of glucosamine units in an acidic solution which is essential to interact with anions or polyanions.

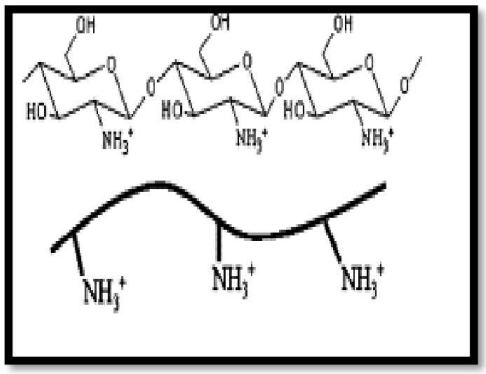

Fig.4: Protonated form of chitosan

TPP in an aqueous solution becomes ionized and all the five O-H bonds change to negatively charged oxygen (O^-) which can interact with NH_3^+ of protonated chitosan**.**

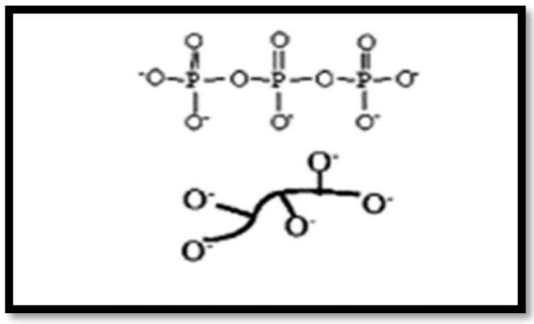

Fig.5: Ionized form of TPP

The ionic gelation technique involves the ionic interaction between NH_3^+ of chitosan and O^- of TPP crosslinker.

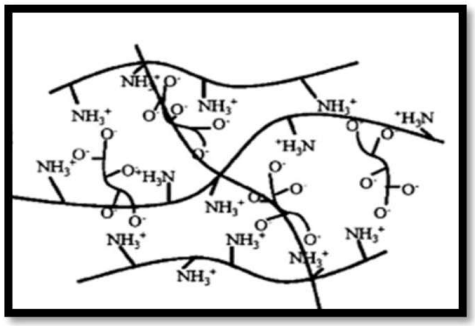

Fig.6: Chitosan crosslinked with TPP

1.12. Solvent [18]

- **Name:** Acetic Acid
- **Synonym:** Ethanoic acid, vinegar, glacial acetic acid
- **Structure:**

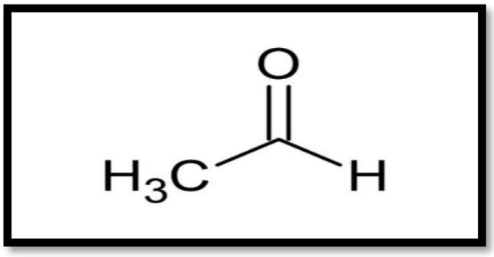

Fig.7: Structure of acetic acid

Properties:

- Acetic acid is a colourless liquid; with a strong vinegar-like odour.
- It is flammable, and at temperatures warmer than 39°C, explosive vapour/air mixtures may be formed.
- **Specific Gravity:** 1.049 at 25°C
- **Melting Point:** 16.7°C
- **Boiling Point:** 118°C
- **Vapour pressure:** 1.5 kPa at 20°C

References

1. Tiwari G, Tiwari R, Sriwastawa B, Bhati L, Pandey S, Pandey P, Bannerjee SK. Drug delivery systems: An updated review. Int. J. Pharm. Investig. 2012;2(1):2-11.
2. Homayun B, Lin X, Choi HJ. Challenges and recent progress in oral drug delivery systems for biopharmaceuticals. Pharm. 2019;11(3):1-29.
3. Lou J, Duan H, Qin Q, Teng Z, Gan F, Zhou X, Zhou X. Advances in oral drug delivery systems: Challenges and opportunities. Pharm. 2023;15(2):1-22.
4. Andrade F, Antunes F, Vanessa Nascimento A, Baptista da Silva S, das Neves J, Ferreira D, Sarmento B. Chitosan formulations as carriers for therapeutic proteins. Curr. Drug Discov. Technol. 2011;8(3):157-72
5. Cano-Cebrian MJ, Zornoza T, Granero L, Polache A. Intestinal absorption enhancement *via* the paracellular route by fatty acids, chitosans and others: a target for drug delivery. Curr Drug Deliv.2005; 2: 9-22.
6. Gupta S, Jain A, Chakraborty M, Sahni JK, Ali J, Dang S. Oral delivery of therapeutic proteins and peptides: a review on recent developments. Drug Deliv. 2013;20(6):237-46.
7. Hou Y, Hu J, Park H, Lee M. Chitosan-based nanoparticles as a sustained protein release carrier for tissue engineering applications. J. Biomed. Mater. Res. 2012;100(4):939-47.
8. Trucillo P. Drug carriers: Classification, administration, release profiles, and industrial approach. Processes. 2021;9(3):470-88.
9. Julienne, V.M.; Benoit, J. Preparation, purification and morphology of polymeric nanoparticles as drug carriers. Pharm. Acta Helv. 1996; 71(2), 121–28
10. Huertas, M.C.; Fessi, H.; Elaissari, A. Polymer-based nanocapsules for drug delivery. Int. J. Pharm. 2010; 385(1-2), 113–42.
11. Patel A, Patel M, Yang X, K Mitra A. Recent advances in protein and peptide drug delivery: a special emphasis on polymeric nanoparticles. PPL. 2014;21(11):1102-20.
12. Ravindran R, Mitra K, Arumugam SK, Doble M. Preparation of Curdlan sulphate-Chitosan nanoparticles as a drug carrier to target Mycobacterium smegmatis infected macrophages. Carbohydrate Polymers. 2021; 258:1176-86.
13. Cao SJ, Xu S, Wang HM, Ling Y, Dong J, Xia RD, Sun XH. Nanoparticles: oral delivery for protein and peptide drugs. AAPS PharmSciTech. 2019; 20:1-11.
14. Jachimska B, Pajor A. Physico-chemical characterization of bovine serum albumin in solution and as deposited on surfaces. Bioelectrochem. 2012; 87:138-46

15. Huang X, Du YZ, Yuan H, Hu FQ. Preparation and pharmacodynamics of low-molecular-weight chitosan nanoparticles containing insulin. Carbohydr. Polym. 2009;76(3):368-73.
16. Joudeh N, Linke D. Nanoparticle classification, physicochemical properties, characterization, and applications: a comprehensive review for biologists. J. Nanobiotechnology. 2022;20(262).1-29
17. Silvestro I, Francolini I, Di Lisio V, Martinelli A, Pietrelli L, Scotto d'Abusco A, Scoppio A, Piozzi A. Preparation and characterization of TPP-chitosan crosslinked scaffolds for tissue engineering. J. Mater. 2020;13(16):1-15.
18. Marti ME. Solvent modification effect on the physical and chemical extraction of acetic acid. Sep. Sci. Technol. 2016;51(11):1806-16.

CHAPTER-II

LITERATURE REVIEW

1. Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system [1]

Maryam Amidi *et al.* (2006)

They have developed protein-loaded N-trimethyl chitosan nanoparticles as a potential nasal delivery system. These nanoparticles were prepared by crosslinking TMC solution with tripolyphosphate, and their size, zeta potential, and morphology were studied. They were found to have a loading efficiency of up to 95% and a loading capacity of up to 50%. The integrity of the entrapped ovalbumin was preserved. Release studies showed that over 70% of the protein remained associated with the nanoparticles for at least 3 hours. Cytotoxicity tests showed no toxic effects, but a partially reversible cilio-inhibiting effect on the ciliary beat frequency of the chicken trachea. In vivo uptake studies indicated the transport of FITC-albumin-associated TMC nanoparticles across the nasal mucosa.

2. Microspheres containing lipid/chitosan nanoparticle complexes for pulmonary delivery of therapeutic proteins [2]:

Ana Grenha *et al.* (2008)-

They created microspheres containing lipid/chitosan nanoparticle complexes for pulmonary delivery of therapeutic proteins. The resulting microspheres are spherical and have suitable aerodynamic characteristics for lung delivery. The physicochemical properties of the L/CS-NP complexes are influenced by the composition of phospholipids, which provide controlled release of the encapsulated protein (insulin). The complexes can be easily recovered from mannitol microspheres, demonstrating that protein-loaded L/CS-NP complexes can be efficiently microencapsulated, resulting in microspheres with adequate properties for deep inhalation.

3. Development of Chitosan Nanoparticles as a Stable Drug Delivery System for Protein/siRNA [3]:

Haliza Katas *et al*. (2013)-

They developed Chitosan Nanoparticles as a Stable Drug Delivery System for Protein/siRNA. CS NPs were fabricated by ionic interaction with dextran sulphate (DS) prior to the determination of their storage stability. The smallest CS NPs of 353 ± 23 nm with a surface charge of +56.2 ± 1.5 mV were produced when CS and DS were mixed at pH 4 and with a DS : CS mass ratio of 0.5: 1. An entrapment efficiency of 98% was achieved when BSA/siRNA was loaded into the nanoparticles. The results also showed that particle size and surface charge of CS NPs were slightly changed up to 2 weeks when stored at 4∘ C. Greater particle size and surface charge were obtained with increasing the concentration of DS. In conclusion, NPs were sufficiently stable when kept at 4∘ C and able to carry and protect protein.

4.Preparation and Characterization of Water-Soluble Chitosan Nanoparticles as Protein Delivery System [4]:

Hong-liang Zhang *et al*. (2010)-

They prepared and characterized Water-Soluble Chitosan Nanoparticles as Protein Delivery System. The objective of this study was to investigate the potential of water-soluble chitosan as a carrier in the preparation of protein-loaded nanoparticles. Nanoparticles were prepared by ionotropic gelation of water-soluble chitosan (WSC) with sodium tripolyphosphate (TPP). Bovine serum albumin (BSA) was applied as a model drug. The size and morphology of the nanoparticles were investigated as a function of the preparation conditions. The particles were spherical in shape and had a smooth surface. The size range of the nanoparticles was between 100 and 400 nm. Results of the in vitro studies showed that the WSC nanoparticles enhance and prolong the intestinal absorption of BSA. These results also indicated that WSC nanoparticles were a potential protein delivery system.

5. Depolymerized Chitosan Nanoparticles for Protein Delivery: Preparation and Characterization [5]:

K. A. Janes *et al*. (2003)-

They prepared and characterized depolymerized chitosan nanoparticles for protein delivery. Using depolymerized fragments generated by NaNO2 degradation of different chitosan salts, they prepared nanoparticle formulations based on ionotropic gelation with sodium tripolyphosphate (TPP). Regardless of the formulation, the nanoparticle size decreased with decreasing molecular weight and the potential values remained unchanged. Similar comparisons were made with the encapsulation of insulin and tetanus toxoid as model proteins. The results indicated that the quantity of TPP in a given formulation has a greater effect on the protein encapsulation than the chitosan molecular weight. In fast-release environments (i.e., buffered media), there was no significant molecular weight effect that could be discerned. These data lead to the conclusion that, under these experimental conditions, the chitosan molecular weight has a measurable effect on the particle properties, although this effect is modest relative to other formulation parameters (e.g., TPP content, type of protein loaded).

6. Analytical characterization of chitosan nanoparticles for peptide drug delivery applications [6]:

E. Ieva et al. (2009)-

Chitosan-cyclodextrin hybrid nanoparticles (NPs) were obtained by the ionic gelation process in the presence of glutathione (GSH), chosen as a model drug. NPs were characterized using transmission electron microscopy and zeta-potential measurements. Furthermore, a detailed X-ray photoelectron spectroscopy study was carried out in both conventional and depth-profile modes. The combination of controlled ion-erosion experiments and a scrupulous curve-fitting approach allowed for the first time the quantitative study of the GSH in-depth distribution in the NPs. NPs were proven to efficiently encapsulate GSH in their inner cores, thus showing promising perspectives as drug carriers.

7. Chitosan Nanoparticles for the Linear Release of Model Cationic Peptide [7]:

Anna Maria Piras et al. (2015)-

The present study is focused on the development of a model drug delivery system (DDS) based on Chitosan (CS) nanoparticles using Renin substrate I (RSI) as a model agent. RSI shares the main chemical-physical features of several biologically active antimicrobial peptides (AMPs). AMPs have a great therapeutic potential that is hampered by their lability in the biological fluids and as such they are perfect candidates for DDS. The development studies of quality DDS loaded with AMPs would require highly sensitive and specific quantification assays. The use of RSI allowed for the fine-tuning and optimization of the formulation parameters to promote the hydrophobic interactions between CS and the cationic peptide, favour the loading of the active ingredient and enhance the release properties of the carrier. RSI was encapsulated in chitosan NPs by mean of ionic gelation and a chromogenic enzymatic essay was carried out for the release kinetics evaluation. The developed formulations displayed almost 100% of encapsulation efficacy, low burst percentages, and a linear release of the model peptide. A release model was created showing a direct dependence on both the amount of RSI and NPs radius.

8. Potential of different salt forming agents on the formation of chitosan nanoparticles as carriers for protein drug delivery systems [8]:

Manee Luangtana-anan et al. (2019)-

The effects of salt forming agents for chitosan on the potential for nanoparticle formation was investigated. The salt forms were prepared from the amino acid group, including glutamic and aspartic acids, and the alpha hydroxyl acid group, including lactic and glycolic acids. All types of chitosan salt could be used to prepare bovine serum albumin (BSA) loaded nanoparticles. The chitosan salts prepared from the amino acid group showed a higher salt formation ability as demonstrated using FTIR, hence a higher %encapsulation efficiency (%EE) and a reduction in zeta potential were obtained. The difference was due to the different organic acids used giving different polymer conformations and pH values in the solution. Chitosan glutamate gave the highest salt formation ability and hence the highest %EE was obtained. The release of protein from all types of chitosan was similar and chitosan glutamate exhibited the highest

release. Chitosan salt is therefore a material of choice for protein-loaded nanoparticles and the characteristics of nanoparticles can be readily modulated by different types of salt form.

