# DEVELOPMENT AND CHARACTERIZATION OF BIPOLYMER BASED NANOPARTICULATE CARRIER SYSTEM AS VACCINE ADJUVANT FOR EFFECTIVE IMMUNIZATION

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## PHARMACEUTICAL SCIENCES

BY

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## PUNJAB

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# DECLARATION

I declare that the thesis entitled "Development and Characterization of Bipolymer based Nanoparticulate Carrier System as Vaccine Adjuvant for Effective Immunization" has been prepared by me under the Supervision of Dr Arun Gupta, Principal R.K.D.F. Institute of Pharmaceutical Sciences and Co-Supervision of Dr Amit Mittal Associate Professor, School of Pharmaceutical Sciences, Lovely Professional University, Punjab. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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# CERTIFICATE

I certify that Mr. Ajay Verma has prepared his thesis entitled "Development and Characterization of Bipolymer based Nanoparticulate Carrier System as Vaccine Adjuvant for Effective Immunization" for the award of PhD degree of the Lovely Professional University, Phagwara, Punjab under my guidance. He has carried out the work at the Department of Pharmaceutics, Smriti College of Pharmaceutical Education, Indore.

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# ABSTRACT

Medical investigations, drug manufacturing and drug delivery has been revolutionized, by breakthroughs in nanotechnology. Man made nanotechnology or nanoscience, however, design small structures that can interact or influence the biological systems on this nanometer scale. Genetic & acquired diseases healing is the aim of Protein therapy at the biomolecular level. The easiest way is the direct injection of naked DNA into the target tissue or organ. Direct injection is not effective enough due to enzymatic degradation and electrostatic repulsion between both negatively charged cell membrane and DNA in most of the cases, results inhibition of efficient cellular uptake. Delivery of the useful proteins by use of any novel carrier like nanoparticles can overcome the drawbacks of the existing technology of protein delivery, currently used conventional vaccines have many limitations due to lack of appropriate adjuvants. For protein and peptide delivery alone hydrophilic or hydrophobic system is ideal. They each have their own advantages and disadvantages. For example, the hydrophilic polymeric systems are biocompatible with the peptide/protein drugs, but have difficulty achieving sustained drug release. When the systems absorb water and swell, peptide /protein molecules will rapidly diffuse out In contrast, the hydrophobic polymeric systems have the capability of yielding sustained drug release. However, they are incompatible with the water soluble protein/peptide drugs. The hydrophobicity of the polymers may induce unfolding of peptide/protein molecules; therefore, the peptide/protein drugs may lose their biological activity after being loaded in and then released from the hydrophobic polymeric systems. In the present study Tetanus Toxoid (TT) was used as the model antigen, and for the study of release and entrapment efficiency, TT was previously conjugated with Fluoresceine-isothiocyanate (FITC). PLGA coated gelatin nanoparticles (PGNPs) were prepared, simultaneously gelatin nanoparticles (GNPs) and PLGA nanoparticles (PNPs) were also prepared for comparative study. GNPs and PNPs were optimized first and the optimized parameters were used for the lamination of gelatin nanoparticles by PLGA. For GNPs Glutaraldehyde concentration, pH, and Temperature and for PNPs Polymer concentration, sonication time and Polyviny alcohol were considered as the process variables. Double emulsion solvent evaporation method is used for the preparation of PGNPs. The prepared formulations were analyzed for size, Size distribution and Polydispersity index (PDI), as per the results obtained   200 µl GTA, 3 pH, and 40º C Temperature were found to be most optimum for GNPs and  for PNPs 5 % Polymer concentration, 2 min

Sonication time and 1% PVA concentration were found to be most optimum and by these parameters gelatin was coated by PLGA with the help of EDC. Shape and morphology of the prepared formulations were studied by TEM and SEM . The cumulative percent release experiment shows the superiority of PGNPs over GNPs that shows the burst release and PNPs that shows the denaturation of protein. The studies revealed that the size of PGNPs are found to be 496.14±34 nm and % entrapment efficiency was 58.2±2.78. After these enthusiastic results, biological studies were performed on BALB/c mice and were analyzed by ELISA. The results shows considerable antibody titre of IgG in both Alum-based marketed preparation and PGNPs. But PGNPs also showed good antibody titre of IgG1 and IgG2a that shows that they also have cell mediated effects. Nasal administration of the formulation showed  sIgA response also. Thus the prepared formulation promotes the advantages and overcomes the disadvantages of both the hydrophilic and the hydrophobic polymeric systems, by a combined hydrophilic-hydrophobic system (gelatin nanoparticles coated with PLGA). This combination can create a new biodegradable system for many other valuable protein and/or peptide drug delivery, that will give benefit to the society by increasing the effects of proteins/peptide drugs.

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"The glory of friendship is not the outstretched hand, nor the kindly smile, nor the joy of companionship, it is the spiritual inspiration that comes to one when he discovers that someone else believes in him and his willing to trust him with friendship", these friends makes us aware that life's completeness and richness depend on the thing we share with our loved ones and friends. Friends like Utpal, Anand, Sanjay, Tabbu, Prabhas, Rakesh, Preet, Prashant and a lot are missing…..

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**Ajay Verma**

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| | |
|---|---|
| AFM | Atomic force microscopy |
| APC | Antigen presenting cell |
| AUC | Area under the curve |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CLSM | Confocal laser scanning microscopy |
| CTL | Cytotoxic T lymphocytes |
| DC | Dendritic cell |
| DLS | Dynamic light scattering |
| DPX | A mixture of disterene (a polystyrene), a plasticizer (tricresylphosphate) and xylene] |
| EDC | 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride |
| ELISA | Enzyme-linked immunosorbent assay |
| FDA US | Food and Drug Administration |
| FITC | Fluoresceine-isothiocyanate |
| GNPs | Gelatin nanoparticle |
| GRAS | Generally recognized as safe |
| GTA | Glutaraldehyde |
| hmw | High molecular weight |
| IEP | Isoelectric point |
| Ig | Immunoglobulin |
| lmw | Low molecular weight |
| Mw | Molecular weight |
| NIBS | Non- Invasive Back-Scatter |
| NK cells | Natural killer cells |
| OD | Optical density |
| OVA | Ovalbumin |
| O/W | Oil in water |

| | |
|---|---|
| PBS | Phosphate-buffered saline |
| PCA | Poly(cyanoacrylate) |
| PCL | Poly($\varepsilon$-caprolactone) |
| PCS | Photon correlation spectroscopy |
| PDI | Polydispersity index |
| PEG | Polyethyleneglycol |
| PLA | Polylactide |
| PLG | Polyglycolide |
| PLGA | Poly(D,L-lactide-co-glycolide) |
| QELS | Quasi-elastic light scattering |
| RES | Reticuloendothelial system |
| RGD | Rayleigh-Gans-Debye |
| RNA | Ribonucleic acid |
| SEM | Scanning electron microscopy |
| TT | Tetanus Toxoid |
| W/O | Water in oil |
| $\zeta$ potential | Zeta potential |

## CHAPTER 1: INTRODUCTION

### 1.1 Nanotechnology and Drug Delivery

Medical investigations, manufacturing of drug & drug delivery has been revolutionized, by breakthroughs in nanotechnology. Biology exists as a natural form of " Nanotechnology" because all elements of living cells fulfill the criteria of technology and size in this respect. Majority of proteins, nucleic acids, lipids and other molecules are in a nano-scale, cellular organelles represent units in a comparable size, organizing functionality and production, leading to self-reproduction and their regulations. Nanotechnology or nanoscience, however, design small structures that can interact or influence the biological systems. **(Gregoriadis et al., 1974; Rehm & Schueler., 2005; Wagner et al, 2006)**

### 1.2 Vaccine and Adjuvants

Immunostimulating compounds are required for vaccines to be effective, e.g. adjuvants, which acts nonspecifically to increase the immune response against defined antigen. Modern vaccinology requires an in-depth search for harmless and effective adjuvants.

Poor immunogenicity is the biggest drawback of subunit vaccines makes them limited for the delivery of highly purified peptides, proteins, DNA and plasmid. An adjuvant is an agent that may stimulate the immune system and increase the response against antigens, but does not have any specific antigenic effect in itself. There are two different types of the most adjuvants, particulate type (exemplified by aluminum salts, oil emulsions and liposomes), non-particulate type (such as saponins, lipid A and muramyl dipeptide derivatives) both exerts their own type of immune modulation**. (Lovgrn-Bengtsson., 1998; Elamanchili and Diwan., 2002; Holmgren et al., 2003; Panda et al., 2006)**

### 1.3 Nanoparticles in Vaccine Delivery.

Genetic and acquired diseases healing is the aim of protein therapy at the biomolecular level. Injection of naked DNA into the target tissue or organ is the easiest way of gene delivery, eventually this technique is constrained to only a few use for example injection of DNA into solid tumors & muscle tissue. Efficient cellular uptake is inhibited by direct injection because of enzymatic degradation and electrostatic repulsion among the charged cell membrane and DNA

in most of the cases. **(Vile & Hart 1993; Wolff *et al.* 2005)**, Delivery of the useful proteins by use of any novel carrier like nanoparticles can overcome the drawbacks of the existing technology of protein delivery, lacking of appropriate adjuvants is the biggest constrain of currently used conventional vaccines.

## 1.4 Polymer: As Adjuvant

Polymers which are intended for internal use when delivered in nano-range can provoke the immune response and can act as adjuvants; currently many polymers established their usefulness in drug delivery systems. Most controlled release technologies have been vitalized by a continued improvement and accelerating research and development in polymeric materials. In the past 25 years, Polymer science gained a considerable interest in the field of novel drug delivery system, where USFDA approved polymers are used for the delivery of drugs e.g. PLA (Poly Lactic Acid), PLGA (Poly Lactide/glycolic Acid), gelatin etc. PLGA, PLA and gelatin are naturally occurring polymers. After recent thorough study of behavior of polymers,
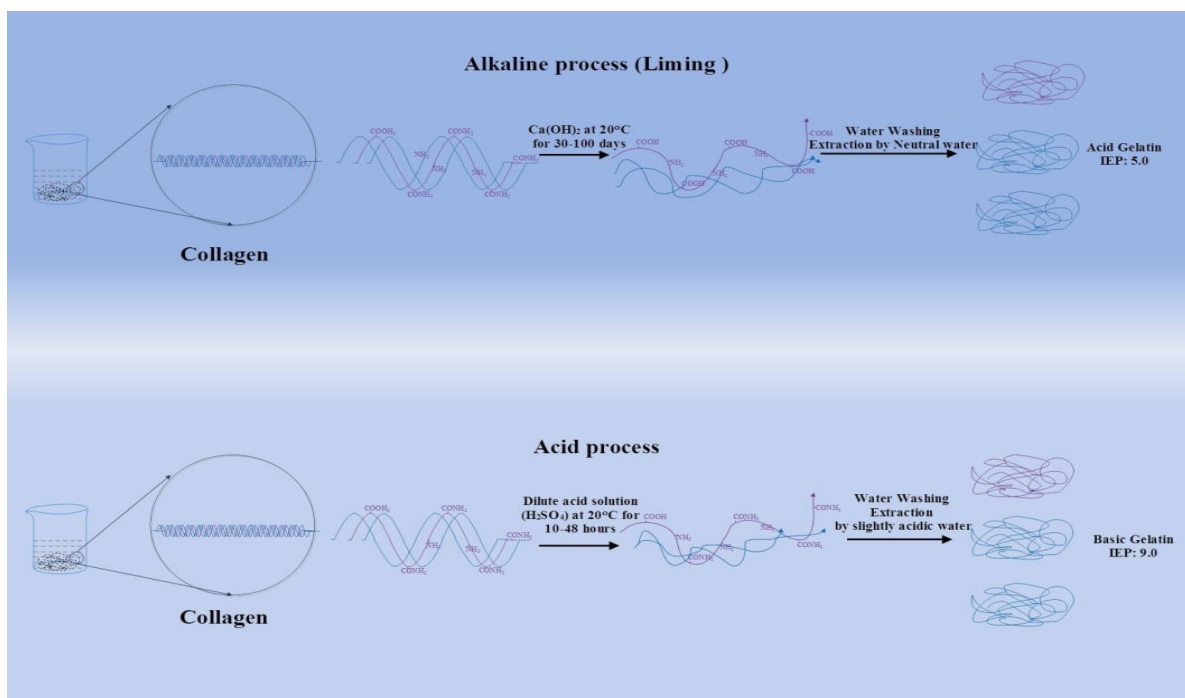


**Fig.1.1: Preparative process for acidic and basic gelatins (Type A and Type B).**

currently available polymers as adjuvants can be classified into four major categories as per the kind of delivery systems, and these include: Diffusion-controlled systems, Solvent-activated

systems, chemically controlled systems, magnetically controlled systems. **(Allison et al., 1991; William et al., 1994; Illum et al., 1997, Pascual et al., 2006).**

## 1.5 Gelatin

Gelatin is a polymer with biological origin, 96-98% part of gelatin is protein in nature which is water soluble. Gelatin based delivery system are biodegradable and without toxic degradation products, they have high physiological tolerance and low immunogenicity as they are biocompatible. US Food and Drug Administration (FDA) approved gelatin as safe and documented by the classification as "Generally Recognized as Safe" (GRAS). **(Friess 1998; Tabata & Ikada 1998 ; Farrugia & Groves 1999; Kelso 1999; Kuijpers et al., 2000; Saito et al., 2005)**

## 1.5.1 Release of Protein from Gelatin

Fig. 1.2 shows a how the protein, peptide or drug release from gelatin occurs. **(Tabata, Y. and Ikada, Y., 1998)**. If an environmental change occurs, the fraction of gelatin such as collagen, changes its ionic strength, and releases protein from the drug/protein–carrier complex. Both type A and type B gelatins are water soluble. The profile of protein and peptide release for example, with water-soluble carbodiimides protein–carrier system is regulated by the glutaraldehyde, this directly indicates that the level of crosslinking is very crucial for release.
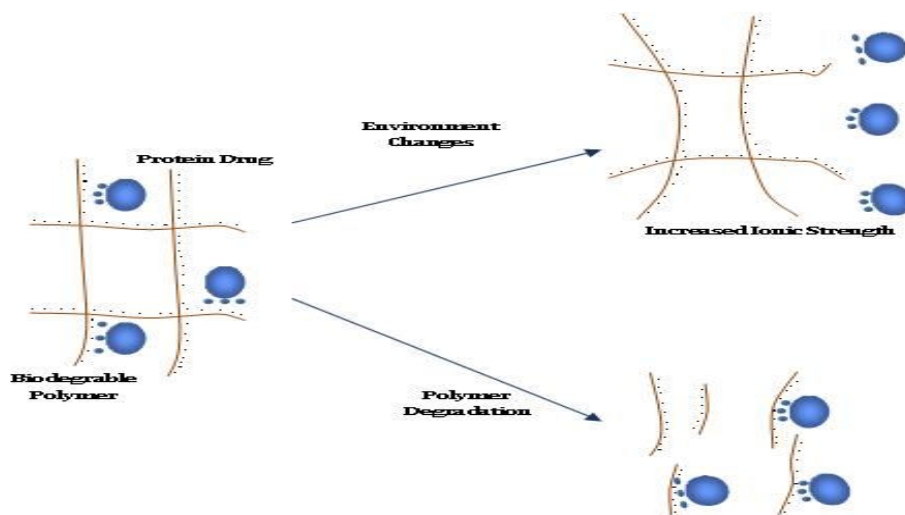
**Fig.1.2. Release of protein and peptide from biodegradable polymer.**

### 1.5.2 Limitations of gelatin as delivery system.

When native gelatin is used it produces a wide molecular weight range, which distress robustness in reproducibility and homogeneity of the produced particles, gelatin alone when used as the carrier system may cause problem of homogeneity as well as release uniformity, which might be reason why gelatin based nanoparticles were not extensively investigated.

### 1.6 PLGA (Poly lactic co-glycolic acid)

Biomaterials mainly biopolymers play an important role in human health, because of the degradation range from 3 weeks to over a year Lactide/glycolide copolymers have had such strong success in drug delivery formulations, depending on the composition of the copolymer as well as the method of preparation and formulation. PLA and its co polymer PLGA is the polymer of choice for the controlled and sustain drug delivery. PLGA is the copolymer of lactic acid and glycolic acid. The ratio of lactic to glycolic acid offers a useful characteristic of PLGA in the novel drug delivery. The composition of lactide and glycolide vary as 25:75, 40:60, 50:50, 75:25, 85:15. The fastest degradation is seen for copolymers with 50:50 of lactide to glycolide and with low molecular weights. Decreasing the degree of crystallinity results increase in degradation rate of the polymer., while increase in the degree of crystallinity results in decrease in degradation rate. The ability of PLA/PLGA polymers to dissolve in a variety of organic solvents as well as to be extruded into a number of shapes has been instrumental in exploring their use from biodegradable sutures into implants, microparticles, nanoparticles, **(Wise et al., 2000 ; Janganathan et. Al., 2004)** and fibers for an ever-increasing number of controlled release formulations and devices.

Because of significant different properties of PLA and PGA careful choice of copolymer composition allows for the optimization of PLGA for intended applications. PLGA copolymers with 25:75 lactide composition is considered as the best suitable for sustained release, PLGA forms amorphous polymers, which are very hydrolytically unstable as compared to the more stable homopolymers. **(Allemann et al., 1993; Mohsin et al., 2008; Ulery et al., 2011)** This is obvious in the degradation times of 50:50 PLGA, 75:25 PLGA, and 85:15 PLGA being 1-2 months, 4-5 months, and 5-6 months.

## 1.7  Surface modification.

Nanoparticles extolled its most captivating position in the nano world. Its uniqness in the conformational characteristics, nanoparticles offers ease in the fabrication of nanoparticles. Multifunctional platform provides ligand conjugation and surface modification possibilities. Nanoparticles manifests various clinical applications (**Tomalia et al., 1985; Hawker et al., 1990; Lee et al., 2000; Kommareddy and Amiji., 2005; Jaganathan  et al., 2006; Surya et al., 2009; Sudhees et al., 2011**). Versatility of nanoparticles, showing promise in the therapeutics arena, and there is a great deal of commercial activity, with a few products already available in the market. The potentiality to engineer & control these parameters creates enormous possibilities for capitalizing nanoparticles as modules for nanodevice design.
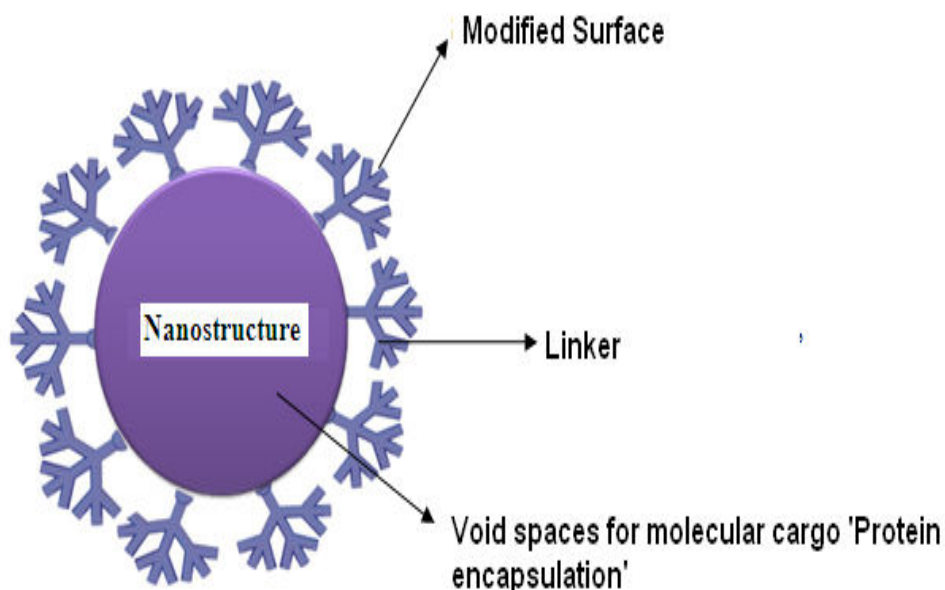


**Fig. 1.3:   Surface modification of nanoparticles**

## 1.7.1 Uniqueness of nanoparticles surface modification:

The system serves to fulfill following objectives:

1.7.1.1. Enhanced permeability and retention effect: Size of nanoparticles i.e. (10-1000 nm) is in nano range. Cancer cells have leaky membranes and having higher                 bio-permeability, lymphatic system is one way and protein loaded nanoparticles get retained

inside **(Malik et al., 2000; Jamie  et al., 2001; Jevprasesphant et al., 2003; Kolhe et al., 2004)**,

1.7.1.2. High permeability: nanoparticles cross bio- barriers like blood brain barrier, cell membrane. Homogeneity and nanometer range in size, enhance their ability to cross cell membranes, this reduces the risk of undesired clearance from the body by the liver or spleen. **(Kojima et al., 2000; Yang et al., 2004; Chauhan et al., 2004)**

1.7.1.3. Sustained /extended effect: Dendrimer having 3-D network structure release drug in a sustained manner. Slower release, higher accumulation in solid tumors, and lower toxicity is displayed by surface modified nanoparticles. **(Devarakonda et al., 2004)**

1.7.1.4. Most anticancer drugs have poor solubility, and surface modification offer higher solubilization potential, ionic interaction, hydrogen bonding and hydrophobic interactions may be possible mechanism by which nanoparticles shows its solubilizing property.

1.7.1.5. More precise protein delivery is achieved by surface modified nanoparticles. High uniformity and purity are obtained by synthetic process used which produces nanoparticles with uniform sizes, precisely defined surface functionality, and very low impurity levels.

1.7.1.6. Multifunctional platform.

1.7.1.7. By encapsulation and absorption nanoparticles structures can be used to carry and store a wide range of metals, organic or inorganic molecules. Drug can get entrapped inside the internal cavities as well as electro statically in the surface of nanoparticles.

1.7.1.8. High stability: Nanoparticles drug complex or conjugate exhibits better stability.

1.7.1.9. Nanoparticles commonly show a very low or negligible immunogenic response when injected or used topically. Most nanoparticles systems display very low cytotoxicity levels, low immunogenicity as the characteristic property.

## 1.8  REVIEW OF LITERATURE

**Sudhees et al., 2011,** studied the effect of gelatin nanoparticles. They prepared gelatin nanoparticles alongwith aminated gelatin nanoparticles (AGNPs) and delivered them intranasaly for enhance delivery of TT, the result were observed by measuring anti-TT IgG, IgG1 and IgG2a isotype. The results showed a fast release of protein from the polymer which was retarded by preparing  aminated gelatin nanoparticles.

**Arvind et al., 2009,** prepared a novel triblock copolymer planted nanoparticles for vaccine delivery against hepatitis B, the study reveals that PLGA is one of most preferred biodegradable polymer in vaccine delivery. However,  hydrophobic nature and generation of acidic microenvironment upon its degradation makes its use deprived, limiting it unfavorable to the entrapment of highly expensive proteins and peptides.

