Novel growth factor delivery system for regenerative medicine

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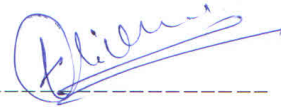The Degree of Master of Technology

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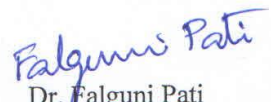
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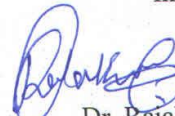IIT Hyderabad

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I am thankful to all my friends for their support and family members for their love, support and care.

Dedicated to

My parents and family members.

Abstract

Growth factors (GFs) are naturally occurring protenatious substance, secreted by surrounding cells essentially required for cellular growth, maturation, proliferation, differentiation and healing of injury. However, we have limited clinical applications due to lack of appropriate delivery systems and biomaterial carriers. Many conventional delivery systems are there to deliver GFs like polymeric particle, emulsions, and dendrimers. In conventional approaches proteins (GFs, enzyme, etc.) undergo several process and storage-related stresses throughout the life of the product that can result in significant degradation and loss of activity. Usually protein-containing microparticles are prepared by the double emulsion method, such as water/oil/water (W/O/W), solid/oil/water (S/O/W) and water/oil/oil (W/O/O). In the primary emulsion the protein encounters an aqueous organic interface which causes denaturation of the protein. Added to this are other process related stresses like, high homogenization speed, sonication and temperature. In order to address the aforementioned problems, we have introduced a novel method of protein encapsulation. The sugar glass nanoparticle system (SGnPs) is produced by incorporating sugars and other stabilizers along with the proteins in inverse micelles. This will ensure the preservation of activity of the encapsulated protein, by protecting it from the various process related stresses. Further these SGnPs are used for preparation of polymeric microparticles using the aforementioned conventional methods (W/O/W and W/O/O) for long time storage and control the release pattern. By using of SGnPs for GFs delivery we have achieved 100% encapsulation efficiency and sustained release, only 18% has released in 30 days.

Nomenclature

GFs- Growth Factors

SGnPs- Sugar Glass nanoparticle

W-O-W- Water in Oil in Water emulsion

W-O-O- Water in Oil in Oil emulsion

SGnPs-O-W- SGnPs in Oil in Water emulsion (Novel approach)

SGnPs-O-O- SGnPs in Oil in Oil (Novel approach)

PLGA- Poly (lactic-co-glycolic acid)

SEM- Scanning electron microscopy

DLS- Dynamic light scattering

PDS- Protein delivery system

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Chapter 1

Introduction

1.1 Tissue engineering and Regenerative medicine

Regenerative medicine is a branch of translational research in field of tissue engineering which combines the principles of biology and engineering to achieve the goal of the regeneration, replacement, restore or establish normal function of damaged or injured body tissues[1]. Regenerative medicine promises not only the engineering of damaged cells, tissues and organs via stimulating the body's own repair mechanisms to restore its normal function but also includes the possibility of growing cells, tissues and organs in the laboratory and safely implanting them when the body cannot heal itself.[2,3]

Regenerative medicine for tissue regeneration consists of cells with proliferative and differentiative potential, Signaling molecules (GFs), GF delivery vehicle, culture medium and bioreactor [4]. In the most frequent paradigm of tissue engineering, isolated living cells are used to develop biological substitutes for the replacement or restoration of cell, tissue or organ functions. Generally, cells are seeded on bio-absorbable scaffolds, a tissue is developed in vitro, and the construct is implanted in the appropriate anatomic location as a prosthesis [4, 5]. Cells used in tissue engineering may come from a variety of sources including application-specific differentiated cells from the patients themselves (autologous), human donors (allogeneic) or animal sources (xenogeneic), or undifferentiated cells comprising progenitor or stem cells [5, 6].

1.2 Growth Factors

Growth factors are naturally occurring a group of proteins that stimulate the growth of specific tissues and play an important role in promoting cellular differentiation, cell division, migration, metabolism and healing [7, 8]. During the study of different biological substances on cell/tissue researchers found some peptides whose functions were totally different form previous known functions i.e. hormone and enzymatic and were involved in stimulating cellular growth so they called Growth factor. GFs play an important role in wound healing, tissue regeneration by stimulating granulation tissue formation, modulating the inflammatory responses, matrix formation, remodeling, re-epithelization process and promoting angiogenesis

[9, 10]. In culture, growth factors are rate limiting factor for proliferation because they deplete more rapidly than any other media components [11, 12].

GFs stimulate cell proliferation and other responses through binding to specific cell membrane receptors (Fig-1.1). The interaction of the GFs to its corresponding receptor is highly specific, which ensures delivery of a particular message to a distinct population of cells. GFs stimulate receptor to transduce secondary signals and activate intracellular signal transduction pathways which controls various aspects of subcellular biology and cellular function (fig- 1.1) [13]. Unlike hormones GFs do not usually act in an endocrine manner; they presumably diffuse short-range through intercellular spaces and act locally [14].