9. Preparation and characterization of BSA as a model protein loaded chitosan nanoparticles for the development of protein-/ peptide-based drug delivery system [9]:

Preeti Yadav et al. (2021)-

The purpose of this study was to develop a protein-/peptide-loaded nanoparticle-based delivery system, which can efficiently deliver therapeutic molecules to the lung via pulmonary delivery. The chitosan nanoparticles were prepared by the ionic gelation method, and bovine serum albumin was used as a model protein. These nanoparticles were characterized for size, zeta potential, encapsulation efficiency, cell cytotoxicity, uptake study, release profile and size distribution and uniformity. The chemical interaction of chitosan and protein was studied by XRD and FTIR. The integrity assessment of encapsulated protein into nanoparticles was studied by native and SDS-PAGE gel electrophoresis. The size and zeta potential of BSA nanoparticles were 193.53±44.97 to 336.36±94.63 and 12.73±0.41 to 18.33±0.96, respectively, with PDI values of 0.35–0.45. The encapsulation efficiency was in the range of 80.73±6.37% to 92.34±1.72%. The cumulative release of the BSA from the nanoparticles was 72.56±6.67% in 2 weeks. The BSA-loaded nanoparticles showed good uptake and no significant cytotoxicity was observed into the A549 cell line. In this study, it was also observed that during nanoparticles' synthesis protein structure and integrity is not compromised. The nanoparticles showed controlled and sustained release with initial burst release. In TEM images, it was shown that nanoparticles' distribution is uniform within the nanometre range.

10. The use of chitosan-6-mercaptonicotinic acid nanoparticles for oral peptide drug delivery [10]:

Gioconda Millotti et al. (2011)-

The aim of this study was to develop a novel nanoparticulate formulation and test its potential for oral peptide drug delivery. Chitosan-6-mercaptonicotinic acid is a novel thiolated chitosan with strong mucoadhesive properties. Nanoparticles were developed by an ionic gelation

method. The obtained particles were characterized in terms of mucoadhesion, stability, toxicity, and in vitro release. Human insulin (HI) was chosen as a model peptide drug, incorporated in the particles and orally administered to rats. Human insulin was quantified in the blood by means of ELISA. The size of the obtained particles was in the range of 200–300nm and the zeta potential was determined to be +8 to +23 depending on the amount of thiol groups attached on the polymer. After 3h of incubation, up to 60% of the thiolated chitosan nanoparticles remained attached to the mucosa in contrast to 20% of unmodified chitosan particles. The AUC of HI after oral administration of thiolated chitosan nanoparticles was 4-fold improved compared to unmodified chitosan nanoparticles. Due to these improvements, chitosan-6-mercapto nicotinic acid nanoparticles are promising vehicles for oral delivery of peptide drugs.

11. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo[11]:

Yan Pan et al. (2002)-

There are many ongoing investigations to improve the oral bioavailability of peptide and protein formulations. Bioadhesive polysaccharide chitosan nanoparticles (CS-NPs) would seem to further enhance intestinal absorption of them. In this study, Insulin-loaded CS-NPs were prepared by ionotropic gelation of CS with tripolyphosphate anions. Its particle size distribution and zeta potential were determined by photon correction spectroscopy and laser Dopper anemometry. The ability of CS-NPs to enhance intestinal absorption of insulin and increase the relative pharmacological bioavailability of insulin was investigated by monitoring the plasma glucose level of alloxan-induced diabetic rats after oral administration of various doses of insulin-loaded CS-NPs. CS-NPs had a particle size in the range of 250/400 nm and its polydispersity index was smaller than 0.1, positively charged, stable. Insulin association was found up to 80% and its in vitro release showed a great initial burst with a pH-sensitivity property. CS-NPs enhanced the intestinal absorption of insulin to a greater extent than the aqueous solution of CS in vivo. Above all, after administration of 21 I.U./kg insulin in the CS-NPs, the hypoglycemia was prolonged over 15 h and the average pharmacological bioavailability relative to SC injection of insulin solution was up to 14.9%

12. Chitosan based nanoparticles as protein carriers for efficient oral antigen delivery[12] :

Ping Gao et al. (2016)-

This study aimed to investigate the efficacy of nanoparticles based on chitosan as a vehicle for oral antigen delivery in fish vaccination. Carboxymethyl chitosan/chitosan nanoparticles (CMCS/CS-NPs) loaded extracellular products (ECPs) of Vibrio anguillarum were successfully developed by ionic gelation method. The prepared ECPs-loaded CMCS/CS-NPs were characterized for various parameters including morphology, particle size (312 ± 7.18 nm), zeta potential (+17.4 ± 0.38 mV), loading efficiency (57.8 ± 2.54%) and stability under the simulated gastrointestinal (GI) tract conditions in turbot. The in vitro profile showed that the cumulative release of ECPs from nanoparticles was higher in pH 7.4 (58%) than in pH 2.0 (37%) and pH 4.5 (29%) after 48 h. Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) was used as model protein antigen and encapsulated in CMCS/CS-NPs for investigating the biodistribution of antigen after oral delivery to turbot in 24 h. Oral immunization of ECPs-loaded CMCS/CS-NPs group in turbot showed elevated specific antibody and higher concentrations of lysozyme activity and complement activity in fish serum than ECPs solution. CMCS/CS-NPs loaded with ECPs could enhance both adaptive and innate immune responses than the group treated with ECPs solution and suggested to be a potential antigen delivery system.

13. Chitosan-based oral nanoparticles as an efficient platform for kidney-targeted drug delivery in the treatment of renal fibrosis[13]:

Qian Zhang et al.(2024)-

There is increasingly keen interest in developing orally delivered targeted drugs, especially for diseases that require long-term medication. Hence, we manufactured nanoparticles derived from methoxypolyethylene glycolchitosan (PCS) to enhance the oral delivery and kidney-targeted distribution of salvianolic acid B (SalB), a naturally occurring renoprotective and anti-fibrotic compound, as a model drug for the treatment of renal fibrosis. Orally administered SalB-loaded PCS nanoparticles (SalB-PCS-NPs) maintained good stability in the gastrointestinal environment, improved mucus-penetrating capacity, and enhanced transmembrane transport through a Caco-2 cell monolayer. The relative oral bioavailability of SalB-PCS-NPs to free SalB and SalB-loaded chitosan nanoparticles (SalB-CS-NPs) was 367.0

% and 206.2 %, respectively. The structural integrity of SalBPCS-NPs after crossing the intestinal barrier was also validated by Forster ¨ resonance energy transfer (FRET) in vitro and in vivo. Fluorescein isothiocyanate (FITC)-labeled SalB-PCS-NPs showed higher kidney accumulation than free FITC and FITC-labeled SalB-CS-NPs (4.6-fold and 2.1-fold, respectively). Significant improvements in kidney function, extracellular matrix accumulation, and pathological changes were observed in a unilateral ureter obstruction mouse model of renal fibrosis after once daily oral treatment with SalB-PCS-NPs for 14 days. Thus, oral administration of SalB-PCS-NPs represents a promising new strategy for kidney-targeted drug delivery.

14. Synthesis of β-acids loaded chitosan-sodium tripolyphosphate nanoparticle towards controlled release, antibacterial and anticancer activity[14]:

Bingren Tian et al.(2024)-

The development of nanoparticles loaded with natural active ingredients is one of the hot trends in the pharmaceutical industry. Herein, chitosan was selected as the base material, and sodium tripolyphosphate was chosen as the cross-linking agent. Chitosan nanoparticles loaded with β-acids from hops were prepared by the ionic cross-linking method. The results of Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) indicated that chitosan nanoparticles successfully encapsulated β-acids. The loading capacity of chitosan nanoparticles with β-acids was 2.00 %–18.26 %, and the encapsulation efficiency was 0.58 %–55.94 %. Scanning electron microscopy (SEM), transmission electron microscope (TEM), particle size, and zeta potential results displayed that the nanoparticles revealed a sphere-like distribution with a particle size range of 241–261 nm, and the potential exhibited positive potential (+14.47–+16.27 mV). The chitosan nanoparticles could slowly release β-acids from different simulated release media. Notably, the β-acids-loaded nanoparticles significantly inhibited Staphylococcus aureus ATCC25923 (S. aureus) and Escherichia coli ATCC25922 (E. coli). Besides, β-acids-loaded chitosan nanoparticles were cytotoxic to colorectal cancer cells (HT-29 and HCT-116). Therefore, applying chitosan nanoparticles can further expand the application of β-acids in biomedical fields.

15. Size-dependent absorption mechanism of polymeric nanoparticles for oral delivery of protein drugs [15]:

Chunbai He et al. (2012)-

Polymeric nanoparticles have been widely applied to oral delivery of protein drugs, however, few studies focused on the systematical elucidation of the size-dependent oral absorption mechanism with well-defined polymeric nanoparticles. Rhodamine B labelled carboxylated chitosan grafted nanoparticles (RhB-CCNP) with different particle sizes (300, 600, and 1000 nm) and similar Zeta potentials (-35 mV) were developed. FITC-labelled bovine serum albumin (FITC-BSA) was encapsulated into RhB-CCNP to form drug-loaded polymeric nanoparticles (RhB-CCNP-BSA). RhB-CCNP-BSA with uniform particle size and similar surface charge possessed desired structural stability in simulated physiological environments to substantially guarantee the validation of elucidation on size-dependent absorption mechanisms of polymeric nanoparticles using in vitro, in situ, and ex vivo models. RhB-CCNP-BSA with smaller sizes (300 nm) demonstrated elevated intestinal absorption, as mechanistically evidenced by higher mucoadhesion in rat ileum, release amount of the payload into the mucus layer, Caco-2 cell internalization, transport across Caco-2 cell monolayers and rat ileum, and systemic biodistribution after oral gavage. Peyer's patches could play a role in the mucoadhesion of nanoparticles, resulting in their close association with the intestinal absorption of nanoparticles. These results provided guidelines for the rational design of oral nanocarriers for protein drugs in terms of particle size.

16. Polymeric nanoparticles for pulmonary protein and DNA delivery [16]:

Jyothi U. Menon et al. (2014)-

Polymeric nanoparticles (NPs) are promising carriers of biological agents to the lung due to advantages including biocompatibility, ease of surface modification, localized action and reduced systemic toxicity. However, there have been no studies extensively characterizing and comparing the behaviour of polymeric NPs for pulmonary protein/DNA delivery both in vitro and in vitro. We screened six polymeric NPs: gelatin, chitosan, alginate, poly(lactic-co-glycolic) acid (PLGA), PLGA–chitosan and PLGA–poly(ethylene glycol) (PEG), for inhalational protein/DNA delivery. All NPs except PLGA–PEG and alginate were <300 nm in size with a bi-phasic core compound release profile. Gelatin, PLGA NPs and PLGA–PEG NPs

remained stable in deionized water, serum, saline and simulated lung fluid (Gamble's solution) over 5 days. PLGA-based NPs and natural polymer NPs exhibited the highest cytocompatibility and dose-dependent in vitro uptake, respectively, by human alveolar type-1 epithelial cells. Based on these profiles, gelatin and PLGA NPs were used to encapsulate plasmid DNA encoding yellow fluorescent protein (YFP) or rhodamine-conjugated erythropoietin (EPO) for inhalational delivery to rats. Following a single inhalation, widespread pulmonary EPO distribution persisted for up to 10 days while increasing YFP expression was observed for at least 7 days for both NPs. The overall results support both PLGA and gelatin NPs as promising carriers for pulmonary protein/DNA delivery.

17. Development and characterization of O-methylated free N,N,N-Trimethylated chitosan nanoparticles as new carriers for oral vaccine delivery in mice [17]:

Subrata Biswas et al. (2014)-

Chitosan (CS) has already gained considerable attentions as vehicles for mucosal immunizations due to its excellent biocompatibility, biodegradability and non-toxicity. However, poor aqueous solubility and loss of penetration-enhancing above pH 6 are major drawbacks for its use as oral vaccine carrier. The study aims to investigate the potential utility of O-methylated free trimethylated chitosan (CS-TM) nanoparticles as an effective adjuvant for oral vaccine delivery. Nanoparticles were formulated by modified coacervation method using different MW of CS consisting two different degree of quaternization (DQ) to which measles antigen was entrapped by an ionic cross-linking technique. Drug loading, encapsulation efficiency, and particle properties such as SEM, size distribution and zeta potential were evaluated. In vitro release studies showed an initial burst followed by extended release of antigen from all formulations, best fitted the Higuchi model. SDS-PAGE assay showed that CS-TM nanoparticles could effectively protect the antigen from degradation in acidic condition for at least 2 h. Cell viability was assessed using MTT assay into HT29 cell line. CS-TM nanoparticles of different formulations were orally administered to mice and immunological responses were evaluated using dried blood spot ELISA method. Obtained results showed that antigen-loaded CS nanoparticles induced strong humoral immune response and significant correlation was observed between the immune response with DQ. Protecting ability of antigen in the gastric environment, sustained release kinetics, enhancement of the

systemic and mucosal immune responses and low cytotoxicity observed for the CS-TM nanoparticles demonstrated that it could be a promising platform for oral vaccine delivery.

18. A comparative study of chitosan and chitosan/cyclodextrin nanoparticles as potential carriers for the oral delivery of small peptides [18]:

Adriana Trapani et al. (2010)-

The aim of this study was to characterize new nanoparticles (NPs) containing chitosan (CS), or CS/cyclodextrin (CDs), and evaluate their potential for the oral delivery of the peptide glutathione (GSH). More precisely, NP formulations composed of CS, CS/a-CD and CS/sulphobutyl ether-b-cyclodextrin (SBE7m-CD) were investigated for this application. CS/CD NPs showed particle sizes ranging from 200 to 500 nm. GSH was loaded more efficiently in CS/SBE7m-b-CD NPs by forming a complex between the tri-peptide and the CD. X-ray Photoelectron Spectroscopy (XPS) analysis suggested that GSH is located in the core of CS/SBE7m-b-CD NPs and that it is almost absent from the NP surface. Release studies performed in vitro at pH 1.2 and pH 6.8 showed that NP release properties can be modulated by selecting an appropriate CD. Transport studies performed in the frog intestine model confirmed that both CS and CS/CD nanoparticles could induce permeabilization of the intestinal epithelia. However, CS/SBE7m-b-CD NPs provided absorption-enhancing properties in all segments of the duodenum, whereas CS NPs effect was restricted to the first segment of the duodenum. From the data obtained, we believe that CS/CD nanoparticles might represent an interesting technological platform for the oral administration of small peptides.