**Chen et al., 2009,** prepared a combination system, composite of glycidyl methacrylated dextran and gelatin nanoparticles for localized protein delivery, study reveals that alone gelatin causes burst release of the protein encapsulated in the nanoparticles. Results of the study confirms about the burst release of the protein from gelatin nanoparticles a potential drawback of the GNPs.

**Jawahar et al., 2009,** prepared PLGA nanoparticles of carvedilol to improve the bioavailability of carvedilol and sustain the release of drug to reduce the initial hypotensive peak and enhance the antihypertensive effect. In vivo biodistribution studies in rats proved that these nanoparticles are distributed in heart, liver and kidney with higher concentration.

**Muthu et al., 2009,** reviewed PLGA and its copolymers based polymeric nanoparticles updates. PLGA loaded with different drugs shows effectiveness in their respective therapies. Opsonization process is overcome by preparing stealth nanoparticles of PLGA co-polymers during I.V. administration for an enhanced  and targeted delivery.

**Morimoto et al., 2008,** investigated two different types of cationized nanoparticles dependent on a native cation gelatin and aminated gelatin with EDA for the controlled release of three model acidic peptide/protein with different molecular weights and IEPs (Isoelectric point). The study gave an idea about the control of the release rate of acidic peptide/protein drugs.

**Zillies et al., 2008,** assessed the short- term storage stability as per ICH guidelines. Dried gelatin nanoparticles formulated in mannitol/sucrose, sucrose, trehalose, and mannitol. The

results were analysed by size, size distribution, and residual moisture content. The results of the study indicated that the sucrose and trehalose containing formulations exhibits highest stability and mannitol containing formulations showed remarkable stabilization in contempt with their crystalline nature.

**Anguilar et al., 2007,** reported that development of novel adjuvant strategies converging the requirements for their clinical applications have been hindered by obstruction like adjuvant toxicity which remains to be fully surpassed. Major bottlenecks hindering the commercialization of nanoparticulate vaccines that include the instability of vaccine protein during fabrication process, storage and the rigorous constrains on toxicity by the regulatory authorities.

**Banu et al., 2007,** prepared two variable molecular weight formulation of PLGA nanoparticles to check the release kinetic of drug. They investigated process of polymer degradation and drug release kinetics from PLGA nanoparticles under neutral and acidic pH conditions. The results showed that on increasing the percentage of glycolide increase the release duration while on increasing lactide percent shows a more hydrolytic degradation.

**Jaganathan et al., 2007,** prepared a stable polymeric microspheres that can release TT over a period ranging from days to over months. They encapsulated TT in PLGA, chitosan and the protein were stabilized by a protein stabilizer (trehalose) and the immune responses were compared. They also utilize $(Mg(OH)_2)$ to neutralize the acids liberated by the biodegradable PLGA polymer, they also added an antacid, which neutralizes the acidity during degradation of the polymer and prevented aggregation caused by PLGA, this study help us to understand the biggest drawback of PLGA polymer.

**Majeti, N. et al.,2007,** performed intranasal immunization with tetanus toxoid and CNF1, this showed that new mucosal adjuvant protects BALB/c mice against lethal challenges hence proved the importance of sIgA along with the IgG responses.

**Coester et al., 2006,** studied the in vitro delivery of gelatin nanoparticles by murine dendritic cells and their localization was obsersed intracellularly by CLSM they checked the in-vitro uptake of the protein by the DCs. Flow cytometry showed that 92% of the cells found to be positive for the specific murine DCs. Results suggested that gelatin nanoparticles are biocompatible and can be useful for vaccine delivery to DCs.

**Owens and Pepas, 2006,** Studied opsonization, biodistribution and pharmacokinetics of polymeric nanoparticles. They investigated that njectable polymeric nanoparticle carriers have

the ability to revolutionize disease treatment through spatially and temporally controlled drug delivery. However, opsonin proteins present in the blood serum quickly bind to conventional non-stealth nanoparticles, allows macrophages of the mononuclear phagocytic system (MPS) to easily recognize and remove these drug delivery devices before they can perform their designed therapeutic function. In order to remove these limitations, several methods have been developed to mask or camouflage nanoparticles from the MPS. They developed the method that creates a hydrophilic protective layer around nanoparticles that repels the absorption of opsonin proteins by steric repulsion forces, thereby blocking and delaying the first step in the opsonization process.

**Brandtzaeg et al., 2006,** reviewed on induction of sIgA and long term memory at mucosal surfaces. They identified that the IN (Intra-Nasal) application targeting NALT (Nasal Associated Lymphoid Tissue) is more advantageous for certain infections, but this can be achieved only on successful stimulation without the use of toxic adjuvants to avoid movement in the central nervous system.

**Diez et al., 2006** prepared the delivery system of PLGA nano-particulate carrier intended for plasmid DNA delivery. They optimized various process variables in different formulations of DNA encapsulated into polymeric particulate this gave the information about the effect of various process variables used in the formulation of PLGA based nanoparticles.

**Alpar et al., 2005,** formulated biodegradable nanoparticles for mucosal antigen and DNA delivery. The formulation showed effective lung delivery for olfactory nerves of the nasal cavity and play an potential role in uptake of vaccine formulations. The results of the study showed the potential of muco- and bioadhesive agents when combined with liposomes IN delivery.

**Katare et al., 2005,** observed that, by a judicious use of factors such appropriate selection of the polymer, particle size, varying dose, prevents the immunogenicity of the Ag during encapsulation and designing an immunization protocol.

**Bootz et al., 2004,** prepared nanoparticles of Poly (butyleyanoacrylate) and compared different techniques like scanning electron microscopy, Photon correlation spectroscopy and analytical ultracentrifugation to study the size of nanoparticles. Results suggested that the identification of size and size distribution can be done most suitably by SEM and PCS.

**Gupta et al., 2004,** studied the gelatin nanoparticles effect of cellular uptake on adhesion, morphology and cell organization in the fibroblast of humans. The study showed that with small

size and large concentration of gelatin nanoparticles can be phagocytosed by the fibroblasts and does not show any toxicity to cells.

**Rubiana et al., 2004,** developed PLGA based spherical nanoparticulate drug carriers with controlled size. Praziquantel, a hydrophobic molecule, was encapsulated in that. This study observed the effects of some process variables on the size and size distribution of nanoparticles. Sonication time, PLGA concentration and PVA concentration was found be the process variables that can influence the design of nanoparticels when prepared by double emulsion solvent evaporation method.

Vila et al., 2003, prepared the nanoparticles of PLGA & chitosan, they compared the size and entrapment, the study suggested the potential utility of SEM and TEM for the identification of particle size and morphology of the nanoparticles.

**Eyles et al., 2003,** studied the stimulation of spleen cells in vitro by nanospheric particle containing protein/peptide. Shows the potential of TT as the strong antigenic in nature and hence can be utilized for the study of proteins delivery as the model antigen.

**Kaul et al., 2002,** studied long-circulating PEG-modified gelatin nanoparticles. They performed the surface modification of PEG and articulated PEG-modified (PEGylated) gelatin derivative. The results showed a positive path towards the development of an delivery system for drugs after surface modification of polymer, surface modification was confirmed by the transmission electron microscopy.

**Raghuvanshi et al., 2002,** described that physical mixture of nano and microparticles generated the high antibody titers. Anti-TT antibody titer generated after immunization with polymer particles containing stabilized TT persist for more than 5 months and was higher than the titer obtained with saline TT. But the antibody responses generated by single point immunization were lower than the conventional two doses of alum-adsorbed TT.

**Sasaik et al., 2001,** reported that antigen denaturation has been attributed to be a major challenge in the development of PLGA nanoparticles. Immunogenicity of the TT has been found to be denatured by exposure to some organic solvents, since microencapsulation of TT shows less immunogenicity than the alum-adsorbed antigen. Use of high temperature and high shear were found to be the other major factors that influences the integrity of antigens.

**Ryan et al., 2001**, investigated a delivery system and immunomodulation vaccine delivery by mucosal routes. They discussed the mechanism involved in immunization elicitation after

mucosal vaccine development and review the applicability of protective immune responses at mucosal surfaces.

**Coester et al., 2000,** reported a new preparation method for gelatin nanoparticles preparation by two step desolvation and conducted cell uptake studies. In this technique, the higher molecular weight (HMW) fraction is separated during first desolvation step after precipitating out by gentle heating and low molecular weight (LMW) gelatin were retained for the formation of nanoparticles. The result of the study demonstrates that low molecular weight gelatin are most suitable for the effective nanoparticles. That ranges between 50- 200 nm.

 **Li et al., 1997,** firstly attempted a polymer in polymer system they studied biodegradable system of gelatin nanoparticles and PLGA microspheres. Their study concluded that the protein containing PLGA-PVA composite may be suitable for long term protein delivery. As after studying the results it gave an an idea about the degradation pattern of the PLGA polymer, and how protein and peptide can be protected by the denaturation caused by the acid produced by the degradation of PLGA.

**Kumar et al., 1982**, demonstrated that TT is stable at 45ºC and therefore antigen loss may be minimum during production procedure for gelatin nanoparticels. Above 45ºC the degradation of toxoid potency increases. Above 50ºC the toxoid fully deactivated, the study supported the use of low molecular weight gelatin for the formation of nanoparticles, since by discarding high molecular weight gelatin nanoparticles gives stable nanoparticles preparation which can withstand at 45ºC.

## 1.9 RESEARCH   ENVISAGED

The primary objective of the study was to develop a vaccine formulation with stability, sterility and efficacy.

For protein and peptide delivery alone hydrophilic or hydrophobic system is ideal. They each have their own advantages and disadvantages. For example, the hydrophilic polymeric systems are biocompatible with the peptide/protein drugs, but have difficulty achieving sustained drug release. When the systems absorb water and swell, peptide /protein molecules will rapidly diffuse out **(Chen et al., 2009)**. In contrast, the hydrophobic polymeric systems have the capability of yielding sustained release of drug. However, they are incompatible with the water soluble protein/peptide drugs. The hydrophobicity of the polymers may induce unfolding of peptide/protein molecules; therefore, the peptide/protein drugs may lose their biological activity after being loaded in and then released from the hydrophobic polymeric systems **(Arvind et al., 2009)**.

Thus the research work envisaged promotes the advantages and overcomes the disadvantages of both the hydrophilic and the hydrophobic polymeric systems, by a combined hydrophilic-hydrophobic system (gelatin nanoparticles coated with PLGA). This combination can create a new biodegradable system for protein/peptide drug delivery.

## AIM OF THE STUDY

Development and Characterization of Bipolymer based Nanoparticulate Carrier System as Vaccine Adjuvant for Effective Immunization

a.   Behave as protein stabilizer.

b.   Provide prolonged antigen release.

c.   Provide prolonged systemic as well as cell mediated immunity along with effective immunization.

## OBJECTIVE OF WORK

a.   Development and characterization of PLGA coated gelatin nanoparticles (PGNPs) can prevent the denaturation of proteins/peptides and at the same time that can prolong antigen release.

b.   Tetanus Toxoid will be used as model antigen.

c.   Gelatin and PLGA will be used for protection as well as sustained release of proteins/peptides.

### 1.10   PLAN OF WORK

1.   **Antigen and its analytical profile**
    a.   FITC-TT Conjugate formation.Calibration curve of FITC-TT.
    b.   Molecular weight determination (SDS PAGE)

2.   **Formulation and optimization of gelatin nanoparticles**
    a.   Study of size & size distribution by DLS (Dyanamic Light Scattering)
    b.   Study of morphology by TEM (Transmission Electron Microscopy)
    c.   Antigen loading efficiency
    d.   Antigen release profile

3.   **Formulation and Optimization of PLGA nanoparticles**
    a.   Study of size & size distribution by DLS (Dyanamic Light Scattering)
    b.   Study of morphology by TEM & SEM (Transmission Electron Microscopy)
    c.   Antigen loading efficiency
    d.   Antigen release profile

4.   **Coating of gelatin nanoparticles by PLGA**
    a.   Study of size & size distribution by DLS (Dyanamic Light Scattering)
    b.   Study of morphology by TEM & SEM (Transmission Electron Microscopy)
    c.   Antigen loading efficiency
    d.   Antigen release profile

5.   **Biological Evaluation (Balb/c mice)**
    a.   Immunization protocol
    b.   Primary immunization at 0 Day
    c.   Booster dose 28 day
    d.   Collection of blood from retro-orbital plexus
    e.   Estimation of serum IgG level (ELISA)
    f.   Estimation of IgG1 and IgG2a (ELISA)
    g.   Estimation of sIgA (ELISA)

6   **Stability Studies**

## CHAPTER 2: ANTIGEN PROFILE

### 2.1  Selection of Antigen

Antigens can be defined as the foreign substance, which can evoke immunological response in the host. Chemically protein functions as most potent immunogen, followed by polysaccharides. Lipid and nucleic acid of an infectious agent generally do not serve as immunogen unless they are complexed to protein or polysaccharide. Immunogenicity is determined by four properties of an immunogen its foreignness, molecular weight, chemical composition and complexity. Best immunogens have molecular weight of about 10,000 or higher but smaller molecules can also be made antigenic by their adsorption on the surface of inert carrier or adjuvant.

The immune system works by recognizing the receptor site of particular macromolecule of an infectious agent called epitope, generally either protein or polysaccharide. The selection of antigen largely depends upon the requirements of particular experiments. In most experimental studies of humoral immunity, soluble protein or polysaccharides are used as immunogen. List of immunogens commonly used as model antigens is presented in Table 2.1.

**Table 2.1**

**Immunogens commonly used as model antigens**

| S.No. | Immunogen | ~ molecular weight (Da) |
|-------|-----------|-------------------------|
| 1. | Hen Egg White Lysozyme (HEL) | 15,000 |
| 2. | Sperm Whole Myoglobin (SWM) | 17,000 |
| 3. | Flagellin | 40,000 |
| 4. | Ovalbumin (OVA) | 44,000 |
| 5. | Bovine Serum Albumin (BSA) | 69,000 |
| 6. | Bovine Gamma Globulin (BGG) | 1,50,000 |
| 7. | Tetanus Toxoid | 1,50,000 |
| 8. | Keyhole Limpet Hemocyanin | >20,00,000 |

**2.1.1 Tetanus toxoid vaccine: An Overview.**

In the present study Tetanus Toxoid (TT) **(Tobio et al., 1997; Panda et al., 2006)** was used as model antigen .TT is a useful antigen because of its low cost, high purity, high antigenicity and ready availability for investigation of immunogenic properties of carrier associated, covalently linked or the entrapped macromolecular antigen within the carrier. Every Pharmacopoeia requires independent potency tests of every new batch of vaccine mandatory for the release of final product by the manufacturer.

The protocol of the EU/EEA Official Medicine Control Laboratory Batch Release system in Europe requires the assay to be performed whenever a new final bulk is used. A reference preparation of tetanus toxoid (adsorbed) calibrated in International Unit and a suitable preparation of tetanus toxin is required for this testing.

Thus calibration of tetanus vaccines is currently dependent on the use of an International Standard, or another reference preparation calibrated against it, and expression of vaccine potency in International Units (IU). International biological standards and reference materials are tolls that allow calibration of the activities of biological preparations in International Units, Thus ensuring global comparability of the expressed activity. The International Standard (I.S.) for tetanus toxoid has played an important part in ensuring the quality and efficacy of tetanus vaccines world- wide for many years **(Montecucco et al., 1993).** Almost 40 years ago the WHO established the First International Standard for Tetanus Toxoid , Adsorbed (TEXA-1). The activity assigned to 1 mg of this formulation was determined as equivalent to 1.5 IU of previously established I.S. for nanoadsorbed tetanus toxoid.

**2.1.2 Tetanus Toxoid (TT)**

A white to light tan colour powder that contains not more than 3.0% w/w of water, containing about 96% proteins, **Molecular weight (Mw):** 150,000 Daltons

**Isoelectric point (IEP):** pH 4.7, **Solubility:** Soluble in pure water. When a volume of water sufficient to give a protein concentration of 5% w/v is added, the substance dissolves completely within ten minutes at 20-28$^{\circ}$C. Coagulates by heat. Precipitates from solution only at high ammonium sulphate concentration, **Storage:** Protect from light and moisture, store at temperature between 2-8$^{\circ}$C.

## 2.2 Conjugation of FITC-TT

To calculate the % entrapment efficiency tetanus toxoid (TT) was conjugated to flourescein isothiocyanate (FITC) before preparation of antigen loaded nanoparticles because routine protein estimation methods could be interfered by the presence of soluble gelatin. In order to avoid this fluorescent labeling of antigen was carried out. The entrapment and release were evaluated by spectrofluoremetric determination. FITC- labeled TT particles were prepared by a reported procedure **(Copland et al., 2003)**. 20 mg FITC was dissolved in 10ml of carbonate buffer pH 9.5 along with 5 ml of tetanus toxoid solution (TT). At $4^0$C for 18 hr the mixture was gently stirred in the dark. Unbound FITC was removed by repeated dilution with water using dialysis membrane of 10,000- molecular weight cut off . The prepared FITC-TT solution was freeze-dried, well protected from light and then stored at $4^0$C for further use.

## 2.3 Integrity testing of the received antigens by SDS-PAGE.

Tetanus toxoid was procured from Serum Institute of India, Pune. The integrity and molecular weight determination of pure TT and FITC-TT conjugate was carried out by SDS-PAGE of antigen (Photograph. 2.1). For analysis and characterization of protein samples SDS-PAGE is widely used. It is useful technique in determining molecular weight, relative estimation of purity and amino acid sequencing of proteins **(Wilson and Walker, 1997; Arvind et al., 2009)**. In the present study, SDS-PAGE was performed at Pharmbio Research Centre, Indore M.P. India.
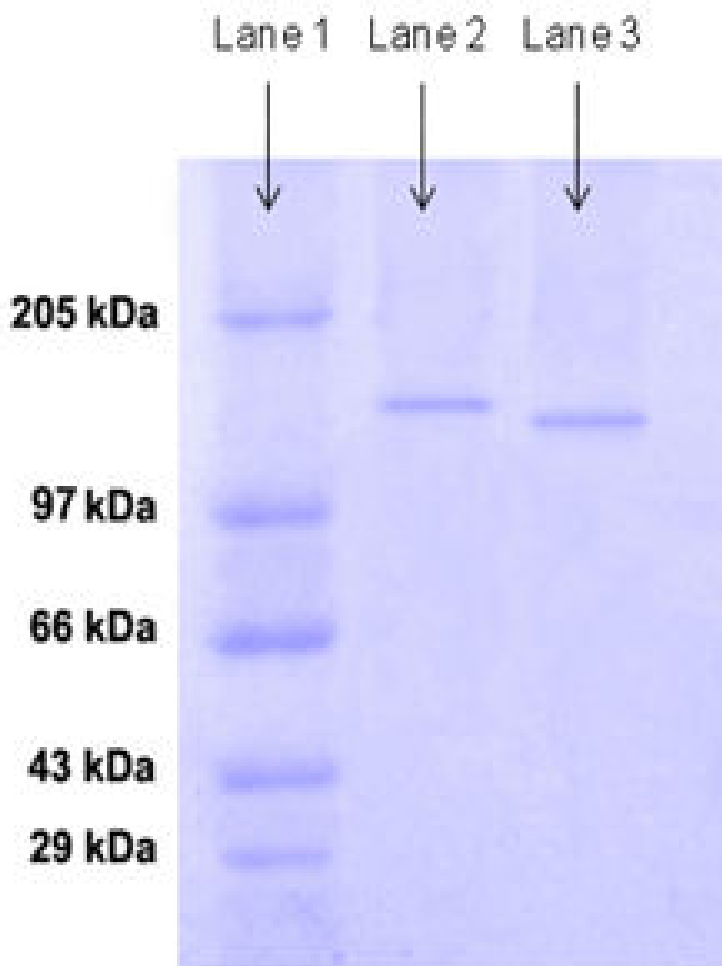
**Photograph 2.1 SDS-PAGE: Lane 1; Molecular Markers, Lane 2; Pure TT (Tetanus Toxoid), Lane 3; FITC-TT.**

## 2.4 Calibration curve of FITC-TT

Spectrofluorometer (RF-5301PC spectrofluorophotometer Shimdzu, Japan) was used for the detection of fluorescence intensity. Calibration curve for FITC-TT was made at five measurement points ranging 0.1 to 1µg/ml protein solution in PBS 7.4 sol. At about 495 nm fluorescence spectra were measured with a spectrofluorometer using a $1cm^2$ quartz cell. The emission and excitation slit widths equaled 5 and 5nm, respectively. Table 2.2 summarizes the regressed calibration curve readings of FITC-TT, which is graphically shown in fig. 2.1.

**Table 2.2**

**Calibration curve FITC-TT in PBS ($\lambda_{ex}$ = 493nm, $\lambda_{em}$ = 520 nm, pH 7.4): emission & excitation wavelength, respectively.**

| S.No. | Concentration (µg/ml) | Normalized Intensity | Normalized Intensity (Regressed) |
|-------|----------------------|----------------------|----------------------------------|
| 1 | 0.15 | 0.070 | 0.06824 |
| 2 | 0.30 | 0.2204 | 0.22298 |
| 3 | 0.45 | 0.3713 | 0.37772 |
| 4 | 0.60 | 0.5249 | 0.53246 |
| 5 | 0.75 | 0.7071 | 0.6872 |
| 6 | 0.90 | 0.8521 | 0.84194 |
| 7 | 1.05 | 0.9812 | 0.99668 |



$y = 1.0316x - 0.0865$

$R^2 = 0.998$
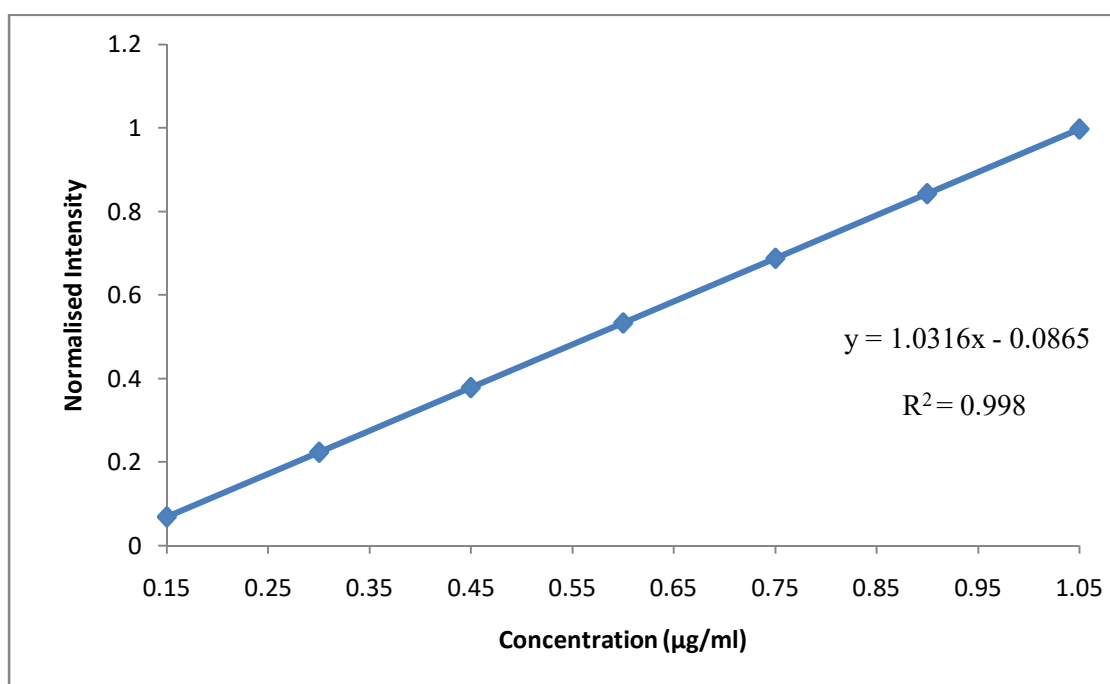
**Fig. 2.1. Calibration Curve of FITC-TT in PBS. ($\lambda_{ex}$ = 493 nm, $\lambda_{em}$ = 520nm, pH 7.4)**

## 2.1.6   RESULT AND DISCUSSION

In the present study TT was selected as model antigen because of ease of availability and its inherent property of high antigenicity. The received antigens integrity and molecular weight of FITC-TT prepared was assessed by SDS-PAGE (Photograph; 2.1). For analysis and characterization of protein samples SDS-PAGE is widely used. Sodium dodecyl sulphate binds with the polypeptide unit of proteins. The molecular weight of the protein can be identified by amount bound to SDS which is directly proportional is not dependent of its sequence. The SDS-protein complexes formed migrates through the polyacrylamide gels in accordance with the molecular weight of protein part. The SDS-PAGE was performed as per the reported protocol.