19. Chitosan/cyclodextrin nanoparticles as macromolecular drug delivery system [19]:

Alexander H. Krauland et al. (2007)-

The aim of this study was to generate a new type of nanoparticles made of chitosan (CS) and carboxymethyl-β-cyclodextrin (CM-β-CD) and to evaluate their potential for the association and delivery of macromolecular drugs. CS and CM-β-CD or mixtures of CM-β-CD/tripolyphosphate (TPP) were processed to nanoparticles via the ionotropic gelation technique. The resulting nanoparticles were in the size range of 231–383 nm and showed a positive zeta potential ranging from +20.6 to +39.7 mV. These nanoparticles were stable in

simulated intestinal fluid pH 6.8 at 37 ◦C for at least 4 h. Elemental analysis studies revealed the actual integration of CM-β-CD to CS nanoparticles. Insulin and heparin used as macromolecular model drugs, could be incorporated into the different nanocarriers with association efficiencies of 85.5–93.3 and 69.3–70.6%, respectively. The association of these compounds led to an increase of the size of the nanoparticles (366–613 nm), with no significant modification of their zeta potentials (+23.3 to +37.1 mV). The release profiles of the associated macromolecules were highly dependent on the type of molecule and its interaction with the nano matrix: insulin was very fast released (84–97% insulin within 15 min) whereas heparin remained highly associated to the nanoparticles for several hours (8.3–9.1% heparin within 8 h). In summary, CS-CD (cyclodextrin) nanoparticles may be considered as nanocarriers for the fast or slow delivery of macromolecules.

20. Carboxymethyl-β-cyclodextrin grafted chitosan nanoparticles as oral delivery carrier of protein drugs [20]:

Mingming Song et al. (2017)-

In this paper, the novel carboxymethyl-β-cyclodextrin grafted chitosan (CMCD-g-CS) nanoparticles were fabricated and their potential as oral delivery carrier of protein drugs was evaluated. The physicochemical properties of the prepared nanocarriers were characterized by Fourier transforms infrared spectroscopy, nuclear magnetic resonance, transmission electron microscopy and dynamic light scattering. Bovine serum albumin (BSA), a model protein drug, was loaded in prepared nanocarriers with ideal entrapment efficiency (EE) and loading content (LC). The drug release profiles of BSA-loaded nanoparticles were studied in simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and simulated colonic fluid (SCF). It was found that the drug-loaded nanovehicles displayed a typical controlled sustained release profiles and the amount of BSA released from the nanocarriers was much higher in SIF and SCF than it in SGF. The research results suggested that the CMCD-g-CS nanoparticles had the potential as promising nanocarriers for oral delivery of protein drugs.

References

1. Amidi M, Romeijn SG, Borchard G, Junginger HE, Hennink WE, Jiskoot W. Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. JCR. 2006;111(1-2):107-16.
2. Grenha A, Remuñán-López C, Carvalho EL, Seijo B. Microspheres containing lipid/chitosan nanoparticles complexes for pulmonary delivery of therapeutic proteins. Eur. J. Pharm. Biopharm. 2008;69(1):83-93.
3. Katas H, Raja MA, Lam KL. Development of chitosan nanoparticles as a stable drug delivery system for protein/siRNA. Int. J. Biomater. 2013;2013:1-9
4. Zhang HL, Wu SH, Tao Y, Zang LQ, Su ZQ. Preparation and characterization of water-soluble chitosan nanoparticles as protein delivery system. J. Nanomater. 2010; 2010:1-5.
5. Janes KA, Alonso MJ. Depolymerized chitosan nanoparticles for protein delivery: preparation and characterization. J. Appl. Polym. Sci. 2003;88(12):2769-76.
6. Ieva E, Trapani A, Cioffi N, Ditaranto N, Monopoli A, Sabbatini L. Analytical characterization of chitosan nanoparticles for peptide drug delivery applications. Anal. Bioanal. Chem. 2009; 393:207-15.
7. Piras AM, Sandreschi S, Maisetta G, Esin S, Batoni G, Chiellini F. Chitosan nanoparticles for the linear release of model cationic peptide. Pharm. Res. 2015; 32:2259-65.
8. Luangtana-anan M, Nunthanid J, Limmatvapirat S. Potential of different salt forming agents on the formation of chitosan nanoparticles as carriers for protein drug delivery systems. J. Pharm. Investig. 2019; 49:37-44.
9. Yadav P, Yadav AB. Preparation and characterization of BSA as a model protein loaded chitosan nanoparticles for the development of protein-/peptide-based drug delivery system. Futur. J. Pharm. Sci.2021; 7:1-9.
10. Millotti G, Perera G, Vigl C, Pickl K, Sinner FM, Bernkop-Schnürch A. The use of chitosan-6-mercaptonicotinic acid nanoparticles for oral peptide drug delivery. Drug Deliv. 2011;18(3):190-7.
11. Pan Y, Li YJ, Zhao HY, Zheng JM, Xu H, Wei G, Hao JS. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. Int. J. Pharm. 2002;249(1-2):139-47.
12. Gao P, Xia G, Bao Z, Feng C, Cheng X, Kong M, Liu Y, Chen X. Chitosan based nanoparticles as protein carriers for efficient oral antigen delivery. Int. J. Biol. Macromol. 2016; 91:716-23.

13. Zhang Q, Li Y, Wang S, Gu D, Zhang C, Xu S, Fang X, Li C, Wu H, Xiong W. Chitosan-based oral nanoparticles as an efficient platform for kidney-targeted drug delivery in the treatment of renal fibrosis. Int. J. Biol. Macromol.2024;256:1-10.
14. Tian B, Qiao X, Guo S, Li A, Xu Y, Cao J, Zhang X, Ma D. Synthesis of β-acids loaded chitosan-sodium tripolyphosphate nanoparticle towards controlled release, antibacterial and anticancer activity. Int. J. Biol. Macromol. 2024; 257:1-12.
15. He C, Yin L, Tang C, Yin C. Size-dependent absorption mechanism of polymeric nanoparticles for oral delivery of protein drugs. Biomater. 2012;33(33):8569-78.
16. Menon JU, Ravikumar P, Pise A, Gyawali D, Hsia CC, Nguyen KT. Polymeric nanoparticles for pulmonary protein and DNA delivery. Acta Biomater. 2014;10(6):2643-52.
17. Biswas S, Sen KK, Saha MK, Roy D. Development and characterization of O-methylated free N, N, N-Trimethylated chitosan nanoparticles as new carriers for oral vaccine delivery in mice. Pharm. Sci.2014;4(1): 01-13
18. Trapani A, Lopedota A, Franco M, Cioffi N, Ieva E, Garcia-Fuentes M, Alonso MJ. A comparative study of chitosan and chitosan/cyclodextrin nanoparticles as potential carriers for the oral delivery of small peptides. Eur. J. Pharm. Biopharm. 2010;75(1):26-32.
19. Krauland AH, Alonso MJ. Chitosan/cyclodextrin nanoparticles as macromolecular drug delivery system. Int. J. Pharm. 2007;340(1-2):134-42.
20. Song M, Li L, Zhang Y, Chen K, Wang H, Gong R. Carboxymethyl-β-cyclodextrin grafted chitosan nanoparticles as oral delivery carrier of protein drugs. React. Funct. Polym. 2017; 117:10-5.

CHAPTER-III

AIM AND OBJECTIVES

Aim:

Oral delivery of protein-based drugs presents several challenges due to the complex environment of the gastrointestinal (GI) tract and the inherent properties of proteins. The acidic pH of the stomach (around pH 1.5 to 3.5) can denature proteins, causing them to lose their functional structure and bioactivity. Addressing these problems requires innovative approaches in formulation science, such as developing protective delivery systems (like chitosan nanoparticles) to improve the efficiency of oral protein delivery.

So, this present study **aims** to develop and characterise chitosan nanoparticles for oral protein delivery.

Objectives:

1. Design and development of polymeric nanoparticles.
2. Drug excipient compatibility study.
3. Physicochemical characterization of the developed nanoparticles.
4. In-vitro drug release study.
5. Release kinetic study.

CHAPTER-IV

MATERIALS AND METHODS

4.1. Materials

Chitosan (high molecular weight) was collected from Gupta College of Technology Sciences, Asansole, BSA (Model drug) was purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Sodium tripolyphosphate anhydrous (TPP) was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). All other reagents and solvents were of analytical grade.

4.2. Instruments used

- Balance: Wenser, Model-PGB220
- pH Meter: Systronics, Model-335
- UV-VIS Spectrophotometer: Shimadzu Corporation, Model-UV-2450
- Magnetic Stirrer: REMI, Model- 2MLH
- Refrigerator: Godrej, Model-RD ERIOPLS205C
- Cooling centrifuge: Sigma Laboratory Centrifuge, Model-3K30
- Lyophilizer: Laboratory Freeze Dryer

4.3. Methods

4.3.1. Preparation of standard curve of Bovine Serum Albumin in phosphate buffer of pH-6.8:

BSA stock solution was prepared by adding 10mg BSA in 100 ml PBS. From the stock solution, different dilutions were made by using a phosphate buffer of pH 6.8 which has 2,4,6,8,10,12 μg/ml of bovine serum albumin. The absorbance was measured at 660 nm wavelength according to the Lowry protein estimation method.

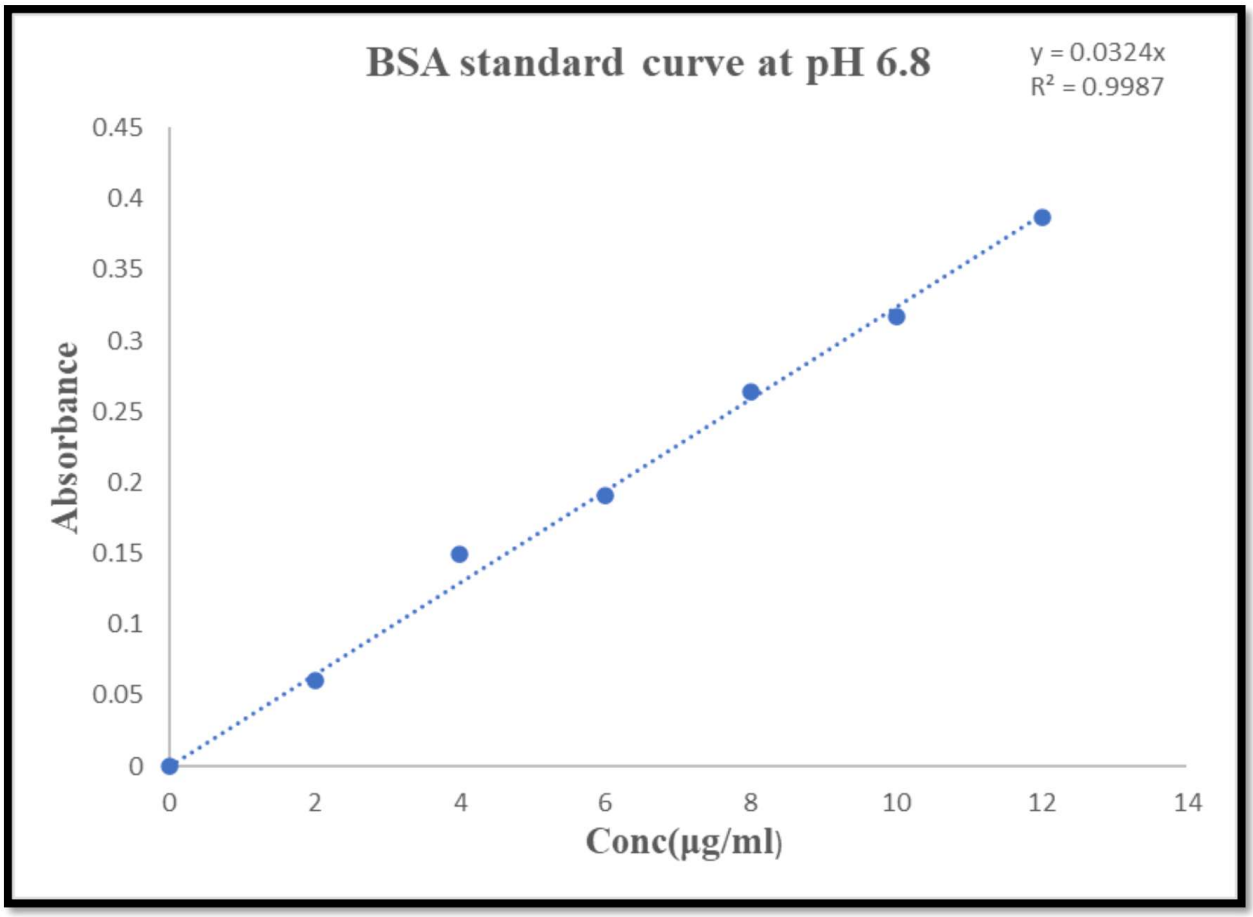

Fig.8: BSA Standard Curve in pH 6.8

4.3.2. Preparation of nanoparticles by ionic gelation technique [1]

CS-NP were prepared based on the ionotropic gelation of CS with TPP, in which the positively charged amino groups of CS interact with the negatively charged TPP. CS was dissolved in 1% (v/v) acetic acid. At first required amount of CS powder (Table 2) was taken and added to deionized water and kept in a magnetic stirrer at 650 rpm to prepare a homogenous solution. The required amount of BSA was added to the prepared CS solution under stirring conditions. A clear solution of TPP was prepared by mixing the required amount of TPP (Table 2) into deionized water. After dissolving the BSA into CS solution the TPP solution was then slowly added dropwise to the BSA containing CS solution under constant stirring at 1200 rpm. After the preparation of nanoparticles, it was centrifuged under cold conditions. Then it was lyophilized for 18-20 hours to get the powder form of nanoparticles (as shown in figure 9).

Table 2: Composition of batches of the BSA-loaded chitosan nanoparticles.

Formulation Code	Drug: Polymer	Cross-linking agent (%)
F1	1:2	0.5
F2	1:2	1
F3	1:2	2
F4	1:2	3
F5	1:3	3
F6	1:4	3
F7	1:5	3

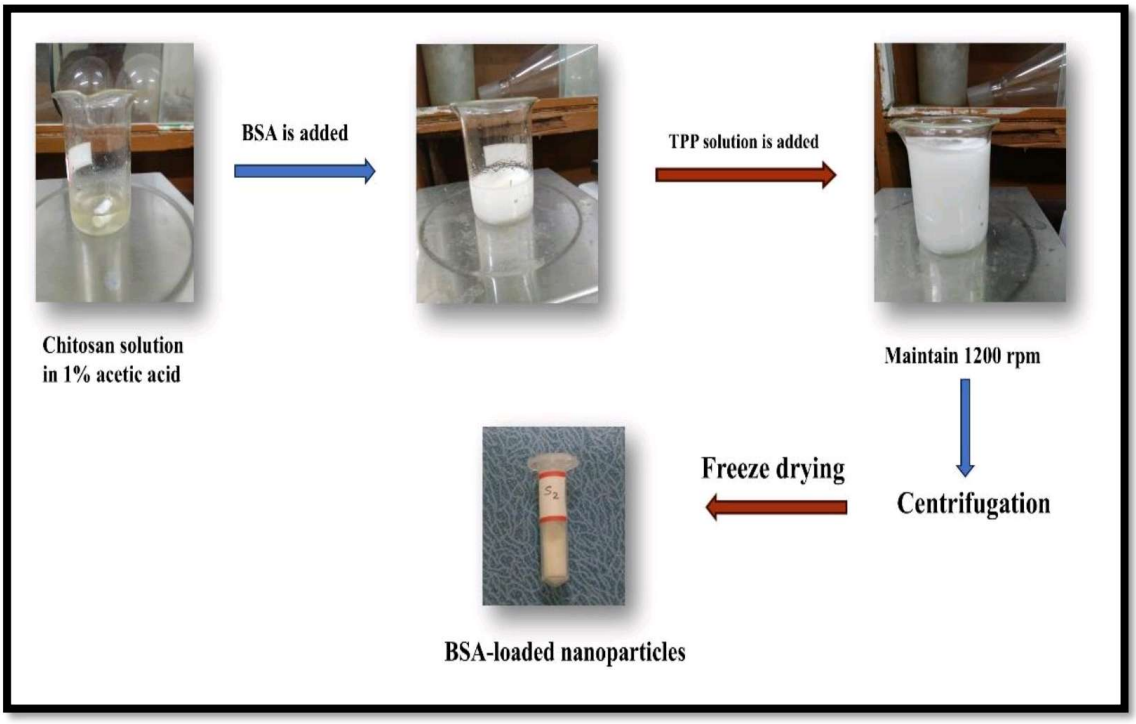

Fig.9: Preparation method of BSA-loaded chitosan nanoparticles

4.4. Optimization of Nanoparticles

4.4.1. Determination of % Yield [2]

The prepared nanoparticles were weighed by using an analytical balance and the percentage yield was calculated by using the following formula:

% Yield = (Actual weight of product/total weight of polymer and drug) × 100

4.4.2. Determination of Encapsulation Efficiency [3]

10 mg of formulation left overnight in 50ml of phosphate buffer solution pH-6.8. After filtration, the amount of BSA present in the filtrate was estimated by using the Lowry method. The absorbance was taken at 660 nm at a UV-VIS Spectrophotometer. The amount of the BSA encapsulated in the nanoparticles was calculated by using the following formula using the standard curve of BSA in PBS ($Y = 0.0324X$, $R^2 = 0.9987$) as a reference.

EE (%) = (Total amount of loaded BSA / Initial of BSA) × 100%

4.4.3. Particle Size and Polydispersity Analysis [4]

The average particle size, PDI and zeta potential of nanoparticles were analysed using Zetasizer (Nano-ZS90, Malvern, UK). The nanoparticle suspension was diluted with distilled water and sonicated for a few minutes. The suspension was then transferred to polystyrene cuvettes and analysed for the size PDI of particles.

4.4.4. *In vitro* drug release study

The release of BSA was done as described by Haliza Katas et al. 2013. A dialysis bag (dialysis membrane-150) containing a known number of freeze-dried nanoparticles and a specific volume of phosphate buffer was placed on a magnetic stirrer with a stirring speed of 100 rpm at 37° C. At predetermined time intervals (0.5,1,2, 3, 4,5,6,7 and 8 hr), 1 ml of sample was removed and was replaced by fresh medium. Then, the supernatant was decanted and replaced with an equivalent volume of fresh buffer solution. The released BSA was determined by UV–visible spectroscopy at a wavelength of 660 nm according to the Lowry method.

4.4.5. Release kinetic study [5]

The *in vitro* drug release data were subjected to various kinetic models to understand the kinetics of drug release from nanoparticles.

4.5. Characterization of Optimized Formulation

4.5.1. Fourier-transformed infrared (FTIR) spectroscopy [5]

The FTIR spectra of the chitosan, BSA drug and BSA nanoparticle were recorded on FTIR spectrometer (NICOLET IS10, Thermo Fisher Scientific) in the range of 4000 and 400cm-1 at a resolution of with scan speed of 1 cm/s.

4.5.2. Shape and Surface Morphology (SEM studies) [5]

The surface morphology of the optimized microsphere (F4) was determined by SEM (Model: ZEISS EVO-18). The samples were dried in a freeze dryer and then the chitosan nanoparticles were coated with a fine gold layer before obtaining the micrographs. The accelerating voltage used was 10 kV.

4.5.3. XRD analysis [6]

The X-ray pattern of BSA and BSA-loaded nanoparticles were obtained by using an X-ray diffractometer (D8, Bruker, Germany). This study was done by maintaining the following parameters: Diffraction angle (2θ) 10– 80° at scan speed 5°/min, current 30 mA, and voltage 45Kv

References

1. Gao P, Xia G, Bao Z, Feng C, Cheng X, Kong M, Liu Y, Chen X. Chitosan-based nanoparticles as protein carriers for efficient oral antigen delivery. International journal of biological macromolecules. 2016 Oct 1;91:716-23.

2. Zhang Q, Li Y, Wang S, Gu D, Zhang C, Xu S, Fang X, Li C, Wu H, Xiong W. Chitosan-based oral nanoparticles as an efficient platform for kidney-targeted drug delivery in the treatment of renal fibrosis. International Journal of Biological Macromolecules. 2024 Jan 1;256:128315.

3. Tian B, Qiao X, Guo S, Li A, Xu Y, Cao J, Zhang X, Ma D. Synthesis of β-acids loaded chitosan-sodium tripolyphosphate nanoparticle towards controlled release, antibacterial and anticancer activity. International Journal of Biological Macromolecules. 2024 Feb 1;257:128719.

4. He C, Yin L, Tang C, Yin C. Size-dependent absorption mechanism of polymeric nanoparticles for oral delivery of protein drugs. Biomaterials. 2012 Nov 1;33(33):8569-78.

5. Menon JU, Ravikumar P, Pise A, Gyawali D, Hsia CC, Nguyen KT. Polymeric nanoparticles for pulmonary protein and DNA delivery. Acta biomaterialia. 2014 Jun 1;10(6):2643-52.

6. Gaur M, Maurya S, Akhtar MS, Yadav AB. Synthesis and Evaluation of BSA-Loaded PLGA–Chitosan Composite Nanoparticles for the Protein-Based Drug Delivery System. ACS omega. 2023 May 17;8(21):18751-9.

CHAPTER-V

RESULTS AND DISCUSSIONS

5.1. Yield of Nanoparticles

In studies on chitosan nanoparticles, the percentage yield is an essential parameter reflecting the effectiveness of the nanoparticle formation. The percentage yield of nanoparticles is in the range of 28.29 to 63.39 (as shown in **Table 3**). Formulation F4 exhibits the highest yield at 63.39%. Polymer concentration and crosslinker concentration are the two factors that affect the percentage yield of nanoparticles. At 3% TPP (crosslinker) when the drug /polymer ratio is 1:2 shows the highest percentage yield. A study by Gan et al. (2005) reported yields between 50% to 75% when optimizing the preparation process of chitosan nanoparticles.

5.2. Encapsulation Efficiency

Encapsulation efficiency of F1 to F7 was found in the range of 29.08 to 64.37 (as shown in **Table 3** and **Figure 10)**. Encapsulation efficiency depends on yield value and the drug/polymer ratio and TPP concentration. Generally, a higher encapsulation efficiency is often associated with a higher percentage yield, as seen in F4, which has the highest EE (64.37%) and the highest yield (63.39%). With the highest encapsulation efficiency (64.37%), F4 is the most effective formulation in encapsulating BSA, which is also reflected in its highest percentage yield. In similar studies, such as by Calvo et al. (1997), encapsulation efficiency of around 50% to 70% was observed.

Table 3: Percentage yield and Encapsulation efficiency table for F1 to F7

Formulation code	**Percentage yield**	**Encapsulation efficiency**
F1	28.29	29.08
F2	33.27	34.27
F3	42.09	41.26
F4	63.39	64.37
F5	52.28	49.77
F6	61.21	58.82
F7	48.24	49.62

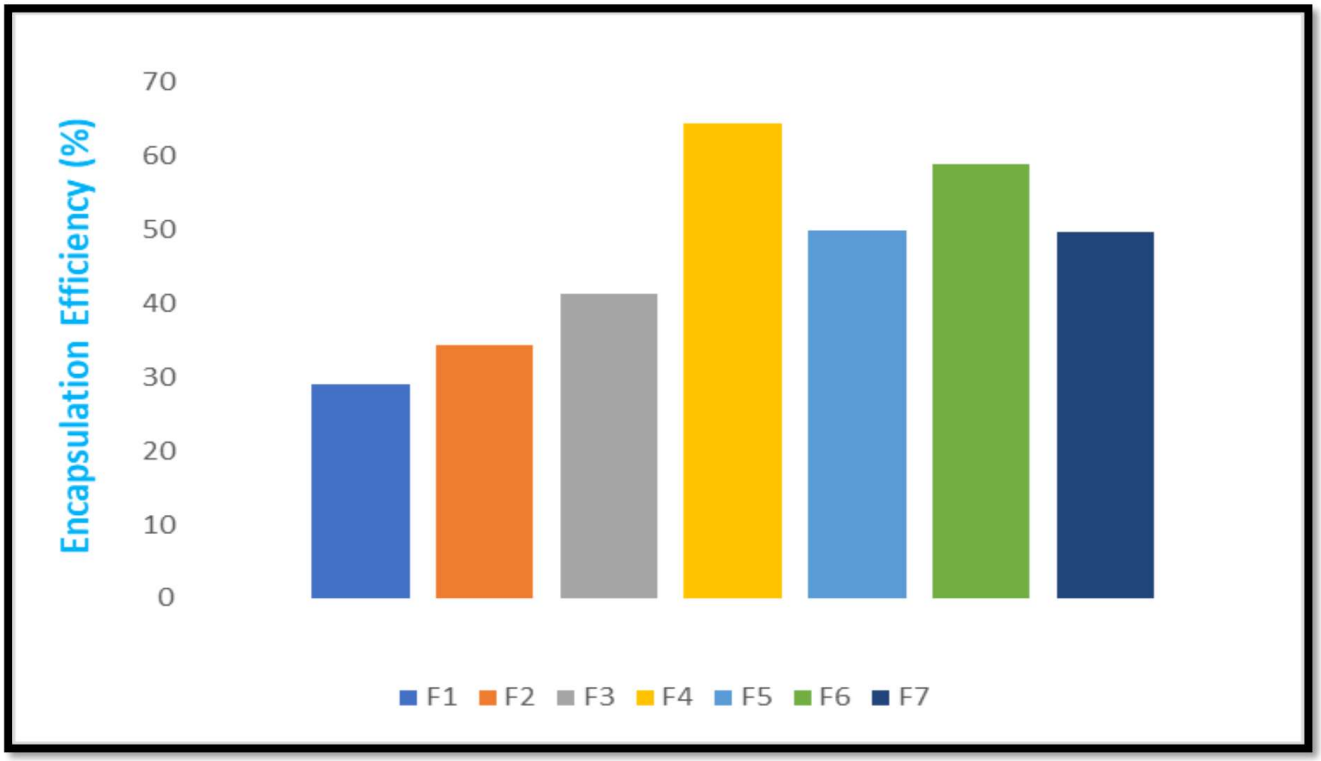

Fig.10: The encapsulation efficiency of BSA-loaded chitosan nanoparticles for F1 to F7

5.3. Particle Size Analysis

The particle size and polydispersity index (PDI) are critical parameters in the characterization of nanoparticles, particularly in ensuring the stability, bioavailability, and controlled release of the encapsulated drug. The particle sizes range from 154.8 nm (F4) to 715.2 nm (F7) as shown in Table 4. Size distribution by intensity for each formulation (F1 to F7) is also shown in Figure 11 to Figure 17. F4 (154.8 nm) and F5 (167.2 nm) exhibit the smallest particle sizes. PDI values range from 0.249 (F5) to 1.000 (F2), reflecting the uniformity of the particle sizes within each formulation. A PDI value below 0.3 indicates a narrow size distribution, which is ideal for nanoparticle formulations. F5 (PDI = 0.249), F4 (PDI = 0.326), and F6 (PDI = 0.315) have the lowest PDIs, indicating these formulations have the most uniform particle sizes. Generally, nanoparticles with sizes below 200 nm and a PDI below 0.3 are considered ideal for systemic drug delivery due to their enhanced permeability [1-3]. Katas et al. (2013) have shown that chitosan nanoparticles with sizes in the range of 100-200 nm and low PDIs (<0.3) are optimal for drug delivery, leading to improved bioavailability and controlled release profiles.