It is clear from Photo. 2.1 that indicates the different lanes of spots, lane 1 corresponds to the molecular markars, lane 2 and lane 3 shows two distinct darker bands at around 150 kDa, and the molecular weight of tetanus toxoid is nearly 150 kDa which corresponds to the subunit of TT and FITC-TT subsequently.

As the prior step of formulation the received antigens were identified and characterized through SDS-PAGE. For the determination of % entrapment efficiency FITC-TT was conjugated and release profile was also estimated by calibration curve of FITC-TT. FITC-TT conjugation was done as it is easy to analyze the protein entrapment and release with this fluorescent dye using fluorescence spectrophotometry because conventional colorimetric assay's such as BCA and Bradford's methods would give erroneous results due to interference by the presence of soluble gelatin which would also give color in the dye binding assay. Typical calibration curve of FITC-TT (PBS 7.4) is shown in figure 2.2 (correlation factor 0.9987). The concentration of FITC-TT in PBS 7.4 was identified at the maximum fluorescence intensity of about 520 nm observed by the calibration curve.

## CHAPTER.3: PREPARATION AND CHARACTERIZATION

### 3.1 General Introduction

With the ever-increasing number of biopharmaceutical products and novel advances in pharmaceutical technology including nanotechnology, which is aimed at stabilization and safe administration of these product have revolutionized the pharmaceutical scenario worldwide. In contrast to the low- molecular-weight drugs processed routinely in the pharmaceutical industry, biopharmaceutical industry, biopharmaceuticals require special attention in their handling, storage and administration. Modern research in the area of biotechnology has made it possible to develop various clinically useful proteins. This technology has brought about the mass production of these bioactive macromolecules, several challenges need to be addressed. In the development process of a nanoparticulate drug delivery system for in vivo application, biodegradability without toxic by-product is one of the major challenges, a potential matrix molecule has to fulfill. Concerning nanodevices suitable for drug delivery, there has already been tremendous progress within the last 40 years, from the first liposomal approach to the recent nanoparticles delivery systems. **(Gregoriadis *et al.* 1974; Couvreur & Vauthier 2006; Kreuter 1978; Allemann *et al.* 1993; Marty *et al.* 1978; Hora et al., 1990; Cohen et al., 1991; Tabata., 1993; Sudhees et al., 2011; Verma et al., 2013)**

Neither the hydrophilic nor the hydrophobic system is ideal for protein/peptide drug delivery. They each have their own advantages and disadvantages. For example, the hydrophilic polymeric systems are biocompatible with the protein/peptide drugs, but have difficulty achieving sustained drug release. When the systems absorb water and swell, protein/peptide molecules will rapidly diffuse out. In contrast, the hydrophobic polymeric systems have the capability of yielding sustained drug release. However, they are incompatible with the water soluble protein/peptide drugs. The hydrophobicity of the polymers may induce unfolding of protein/peptide molecules; therefore, the protein/peptide drugs may lose their biological activity after being loaded in and then released from the hydrophobic polymeric systems.

Gelatin nanoparticles are a promising antigen delivery system candidate. The beneficial properties of gelatin contributed to its proven record of safety which is also documented by the classification as "GRAS" by USFDA. However molecular heterogeneity of gelatin and release studies of gelatin nanoparticles makes it challenging to prepare a protein formulation with

sustain release. On the other hand PLGA is the polymer of choice for delivery of protein, but its acidic nature denatures the proteins on long exposure (**Verma et al., 2013**). Hence the hypothesis prepared to modify the surface of gelatin nanoparticles by PLGA, and tetanus toxoid was used as a therapeutic candidate antigen in the present study.

## 3.2 AIM OF THE STUDY.

Preparation, optimization and characterization of gelatin nanoparticles (GNPs).

Preparation, optimization and characterization of PLGA nanoparticles   (PNPs).

Coating of gelatin nanoparticles by PLGA (PGNPs) and characterization of PGNPs.

## 3.3 Formulation, optimization of gelatin nanoparticles by two-step desolvation (GNPs).

### 3.3.1 Materials

Tetanus Toxoid (TT) received from Serum Institute of India, Pune, (3000 lf/ml Protein content 8mg/ml) Type A Gelatin (from porcine skin, 175 bloom.) was procured from Sigma Chemical Co.  All the chemicals and reagents used for the formulations were of analytical grade.

### 3.3.2 Method of preparation of gelatin nanoparticles (GNPs).

Gelatin nanoparticles (GNPs) were prepared by a two step desolvation method**.** In the process 1.25 g gelatin (Bloom 175 Type A) was dissolved in 25 ml MilliQ$^{TM}$ water (conductivity max. 0.05μS/0.05μMho) and heated constantly at an temperature of 40ºC. 25 ml of acetone was added to the gelatin solution which acts as  desolvating agent to precipitate the high molecular weight gelatin fractions, The supernatant was discarded out and the HMW gelatin was redissolved by adding 25 ml of water and stirring at 500 rpm under constant heating and at the same time pH was adjusted to 3 that fevers the more intense crosslinking. 2 mg of FITC-TT conjugate or TT was added to the gelatin solution just before the second desolvation step. Gelatin nanoparticles were formed during a second desolvation step by drop by drop addition of 50ml acetone under constant stirring at 500rpm. to crosslink the nanoparticles, 200μl of glutaraldehyde (25% w/v) was added to the reaction vessel after 10 min, and further stirred for 6 h at 600 rpm, and  remaining organic solvent was evaporated by using a rotary evaporator ( Strike Stero Glass, Italy) the prepared particles were purified by three fold centrifugation (20,000g for 20 min, Hitachi preparative ultracentrifuge MX series, Japan) and stored as

dispersion in highly purified water ( conductivity < 00.4 µs/cm) at 2-8ºC as per the ICH guidelines of storage conditions. Aseptic conditions were maintained during the manufacturing steps. Time to time sterility testing's were achieved using fluid thioglycollate medium and soybean casein digest medium during the experiment.**(Coester et al., 2000; Sudhees et al., 2011; Verma et al., 2013)** .

### 3.3.3. Entrapment Efficiency of GNPs

FITC-TT loaded nanoparticles were prepared and purified as described above. The concentration of the nanoparticles were dried by  drying 500µl dispersion in an oven at 60ºC overnight, until the weight was constant to determined the concentration of nanoparticles gravimetrically. For determining entrapment, nanoparticles were dispersed in 2 ml (20 mg/ml) and were centrifuged at 20,000 g upto 30 min and the sedimented particles found were mixed with 2.0 ml (0.2 M) NaOH solution to get clear solution. Clear solution was observed after 48 hr at 4ºC **(Sharif et al., 1995)**. The presence of any insoluble polymer was checked by centrifugation at 20,000 g for 30 min. Protein amount  was determined by fluorescence measurement ( excitation wavelength; 493 nm, emission wavelength; 520 nm) in the supernatant using a fluorescence spectrophotometer (RF-5301PC spectrofluorophotometer Shimadzu, Japan). Readings of all fluorescence were analyzed using a standard curve of FITC-TT in PBS (pH 7.4). To correct for interferences, background fluorescent intensity reading was subtracted (i.e. fluorescent intensity readings for the unloaded nanoparticles treated in the same way as FITC-TT loaded nanoparticles).

### 3.3.4 Optimization of GNPs.

Our first goal was to achieve reproducible preparation of gelatin nanoparticles in a narrow size range with high entrapment efficiency. Various formulation variables that could influence the preparation of homogeneous nanoparticels and size of the GNPs were investigated. The variables, which could influence the resulting nanoparticulate formulations, are as follows:

3.3.4.1 Influence of pH just before the second desolvation step.

3.3.4.2 Influence of temperature during the second desolvation step

3.3.4.3 Influence of the amount of cross-linking reagent used. (Glutaraldehyde)

### 3.3.4.1     Influence of pH value before second desolvation step.

Under standardized conditions and after review as previously described, gelatin nanoparticles were produced within a range of pH 3-4.5. Since gelatin is a protein, pH has a

significant effect on the particle size. This directly reflects that on changing pH led to strong alterations in final particle size of nanoparticles. The pH region was observed between pH 3-4.5. The pH range was selected taking in to consideration the stability of the antigen to be entrapped. The findings are shown in the table 3.2.

### 3.3.4.2    Influence of temperature during the second desolvation step.

Temperature range of 40-60ºC was used in the present study. As identified from the literature review the upper limit of the tested temp. Spectrum was due to the temperature stability of antigen to be used during work. It is well documented in literature that TT (antigen used ) is stable at 45ºC for one month **(Galazka et al., 1998).** The lowest (35ºC) temperature condition led to the most nonhomogeneous particles while uniform particles were formed in the other tested temperature. During the second desolvation step, 40-45ºC temperature was maintained as optimal temperature for the production of homogeneous nanoparticles.

### 3.3.4.3    Influence of amount of cross linking reagent (glutaraldehyde) used.

Glutaraldehyde (GTA) was used to crosslink preformed nanoparticles. GTA is a bi-functional agent. It can effectively hook up the amino group of two adjacent molecules on the surface of the nanoparticles. This bi-functional agent can therefore lead to cross-linking as well as cross-binding among the surface free amine groups. **(Lin et al., 1998; Ulubayram; et al., 2002; Zeiger et al., 2005)** Results of the experiment are shown in the table 3.3.

**Table 3.1**

**Effect of the temperature on the particle size, % entrapment & PDI (n=3)**

| S.No. | Temperature (°C) | Size (nm) | Entrapment efficiency (%) | Polydispersity Index (PDI) |
|-------|------------------|-----------|---------------------------|----------------------------|
| 1 | 30 | 114.7±10.1 | 85.14±8.16 | 0.199±0.012 |
| 2 | **40** | **63.4±5.8** | **91.14 ±4.36** | **0.135±0.09** |
| 3 | 50 | 303.7±23.87 | 86.35±9.32 | 0.212±0.015 |

pH 3.0, glutaraldehyde: 200µl
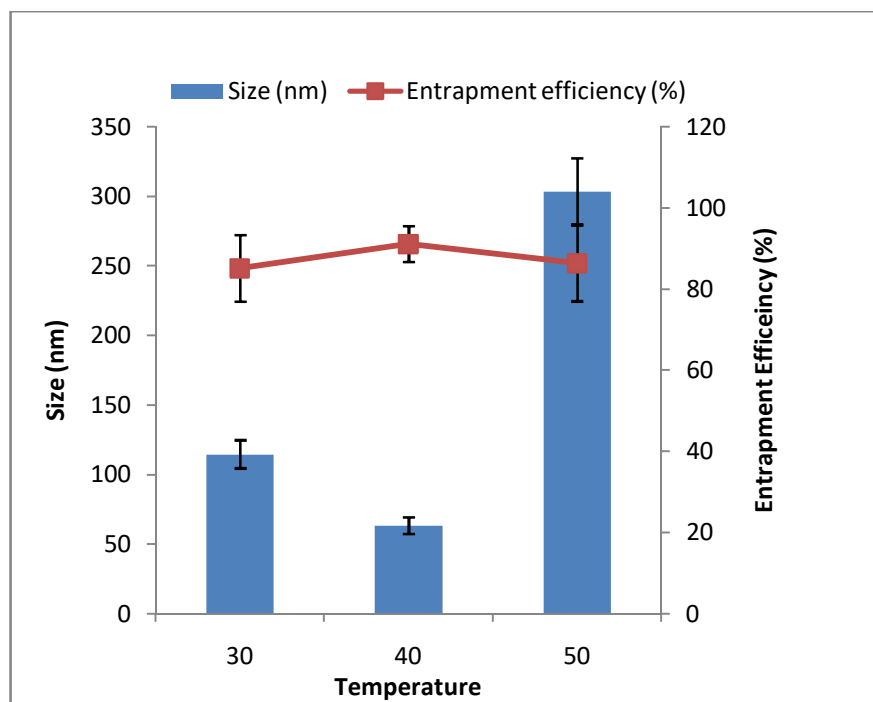


**Fig. 3.1: Influence of the temperature on the particle size, % entrapment.**

**Table 3.2**

**Effect of pH on the particle size, % entrapment efficiency & PDI (n=3)**

| S.No. | pH | Size (nm) | Entrapment efficiency (%) | Polydispersity Index (PDI) |
|-------|-----|-----------|---------------------------|----------------------------|
| 1 | **3.0** | **62.4±5.8** | **88.17±4.36** | **0.120±0.06** |
| 2 | 4.0 | 129.6±9.3 | 86.35±9.32 | 0.231±0.09 |
| 3 | 4.5 | 212.2±17.3 | 85.14±8.16 | 0.181±0.05 |

Temp. 40 ºC, glutaraldehyde: 200µl
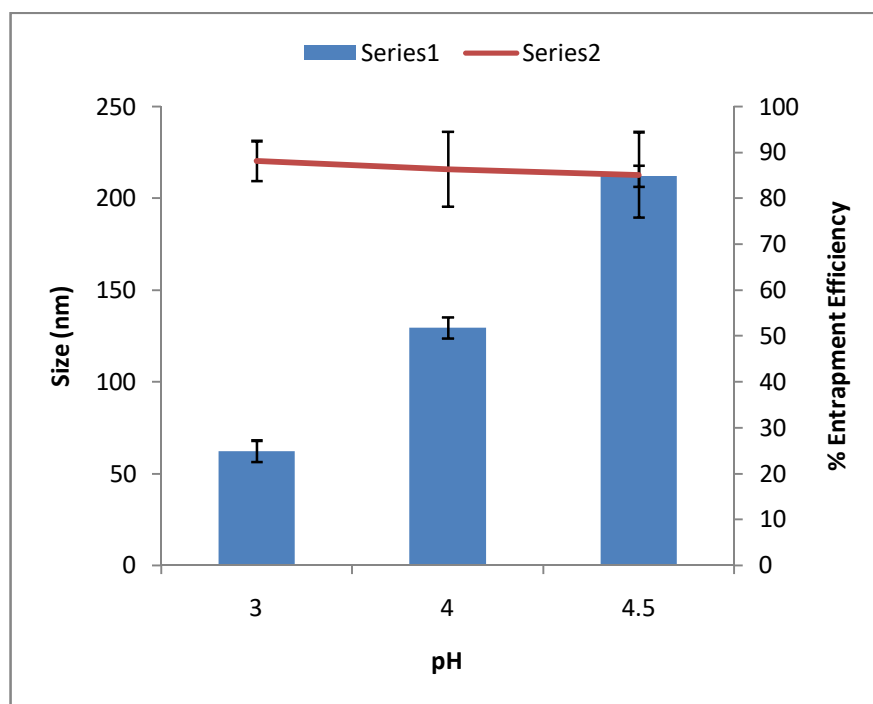


**Fig. 3.2 Influence of pH value before second desolvation step.**

**Table 3.3**

**Effect of the glutaraldehyde (GTA) concentration on particle size, % entrapment efficiency & PDI (n=3).**

| S.No. | Volume of GTA (μl) | Size (nm) | Entrapment efficiency (%) | Polydispersity Index (PDI) |
|-------|--------------------|-----------|---------------------------|----------------------------|
| 1 | 100 | 61.4±5.9 | 87.14±8.16 | 0.247±0.03 |
| **2** | **200** | **64.4±5.8** | **89.14 ±4.36** | **0.120±0.06** |
| 3 | 400 | 305.4±6.9 | 92.35±9.32 | 0.167±0.02 |

Temp. 40 ºC, pH 3.0
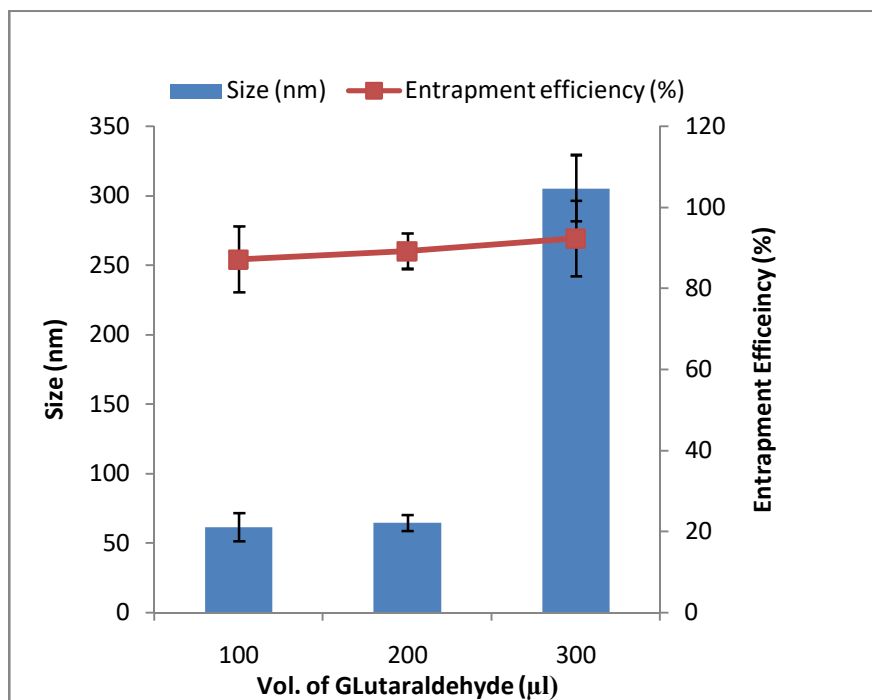


**Fig. 3.3. Effect of the glutaraldehyde (GTA) concentration .**

**Table. 3.4**

**Optimization of gelatin nanoparticels (GNPs)**

| Code | Effect of temperature (°C) | Conc. of GTA (µl) | Effect of pH | Particle Size (nm) | Polydispersivity Index (PDI) | Entrapment efficiency (%) |
|------|------|------|------|------|------|------|
| TM-1 | 30 | 200 | 3 | 114.7±10.1 | 0.199±0.012 | 85.14±8.16 |
| TM-2 | 40 | 200 | 3 | **63.4±5.8** | **0.135±0.09** | **91.14 ±4.36** |
| TM-3 | 50 | 200 | 3 | 303.7±23.87 | 0.212±0.015 | 86.35±9.32 |
| GTD-1 | 40 | 100 | 3 | 61.4±5.9 | 0.247±0.03 | 87.14±8.16 |
| GTD-2 | 40 | 200 | 3 | **64.4±5.8** | **0.120±0.06** | **89.14 ±4.36** |
| GTD-3 | 40 | 400 | 3 | 305.4±6.9 | 0.167±0.02 | 92.35±9.32 |
| pH-1 | 40 | 200 | 3 | **62.4±5.8** | **0.130±0.09** | **88.17 ±4.36** |
| pH-2 | 40 | 200 | 4 | 129.6±9.3 | 0.231±0.09 | 86.35±9.32 |
| pH-3 | 40 | 200 | 4.5 | 212.2±17.3 | 0.181±0.05 | 85.14±8.16 |

## 3.4  Method of PLGA nanoparticels Preparation of by double emulsification method. (PNPs).

### 3.4.1  Materials

PLGA with a lactide/glycolide ratio of 50:5 (40-75 kDa; NII ,New Delhi) Polyvinyl alcohol (13-23 kDa; 88% hydrolyzed;) Tetanus Toxoid (TT) received from Serum Institute of India, Pune . All the chemicals and reagents used during experiment were of analytical grade.

### 3.4.2  Method of preparation of PLGA nanoparticles (PNPs)

In the present experiment PLGA nanoparticles (PNPs) were prepared by double emulsification method process at RT as reported by **(Lima et al., 2003)** with slight modifications.

PLGA (5%) dissolved in DCM (1 ml) by sonication for 30 seconds (Branson Sonicator 250, New Delhi, India) 200µl TT sol. Water-in-oil primary emulsion forms, this primary emulsion was transferred to aqueous solution of polyvinyl alcohol (10 ml, 1% w/v) and sonicated for 2 min. and  mixed with stirrer at 500 rpm for 3 hrs.  (RQ-122, Remi Motors, Mumbai) to obtain a w/o/w emulsion. Centrifuged for 20 min. at 20000 g, following solvent evaporation overnight, hardened PNPs were harvested by centrifugation. PVA was washed with heptane.

### 3.4.3  Entrapment Efficiency of PNPs.

FITC-TT loaded PLGA nanoparticles were prepared and purified as described above. The concentration of the nanoparticles were dried by  by drying 500µl dispersion in an oven at 60ºC overnight, until the weight was constant to determined the concentration of nanoparticles gravimetrically. 20 mg of PNPs was dissolved in 2 mL of 5% w/v sodium dodecyl sulfate (SDS) in 0.1 M NaOH sol to identify entrapment efficiency. **(Arvind et al., 2009)**. The solution was centrifuged at 20,000 g for 30 min to check the presence of any insoluble polymer. Amount of the protein was determined by measuring fluorescence ( excitation wavelength; 488 nm, emission wavelength ; 520 nm) in the supernatant using a fluorescence spectrophotometer (RF-5301 PC spectrofluorophotometer Shimadzu, Japan). All fluorescence readings were analyzed using a standard curve of FITC-TT in PBS (pH 7.4). To correct for interferences, background fluorescent intensity reading was subtracted (i.e. fluorescent intensity readings for the unloaded nanoparticles treated in the same way as FITC-TT loaded nanoparticle).

### 3.4.4  Optimization of PNPs.

To achieve reproducible preparation of PLGA nanoparticles in a narrow size range with high entrapment efficiency. Various formulation variables that could influence the preparation of homogeneous nanoparticels and size of the PNPs were investigated. The variables, which could influence the resulting nanoparticulate formulations, are as follows:

3.4.4.1 Influence of polymer concentration (PLGA).

3.4.4.2 Influence of polyvinyl alcohol (PVA) concentration.

3.4.4.3 Influence of sonication time during secondary emulsion.

### 3.4.4.1 Influence of polymer (PLGA) concentration.

PNPs are prepared by double emulsification method. Different concentration PLGA is used to check the effect on the size, percent entrapment efficiency and polydispersity index of the formulation. The results of different formulations suggested that on increasing in the polymer concentration while keeping similar other parameters, the viscosity of the organic phase increases results poor emulsification that gives higher particle size with higher polydispersity index. Hence 5% of PLGA concentration was considered as the optimum for PNPs.

### 3.4.4.2 Influence of polyvinyl alcohol (PVA) concentration.

Polyvinyl alcohol (PVA) was used in the double emulsification method as an emulsifying agent, different percent PVA concentrations were studies by keeping similar other parameters, PVA was used as emulsifying agent in the external aqueous phase that stabilizes the secondary emulsion as it produce relatively uniform and smaller size particles. **(Zambaux et al., 1998)**. On the other hand it is difficult to remove the PVA completely from the formulations as it is supposed to get absorbed irreversibly at the interface. Thus, 1% PVA concentration was considered to be optimize, as it was capable to stabilize the desired size range.

### 3.4.4.3 Influence of sonication time during secondary emulsion.

The 5% sol of polymer and internal aqueous phase and sonication was done by probe sonication (Soniweld, India) in ice bath for 30 seconds (40W) to form primary emulsion. After adding primary emulsion into 1% PVA solution, again sonicated to form secondary emulsion. Different duration of sonication were observed keeping similar other parameters. Results indicated (Table. 3.8) that when sonicated for 1 min. larger particle size are formed while size decreases on increasing the duration, but of further increase size increases this may be due to agglomerate formation of stabilizer, hence the most optimum sonication time is found to be 2 min. that gives better size with effective entrapment and polydispersity index.

**Table 3.5**

**Effect of the polymer (PLGA) concentration on size of particle, % entrapment efficiency & PDI (n=3).**

| S.No. | Polymer Conc. (mg/ml) | Size (nm) | Polydispersity Index (PDI) | Entrapment efficiency (%) |
|-------|------------------------|-----------|-----------------------------|----------------------------|
| 1 | 25 | 250±53 | 0.131±0.09 | 47.14±3.19 |
| **2** | **50** | **497±28** | **0.129±0.01** | **58.23±3.92** |
| 3 | 75 | 683±45 | 0.243±0.05 | 33.05±3.32 |

PVA 1%, sonication time 2 min.
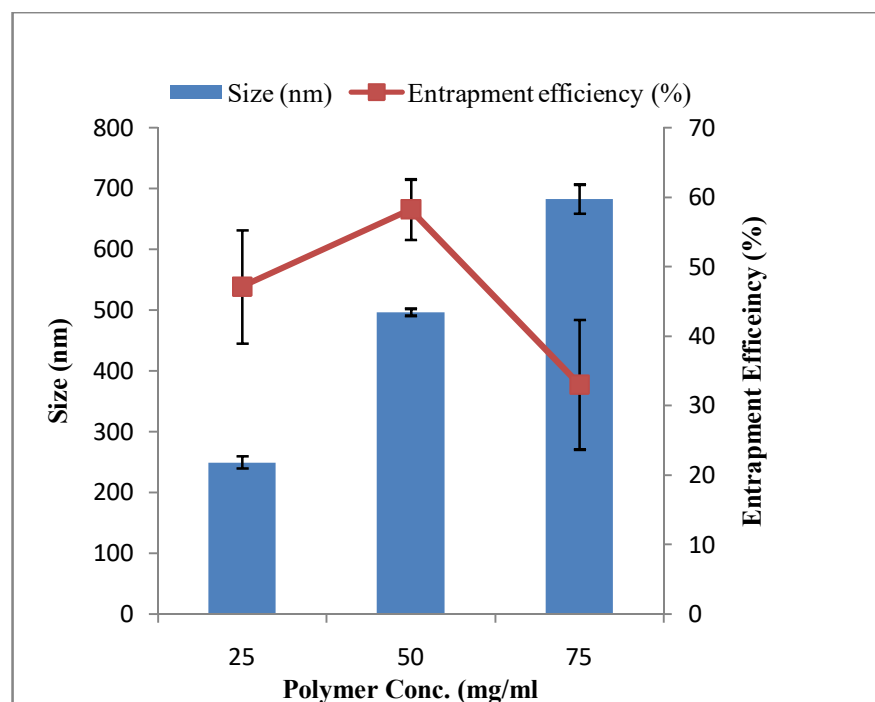


**Fig. 3.4; Effect of the polymer (PLGA) concentration on size of particle, % entrapment efficiency.**

**Table 3.6**

**Effect of the polyvinyl alcohol (PVA) concentration on particle size, % entrapment efficiency & PDI (n=3).**

| S.No. | % PVA | Size (nm) | Polydispersity Index (PDI) | Entrapment efficiency (%) |
|-------|-------|-----------|----------------------------|---------------------------|
| 1 | 0.5 | 694±41 | 0.134±0.04 | 49.14±3.91 |
| **2** | **1.0** | **474±19** | **0.121±0.03** | **57.28 ±2.78** |
| 3 | 2.0 | 689±22 | 0.151±0.09 | 37.05±3.34 |

Polymer conc. 50mg, sonication time 2 min.
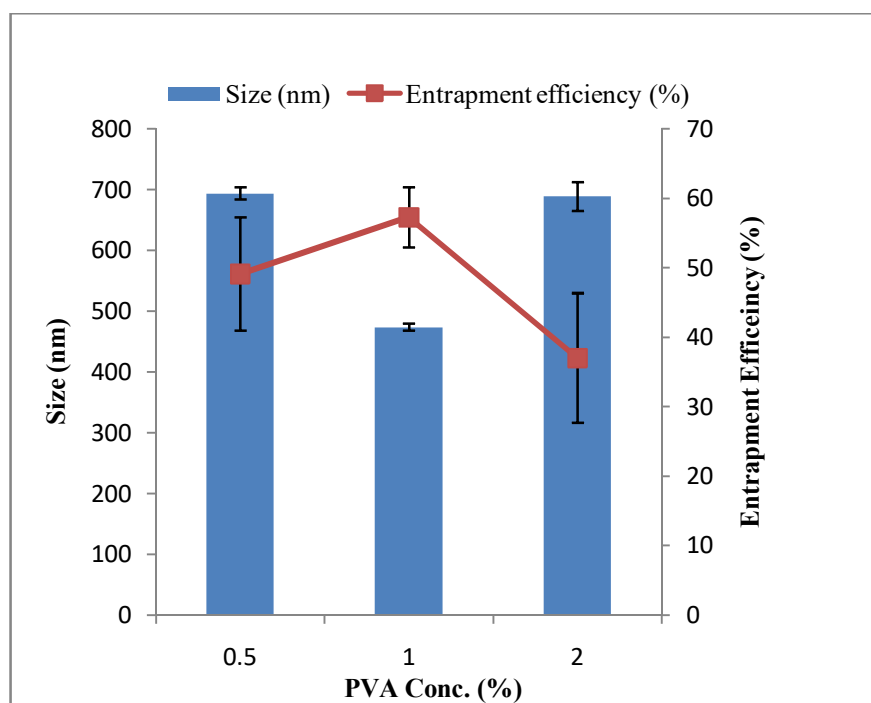


**Fig.3.5; Influence of the polyvinyl alcohol (PVA) concentration on particle size, % entrapment efficiency.**

**Table 3.7**

**Influence of sonication time  on particle size, % entrapment efficiency & PDI (n=3).**

| S.No. | SonicationTime (min.) | Size (nm) | Polydispersity Index (PDI) | Entrapment efficiency (%) |
|-------|------------------------|-----------|-----------------------------|----------------------------|
| 1 | 1 | 519±49 | 0.141±0.05 | 51.14±3.92 |
| **2** | **2** | **482±24** | **0.127±0.01** | **53.21±3.32** |
| 3 | 3 | 493±34 | 0.153±0.05 | 45.05±3.16 |

PVA 1%, Polymer Conc. 50 mg
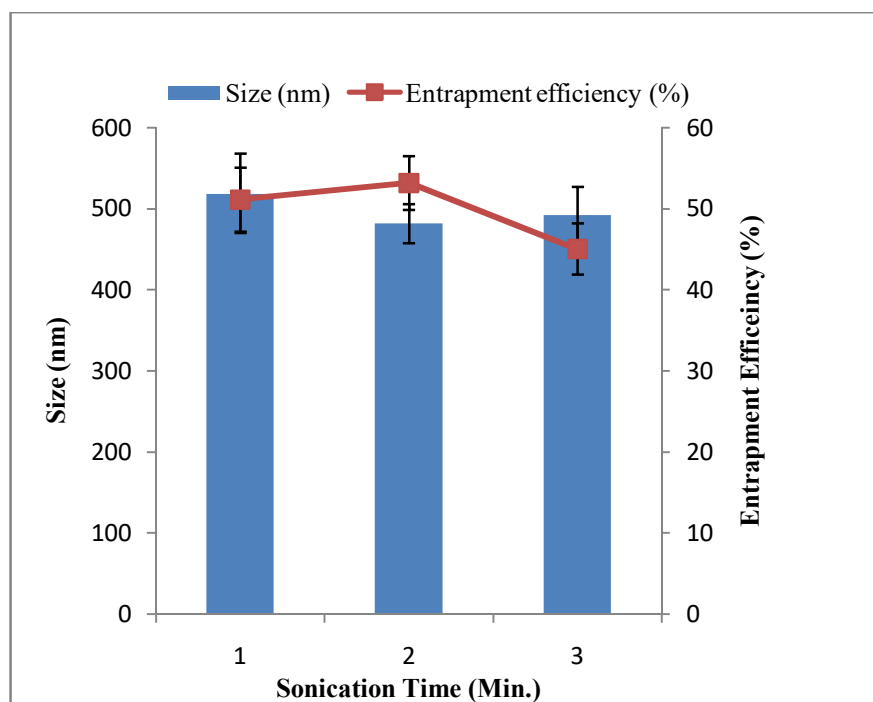


**Fig.3.6; Influence of sonication time  on particle size, % entrapment efficiency.**

**Table 3.8**

**Optimization of PLGA nanoparticles (PNPs).**

| Code | Polymer Conc. (mg/ml) | PVA Conc. (%) | Sonication Time (Min.) | Particle Size (nm) | Poly-dispersivity Index (PDI) | Entrapment efficiency (%) |
|---|---|---|---|---|---|---|
| PG-1 | 25 | 1.0 | 2 | 250±53 | 0.131±0.09 | 47.14±3.19 |
| **PG-2** | **50** | **1.0** | **2** | **497±28** | **0.129±0.01** | **58.23±3.92** |
| PG-3 | 75 | 1.0 | 2 | 683±45 | 0.243±0.05 | 33.05±3.32 |
| PV-1 | 50 | 0.5 | 2 | 694±41 | 0.134±0.04 | 49.14±3.91 |
| **PV-2** | **50** | **1.0** | **2** | **474±19** | **0.121±0.03** | **57.28 ±2.78** |
| PV-3 | 50 | 2.0 | 2 | 689±22 | 0.151±0.09 | 37.05±3.34 |
| ST-1 | 50 | 1.0 | 1 | 519±49 | 0.141±0.05 | 51.14±3.92 |
| **ST-2** | **50** | **1.0** | **2** | **482±24** | **0.127±0.01** | **53.21±3.32** |
| ST-3 | 50 | 1.0 | 3 | 493±34 | 0.153±0.05 | 45.05±3.16 |

## 3.5  Coating  of  gelatin nanoparticles by PLGA (PGNPs)

Conventional drugs have difference in the  physicochemical and biological properties of protein and peptide drugs. One can not establish a concept that the delivery systems that function well for conventional drugs can be suitable for protein and peptide drugs also. Hence the  design and manufacturing of truly unique protein and peptide drug delivery systems is required for effective treatment. To reconcile this desire, different-different types of nonconventional systems have been developed and examined. For example biodegradable systems for protein and peptide drug delivery are injectable nanoparticles based on PLGA. Recent researches had proved that biodegradable systems have many advantages over nondegradable products **(Kaul and Amiji, 2005; Arvind et al., 2009)**.

To endorse the advantages and the disadvantages of both the hydrophilic and hydrophobic polymeric systems, an combined  hydrophilic system, gelatin nanoparticles, laminated with a hydrophobic polymeric system of PLGA was articulated. Gelatin nanoparticles containing tetanus toxoid (TT), were prepared first, and then with the help of EDC, PLGA was coated over gelatin nanoparticles (PGNPs). The present formulation acts  as protein stabilizer, and provide prolonged antigen release, with prolonged systemic immunity along with effective cellular immune response. Since gelatin being a hydrophilic polymer and PLGA being a hydrophobic polymer alone cannot **(Tsung et al., 2001; Kaul and Amiji, 2005)** achieve the above discussed objectives hence a  combination of both these polymer is hypothesized. The coating of PLGA over gelatin was conducted using a double emulsion-solvent evaporation method. The average diameter of the PGNPs is between 400-600 nm. Protein loading efficiency was nearly 58%.

### 3.5.1  Materials.

PLGA with a lactide/glycolide ratio of 50:5 (40-75 kDa; NII, New Delhi), Type A Gelatin (from porcine skin, 175 bloom.) was obtained from Sigma Chemical Co., Polyvinyl alcohol (13-23 kDa; 88% hydrolyzed;), Tetanus Toxoid (TT) received from Serum Institute of India, Pune . All the chemicals and reagents used for the formulations were of analytical grade.

### 3.5.2 Coating of gelatin nanoparticles by PLGA. (Double emulsion solvent evaporation method)

By taking all the parameters of optimized formulations of GNPs and PNPs, PGNPs are prepared. They are prepared by carful use of polymers and reagents with previously calibrated instruments and double emulsion solvent evaporation method is used for the formulation. 5% PLGA weighed accurately and dissolved in 1 ml dichloromethane to make 1 ml 5% w/v of PLGA solution in a sonication tube. 200µl, 25 mg/ml lyophilized FITC-TT loaded gelatin nanoparticles were taken separately and 0.5mg/ml of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide commonly known as EDC was added to GNPs solution and kept aside for 1 hr **(Fields et al., 1971; Jamie et al., 2001)**. Then this solution was added in 5% solution of PLGA and allowed to sonicate for 30 sec., w/o type a primary emulsion was formed. This w/o type primary emulsion was transferred to 10 ml of 1 % polyvinyl alcohol (PVA) and allowed to sonicate for 2 min. Then the solution was directed to continuous stirring for 3 hrs. (500 rpm) on

stirrer. PLGA coated gelatin nanoparticles (PGNPs) were recovered by centrifugation for 20 min at 20000 g, the recovered PGNPs were freeze dried for further investigations.
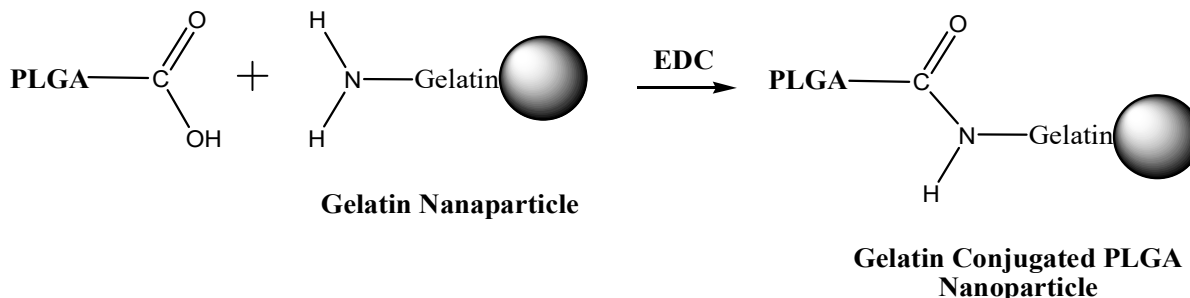


**Fig.3.7. Conjugation by carboxyl group of PLGA and the amine-group of Gelatin.**

### 3.5.3 Entrapment Efficiency of PGNPs.

FITC-TT loaded PLGA nanoparticles were prepared and purified as described above. The concentration of the nanoparticles were dried by drying 500µl dispersion in an oven at 60ºC overnight, until the weight was constant to determined the concentration of nanoparticles gravimetrically. 20 mg of PGNPs was dissolved in 2 mL of 5% w/v sodium dodecyl sulfate (SDS) in 0.1 M NaOH sol to identify entrapment efficiency. **(Arvind et al., 2009)**. The solution was centrifuged at 20,000 g for 30 min to check the presence of any insoluble polymer. Amount of the protein was determined by measuring fluorescence (excitation wavelength; 488 nm, emission wavelength ; 520 nm) in the supernatant using a fluorescence spectrophotometer (RF-5301 PC spectrofluorophotometer Shimadzu, Japan). All fluorescence readings were analyzed using a standard curve of FITC-TT (Fig.2.1). To correct for interferences, background fluorescent intensity reading was subtracted (i.e. fluorescent intensity readings for the unloaded nanoparticles treated in the same way as FITC-TT loaded nanoparticle).

### 3.5.4 Determination of particles size, shape and morphology of nanoparticles

Particle size determination of final optimized formulation was performed by dynamic light scattering (DLS). To determine the shape and morphology of the prepared formulation, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used.

### 3.5.4.1 Dynamic light scattering

A particle in the range of 1 nm or lower can be measured by use of Dynamic light scattering (DLS), also called as Photon correlation spectroscopy (PCS) and sometimes called as Quasi-elastic light scattering (QELS), it is a non-invasive technique.

Only intensity-based average values are shown by these mean sizes and do not further represents any clarity about the size distributions. Information about the actual distortion of monomodal light scattering signal is given by, the polydispersity index (PDI). The PDI can have values ranging from 0-1 and it is equivalent to the variance $\sigma^2$ of the distribution **(Rawle et al., 1993)**. A Zetasizer Nano ZS 90 ( Malvern Instruments, UK) using NIBS[TM] technology was used to analyze the samples. This technology extends the range of sizes and concentration of samples than can be measured i.e. by NIBS (Non- Invasive Back-Scatter). NIBS[TM] technology has the advantages of improved sensitivity, simplified sample preparation and a broder range of specimen concentrations can be measured. Except stated otherwise, the nanoparticles were diluted in MilliQ[TM] (conductivity max. 0.05µS/0.05µMho) and measured in the range 40-100 µg/ml of concentration.

**Table 3.9  Particle size, % entrapment & PDI of optimized formulation of PGNPs.**

| S.No. | Formulation Code | Size (nm) | Polydispersity Index (PDI) | Entrapment efficiency (%) |
|-------|------------------|-----------|----------------------------|---------------------------|
| 1 | Blank PGNPs | 418±19 | 0.128±0.07 | - |
| 2 | Loaded PGNPs | 496±34 | 0.141±0.05 | 58.21±2.78 |

### 3.5.4.2 Transmission Electron Microscopy (TEM)

By using conventional negative staining method, samples for transmission electron microscopy (TEM) were prepared at room temperature by adding 0.2% phosphotungstic acid (PTA). Carbon coated grid were placed on a drop of sample for 2 minutes. The grid was then transferred to air dry on a filter paper. The grids were immediately observed under a

Transmission microscope commonly known as TEM. (Phillips Morgagni D-268, Netherlands). The Images are shown in Photomicrograph. 3.1.
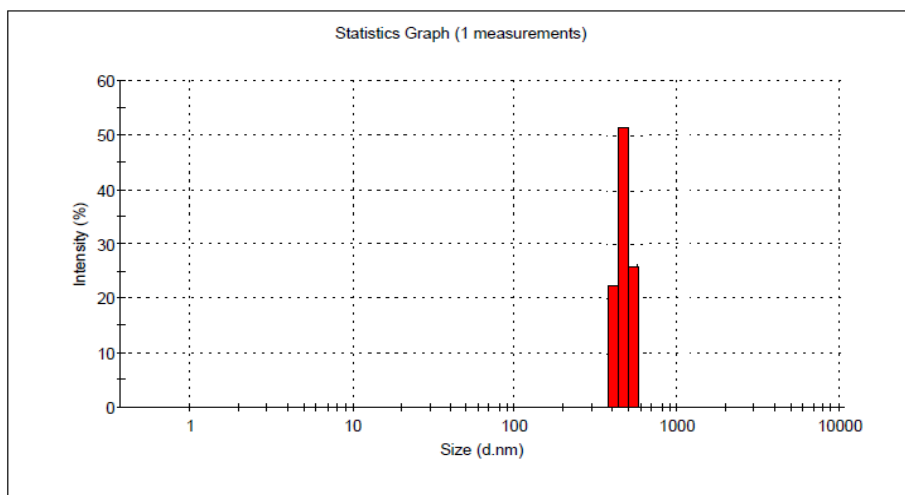
**Fig. 3.8; Size distribution of the optimized formulation of PGNPs (496.81 nm).**



(a)