Table 4: Particle size and PDI value for F1 to F7

Formulation code	Particle size (nm)	PDI
F1	168.7	0.864
F2	208.9	1.000
F3	302.8	0.899
F4	154.8	0.326
F5	167.2	0.249
F6	268.4	0.315
F7	715.2	0.531

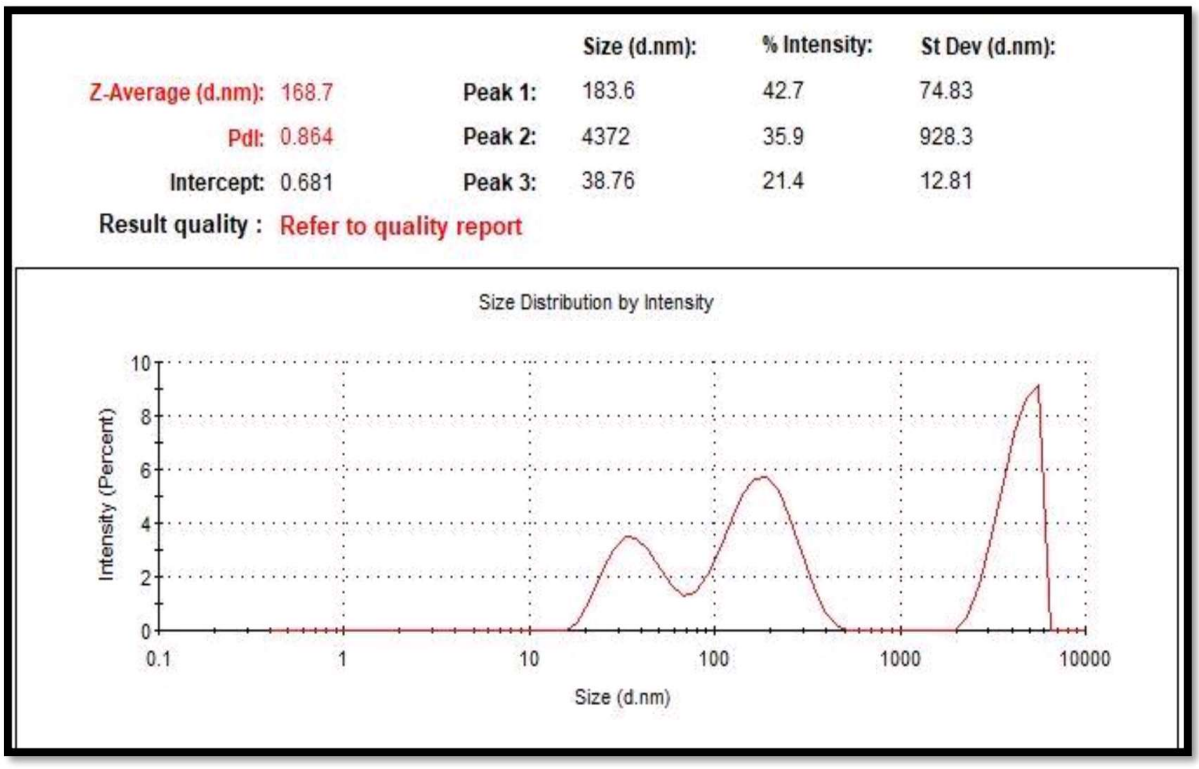

Fig.11: Particle size of F1 formulation

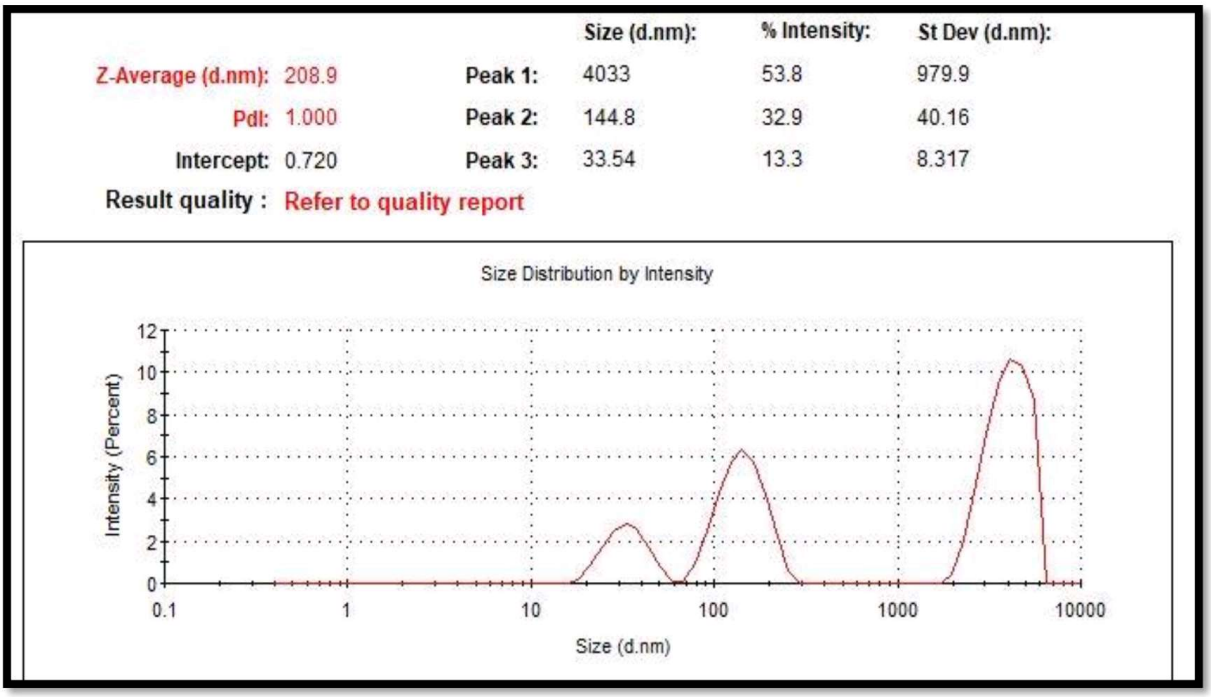

Fig.12: Particle size of F2 formulation

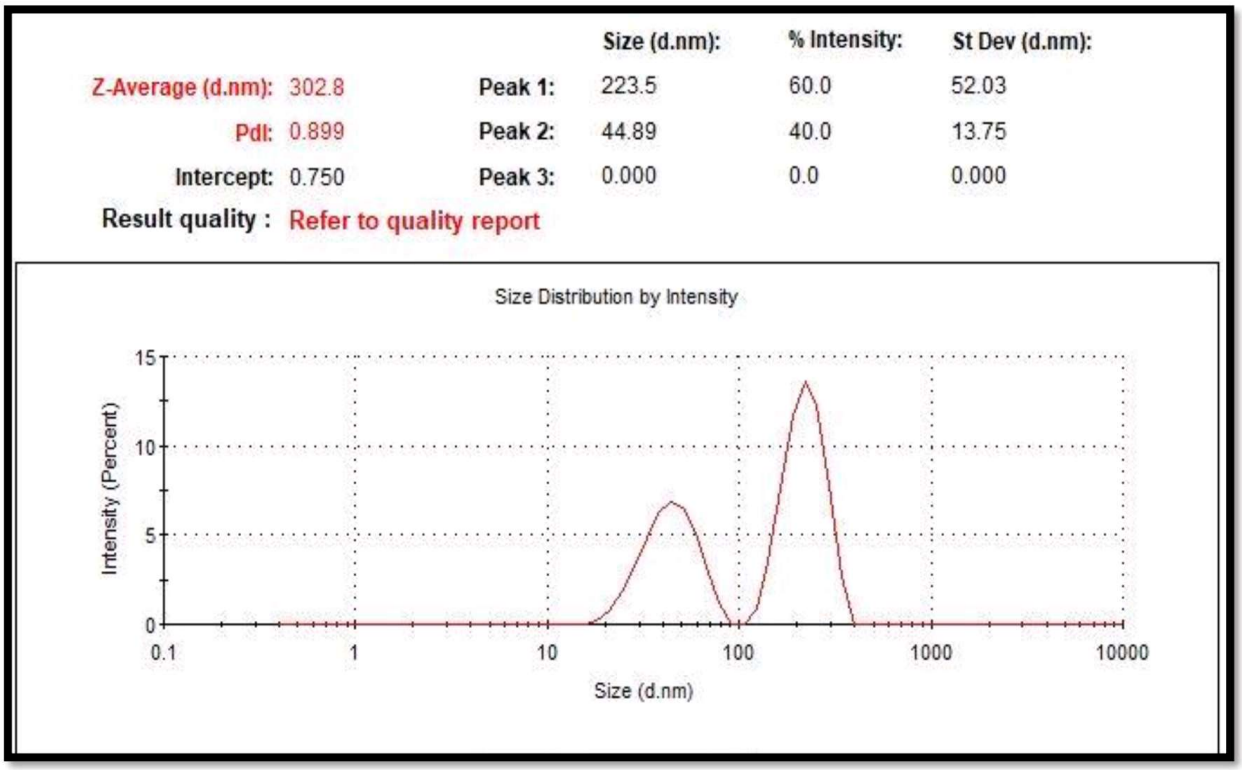

Fig.13: Particle size of F3 formulation

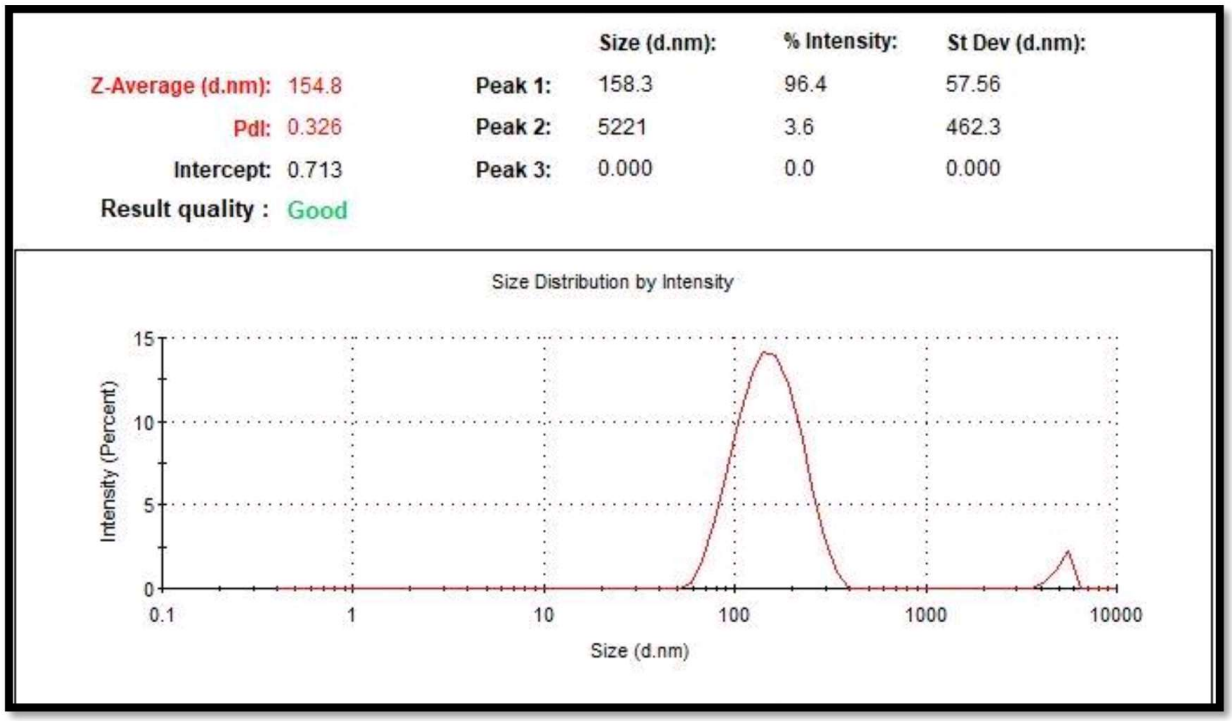

Fig.14: Particle size of F4 formulation

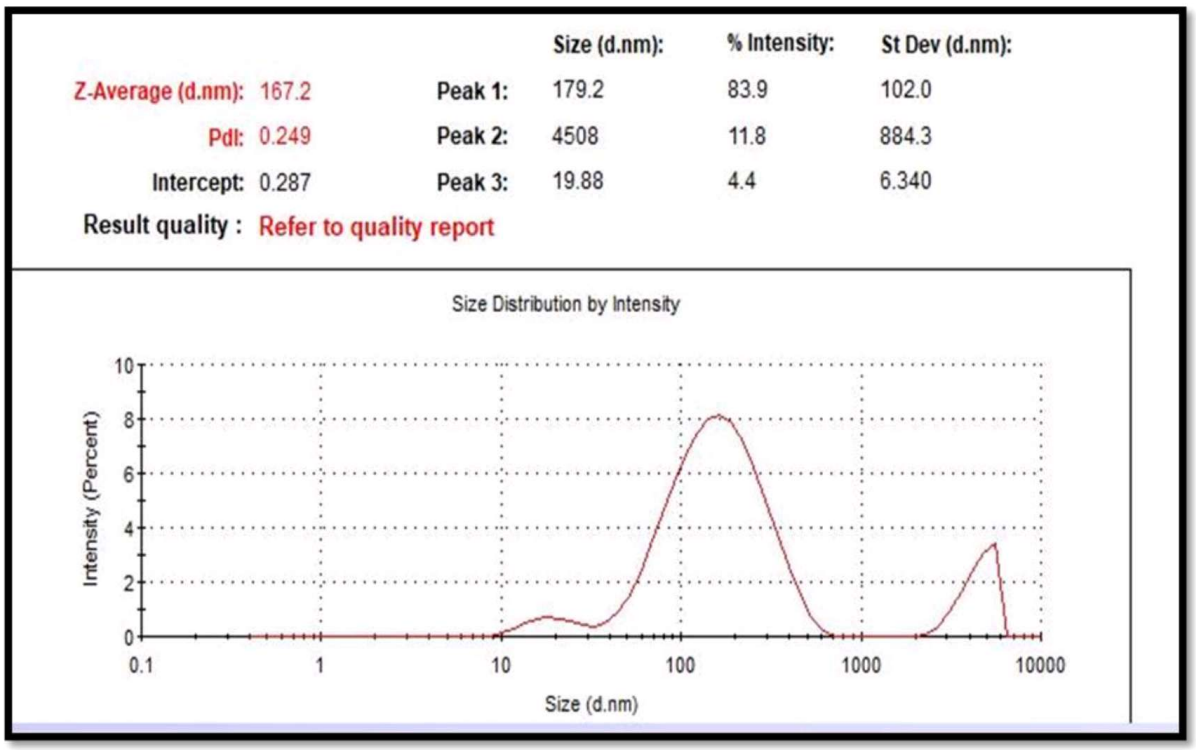

Fig.15: Particle size of F5 formulation

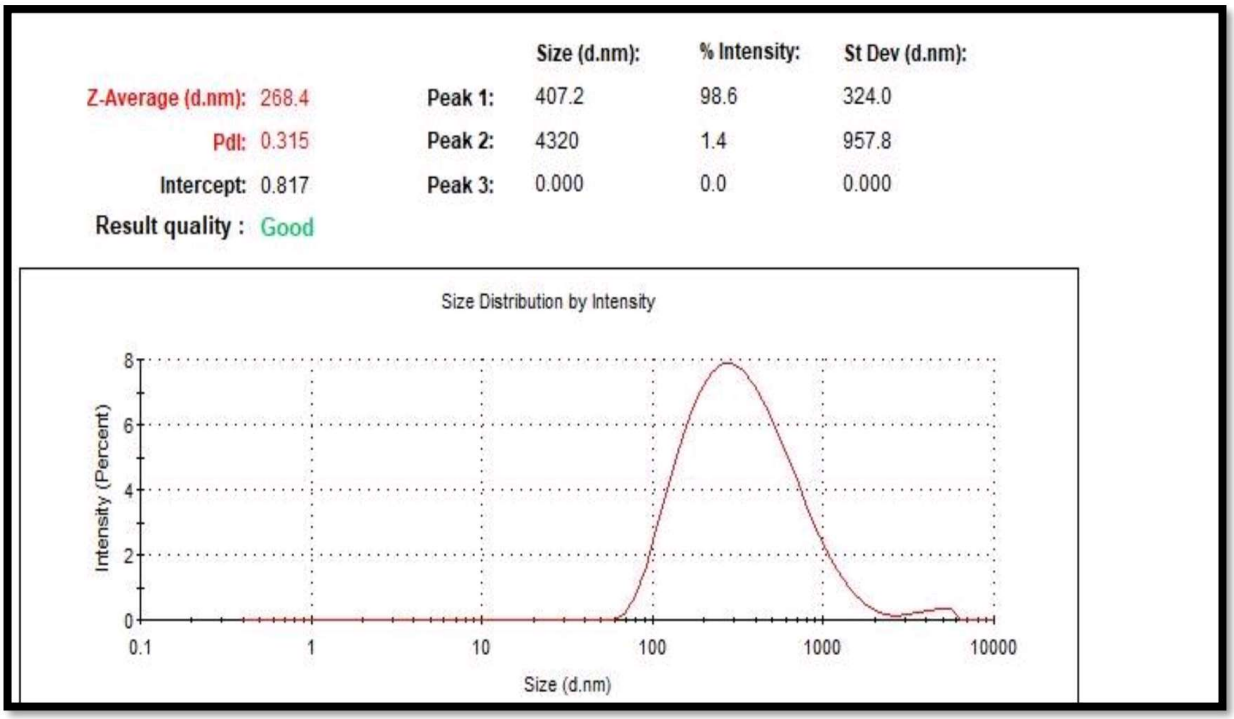

Fig.16: Particle size of F6 formulation

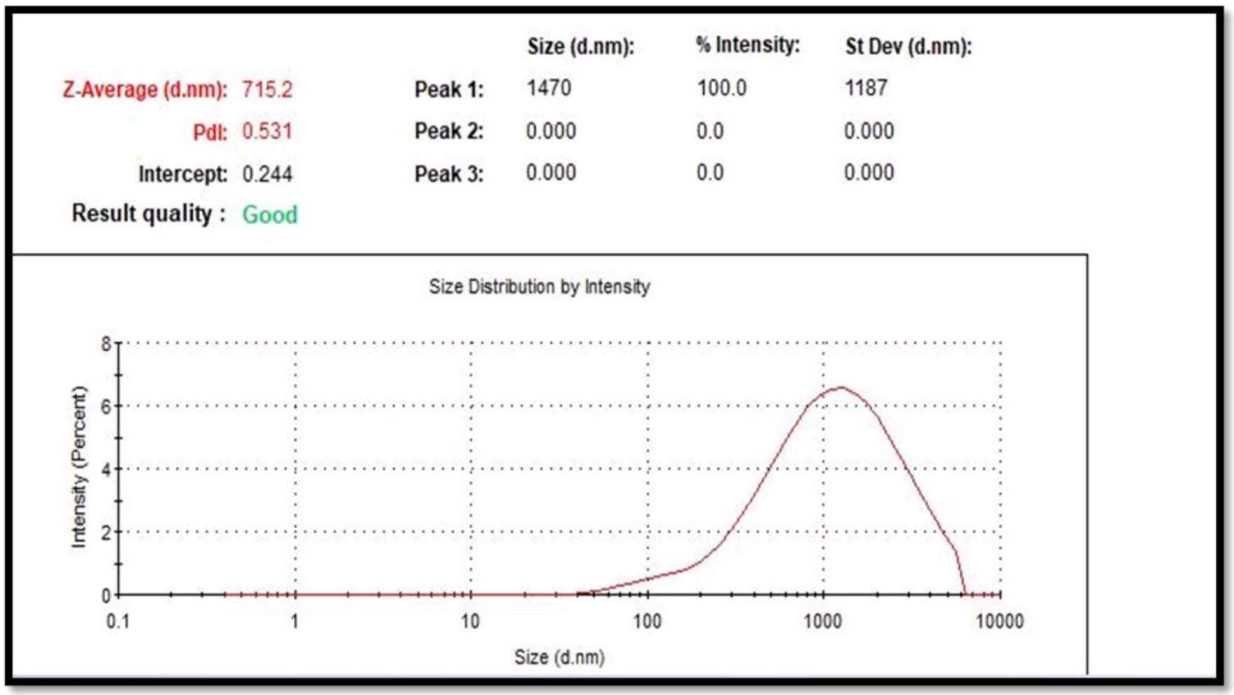

Fig.17: Particle size of F7 formulation

5.4. *In vitro* drug release study

The Cumulative Percentage Release (CPR) data for the different formulations (F1 to F7) of chitosan nanoparticles indicates how much of the encapsulated drug (BSA) is released over time. The cumulative release profile for chitosan nanoparticles is highly dependent on factors like the degree of cross-linking, the drug's interaction with the polymer, and the particle size initial release of BSA protein is in the range of 5.19%(F3) to 8.22% (F1). After 8 hours of release, the CPR value reaches in the range of 15.63% (F7) to 39.41% (F1). All formulations (F1 to F7) show an increase in CPR over time. F1 consistently shows higher CPR values as the polymer concentration is low in the F1 formulation which indicates that sustained release can be achieved with an increase in polymer concentration. F7 reflect the lowest CPR as it contains the highest concentration of polymer which indicates the sustained release of therapeutics. CPR of Formulation F4 indicates controlled release, balancing between immediate and sustained release, which is beneficial for maintaining consistent plasma levels of the drug over time. The *in vitro* drug release data for all the formulations (F1 to F7) is given in **Table 5** and the graphical representation is shown in Figure 18. Agnihotri et al. (2004) have shown that higher degrees of cross-linking lead to slower drug release due to a denser matrix, which is consistent with the slower release profiles seen in formulations F3, F4 and F7.

Table 5: *In vitro* release of BSA for F1 to F7

Time (Hours)	F1(CPR)	F2(CPR)	F3(CPR)	F4(CPR)	F5(CPR)	F6(CPR)	F7(CPR)
0	0	0	0	0	0	0	0
0.5	8.22	6.11	5.19	7.72	8.11	5.89	6.72
1	11.32	8.33	6.72	9.22	8.22	6.11	6.78
2	14.18	12.36	6.29	11.38	9.57	6.22	7.29
3	16.27	14.29	8.71	12.62	10.62	7.28	8.82
4	20.36	18.25	10.26	13.29	11.36	9.23	9.81
5	24.21	20.97	12.52	15.66	14.29	12.36	10.26
6	29.36	22.72	14.62	20.29	15.62	14.22	12.66
7	34.2	26.27	17.92	20.62	16.33	15.29	14.29