**(b)**



(c)
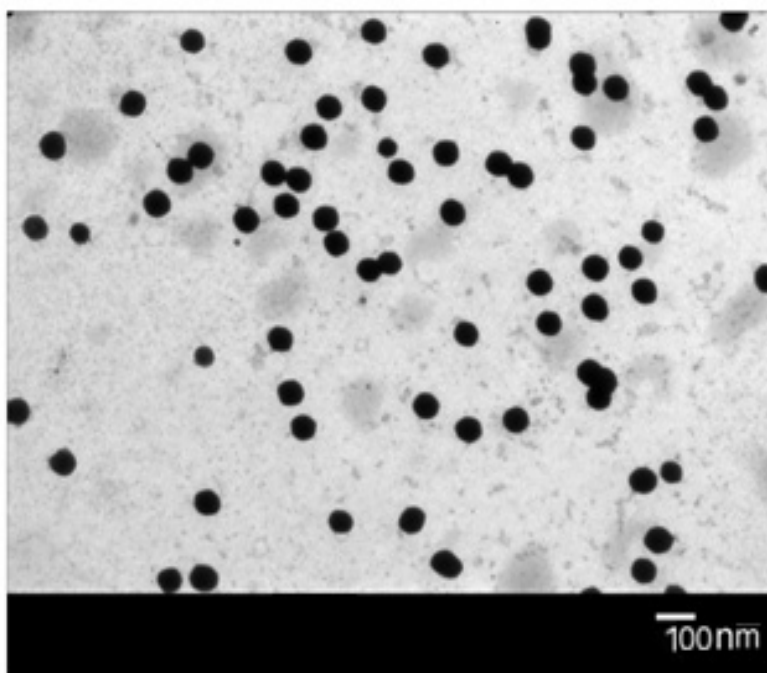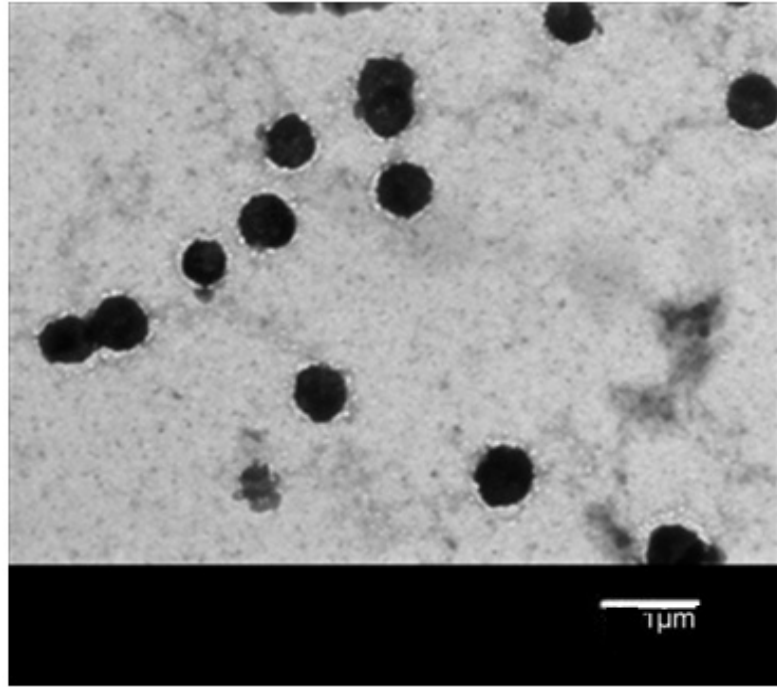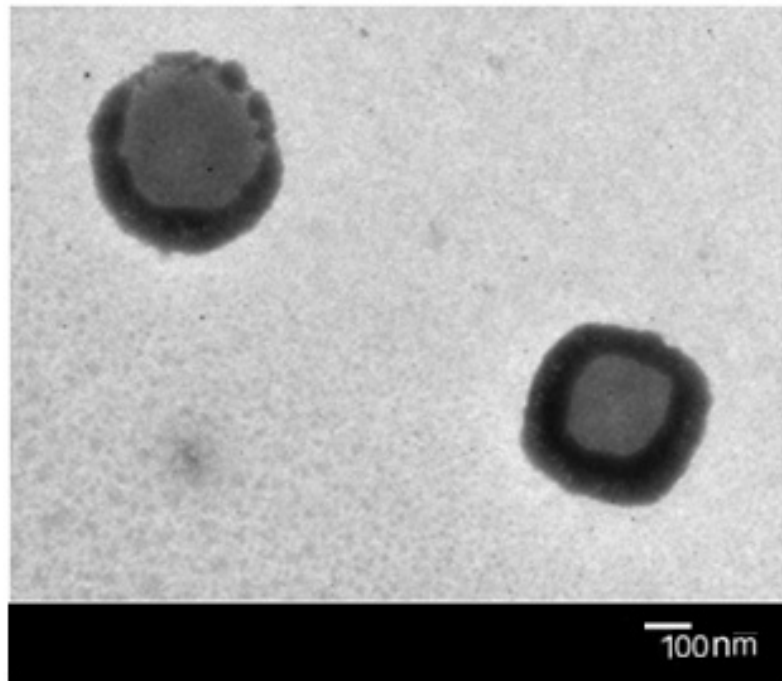
**Photomicrograph 3.1 Transmission Electron Microscopy (TEM); a). GNPs, b). PNPs, c). PGNPs.**

### 3.5.4.3    Scanning Electron Microscopy (SEM)

To characterize the surface morphology of dry un- dispersed nanoparticles, (PGNPs) were analyzed by SEM (Photomicrograph 3.2). Field emission scanning electron microscope (Philips CM12, Eindhoven Netherlands) was used to take the pictures at 5.0kV and working distance was kept about 9.7 mm, nanoparticles were dispersed in acetone at an concentration of 40µg/ml for sample preparation, and placed on a specifically polished sample grid. The samples were finally sputter coated with a 20 nm gold layer before microscopical analysis after 18 hrs of vaccume drying. The Photomicrograph of unloaded and loaded FITC-TT, PGNPs are shown in Photomicrograph 3.2..



**(a)**

**(b)**



**(c)**
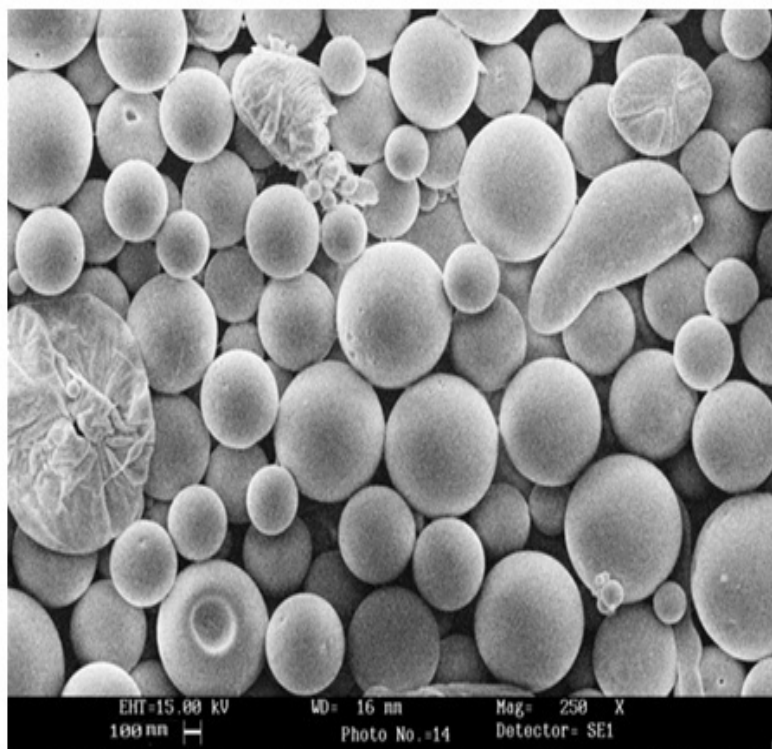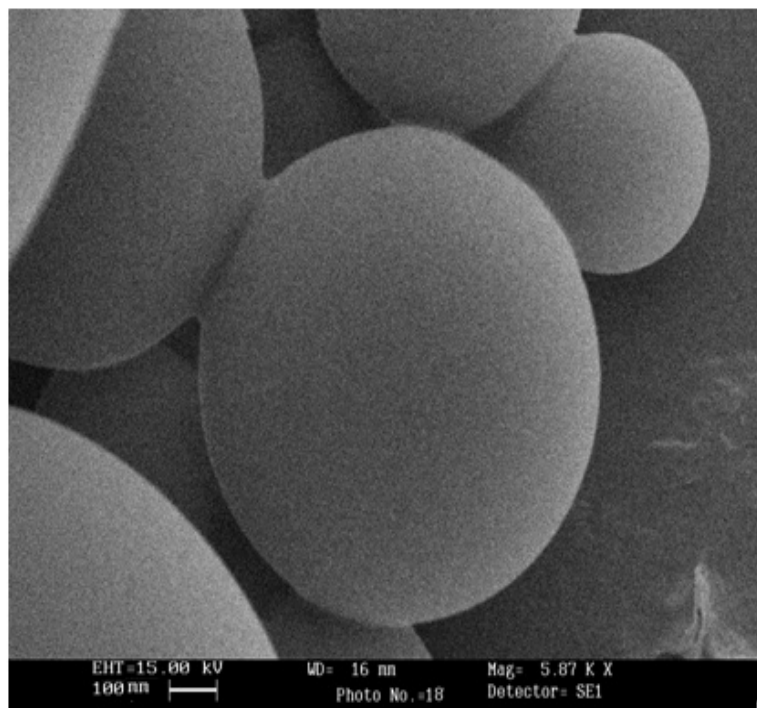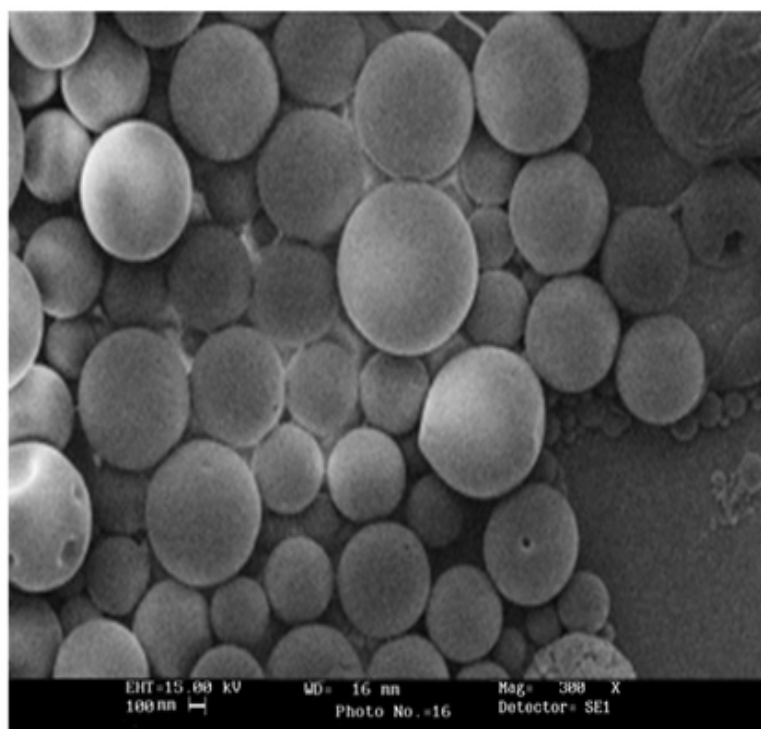
**Photomicrograph. 3.2; Scanning Electron Microscopy (SEM) images; (a) Unloaded PGNPs : (b) & (c) FITC-TT loaded PGNPs.**
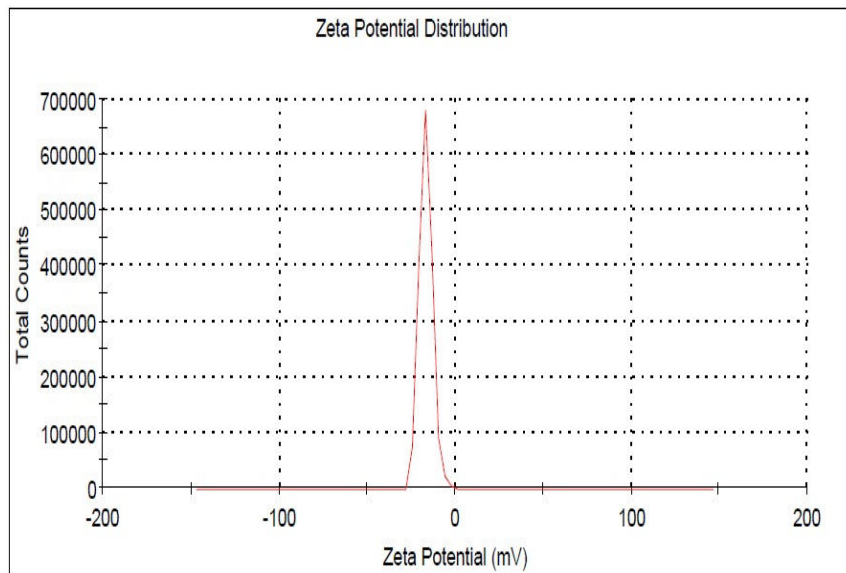


**Fig. 3.9; Zeta potential analysis of final optimized formulation = -11.8mV.**

### 3.5.5  Zeta potential (ξ Potential) determination of PGNPs.

Zeta potential of final formulation of PGNPs was determined. (Fig. 3.9) An electric field across dispersion was applied to measure Zeta potential. With the velocity proportional to the magnitude of zeta potential the particles within the dispersion migrates towards the electrode of opposite charge. Laser Doppler Anemometry technique is used to measure this velocity which is directly proportional to the Zeta potential. In specific disposable cuvettes at concentrations of 20μg/ml all these measurements were performed.

### 3.6 Cumulative percent release (In Vitro release profile) of GNPs, PNPs and PGNPs.

A 56 days release of FITC-TT loaded GNPs, PNPs and PGNPs  was carried out in pH 7.4, 0.05 M at 37ºC in a shaking water bath in dark with speed 36 strokes/ mom. 20 mg of FITC-TT loaded formulations were vortexed after resuspending in 1.0 ml of PBS (pH 7.4), at different time intervals the suspension was subjected to centrifugation (40,000g for 20 min) to get supernatants and the amount of FITC-TT released was determined by measuring the fluorescent intensity of the supernatant. PBS solution was replaced each time in the respective vial containing GNPs to maintain a constant volume. The fluorescent intensity of the supernatant

obtained at different time intervals were measured under the same conditions as described above in the entrapment study and the cumulative amount released was calculated from the appropriate calibration curve (Fig. 2.1). For the release studies, background fluorescent intensity reading was subtracted (i.e. fluorescent intensity readings for the unloaded nanoparticles treated in the same way as FITC-TT loaded nanoparticles). Table.3.11 and Fig. 3.10, Summarized the cumulative release profile of the GNPs, PNPs and PGNPs.

## 3.7  RESULTS AND DISCUSSIONS

The primary objective of the study was to develop a vaccine formulation with stability, sterility and efficacy. For protein and peptide delivery alone hydrophilic or hydrophobic system is not ideal. They each have their own advantages and disadvantages. For example, the hydrophilic polymeric systems are biocompatible with the peptide/protein drugs, but have difficulty achieving sustained drug release. When the systems absorb water and swell, peptide /protein molecules will rapidly diffuse out **(Lee et al., 2000; Kaul and Amiji, 2005; Chen et al., 2009; Arvind et al., 2009)**. In contrast, the hydrophobic polymeric systems have the capability of yielding sustained drug release. However, they are incompatible with the water soluble protein/peptide drugs. The hydrophobicity of the polymers may induce unfolding of peptide/protein molecules; therefore, the peptide/protein drugs may lose their biological activity after being loaded in and then released from the hydrophobic polymeric systems. Hence to promotes the advantages and overcomes the disadvantages of both the hydrophilic and the hydrophobic polymeric systems, by a combined hydrophilic-hydrophobic system (gelatin nanoparticles coated with PLGA). This combination can create a new biodegradable system for protein and/or peptide drug delivery. That gives benefit to the society by increasing the effects of proteins/peptide drugs.

A combined two polymer based system the hydrophilic gelatin nanoparticles, surface modified with hydrophobic PLGA was articulated. The prepared combination creates novel biodegradable system for protein-peptide drug delivery. The gelatin nanoparticles containing tetanus toxoid (TT) were prepared first, and then with the help of EDC, PLGA was coated over gelatin nanoparticles (PGNPs). Since gelatin being a hydrophilic polymer and PLGA being a hydrophobic polymer alone cannot **(Tsung et al., 2001; Kaul and Amiji, 2005)** achieve the above discussed objectives hence a combination of both these polymer is hypothesized. The

**Table.3.10**

*In Vitro* **release profile of FITC-TT loaded GNPs, PNPs and PGNPs. (n=3)**

| S.No. | Time (Days) | % Cumulative Release | | |
|-------|-------------|------|------|-------|
| | | **GNPs** | **PNPs** | **PGNPs** |
| 1 | 7 | 70.3±5.6 | 30.6±2.8 | 11.3±0.8 |
| 2 | 14 | 79.1±4.2 | 42.4±5.6 | 31.1±1.2 |
| 3 | 28 | 81.2±4.1 | 50.1±5.2 | 47.7±2.3 |
| 4 | 56 | 89.7±5.1 | 54.2±5.3 | 73.3±4.6 |
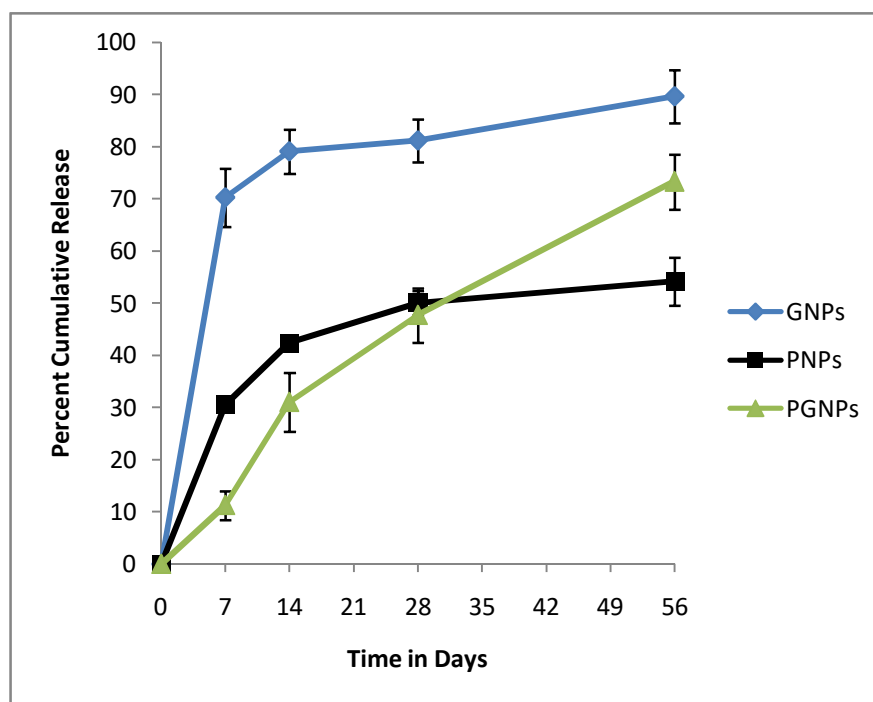


**Fig.3.10; In-Vitro release profile of GNPs, PNPs and PGNPs.**

coating was conducted by double emulsion solvent evaporation method. The average diameter of the PGNPs is between 496 nm. Protein loading efficiency was nearly 58%. Before preparing this gelatin nanoparticles and PLGA nanoparticleswere optimized.

By the use of two-step desolvation method homogeneous GNPs were prepared. This method shows much less aggregation as compared with the one-step desolvation method **(Marty et al., 1978).** High molecular- weight fraction of the gelatin used was collected in the sediment after first desolvation step while in the supernatant the low molecular weight gelatin is precipitated out, this proves  that the removal of the low molecular weight gelatin fraction in the supernatant after the first desolvation step not only reduced the formation of aggregate during cross-linking because of an enhanced stability of particles formed before cross-linking but also prevented further secondary aggregation and flocculation of particles during storage. The PLGA nanoparticles (PNPs) were prepared by double emulsification method, polymer was first dissolved in the dichloromethane as hydrophobic polymer well dissolves in the organic solvents, PVA was used a emulsifying agent to stabilize the system. Various process parameters were optimized, by using the most optimum parameters of the formulations hypothesized system PGNPs were prepared by the use of double emulsion solvent evaporation method with slight modifications.  The working area was sterilized and monitored regularly by sampling the air and the surface of the working area to ensure endotoxin- free conditions of the preparation process. FITC-TT conjugation was done first to determine % entrapment efficiency (EE) and release. The entrapment of FITC-TT in the formation of strong interpenetrating network between gelatin and TT. FITC-TT would, therefore, be encapsulated during the coacervation phase at an acidic pH in the last desolvation step. Measurements of the resulting nanoparticles was done by DLS, the software calculated average mean size of GNPs $64.4\pm5.8$ nm and PDI of $0.135\pm0.09$, PNPs $497\pm28$ and PDI of $0.129\pm0.013$ and for PGNPs $498\pm24$ with PDI $0.141\pm0.05$ (mean of 3 measurements with 22 individual subruns for each) in the optimized formulations PDI obtained were well within the acceptable limit ($<0.1$). We additionally analyzed these nanoparticles by TEM and SEM to visualize the particles and investigate their morphology. TEM (photograph) and SEM (photograph) confirmed that the sample analyzed were spherical and smooth.

Effect of varying production parameters on the nanoparticles properties were studied in our experiment to make the optimized formulation. For GNPs the synthesis parameters altered were temperature, pH, and concentration of glutaraldehyde, and for PNPs polymer concentration,

percent PVA concentration, and duration of sonication were altered. The basic goal was to prepare nanoparticles having good entrapment efficiency and narrow size distribution. We studied the effect of above discussed parameters on the particle size, % entrapment and the polydispersity index, the polydispersity index indicates about the size distribution of the nanoparticles population. If polydispersity index is low it indicates a narrower size distribution of the system. Nanoparticles with maximum entrapment efficiency and PDI below 0.1 were considered as optimized batches and were taken for further studies.

After making first formulations and to study the effect of temperature on system, all other parameters were kept constant while the temperature were changed during the experiments, 50 ml acetone was used as desolvating agent and 200µl glutaraldehyde as crosslinker in the technique used. Preparation of gelatin nanoparticles was not possible at ambient temperature (30ºC) because the gelatin formed a highly viscous gel on this temperature. Temperature plays an important role in the sedimentation of gelatin in the first desolvation and formation of nanoparticles in the second desolvation step due to occurrence of denaturation and renaturation process as evident from literature **(Farrugia, 1998 and Groves, 1999).** At higher temperature (60ºC) gelatin exhibits partial reversible denaturation of HMW gelatin fractions. Sol-gel transition of gelatin solutions results which is due to increase in renaturation at lower temperatures. 35-40ºC is the typical sol-gel transition temperature of gelatin solution (**Peyrelasse et al., 1996).** The results at 30ºC, 40ºC and 50ºC showed that temperature has a strong influence on the particles size. Homogeneous batch of nanoparticles with maximum entrapment efficiency were prepared at 40ºC with gelatin A. A better-controlled precipitation of the macromolecules is found at 40ºC, after addition of the desolvating agent the chains seem to be sufficiently uncoiled at this temp. Compared to higher temperature.

In all the tested pH range, gelatin nanoparticles were successfully prepared, although at pH above 4, formulation produced deviated strongly towards aggregation and precipitation the reason is the pH value is too close to IEP (Isoelectric point) of gelatin type A (approximately 6.1). Thus the net charge that remains on gelatin is too weak to prevent the nanoparticles from instabilities. During the second desolvation step to form nanoparticles, the pH of the gelatin solution was adjusted which was away from isoelectric points of gelatin. On addition of the desolvating agent water available to keep the gelatin in solution reduces resulted in shrinkage of the hydrated gelatin chains. At a certain point the protein chains precipitate as nanoparticles as

the hydration is too low. pH 3 was found to be most the optimum pH for preparing the nanoparticles concluded from the results demonstrated. As the pH is increased to 4 it causes early agglomeration of gelatin when the desolvating agent was added. A possible rational of this finding is that at pH 3 protein chains are highly positively or negatively charged, that favors the cross-linking. These electrostatic repulsions devoid the polymer chains from unwanted agglomeration. Surfaces of the nanoparticles acquire sufficient zeta potential to prevent further agglomeration of the particles after formation. The produced nanoparticles had size range between 60 to 300 nm. At pH 3 homogeneous nanoparticles with PDI below 0.1 were prepared. By drop-wise addition of 50 ml acetone to nanoparticles were prepared, first turbidity noticed, indicates phase separation, and nanoparticles were generates at a later point after acetone addition by allowing the pH towards lower values. The intermolecular repulsion forces stabilizing the characteristic three-dimensional protein shape and prevented collapse and aggregation induced by the desolvation agent because at lower pH values the net charge of the gelatin molecules was apparently too intense.