8	39.41	30.32	20.22	28.92	17.23	16.38	15.63

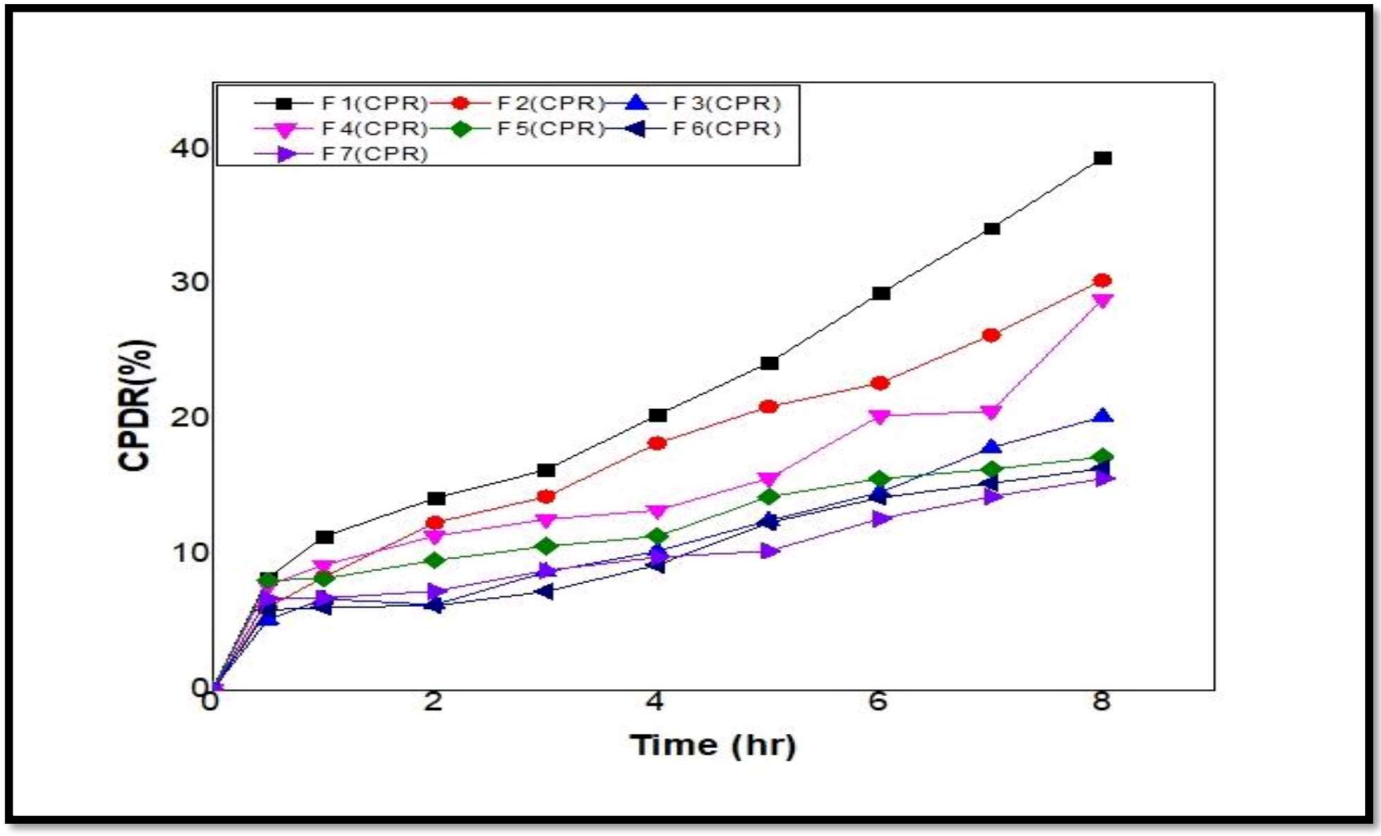

Fig.18: *In vitro* release of BSA for F1 to F7

5.5. Release kinetic study of BSA

The *in vitro* release data of all the formulations was subjected to various kinetic models of drug release. The data relates to the goodness of fit (R^2 values) for different kinetic models (Zero Order, First Order, Higuchi, Korsmeyer-Peppas) and the release exponent (n) for all formulations (F1 to F7) as shown in **Table 6**. These models help to understand the drug release mechanisms from the nanoparticles. Zero-order kinetics implies a constant drug release rate over time, independent of the concentration. F1 shows the highest R^2 value (0.9906), indicating the strongest correlation with Zero Order kinetics, which indicates a consistent release rate that is ideal for maintaining constant drug levels. F2 to F7 also exhibit high R^2 values (ranging from 0.980 to 0.9886), indicating that Zero Order kinetics is a significant model for all formulations, although slightly less so for F7 (0.980). First-order kinetics means the release rate is concentration-dependent, it decreases as the concentration of the drug within the nanoparticles decreases [4-6]. The R^2 values for First Order are generally lower across all formulations, with F4 (0.9313) showing the best fit. The Higuchi model describes drug release as a diffusion process based on Fick's law. F6 shows the highest R^2 value (0.9756), suggesting that diffusion is the dominant release mechanism for this formulation. F1 (0.9437) and F4 (0.9561), also fit well with the Higuchi model, indicating that diffusion contributes significantly to drug release. The Korsmeyer-Peppas model is used to describe drug release from polymeric systems. F6 has the highest R^2 value (0.9932), indicating a strong fit with this model, suggesting a complex release mechanism involving more than one type of release kinetics. F1 has an n value of 0.8764, indicating non-Fickian transport, suggesting a combination of diffusion and erosion mechanisms. F6 (n = 0.7055) and F5 (n = 0.7293) also indicate non-Fickian transport, while F4 (n = 0.7305) is closer to the non-Fickian region but tends towards Fickian behaviour. F3 (n = 0.7838) and F7 (n = 0.8548) also exhibit non-Fickian transport, indicating mixed release mechanisms. F1 and F4 are particularly excellent as they show strong correlations with zero-order kinetics, indicating consistent drug release, making them ideal for applications where maintaining constant drug levels is crucial. F6 stands out with the highest correlation to the Higuchi and Korsmeyer-Peppas models, suggesting a more complex release mechanism dominated by diffusion.