100, 200 and 400µl aliquots of a 25% v/v aqueous glutaraldehyde solution were added to the nanoparticles to study the effect of the concentration of glutaraldehyde as a cross-linking agent. As per the conditions discussed above the nanoparticles were prepared at 40ºC using gelatin Type A, acetone was used as the desolvating agent. On increasing GTA amount as presented in figure 3.3, there is a steady decrease in particle size found to support the study of researchers **(Nakaoka et al., 1995; Balthasar et al., 2005)** that increasing amounts of cross-links lead to smaller and denser nanoparticels. The particle size at 100, 200, and 400 µl of GTA were found to be 61.4±5.9, 64.4±5.8 and 305.4±6.9 and % entrapment efficiency was 87.14±8.16, 89.14±4.36, and 92.35±9.32 with a PDI of 0.247±0.03, 0.120±0.06, and 0.167±0.02. The most optimum amount with the most homogeneous nanoparticles resulting (as suggested by PDI values). By higher amounts of GTA there is a slight increase of the PDI values beyond this point that might be accounted to stronger interparticulate aggregation tendencies, it would be advisable to keep the amount of GTA as low as possible from a toxicological point of view since one of the intended routes of administration is nasal. 200 µl was chosen to be optimum GTA for the formulation.

For the preparation of PLGA nanoparticles formulation parameters were checked for the optimum preparation. Different concentration of PLGA was used to check the effect on the size,

entrapment efficiency and polydispersity index (PDI) of the formulations. To check the effect of polymer concentration all other parameters were kept constant. 25, 50, and 75 µl of PLGA were used. The size, entrapment efficiency and polydispersity index (PDI) were found to be 250±53, 497±28, and 683±45 nm, 47.14±3.92, 58.23±3.92, and 33.05±3.32%,  0.131±0.09, 0.129±0.01, and 0.243±0.05. Nanoparticles formed by 25 mg of PLGA achieve a size of 250nm but the formulation was non-spherical in shape, and shows agglomerates. Since the main objective was to laminate gelatin nanoparticles by PLGA this size does not fit to laminate it properly and as the polymer concentration was doubled particle diameter also increases but on further increase of polymer the viscosity  of the dispersed phase also increases that results a strapped dispersion of the PLGA. Increased polymer conc. shows high viscous resistance against the shear forces involved in the emulsion during the emulsification that causes coalescence of the particles, Hence it is clear from the results of different formulations that with the increase in the polymer concentration while keeping similar other parameters, the viscosity of the organic phase increases that causes poor emulsion with higher particle size and higher polydispersity index. Hence 5% of PLGA concentration was considered as the optimum for nanoparticles.

To study the effect of surfactant, Polyvinyl alcohol (PVA) was used in the double emulsification method as an emulsifying agent, different percent PVA concentrations were studies by keeping similar other parameters, to stabilize the secondary emulsion PVA was used as emulsifying agent in the external aqueous phase as it produce relatively smaller and uniform particles **(Hueper et al., 1959; Sahoo et al., 2002; Zambaux et al., 1998)**. The emulsification and stabilization of the globules are crucial factors in double emulsification method, in the emulsification process of nanoparticles formation and in the protection of the globules the amount of surfactant used plays an exclusive role as it has the ability to avoid the coalescence of globules. In our formulation we received the analogous outcomes were found in the present study, as on increasing the PVA concentration there is a decrease in the particles size with an increase in entrapment efficiency. 694±53, 474±28, and 689±22, 49.14 ±3.92, 57.28±2.78, and 37.05±3.34%  with PDI 0.134±004, 0.121±0.03, and 0.151±0.09.

It is found that it is difficult to remove the PVA completely from the formulations as it is expected to get absorbed irreversibly at the interface of globules this may lead to increase in the particle size also as shown in fig 3.5. Thus, from the results obtained in the study 1% PVA

concentration was considered to be optimum that shows the capability to produce the desired size range.

In order to prepare stable emulsified systems, energy incorporation in the system is a fundamental step. To form primary emulsion the optimized 5% sol of polymer and internal aqueous phase was sonicated by probe sonication (Soniweld, India) in ice bath for 30 seconds at 40W. After adding primary emulsion into 1% PVA solution, again sonicated to form secondary emulsion. To check the effect of sonication time on nanoparticles shape, entrapment efficiency and poly-dispersity index sonication time was altered between 1 to 3 min. The observed parameters of shape, entrapment efficiency and poly-dispersity index after experiments are 519±49, 482±24, and 493±34, 51.14 ±3.92, 53.21±3.32, and 45.05±3.32%, 0.141±0.05, 0.127±0.01, and 0.153±0.05. Different duration of sonication were observed keeping similar other parameters. Results indicated (Table. 3.8) that when sonicated for 1 min. larger particle size are formed while size decreases on increasing the duration, but of further increase size increases this may be due to agglomerate formation of stabilizer, hence the most optimum sonication time is found to be 2 min. that gives better size with effective entrapment and polydispersity index.

The basic idea of the study was to articulate a system that can behave as protein stabilizer, can provide prolonged antigen release, and can provide prolonged systemic immunity along with effective cellular immune response. Since gelatin being a hydrophilic polymer and PLGA being a hydrophobic polymer alone cannot **(Tsung et al., 2001; Kaul and Amiji, 2005)** achieve the above discussed objectives a combination of both these polymer was prepared. The coating was conducted by Double emulsion solvent evaporation technique with slight modification. We finally investigated optimized conditions of GNPs and PNPs, by taking all the parameters in consideration we prepared PLGA coated gelatin nanoparticles, PGNPs, since we had not made any modification in the above discussed methods these parameters can be taken for **(Jain, et al., 2009; Sudhees et al., 2011)** final formulation of PGNPs. 1-ethyl-3(3-dimethylaminopropyl) carbodiimide also known as EDC was used as the zero length linker in the method. EDC is hydrophilic in nature when it is kept with the gelatin it easily forms a bond with the amino group of gelatin. And forms the internal aqueous phase and during the sonication time this makes a bond with the carboxylic group of PLGA as shown in fig. 3.7 supports the lamination of PLGA over gelatin nanoparticles. Obtained zeta potential i.e. -11.2mV proves that the

prepared formulation was quite stable (Fig.3.9). On further characterization formulations were sent for TEM and SEM analysis. TEM images Photomicrograph 3.1(c) clearly shows that the internal particle is laminated by external polymer proves the coating of the PLGA over GNPs, SEM images of loaded and unloaded PGNPs shows smooth surface that clearly indicates that there is no adsorbed gelatin on the surface of the PLGA as only PLGA appears in the Scanning Electron Microscope image these results are also in accordance with those observed by other author **(Lee et al., 2000; Kaul and Amiji et al., 2005).** The studies revealed that the size of PGNPs are found to be 496.14±34 nm and % entrapment efficiency was 58.2±2.78.

The formulations were further characterized by *in vitro* release studies all three different formulations were studies (GNPs, PNPs and PGNPs). The release of FITC-TT was performed in PBS (7.4). Fig.3.10 shows FITC-TT release curves of the GNPs, PNPs and PGNPs. The GNPs show a burst release of antigen, almost 75 percent of antigen was released in the first few days, and PNPs shows slow release which is the characteristic of PLGA to provide sustained release but the after nearly 40 percent antigen release the TT was not detected at all proves that the antigen might be denatured by acidic environment of PLGA as on hydrolysis PLGA is degraded into monomer units i.e. Lactic acid and glycolic acid. The release profile of PGNPs was slow in the initial as the mechanism of slow release of antigen from PGNPs is relatively complicated. The mechanism of slow release is relatively complicated. It usually involve the route as: water permeation through the PLGA matrix and absorption by the gelatin nanoparticles than gelatin nanoparticles swelling occurs and diffusion of FITC-TT conjugate through the swollen gelatin nanoparticles starts on further diffusion of FITC-TT through a tortuous, water-filled path in the PLGA matrix, finally PLGA degradation occurs and the protein releases out. However the new PGNPs system releases the protein slowly. The data shows that in 18 days, only 66% of the loaded protein molecules are released and after 40 days, protein can still be released from the system, hindrance of the burst release and protection from denaturation makes this novel biodegradable system a alternative carrier candidate that can stabilize protein , and provide prolonged antigen release.

## CHAPTER 4  STABILITY STUDIES

### 4.1 General Introduction

Enhancement in the stability of a protein product and efficacy during storage and transportation is the basic goal of biopharmaceutical formulations and research **(Lee et al., 2000).** As per international conference on harmonization a well designed stability testing plan is essential and pertinent part, of the quality assurance programme. In order to cure or prevent any pathological **(Wei et al., 1999)** condition, a well accepted stability testing protocol is an important concern as every bioactive or medicament is required to elicit optimal pharmacodynamic response.

### 4.2 Stability Protocol

Commercialization of every nanoproducts requires formulations that are stable during the course of transportation and storage. Sometimes it is seen that suspended nanoparticulate formulation may lead to aggregation and settling over time results the stability of the delivery system and its transportation may be not up to the mark. To investigate the quality of the nanoparticles after freeze drying along with a cryoprotectant to maintain the size distribution and antigenicity of gelatin nanoparticles during freeze-drying and subsequent rehydration was the primary goal of the present work. Further the freeze-dried nanoparticles were subjected to accelerated stability testing (6 months). The test conditions were maintained as per ICH Guidelines, Q5C which are as follows-

### 4.2.1 Stabilization of protein in the solid state.

Obtaining stable antigenic proteins in the dry state is one of the keystones in the improvement of vaccine formulations. To achieve this goal  various stability studies are required b. The important factors that must be considered during drug development are shelf life and stabilization of the potential products. Firstly lyophilization was proposed in 1978 to enhance the stability of liposomes **(Van Winden and Crommelin, 1997)** which led to the regulatory clearance for Ambisome<sup>TM</sup> as the first freeze-dried liposomal drug formulation in 1992.  At present lyophilized form is very well excepted for the protein and peptide formulations so as to enhance the stability of these preceious therapeutic agents.

**4.2.2. Storage conditions**

**4.2.2.1. Temperature**

As per recommendation of ICH it is strongly suggested that the studies conducted on the proteins and protein products should be under accelerated and stress conditions. Well defined storage temperatures, stability studies can be confined to the proposed storage temperature. Two temperature conditions were chosen as at $4\pm1^{o}C$ and $37\pm1^{o}C$ for the study in accordance with the ICH guidelines.

**4.3 SDS-PAGE of prepared formulation.**

The SDS-PAGE was performed in order to check the integrity of the loaded antigen in the various formulations. Molecules being separated depends upon charge distribution of the electrophoretic separations. In process of electrophoresis, when the anions are allowed to flow towards the anode (positive electrode) or the cations flowing towards the cathode (negative electrode), the system is referred to as anionic or cationic, respectively. Despite the advantages of tube gels, slab gels are preferred due to the advantage of running multiple samples at a time in the same gel and also allowing for two-dimensional analysis. Electrophoresis in polyacrylamide gel is more convenient and simple than any other medium such as paper, agarose and starch gel.

The antigen loaded formulations containing a known amount of antigen were incubated with 0.1% w/v SDS (in PBS) at room temperature for 1 hr with gentle shaking and then centrifuged at 4,000 rpm for 25 minutes. The supernatants were used for sample preparation. The whole procedure of electrophoresis was performed taking antigen loaded formulations (GNPs, PNPs and PGNPs) simultaneously (Photograph 4.1) with pure antigen, instead of antigen alone.
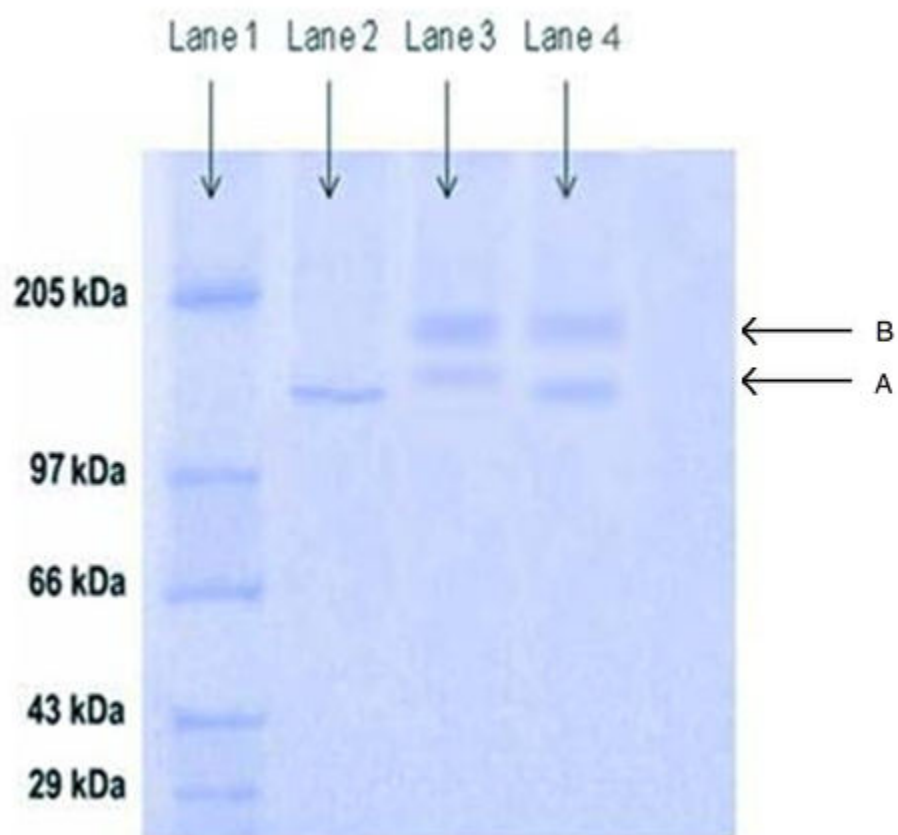
**Photograph 4.1; SDS-PAGE analysis showing structural integrity of release antigen. Lane 1; Marker Proteins (205 kDa myosin; 97 kDa phoporylase B; 67 kDa bovine serum albumin; 43 kDa; ovalbumin; 29 kDa carbonic anhydrase); Lane 2; Pure TT Lane 3; A. Gelatin, B. TT recovered from GNPs , Lane 4 : A. Gelatin, B. TT recovered from PGNPs.**

## 4.4 Effect of storage on residual antigen content at (4±1°C)

In present work stability studies of prepared formulation (loaded with TT) and its solution were carried out after storing the formulations at $4\pm1^{\circ}$C for 28 days in an amber coloured glass bottles. After every 7 days the formulations were evaluated and observed the changes in percent residual antigen content. The percent residual TT was determined by using calibration curve of FITC-TT (Fig. 2.1) by spectrofluorophotometer.

**Table 4.1**

**Effect of storage on residual antigen content at (4±1$^o$C)**

| S.No. | Time (Days) | Percent residual antigen | | |
|-------|-------------|------|------|-------|
| | | **GNPs** | **PNPs** | **PGNPs** |
| 1 | 0 | 100 | 100 | 100 |
| 2 | 7 | 99.58 | 99.74 | 99.48 |
| 3 | 14 | 99.23 | 99.38 | 99.23 |
| 4 | 21 | 99.01 | 98.57 | 98.75 |
| 5 | 28 | 97.72 | 98.14 | 98.15 |
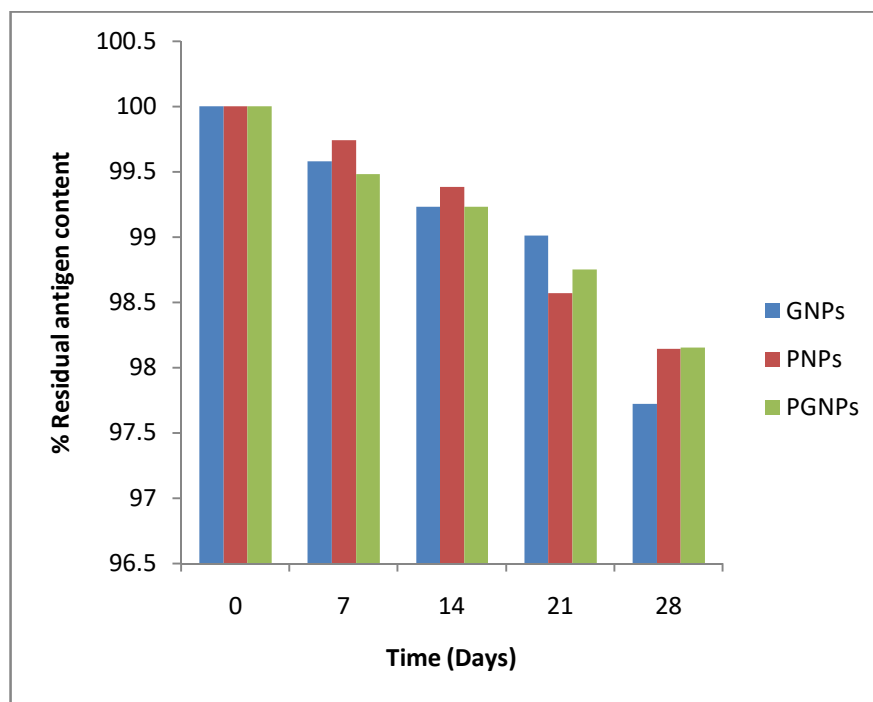


**Fig. 4.1: Effect of storage on residual antigen content (4±1$^o$C).**

## 4.5 Effect of storage on residual antigen content at (37±1°C)

In present work stability studies of prepared formulation (loaded with TT) and its solution were carried out after storing the formulations at 37±1°C for 28 days in an amber coloured glass bottles. After every 7 days the formulations were evaluated and observed the changes in percent residual antigen content. The % residual TT was determined by using calibration curve of FITC-TT (Fig. 2.1) by spectrofluorophotometer.

**Table 4.2**

**Effect of storage on residual antigen content at (37±1°C)**

| S.No | Time (Days) | Percent residual antigen | | |
|---|---|---|---|---|
| | | GNPs | PNPs | PGNPs |
| 1 | 0 | 100 | 100 | 100 |
| 2 | 7 | 99.57 | 99.34 | 99.56 |
| 3 | 14 | 98.91 | 99.27 | 99.19 |
| 4 | 21 | 98.13 | 98.13 | 97.49 |
| 5 | 28 | 81.07 | 82.02 | 85.68 |
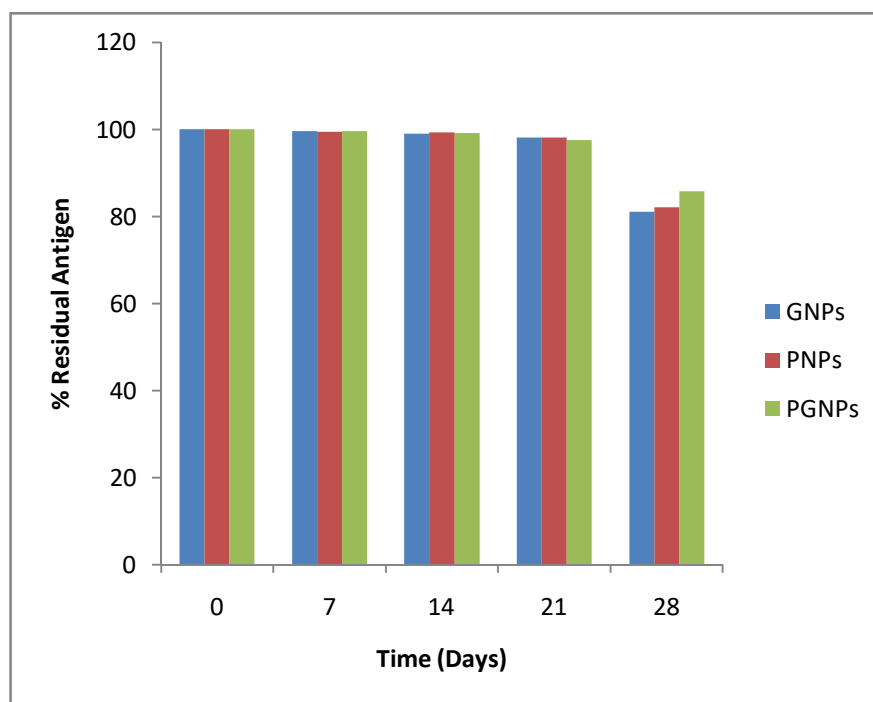


**Fig. 4.2: Effect of storage on residual antigen content (37±1°C) Series 1; PGNPs, Series 2; PNPs, Series 3; GNPs.**

## 4.6  Results and Discussion

Stability studies of the formulations were performed to assure the ability of the developed delivery system to tolerate environmental stress while maintain the antigenicity of the antigen used. The stability of antigen was checked as its integrity following the preparation of formulation. Also the storage stability at different shelf temperature was determined. A stable formulation must exhibit a constant particle size and a constant level of loaded active antigen during the storage. Storage stability testing was also performed at room temperature to investigate the protective effect offered by the polymer on the antigen even though immunological preparations are instructed to be stored under refrigerated conditions,

In present study stability and integrity of TT was assessed using SDS-PAGE. The PAGE was run with spots of pure antigen and antigen extracted from formulations i.e. from GNPs, PGNPs and PNPs. Different band appears in the gel plate, these bands are of first lane the marker lane second was of pure antigen third and fourth were of gelatin and TT recovered from the formulations (Photograph 4.1). Band of TT in different formulations at around 150 kDa and locations respectively. This reveals that the preparation conditions did not cause an irreversible aggregation or cleavage of the protein in gelatin nanoparticles and PGNPs and spots on PNPs lane shows some denaturation of protein by PLGA

The selected FITC-TT loaded formulations after lyophilization were stored in tightly closed amber coloured bottles at $4\pm1^{\circ}$C and elevated temperature $37\pm1^{\circ}$C for 28 days. These were analyzed weekly. The release studies were performed by the method described in sec. 3.3.1, by considering initial antigen content as 100% it was observed that after 28 days at $37\pm1^{\circ}$C, around 98 % found after the tests whereas more than 98% antigen was measured when the formulations were stored at $4\pm1^{\circ}$C. Thus from these results, it can be concluded that for better stability, the formulation should be stored only at refrigerated conditions.

## CHAPTER 5. BIOLOGIACAL STUDIES

### 5.1. Regulatory aspects

Reproducibility of the product performance is a very crucial aspect in  pharmaceutical research, and producing any delivery system for market needs control procedures so as to ensure reproducible product performance. Till date only PLGA based systems have been approved for human use by the major regulatory authorities (**Jaganathan  et al., 2006).**

### 5.1.1 Adjuvant and delivery system: Recent regulatory challenges.

Because of their poor immunogenic properties new generation vaccines, based on synthetics, recombinant proteins, and conjugated antigens have emerged but have limitations, consequently more cooperative adjuvants to evoke the immune response are required. Lots of research on new adjuvants and delivery systems has been done in the last few decades, and different molecules have been identified for their ability to enhance the immunity. Most of the vaccines that are in use presently have the licensed use of aluminium salts or calcium salts. Hence there is a strong regulatory concern for the production of new generation vaccines.

The goal of the present work was to develop a delivery system that can deliver antigen(s) in its immunological active form to their target site following their administration in order to show specific humoral and broad cellular immune responses. After getting encouraging data *in-vivo* immunological responses were checked.