Table 6: Different Release kinetic model for F1 to F7

Formulation Code	**Zero Order**	**First Order**	**Higuchi Model**	**Korsmeyer- Peppas Model**	
	R^2	**R^2**	**R^2**	**R^2**	**Release Exponent (n)**
F1	0.9906	0.908	0.9437	0.9804	0.8764
F2	0.9823	0.8688	0.9257	0.9651	0.8295
F3	0.9899	0.8713	0.9475	0.9714	0.7838
F4	0.9894	0.9313	0.9561	0.9719	0.7305
F5	0.9878	0.8511	0.9420	0.9547	0.7293
F6	0.9886	0.8144	0.9756	0.9932	0.7055
F7	0.980	0.8804	0.9441	0.9773	0.8548

5.6. Fourier-transformed infrared (FTIR) spectroscopy Analysis:

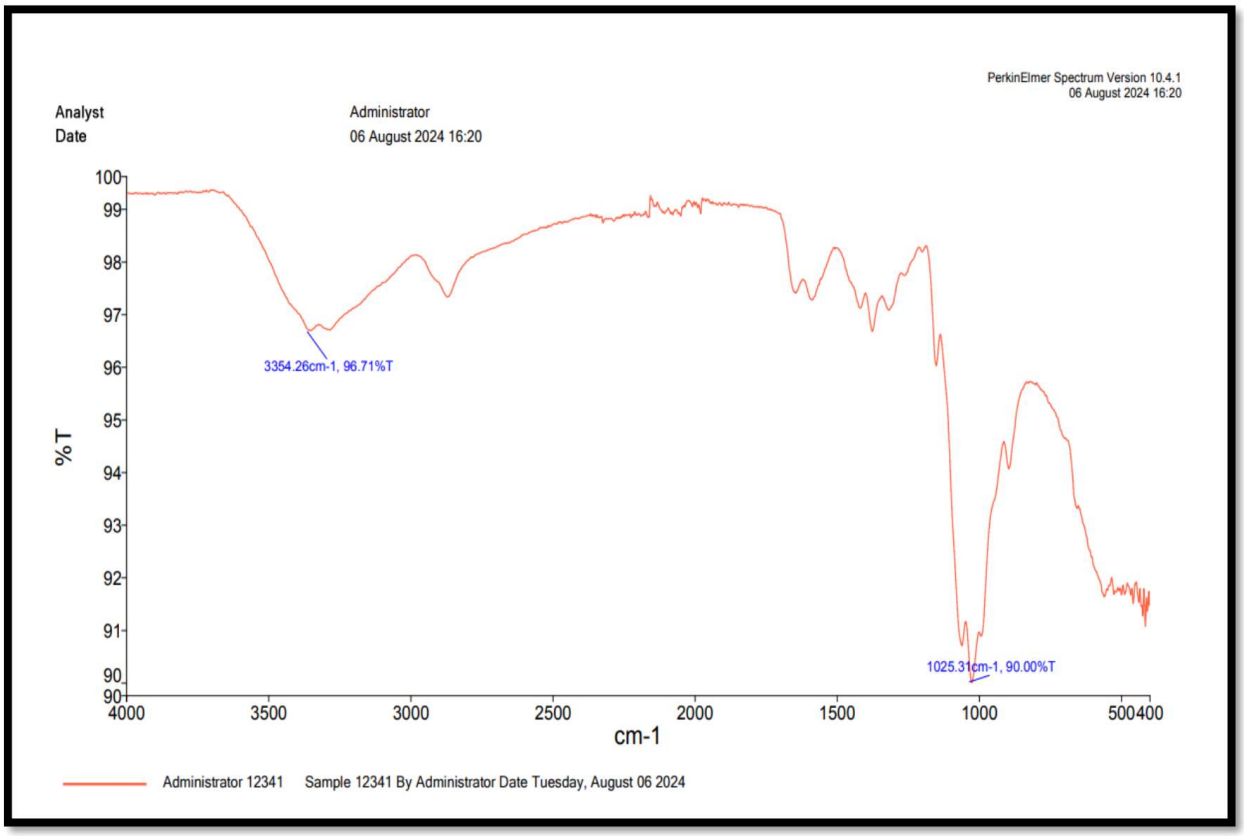

Fig 19: FTIR spectra of Chitosan

5.6.1. FTIR of Chitosan

The broad peak around 3354.26 cm^{-1} corresponds to the O-H and N-H stretching vibrations. This is characteristic of the hydroxyl (O-H) groups and amine (N-H) groups in the chitosan structure. The broadness of this peak is due to hydrogen bonding interactions. A smaller peak in the region of 2875-2925 cm^{-1} is observed, which corresponds to C-H stretching. The peaks around 1655-1550 cm^{-1} are attributed to the amide bands. These are related to the C=O stretching vibrations of the amide group and N-H bending. The region around 1400-1460 cm^{-1} corresponds to C-H bending vibrations. The peak observed at 1025.31 cm^{-1} is caused by the stretching vibrations of the C-O-C bond. This is a typical feature of the polysaccharide backbone of chitosan.[7]

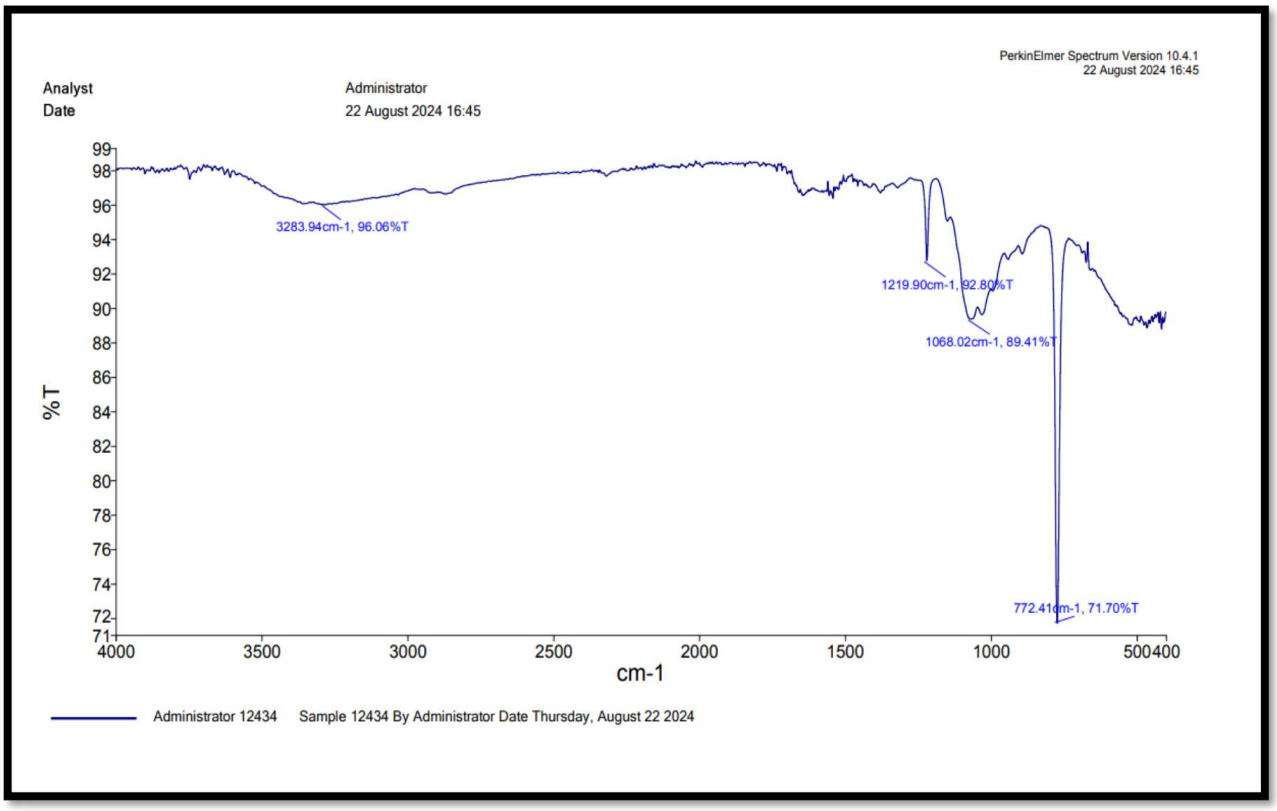

Fig 20: FTIR spectra of BSA

5.6.2. FTIR of BSA

The peak observed at 3284.07 cm^{-1} corresponds to the N-H stretching vibration. This is characteristic of proteins and is primarily due to the stretching of the N-H bond in the peptide linkage. The peak at 1641.85 cm^{-1} represents the Amide band, which is associated with the C=O stretching vibrations of the peptide bond. This peak is critical for identifying secondary protein structures, such as α-helices and β-sheets. The peak at 1524.88 cm^{-1} also indicates the Amide band, which is related to N-H bending and C-N stretching vibrations. The peak at 1220.06 cm^{-1} also indicates the Amide band. This band involves complex interactions between C-N stretching and N-H bending. The peak around 1390.89 cm^{-1} is for C-H bending vibrations, typically from aliphatic side chains in the protein. The peak at 772.38 cm^{-1} may be related to C-N stretching or other out-of-plane bending modes. This can indicate specific interactions within the protein structure.[7]

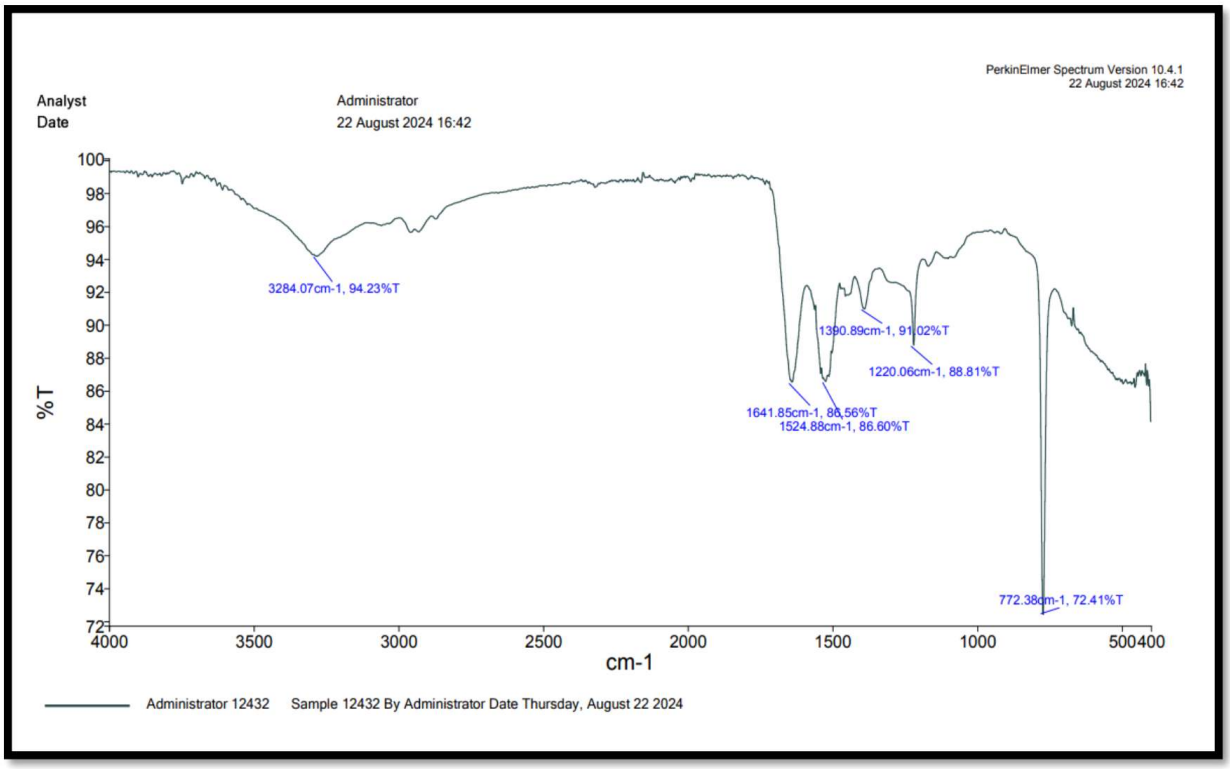

Fig 21: FTIR spectra of BSA nanoparticles

5.6.3. FTIR of BSA nanoparticles

The peak at 3283.94 cm^{-1} corresponds to the O-H and N-H stretching vibrations, which are characteristic of both chitosan and BSA. The slight shift in this peak compared to the pure chitosan (3354 cm^{-1}) and BSA (3284 cm^{-1}) spectra indicate interactions between the BSA and chitosan, likely due to hydrogen bonding. The Amide band, typically observed around 1640-1650 cm^{-1} for BSA, appears less distinct in this spectrum, possibly due to overlapping with the chitosan peaks or broadening due to interactions between BSA and chitosan. The other Amide band is generally observed around 1520-1530 cm^{-1} for BSA. However, this band seems to be less prominent or overlapped by chitosan's characteristic peaks, encapsulation of BSA within the chitosan matrix. The peak at 1068.02 cm^{-1} corresponds to the C-O-C stretching vibrations, which are typical for chitosan. This peak is present in the spectrum of the BSA-loaded chitosan nanoparticles, indicating that the polysaccharide structure of chitosan is retained. The peak at 1219.90 cm^{-1} indicates N-H bending or C-N stretching. The presence of this peak indicates that the amine functionalities of chitosan are intact, with possible interactions with the protein (BSA). The peak at 772.41 cm^{-1} is consistent with the fingerprint region of chitosan, indicating that the structural integrity of the chitosan is maintained in the nanoparticle formulation.[7]

5.6. XRD Analysis

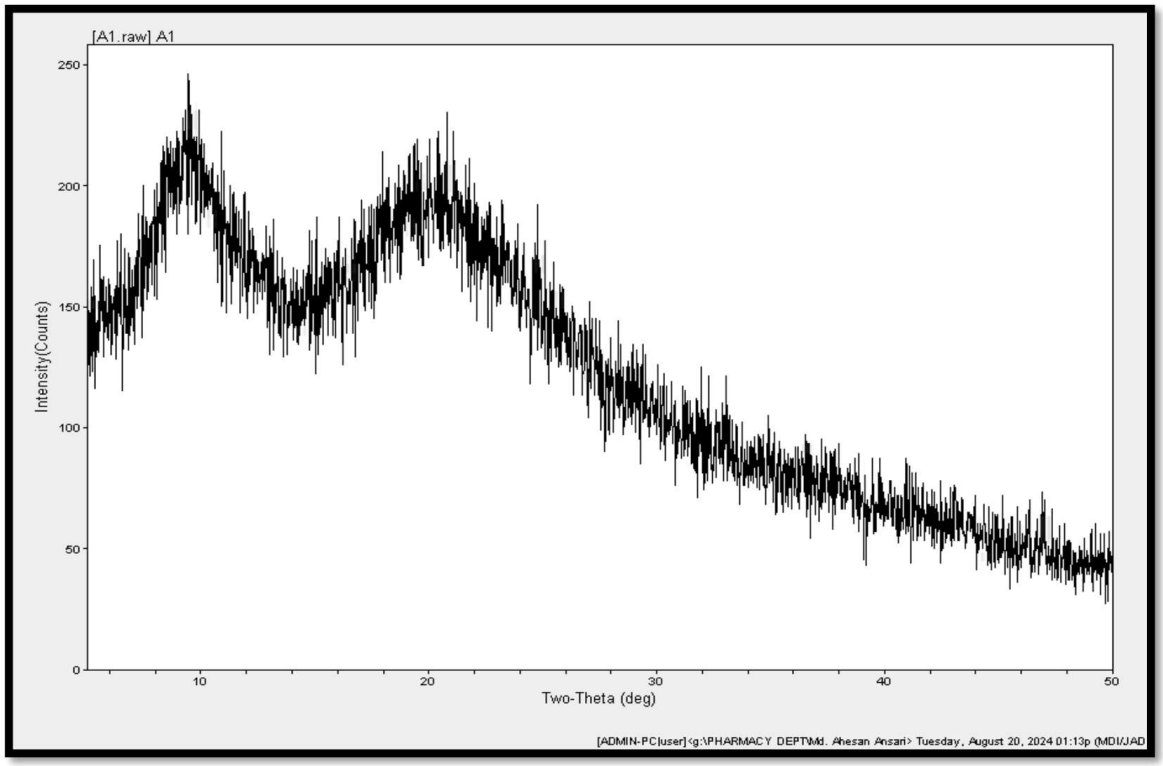

Fig 22: XRD pattern of BSA protein

5.6.1. XRD pattern of BSA protein

The broad peak observed around 10° to 20° in the 2θ range indicates that the BSA sample is largely amorphous. This is consistent with the nature of proteins, which typically exhibit broad diffraction peaks due to their complex, non-crystalline structures. The intensity of the peak is relatively low, which further supports the amorphous nature of BSA. Crystalline materials usually show sharp, well-defined peaks with higher intensities, while amorphous materials display broad and less intense peaks.[8] The absence of sharp peaks indicates that there is no significant crystalline phase present in the BSA sample as shown in Figure 22.

5.6.2. XRD pattern of BSA-loaded chitosan nanoparticle

Similar to the pure BSA sample, the broad peak around 10° to 20° indicates that the BSA within the chitosan nanoparticles retains an amorphous character (shown in Figure 23). This broad peak indicates that there is no significant crystalline order in the BSA or its interaction with chitosan. The intensity of the peaks in this pattern is similar to that of the pure BSA, but there are small changes due to the presence of chitosan. However, the broad nature of the peak indicates that the chitosan matrix does not significantly crystallize in the presence of BSA. The overall amorphous nature of the nanoparticles is indicated by the absence of sharp peaks. The absence of sharp and distinct peaks in the pattern suggests that the BSA-loaded chitosan nanoparticles are predominantly amorphous.[9] This is consistent with the typical behaviour of chitosan, which is known to be semi-crystalline, but when loaded with BSA, the crystallinity is reduced due to the interaction between BSA and chitosan. The broad hump-like feature in the pattern confirms the lack of long-range crystalline order. This is indicative of the successful encapsulation of BSA in a chitosan matrix, resulting in amorphous material.

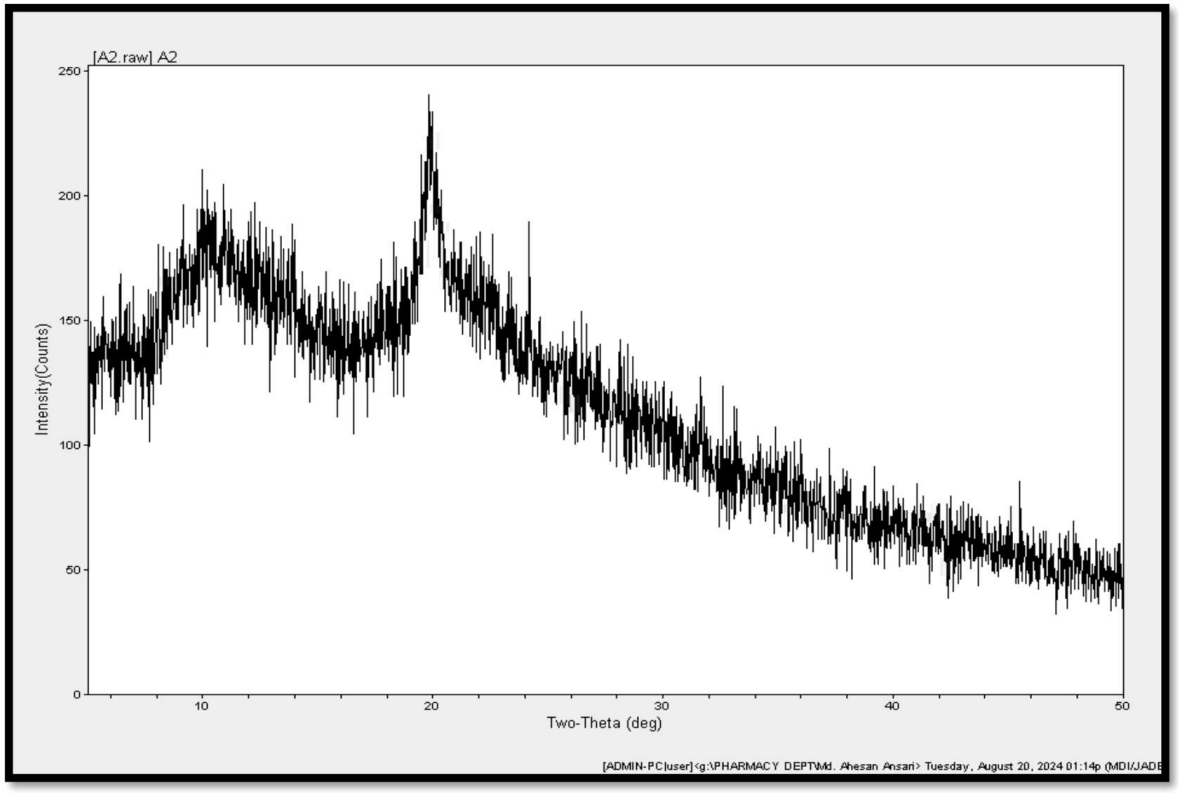

Fig 23: XRD pattern of BSA-loaded chitosan nanoparticle

5.7. Shape and Surface Morphology (SEM studies)

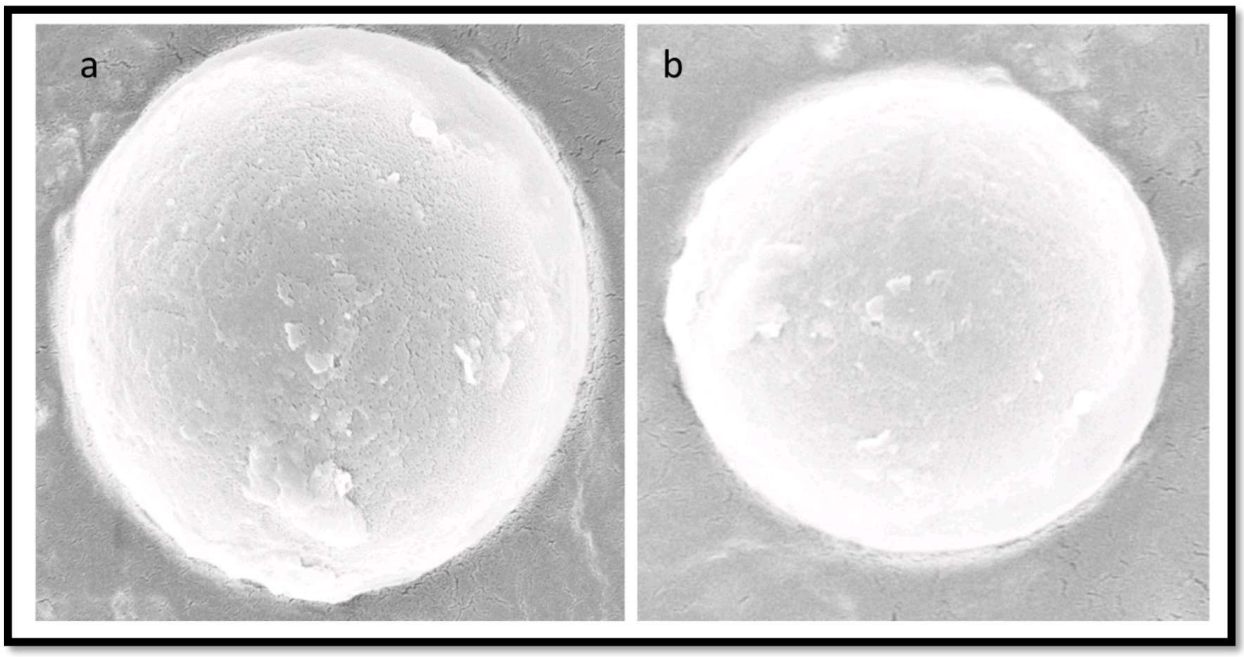

Fig 24: SEM image of Optimized formulation

The BSA-loaded chitosan nanoparticles appear spherical, which is typical for chitosan-based nanoparticles formed by the ionotropic gelation technique. The smoothness indicates that the particle has a well-formed shell, likely due to the successful encapsulation of BSA within the chitosan matrix as shown in Figure 24a. The particle shown in 24b is similar in appearance to the particle as shown in 24a with a smooth surface with negligible irregularities. The uniformity of the surface indicates effective encapsulation of BSA within the chitosan matrix. Both particles show a smooth surface with minor surface features, typical of well-formed chitosan nanoparticles.[10] The smooth surface is indicative of a stable nanoparticle system where the BSA has been effectively encapsulated. The particles are intact and well-formed, with no signs of significant aggregation or collapse. This indicates that the formulation process was controlled, resulting in stable particles. The smooth surface morphology indicates that these nanoparticles exhibit a slower release profile which is justified by earlier discussion *in vitro* drug release study of BSA-loaded chitosan nanoparticles.

References

1. Andrade F, Antunes F, Vanessa Nascimento A, Baptista da Silva S, das Neves J, Ferreira D, Sarmento B. Chitosan formulations as carriers for therapeutic proteins. Curr. Drug Discov. Technol. 2011;8(3):157-72
2. Gupta S, Jain A, Chakraborty M, Sahni JK, Ali J, Dang S. Oral delivery of therapeutic proteins and peptides: a review on recent developments. Drug Deliv. 2013;20(6):237-46.
3. Hou Y, Hu J, Park H, Lee M. Chitosan-based nanoparticles as a sustained protein release carrier for tissue engineering applications. J. Biomed. Mater. Res. 2012;100(4):939-47.
4. Trucillo P. Drug carriers: Classification, administration, release profiles, and industrial approach. Processes. 2021;9(3):470-88.
5. Julienne, V.M.; Benoit, J. Preparation, purification and morphology of polymeric nanoparticles as drug carriers. Pharm. Acta Helv. 1996; 71(2), 121–28
6. Huertas, M.C.; Fessi, H.; Elaissari, A. Polymer-based nanocapsules for drug delivery. Int. J. Pharm. 2010; 385(1-2), 113–42.
7. Patel A, Patel M, Yang X, K Mitra A. Recent advances in protein and peptide drug delivery: a special emphasis on polymeric nanoparticles. PPL. 2014;21(11):1102-20.
8. Ravindran R, Mitra K, Arumugam SK, Doble M. Preparation of Curdlan sulphate-Chitosan nanoparticles as a drug carrier to target Mycobacterium smegmatis infected macrophages. Carbohydrate Polymers. 2021; 258:1176-86.
9. Cao SJ, Xu S, Wang HM, Ling Y, Dong J, Xia RD, Sun XH. Nanoparticles: oral delivery for protein and peptide drugs. AAPS PharmSciTech. 2019; 20:1-11.
10. Jachimska B, Pajor A. Physico-chemical characterization of bovine serum albumin in solution and as deposited on surfaces. Bioelectrochem. 2012; 87:138-46

CHAPTER-VI

SUMMARY AND CONCLUSIONS

6.1. Summary

The chitosan nanoparticles were formulated using ionotropic gelation, a technique that involves the interaction between chitosan and sodium tripolyphosphate (TPP) as a crosslinker. Various formulations (F1 to F7) were developed by varying the drug/polymer ratio and crosslinker concentration. It was found that the percentage yield of nanoparticles ranged from 28.29% to 63.39%, with formulation F4 having the highest yield.

Encapsulation efficiency (EE) of the formulations ranged from 29.08% to 64.37%, with the highest efficiency observed in formulation F4 (64.37%). This indicates that F4 is the most effective in encapsulating BSA, as well as high percentage yield.

The particle size of the nanoparticles ranged from 154.8 nm (F4) to 715.2 nm (F7). Formulations F4 and F5 show the smallest particle sizes, while F5 has the lowest PDI (0.249), indicating a uniform particle size distribution. Smaller particle sizes and low PDI values are advantageous for drug delivery.

The in vitro drug release study showed varying cumulative percentage release (CPR) among the formulations. Formulation F4 demonstrated a controlled release profile which is beneficial for maintaining consistent plasma levels of the drug. The release kinetics were evaluated using different models, with most formulations fitting well to zero-order kinetics, indicating a consistent release rate.

FTIR spectroscopy was used to confirm the successful encapsulation of BSA within the chitosan matrix. The FTIR spectra showed characteristic peaks indicating interactions between BSA and chitosan, indicating the formation of nanoparticles. XRD analysis indicated that the BSA-loaded chitosan nanoparticles are predominantly amorphous which confirms the successful encapsulation of BSA in the chitosan matrix.

Scanning Electron Microscopy (SEM) images revealed that the nanoparticles are spherical with smooth surfaces, indicating successful encapsulation of BSA. The smooth surface morphology also indicates a stable nanoparticle system, correlating with the controlled release profile observed in the *in vitro* drug release study.

6.2. Conclusions

This has been one of the most promising developments in protein drug delivery systems regarding the development and characterization of BSA-loaded chitosan nanoparticles. In this study, a successfully developed BSA-loaded chitosan nanoparticle is seen with the desired characteristics of a protein drug: encapsulation efficiency, control release, and homogeneous particle size distribution. Among all, the formulation F4 is the most promising since it had the highest yield, encapsulation efficiency, and controlled release profile. These results provide evidence that chitosan nanoparticles can be a very potential delivery system for protein drugs. So, this research work makes valuable contributions toward the design and optimization of nanoparticle-based delivery systems of protein therapeutics. This formulation can further be optimized and evaluated *in vivo* for possible therapeutic outcomes in future.