### 5.2 Immunization protocol.

*In vivo* immune response of various TT loaded preparations were tested in BALB/c mice as animal model, after getting permission from Institutional Animal Ethical Committee (IAEC) of Smriti College of Pharmaceutical Education Indore, (IAEC/SCOPE/11-12/80). All the studies were performed as per the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India provides the guidelines for the animal studies and all the studies were performed in accordance with the guidelines provided.

## 5.3 Intranasal immunization

A dose response experiment was conducted in order to optimize the dose of the antigen. Dose response experiment was performed using 20µl of vaccine as per reported by **(Brunel et al., 1999)** with slight modification. Various equivalent doses of TT (5, 7, 9, 11, and 13 Lf) were carried out in the dose response (IgG response) experiments. Dose response of various formulations were summarized in table 5.1, Vaccines were prepared prior to immunization The control group, however, received 5 Lf of alum-adsorbed TT subcutaneously and 7 Lf of Plain TT and blank nanoparticles (GNPs, PNPs and PGNPs) with single dose and respective booster after 4 weeks of primary immunization. Immunizations were performed two times at the interval of four weeks; day 0 and 28. Formulations were administered using a 10µl micropipette (Rankem, India) after moderate anesthesia. Mice were anaesthetized by injecting ketamine (120µl of 10 mg/ml solution in saline) intraperitoneally. Formulations were then administered, 4µl per nare (total of 20µl equivalent to the optimized dose of TT) at an interval of 5 min. Administration of formulations in divided doses at intervals of 5 min leads to complete retention in the nasal cavity **(Vishweshwariah et al., 2002).** Bolus dose may lead to localization of antigen in the esophagus, trachea and/ or stomach. Mice were held in supine position both during and post administration.

## 5.4 Collection of blood Serum

To study the effect of various formulations, after administration of forumulations animals were anesthetized Under mild ether anesthesia the collection of blood was done by retro-orbital puncture at 7, 14, 28, 42, 56 days.  All samples were collected for different different formulation so as to ensure the effect of each formulation . The serum from the collected blood was separated and allowed to coagulate. The serum from the collected blood was separated and allowed to coagulate, Seperated sera were stored at −40°C until tested by ELISA for antibody detection. At week 0 samples were collected before the booster administration to compare primary and secondary immunization. The nasal secretions were collected at week 0 prior to and 8 weeks after the booster immunization. Mice were sacrificed and the nasal washes were collected by cannulation of the trachea. The nasal secretion was stored with 100 mM Phenylmethyl sulfonyl fluoride (PMSF) as a protease inhibitor at -40ºC until tested by ELISA for secretary antibody (sIgA) levels.

**5.5 TT specific IgG response measurements by ELISA.**

Estimation of Anti-TT IgG antibodies in mice sera were done as reported previously **(Raghuvanshi et al., 1998)** with slight modification by using commercially available ELISA Kit (Bangalore Genei, India). IgG1 and IgG2a isotypes were determined by sigma isotyping kit (Sigma-Aldrich Pvt. Ltd., St. Louis, MO, USA). The logarithm of the reciprocal of the last dilution was expressed as end-point titre, which gave an optical density at 450 or 492 nm above the optical density of negative control. The systemic antibody responses (IgG) was evaluated for all the formulations (Fig. 5.3)

**5.6 Measurement of TT specific IgG1 and IgG2a responses by ELISA.**

IgG1 and IgG2a anti-TT titers were determined.by using sigma isotyping kit (Sigma-Aldrich Pvt. Ltd., St. Louis, MO, USA). After booster dose a weak IgG2a antibody titre (Fig.5.3) was elicited by alum-TT on the other hand mice injected with TT entrapped PGNPs produced high levels of IgG2a. That proves the prepared formulation shows both systemic and cell mediated immune response.

**5.7 Measurement of specific IgA response**

Secretary IgA level in nasal fluids was determined by ELISA using a modified method (**Debin et al., 2002**)

**5.8  Fluorescence microscopy of nasal mucosa**

The deposition of prepared carrier system in nasal associated lymphoid tissue (NALT)  was confirmed by fluorescence microscopy after nasal administration. Fluorescent Isothiocynate (FITC) conjugated BSA used for the study. The tissues were blotted and wiped with tissue paper. The wiped tissue were fixed in Carnoy's fluid (absolute alcohol: chloroform: acetic acid, 6:3:1v/v) for 3-4 hours. The tissues were then washed with 90% alcohol for 10-15 minute and again fresh 90% alcohol was used for immersion upto 1 hr. The tissues were transferred to a mixture of absolute alcohol:xylene (1:1v/v) and kept for 15 to 20 minutes. The tissues were then transferred to xylene and kept undisturbed for 24 hr.

The matured molten paraffin wax was filtered through whatman filter paper to remove any suspended particles and was kept in molten state for 24 hr at 62-64 $^{\circ}$C, Thick molten wax was

then poured in infiltration pans, kept at 62-64 $^{o}$C in an oven and tissues were transferred to the first pan for 30 min. then to second and third infiltration pans for 30 min. each at controlled temperature. Glycerin was applied on the upper and side surface of the lid of cufflings jar. Filtered molten wax was poured in the lid up to 4/5 of its total height. The tissues were placed gently into the lid and allowed to stand at RT, untill solidifies. The lid was placed in tray containing water till the block separated and floated on water. The block was cut and trimmed to remove wax.
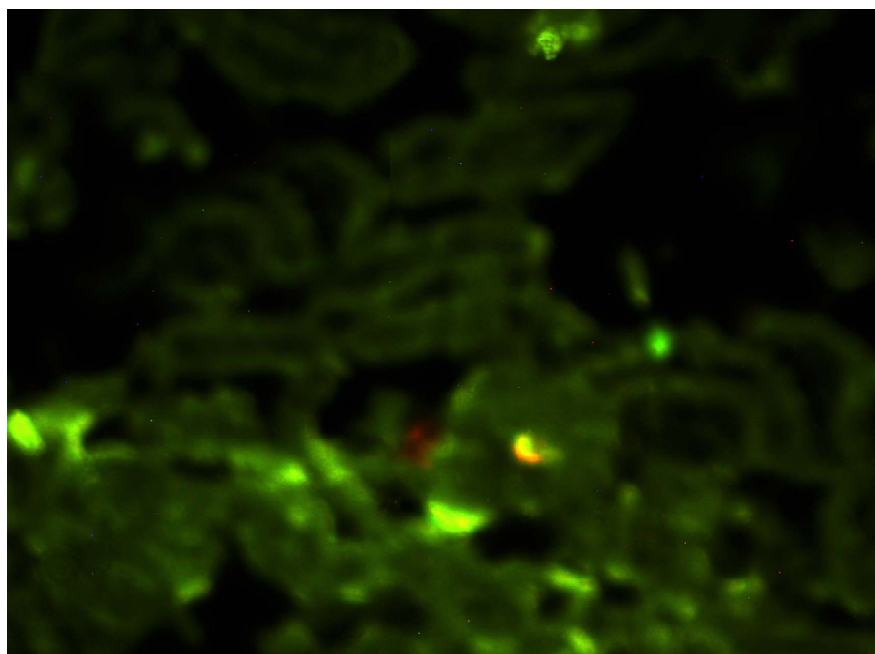


**Photograph 5.1 Fluorescent Microscopy of Nasal Mucosa of BALB/c Mice.**

Microtomy was performed and ribbon of tissue sections of thickness 5µm was fixed on slides by egg albumin solution as fixative. During fixation, mild heating (in the presence of water) was applied to open the wrinkles of the section on slides. Then the slides were kept undisturbed for 1-2 days. The dewaxing was performed with xylene and coverslips were mounted using DPX [A mixture of disterene (a polystyrene), a plasticizer (tricresyl phosphate) and xylene]. After 2 days the slides were cleaned and the sections were viewed under the specialized microscope called fluorescence microscope and photographs (Photograph 5.1).

**Table 5.1**

**Dose response on BALB/c mice (n=6) with various doses of TT loaded GNPs, PNPs.**

| S.No | Dose of TT (Lf) | $Log_{10}$ IgG antibody titers after 4 weeks dose | | |
|------|-----------------|------|------|-------|
|      |                 | **GNPs** | **PNPs** | **PGNPs** |
| 1 | 5.0 | 0.7 | 0.82 | 1.12 |
| 2 | 7.0 | 1.72 | 1.66 | 2.31 |
| 3 | 9.0 | 1.74 | 1.67 | 2.40 |
| 4 | 11.0 | 1.61 | 1.58 | 1.92 |
| 5 | 13.0 | 1.36 | 1.32 | 1.83 |
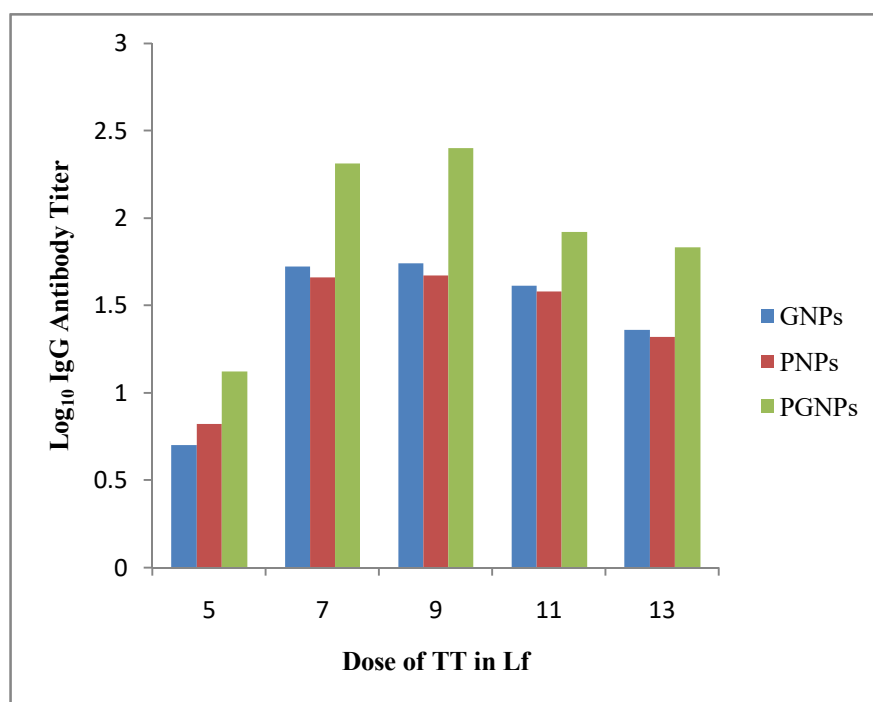


**Fig. 5.1: Dose response on BALB/c mice (n=6) immunized with various doses of TT loaded GNPs, PNPs, and PGNPs: 5,7,9,11,13 Lf**

**Table 5.2**

**Immunization studies serum IgG levels with formulations**

| S.No. | Formulations | IgG Titer at different time interval (Days), $Log_{10}$ | | | | |
|-------|--------------|------|------|------|------|------|
| | | 7 | 14 | 28 | 42 | 56 |
| 1 | Marketed | 4.39±0.85 | 5.01±0.69 | 4.93±0.53 | 5.31±0.62 | 5.09±0.43 |
| 2 | GNPs | 2.21±0.65 | 2.23±0.47 | 2.4±0.64 | 2.12±0.73 | 2.09±0.57 |
| 3 | PNPs | 2.48±0.59 | 2.67±0.61 | 2.98±0.34 | 2.74±0.32 | 2.12±0.86 |
| 4 | PGNPs | 4.09±0.35 | 4.34±0.72 | 4.45±0.85 | 4.4±0.85 | 4.19±0.44 |
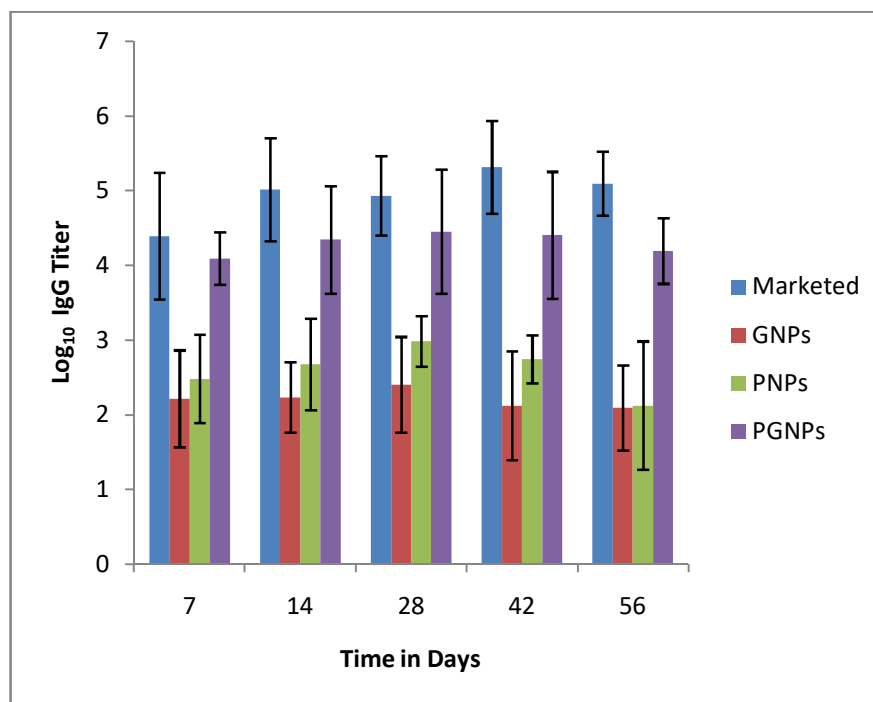


**Fig. 5.2; IgG Titre after intra-nasal administration of different formulation at different time interval.**

**Table 5.3**

**IgG1 and IgG2a response of different formulation**

| S.No. | Antibody Titer | IgG1 | IgG2a |
|---|---|---|---|
| 1 | Alum-TT | 4.2± 0.75 | 0.8±0.2 |
| 2 | GNPs | 2.1±0.32 | 1.5±0.39 |
| 3 | PNPs | 2.8±0.31 | 2.1±0.41 |
| 4 | PGNPs | 3.6±0.28 | 2.5±0.45 |
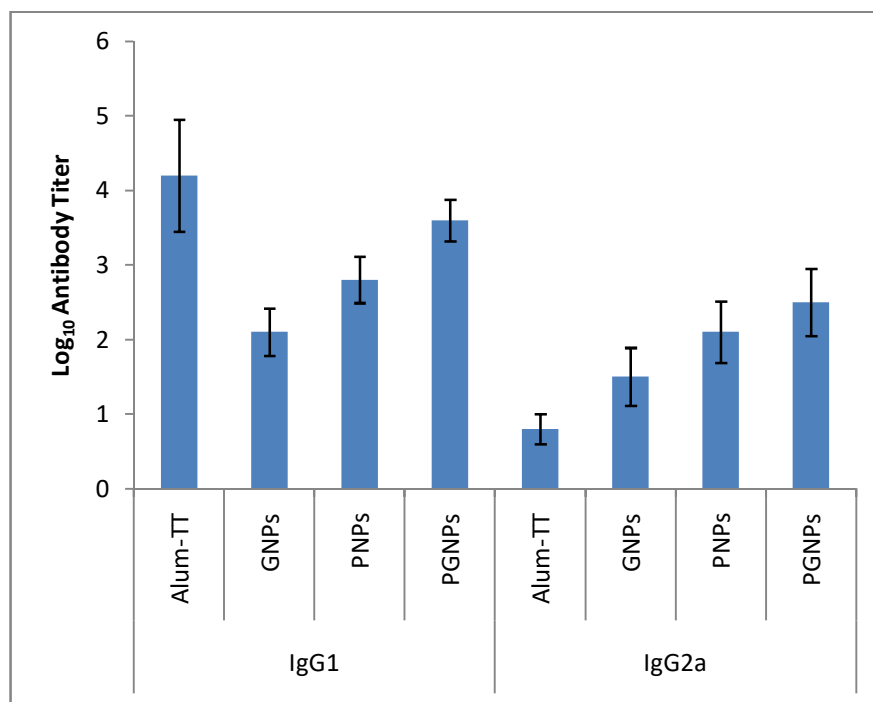


**Fig.5.3:  IgG1 and IgG2a antibody titer of different formulation.**

**Table 5.4**

**IgA Titer at different mucosal surfaces 42 days after immunization by nasal route.**

| S.No. | Code | IgA titre , 42 Days After Immunization, $Log_{10}$ | | |
|-------|------|----------|---------|-------|
| | | **Salivary** | **Vaginal** | **Nasal** |
| 1 | MKT | 0.767±0.19 | 0.757±0.23 | 0.762±0.11 |
| 2 | GNPs | 3.41±0.31 | 3.48±0.42 | 3.44±0.42 |
| 3 | PNPs | 3.21±0.46 | 3.28±0.57 | 3.22±0.52 |
| 4 | PGNPs | 4.25±0.56 | 4.21±0.37 | 4.27±0.33 |
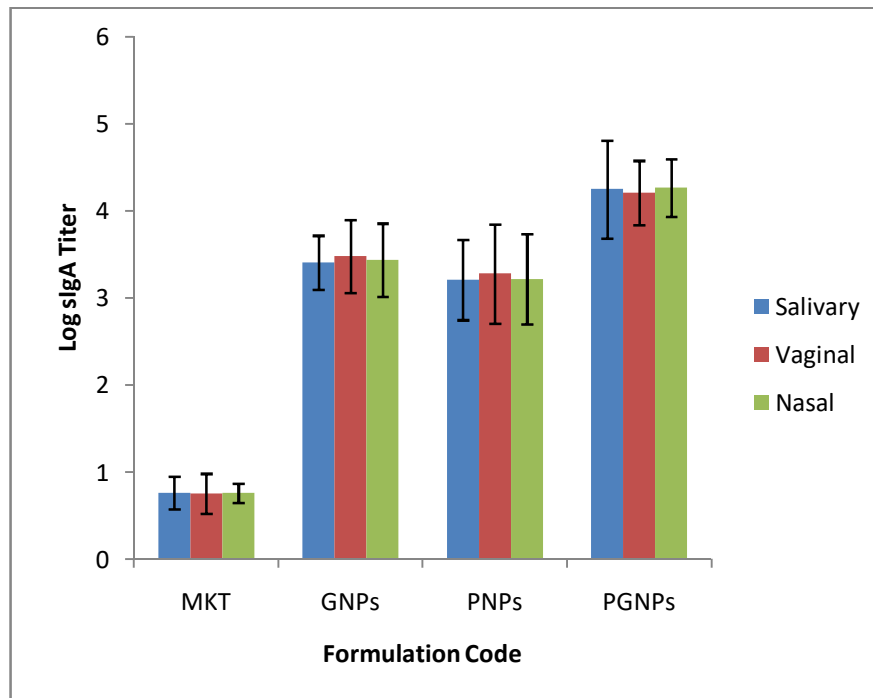


**Fig. 5.4: sIgA Titer at different mucosal surfaces 42 days after immunization by nasal route.**

## 5.10 RESULTS AND DISCUSSION

The enhancement of immune responses by encapsulated human gamma globulin (HGG), a model antigen in gelatin microparticles prepared by emulsification method has been reported (**Nakoka et al., 1995**). Present work is different from this, in the following respect. Double emulsion solvent evaporation technique was used for the formation of articulated system in our study, which is found to be a protein friendly method due to the use of process variables involved and their effect in the technique. Exceptional size homogeneity can be obtained by this method due to removal of unwanted materials i.e. low molecular weight gelatin in the initial GNPs formation and extensive PVA washing which is an crucial step in emulsification method. Further the modification of gelatin was carried out to prepare PGNPs, with slow release, superior biodegradability and protection of protein.

Mucosal administration (nasal) offers several significant advantages over traditional approach of vaccine delivery that normally involves intramuscular injections and is considered as an attractive approach. The advantages of mucosal delivery over the conventional ones are now well established because of their ease of administration, low dose, pricking less, less skill application, etc.

In the present study, fluorescent microscopy was performed and the uptake of developed system by mucosal tissues, or NALT following nasal delivery was confirmed respectively. Bright yellowish green fluorescent is observed in the cross sections of nasal mucosa, that indicates that the developed systems were well taken up and processed by NALT of nasal mucosa.

The immune adjuvant capability of the developed formulations were evaluated by immunization studies performed. Female BALB/c mice were used, animals were procured from CDRI, Lucknow. 24 female BALB/c mice (8-10 week old, weighing 20-25 gms.) were divided into four groups, each comprising of 6 animals. Genetically pure and homogenous animal were selected for the experiment to avoid any unwanted genetic variations in the results. The mice were starved 3 hr before the immunization, Group I received alum adsorbed marketed formulation, This group received a booster dose at $30^{th}$ day, Group II received the GNPs, Group III received PNPs, Group IV received PGNPs. Nasal administration of developed formulation was done by using sterile hemilton syringe i.e. (Group II,III,IV). At initial day blood samples of each animal was collected before the immunization.

To estimate IgG level by the use of ELISA blood samples were collected by retro-orbital plexus and serum was isolated. IgG titre was calculated from the absorbance obtained as the result to ELISA Table. 5.1 shows the IgG titre obtained with different formulations at different time period.

A biphasic response was observed in all the formulation, these results are in favour with the *in-vitro* release study, that showed a sudden higher release around 30 days due to degradation dependent release. The results of *in-vivo* study show second phase release that may have worked as the booster dose which potentiated the IgG titre in the blood plasma. For the single shot vaccine development this kind of response is prerequisite. The antigen delivery ability of PGNPs by nasal administration to evoke systemic and mucosal based antibody responses were evaluated. To determine dose range from the formulations, in BALB/c mice a intranasal delivery was done and dose response experimental results were analyzed. ELISA was performed to study the IgG anti-TT titers and results after 4 weeks of booster immunization were summarized in table 5.2 and represented graphically by fig. 5.2 The data obtained shows that the production of anti-TT antibodies increased up to a dose of 7 Lf (Fig. 5.1 ) The plateau of antibody response was reached with 7 Lf TT. This confirmed the 7 Lf dose and was adopted for the next experiments concerning the adjuvancity of all the nanoparticles.

After the nasal administration, Group IV exhibited good IgG titre throughout the studies where as Group II and III also showed the less effective IgG titre, probably due the stability problems as gelatin nanoparticles shows the burst effect and PLGA nanoparticles shows the denaturation of protein **(Jagannathan et al., 2006).** In the present study alum adsorbed marketed formulation had dominated in the IgG titre throughout the study and PGNPs responses were found to be comparative to them. That showed the superiority of the system over other polymer based delivery. The IgG1 and IgG2a anti-TT titers identified by ELISA are shown in figure 5.3 and table 5.3, the data indicates that alum induces a Th2 response, characterized by very low IgG2a response. Alum-TT after a booster dose shows weak IgG2a antibody titre, In contrast the mice administered the PGNPs shows high levels of IgG2a. These results shows that alum induces a Th2 response, while PGNPs shows both Th1 and Th2 responses which are demonstrated by the strong IgG2a and IgG1 anti-TT antibody responses in the table 5.3.

Developed formulations also showed the effective level of sIgA at the mucosal surfaces (Fig.5.4). Whereas alum adsorbed marketed formulation failed to do so. The comparative results

were somewhat similar to IgG titre as PGNPs were found to be most effective and gelatin was found to be less efficient in generation of sIgA antibodies. On the basis of the results and analysis it can be concluded that the prepared systems is a system of choice as effective adjuvant for non-invasive mucosal immunization through intra-nasal route. The hypothesized system when formulated shows the capability to elicit humoral and mucosal immune responses and can be used as a potential carrier system for a number of proteins and peptide based delivery system. Although humoral response obtained were only comparative to conventional alum adsorbed I.M. vaccine but the developed formulation showed these results after single dose compared to the booster dose given, also elicit significant mucosal immune response which was not elicited by conventional alum adsorbed I.M. vaccine, This is the additional advantage of the developed carrier system, this system also overcomes the disadvantages associated with the classical invasive method.

## CHAPTER 6:  SUMMARY AND CONCLUSION

### 6.1 Summary

Hallmark of a successful vaccination strategy is the production of "sterilizing immunity" in other words prevention of infections and this can be achieved by high and long lasting antibody titre. Even though the aluminum salt precipitates often referred to as 'alum' are  relatively poor adjuvants in many situations, still they are  most widely used adjuvants in humans. Sometimes these vaccines are found to be associated with severe local reactions. These local reactions of alum salts are always arguable and regulatory requirements have been raised since their development and use, although they had been used for many years but their toxicity and side effects may preclude its registration for human use by regulatory bodies it they would be newly discovered as an adjuvant at present.

Over the past decades a large number of potentially useful immunostimulating formulations have been explored and broadly they can be divided into adjuvants and antigen-carriers. Antigen carriers enhance antibody responses by the influence of associated antigens and adjuvants have immunostimulatory properties. The most efficient, cost effective means for the prevention of a wide variety of infectious diseases are claimed to be vaccines. Vaccination remains to be a major challenge due to problems associated with vaccine delivery, stability and cost. Major problem in vaccination is the optimum and appropriate antigen presentation by the system.

 In the present study TT (Tetanus Toxoid) was selected as model antigen because of ease of availability and its inherent property of high antigenicity. Tetanus toxoid represents a protein that could be very suitable for encapsulation in microencapsulation technologies.
After extensive literature review the goal was set,  to develop reproducible and homogeneous preparation of TT loaded PLGA coated gelatin nanoparticles (PGNPs), These formulations can behave as protein stabilizer, can provide prolonged antigen release, and can provide prolonged systemic immunity along with effective cellular immune response, to achiev the goal further studies were preformed  to study the immune stimulating property of the formulations in an animal model, gelatin nanoparticles and PLGA nanoparticles were also prepared for comparative study and for checking the effect of process variables.

To achieve the above mentioned goals firstly gelatin nanoparticles were prepared (GNPs), molecular heterogeneity of gelatin makes it challenging to prepare homogeneous

nanoparticulate formulations. Most of the emulsification techniques use simple w/o emulsions. Emulsification techniques suffer from major drawbacks, such as low yield sometimes less than 30%, broad size distributions etc.

The received antigens integrity and molecular weight of FITC-TT prepared was assessed by SDS-Page (Photograph; 2.1). For analysis and characterization of protein samples SDS-PAGE is widely used. It is clear from Photograph 2.1 that two distinct darker bands were seen at around 150 kDa and the molecular weight of tetanus toxoid is nearly 150 kDa which corresponds to the subunit of TT and FITC-TT subsequently.

 As the prior step of formulation the received antigens were identified and characterized through SDS-PAGE. For the determination of % entrapment efficiency FITC-TT was conjugated and release profile was also estimated by calibration curve of FITC-TT. FITC-TT conjugation was done as it was easy to analyze the protein entrapment and release with this fluorescent dye using fluorescence spectrophotometry because conventional colorimetric assay's such as BCA and Bradford's methods would give erroneous results due to interference by the presence of soluble gelatin which would also give color in the dye binding assay. Typical calibration curve of FITC-TT in PBS pH 7.4 is shown in figure 2.2. Good linearity is achieved in the calibration (correlation factor ($R^2$) approximately equals 0.9987). The concentration of FITC-TT in PBS 7.4 was identified at the maximum fluorescence intensity of about 520 nm observed by the calibration curve (Fig. 2.2).

By the use of two- step desolvation method homogeneous GNPs were prepared. This method shows much less aggregation as compared with the one-step desolvation method **(Marty et al., 1978).** High molecular- weight fraction of the gelatin used was collected in the sediment after first desolvation step while in the supernatant the low molecular weight gelatin is precipitated out, this proves  that the removal of the low molecular weight gelatin fraction in the supernatant after the first desolvation step not only reduced the formation of aggregate during cross-linking because of an enhanced stability of particles formed before cross-linking but also prevented further secondary aggregation and flocculation of particles during storage. The PLGA nanoparticles (PNPs) were prepared by double emulsification method, Polymer was first dissolved in the dichloromethane as hydrophobic polymer well dissolves in the organic solvents, PVA was used a emulsifying agent to stabilize the system. Various process parameters were optimized, by using the most optimum parameters of the formulations hypothesized system

PGNPs were prepared by the use of double emulsion solvent evaporation method with slight modifications. The working area was sterilized and monitored regularly by sampling the air and the surface of the working area to ensure endotoxin- free conditions of the preparation process. FITC-TT conjugation was done first to determine % entrapment efficiency (EE) and release. The entrapment of FITC-TT in the formation of strong interpenetrating network between gelatin and TT (Tetanus Toxoid). FITC-TT would, therefore, be encapsulated during the coacervation phase at an acidic pH in the last desolvation step. Measurements of the resulting nanoparticles was done by DLS, the software calculated average mean size of GNPs 64.4±5.8 nm and PDI of 0.135±0.09, PNPs 497±28 and PDI of 0.129±0.013 and for PGNPs 498±24 with PDI 0.141±0.05 (mean of 3 measurements with 22 individual subruns for each) in the optimized formulations PDI obtained were well within the acceptable limit (<0.1). We additionally analyzed these nanoparticles by TEM and SEM to visualize the particles and investigate their morphology. TEM (photograph) and SEM (photograph) confirmed that the sample analyzed were spherical and smooth.

Various process variables for the formulations were checked for their influence. Most optimum variables were selected for the final formulation, for GNPs the process variables were temperature, pH, and concentration of glutaraldehyde, and for PNPs the process variables were polymer concentration, percent PVA concentration, and duration of sonication. The basic goal was to prepare nanoparticles having good entrapment efficiency and narrow size distribution. We studied the effect of above discussed parameters on the particle size, % entrapment and polydispersity index. If polydispersity index is low it indicates a narrower size distribution of the system. Nanoparticles with maximum entrapment efficiency and PDI below 0.1 were considered as optimized batches and were taken for further studies. To study the effect of temperature on system, all other parameters were kept constant while temperature was changed during the experiment, 50 ml acetone was used as desolvating agent and 200µl glutaraldehyde as crosslinker in the technique used. Temperature plays an important role in the sedimentation of gelatin in the first desolvation and formation of nanoparticles in the second desolvation step due to occurrence of denaturation and renaturation process as evident from literature (**Farrugia and Groves, 1999).** At higher temperature (60ºC) gelatin exhibits partial reversible denaturation of HMW gelatin fractions. Sol-gel transition of gelatin solutions results which is due to increase in renaturation at lower temperatures. 35-40ºC is the typical sol-gel transition temperature of

gelatin solution (**Peyrelasse et al., 1996).** The results at 30ºC, 40ºC and 50ºC showed that temperature has an strong influence on the particles size. Homogeneous batch of nanoparticles with maximum entrapment efficiency were prepared at 40ºC with gelatin A. Better-controlled precipitation of the macromolecules is found at 40ºC, after addition of the desolvating agent the chains seem to be sufficiently uncoiled at this temperature compared to higher temperature.

In all the tested pH range, gelatin nanoparticles were successfully prepared, although at pH above 4, formulation produced deviated strongly towards aggregation and precipitation the reason is the pH value is too close to IEP of gelatin type A (approximately 6.1). Thus the net charge that remains on gelatin is too weak to prevent the nanoparticles from instabilities. During the second desolvation step to form nanoparticles, pH was adjusted and kept away from isoelectric points of gelatin. As per the conditions discussed above the nanoparticles were prepared at 40ºC using gelatin Type A, acetone was used as the desolvating agent. On increasing GTA amount as presented in figure 3.3, there is a steady decrease in particle size found to support the study of researchers **(Nakaoka et al., 1995; Balthasar et al., 2005)** that increasing amounts of cross-links lead to smaller and denser nanoparticels. The particle size at 100, 200, and 400 µl of GTA were found to be 61.4±5.9, 64.4±5.8 and 305.4±6.9 nm and % entrapment efficiency was 87.14±8.16, 89.14±4.36, and 92.35±9.32 with a PDI of 0.247±0.03, 0.120±0.06, and 0.167±0.02. The most optimum amount with the most homogeneous nanoparticles resulting (as suggested by PDI values). By higher amounts of GTA there is a slight increase of the PDI values beyond this point that might be accounted to stronger interparticulate aggregation tendencies, it would be advisable to keep the amount of GTA as low as possible from a toxicological point of view since one of the intended routes of administration is nasal. 200 µl was chosen to be optimum GTA for the formulation.

For the preparation of PLGA nanoparticles formulation parameters were studied for the optimum preparation. Different concentration of PLGA was used to check the effect on the size, entrapment efficiency and polydispersity index (PDI) of the formulations. To check the effect of polymer concentration all other parameters were kept constant. 25, 50, and 75 µl of PLGA were used. The size, entrapment efficiency and polydispersity index (PDI) were found to be 250±53, 497±28, and 683±45 nm, 47.14±3.92, 58.23±3.92, and 33.05±3.32%, 0.131±0.09, 0.129±0.01, and 0.243±0.05. Nanoparticles formed by 25 mg of PLGA achieves a size of 250 nm but the particles were non-spherical in shape, and shows agglomerates. Since the main objective was to

laminate gelatin nanoparticles by PLGA this size does not fit to laminate it properly and as the polymer concentration was doubled particle diameter also increases but on further increase of polymer the viscosity  of the dispersed phase also increases that results a strapped dispersion of the PLGA. Increased polymer concentration shows high viscous resistance against the shear forces involved in the emulsion during the emulsification that causes coalescence of the particles. Hence it is clear from the results of different formulations that with the increase in the polymer concentration while keeping similar other parameters, the viscosity of the organic phase increases that causes poor emulsion with higher particle size and higher polydispersity index. Hence 5% of PLGA concentration was considered as the optimum for nanoparticles.

To study the effect of surfactant, polyvinyl alcohol (PVA) was used in the double emulsification method, different percent PVA concentrations were studies by keeping similar other parameters, to stabilize the secondary emulsion PVA was used as surgfactant in the external aqueous phase as it produce relatively smaller and uniform particles **(Hueper et al., 1959; Sahoo et al., 2002; Zambaux et al., 1998)**. The emulsification and stabilization of the globules are crucial factors in double emulsification method, in the emulsification process of nanoparticles formation and in the protection of the globules the amount of surfactant used plays an exclusive role as it has the ability to avoid the coalescence of globules. In our formulation we received the analogous outcomes were found in the present study, as on increasing the PVA concentration there is a decrease in the particles size with an increase in entrapment efficiency. 694±53, 474±28, and 689±22 nm 49.14 ±3.92, 57.28±2.78 and 37.05±3.34 % entrapment, with PDI 0.134±004, 0.121±0.03, and 0.151±0.09.

To remove the PVA completely from the formulations was very difficult as it was expected to get absorbed irreversibly at the interface of globules that may lead to increase in the particle size, as shown in Fig. 3.5. Thus, from the results obtained in the study 1% PVA concentration was considered to be optimum, that shows the capability to produce the desired size range.

In order to prepare stable emulsified systems, energy incorporation in the system is a fundamental step. To form primary emulsion the optimized 5% sol of polymer and internal aqueous phase was sonicated by probe sonication (Soniweld, India) in ice bath for 30 seconds at 40W.  After adding primary emulsion into 1% PVA solution, again sonicated to form secondary emulsion. To check the effect of sonication time on nanoparticles shape, entrapment efficiency and poly-dispersity index, sonication time was altered between 1 to 3 min. The observed

parameters of shape, entrapment efficiency and poly-dispersity index after experiments are 519±49, 482±24, and 493±34, 51.14 ±3.92, 53.21±3.32, and 45.05±3.32, 0.141±0.05, 0.127±0.01, and 0.153±0.05. Different duration of sonication were observed keeping similar other parameters. Results indicated (Table. 3.8) that when sonicated for 1 min. larger particle size are formed while size decreases on increasing the duration, but of further increase size increases this may be due to agglomerate formation of stabilizer, hence the most optimum sonication time was found to be 2 min. that gives better size with effective entrapment and polydispersity index.

The basic idea of the study was to articulate a system that can behave as protein stabilizer, can provide prolonged antigen release, and can provide prolonged systemic immunity along with effective cellular immune response. Since gelatin being a hydrophilic polymer and PLGA being a hydrophobic polymer alone cannot **(Tsung et al., 2001; Kaul and Amiji, 2005)** achieve the above discussed objectives a combination of both these polymer was prepared. The coating was performed by double emulsion solvent evaporation technique with slight modification. We finally investigated optimized conditions of GNPs and PNPs, by taking all the parameters in consideration we prepared PLGA coated gelatin nanoparticles, PGNPs, since we had not made any modification in the above discussed methods these parameters can be taken for (**Jain, et al., 2009; Sudhees et al., 2011**) final formulation of PGNPs. 1-ethyl-3(3-dimethylaminopropyl) carbodiimide also known as EDC was used as the zero length linker in the method. EDC is hydrophilic in nature when it is kept with the gelatin it easily forms a bond with the amino group of gelatin, and forms the internal aqueous phase and during the sonication time this makes a bond with the carboxylic group of PLGA as shown in Fig. 3.7, this supports the lamination of PLGA over gelatin nanoparticles. Obtained zeta potential i.e. -11.2mV proves that the prepared formulation was quite stable (Fig.3.9).  On further characterization formulations were sent for TEM and SEM analysis. TEM images Photomicrograph 3.1(c) clearly shows that the internal particle is laminated by external polymer proves the coating of the PLGA over GNPs, SEM images of loaded and unloaded PGNPs shows smooth surface that clearly indicates that there is no adsorbed gelatin on the surface of the PLGA as only PLGA appears in the scanning electron microscope image these results are also in accordance with those observed by other author (**Lee et al., 2000; Kaul and Amiji et al., 2005).** These studies revealed that the size of PGNPs were found to be 496.14±34 nm and % entrapment efficiency was 58.2±2.78.

The formulations were further characterized by *in vitro* release studies all three different formulations were studies (GNPs, PNPs and PGNPs). The release of FITC-TT was performed in PBS 7.4. Fig. 3.10 shows FITC-TT release curves of the GNPs, PNPs and PGNPs. The GNPs show a burst release of antigen, almost 75 % of antigen was released in the first few days, and PNPs shows slow release which is the characteristic of PLGA to provide sustained release but the after nearly 40 % antigen release the TT was not detected at all proves that the antigen might be denatured by acidic environment of PLGA as on hydrolysis PLGA is degraded into monomer units i.e. lactic acid and glycolic acid. The release profile of PGNPs was slow in the initial as the mechanism of slow release of antigen from PGNPs is relatively complicated. The mechanism of slow release is relatively complicated. It usually involve the route as: water permeation through the PLGA matrix and absorption by the gelatin nanoparticles than gelatin nanoparticles swelling occurs and diffusion of FITC-TT conjugate through the swollen gelatin nanoparticles starts on further diffusion of FITC-TT through a tortuous, water-filled path in the PLGA matrix, finally PLGA degradation occurs and the protein releases out. However the new PGNPs system releases the protein slowly. The data shows that in 18 days, only 66% of the loaded protein molecules are released and after 40 days, protein can still be released from the system, hindrance of the burst release and protection from denaturation makes this novel biodegradable system a alternative carrier candidate that can stabilize protein and provide prolonged antigen release.

In present study stability and integrity of TT was assessed using SDS-PAGE. The PAGE was run with spots of pure antigen and antigen extracted from formulations i.e. from GNPs, PGNPs and PNPs. Clearly visible bands for pure as well as extracted antigens can be seen in Photograph 4.1, for TT in different formulations at around 150 kDa and location respectively. This reveals that the preparation conditions did not cause an irreversible aggregation or cleavage of the protein in gelatin nanoparticles and PGNPs and spots on PNPs lane shows some denaturation of protein by PLGA.

The selected FITC-TT loaded formulations after lyophilization were stored in tightly closed amber colored bottles at $4\pm1^{o}C$ and elevated temperature $37\pm1^{o}C$ for 28 days. These were analyzed weekly. The release studies were performed by the method described in sec. 3.3.1, by considering initial antigen content as 100% it was observed that after 28 days at $37\pm1^{o}C$, around 96% found after the tests whereas more than 98% antigen was measured when the formulations

were stored at 4±1$^o$C. Thus from these results, it was concluded that for better stability, the formulation should be stored at refrigerated conditions.

The enhancement of immune responses by encapsulated human gamma globulin (HGG), a model antigen in gelatin microparticles prepared by emulsification method has been reported (**Nakoka et al., 1995**). Present work is different from this, in the following respect. Double emulsion solvent evaporation technique was used for the formation of articulated system in our study, which is found to be a protein friendly method due to the use of process variables involved and their effect in the technique. Exceptional size homogeneity can be obtained by this method due to removal of unwanted materials i.e. low molecular weight gelatin in the initial GNPs formation, extensive PVA washing etc. which is crucial in emulsification method make it a tedious process. Further the modification of gelatin was carried out to prepare PGNPs, with slow release and superior biodegradability with protection of protein. Mucosal administration (nasal) offers several significant advantages over traditional approach of vaccine delivery that normally involves intramuscular injections and is considered as an attractive approach. The advantages of mucosal delivery over the conventional ones are now well established they are ease of administration, low dose, pricking less, less skill application, etc.

In the present study, fluorescent microscopy was performed and the uptake of developed system by mucosal tissues, or NALT following nasal delivery was confirmed respectively. Bright yellowish green fluorescent is observed in the cross sections of nasal mucosa, that indicates that the developed systems were well taken up and processed by NALT of nasal mucosa.

The immune adjuvant capability of the developed formulations were evaluated by immunization studies performed. Female BALB/c mice were used for the experiment and animal were procured from CDRI, Lucknow. 24 female BALB/c mice (8-10 week old, weighing 20-25 gms.) were divided into four groups, each comprising of 6 animals. Genetically pure and homogenous animal were selected for the experiment to avoid any unwanted genetic variations in the results. The mice were starved 3hr before the immunization, Group I received alum adsorbed marketed formulation, This group received a booster dose at 30$^{th}$ day, Group II received the GNPs, Group III received PNPs, Group IV received PGNPs. nasal administration of developed formulation was done by using sterile hemilton syringe i.e.(Group II,III,IV). At initial day blood samples of each animal was collected before the immunization.

To estimate IgG level by the use of ELISA blood samples were collected by retro-orbital plexus and serum was isolated. The statistical analysis methods were used after the completion of immunization study. IgG titre was calculated from the absorbance obtained as the result to ELISA Tab. 5.2 and Figure 5.2 shows the IgG titre obtained with different formulations at different time period.

In the study a biphasic response was observed in all the formulation this result is in fever with the *in-vitro* release study that showed a sudden higher release around 30 days due to degradation dependent release. The results of *in-vivo* study show second phase release that may have worked as the booster dose which potentiated the IgG titre in the blood plasma. To determine dose range from the formulations, in BALB/c mice a intranasal delivery was done and dose response experimental results were analyzed. ELISA was performed to study the IgG anti-TT titers and results after 4 weeks of booster immunization were summarized in table 5.1 and represented graphically by fig. 5.1 The data obtained shows that the production of anti-TT antibodies increased up to a dose of 7 Lf, The plateau of antibody response was reached with 7 Lf TT. This confirmed the 7 Lf dose and was adopted for the next experiments concerning the adjuvancity of all the nanoparticles.

After the nasal administration, Group IV exhibited good IgG titre throughout the studies where as group II and III also showed the less effective IgG titre, probably due the stability problems as gelatin nanoparticles shows the burst effect and PLGA nanoparticles shows the denaturation of protein **(Jagannathan et al., 2006).** In the present study alum adsorbed marketed formulation had dominated in the IgG titre throughout the study and PGNPs responses were found to be comparative to them. That showed the superiority of the system over other polymer based delivery. The IgG1 and IgG2a anti-TT titers identified by ELISA are shown in fig. 5.3 and table 5.3, the data indicates that alum induces a Th2 response, characterized by very low IgG2a response. Alum-TT after a booster dose shows weak IgG2a antibody titre; In contrast the mice administered the PGNPs shows high levels of IgG2a. These results shows that Alum induces a Th2 response, while PGNPs shows both Th1 and Th2 responses which are demonstrated by the strong IgG2a and IgG1 anti-TT antibody responses in the table 5.3.
Developed formulations also showed the effective level of sIgA at the mucosal surfaces (Fig.5.4). Whereas alum adsorbed marketed formulation failed to do so. The comparative results

were somewhat similar to IgG titre as PGNPs were found to be most effective and gelatin was found to be less efficient in generation of sIgA antibodies.

On the basis of the results and analysis it can be concluded that the prepared systems is a system of choice as effective adjuvant for non-invasive mucosal immunization through intra-nasal route. The hypothesized system when formulated shows the capability to elicit humoral and mucosal immune responses and can be used as a potential carrier system for a number of protein and peptide based delivery system. Although humoral response obtained were only comparative to I.M. standard but the developed formulation showed these results after single dose compare to the booster dose given in case of standard and also elicit significant mucosal immune response, which was not elicited by conventional alum adsorbed I.M. vaccine, This is the additional advantage of the developed carrier system, this system also overcome the disadvantage of classical invasive method.

**In conclusion the major outcomes of our research are:**

The preparation parameters were well optimized first and on the basis of the data generated we were able to find the  customized conditions with reproducible batches of high quality nanoparticles and with the  predictability of better than ±15 nm size within the size range of 400-500nm.

PGNPs have demonstrated to be highly valuable carrier system for immunization. Since the results shows that Th1/CTL responses were up to the mark with specificity, hence this new adjuvant perfectly fits to comply all the needs of antiviral or anti humoral delivery where alum has it disadvantage.

Major advantage in our study is that intra-nasal vaccination has the potential to induction sIgA antibodies response at the mucosal epithelium. Freeze-drying was found to provide excellent stabilization of nanoparticles in conjugation with trehalose, used as a cryoprotectant. Increased concentration of cryoprotectants correlated with enhanced stability and antigenicity of TT. Fluorescence study reveals that not only NALT but nasal epithelium is involved in the transport of nanoparticles. Preliminary investigation reveals that PGNPs are nontoxic as adjuvants.

**Chapter 7  Bibliography**

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