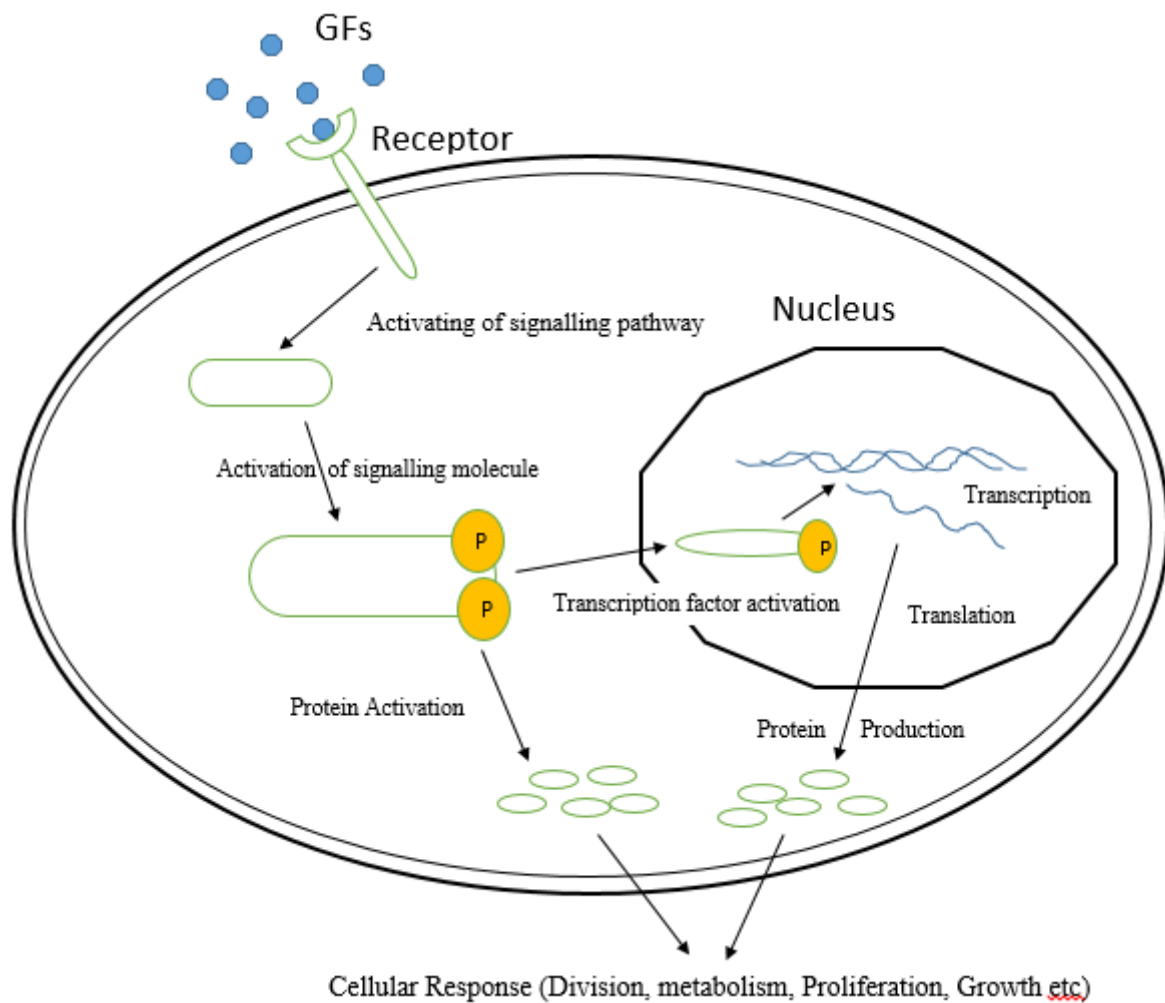**Fig.1.1**- Mechanism of action of growth factors. GFs bind to their respective receptors and initiate the signaling pathways that activate signaling molecules. Signaling molecules can either activate proteins present in the cytoplasm or induce transcription of new proteins through the activation of transcription factors for different cellular responses such as growth, differentiation, migration, etc. P= phosphorylation.

Application of growth factors may not be much effective without proper delivery vehicle, because of their short half-life in vivo and the potential toxicity and systemic effects of bolus delivery [15].

Organs transplantation or synthetic implants are the currently available methods to treat loss of tissues and organs but still there is a continue demand of a new solutions and approaches for tissues failure since the definitive solution is too far to be achieved, and for this reason regenerative medicine and tissue engineering are becoming as the alternative solution to repair or regenerate damaged tissue [16, 17].

The main goal of this experiment is to develop a vehicle releasing growth factors to stimulate desired cell response in order to enhance and accelerated tissue healing [16].

1.3 Biomaterials for Growth Factor Delivery

A biomaterial can be defined as a nonviable substance that has been engineered to interact biological system, used alone or as part of a complex system, for a medical purpose either as therapeutic or diagnostic substance [18. 19]. Many biomaterials have been discovered, developed and used by researchers over the years as carrier for growth factors [19].

These materials can be derived either from natural resources or synthesize in laboratory using a variety of chemical approaches.

1.3.1 Natural Materials

Being natural originated, these materials minimizes chronic inflammation and rejection by immune system and also ensure to develop a delivery systems that functions at the molecular level. Most of natural materials are water soluble that allows mild fabrication conditions which are harmless to the bioactivity of the loaded growth factor. Sometimes these natural material also have some intrinsic biological activity that may be induce to regeneration.

Commonly used natural material as growth factor carriers are silk, collagen, gelatin, chitosan, alginate, agarose, hyaluronic acids, fibrin, elastin, starch, and carrageenan [20, 21, 22].
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1.3.2 Synthetic Materials

The major advantage of using synthetic materials over natural is that they can be synthesized or modified based on the desired properties beneficial to clinical outcomes and additionally composites of different materials can be used to optimize the individual characteristics of biomaterial and improve the efficiency of growth factor delivery system. Commonly used

synthetic biomaterial as GF carrier are Poly-l-lysine (PLL), poly-lactic acid (PLA), polyglycolic acid (PGA) their copolymers polylactic-co-glycolic acid (PLGA), poly-gammaglutamic acid polyethyleneimine (PEI), polyethylene glycol poly-N-isopropylacrylamide (pNiPAAm), poly-hydroxyethylmethacrylate (pHEMA), poly-caprolactone (PCL) [20, 21, 23].

1.4 Strategies for Growth Factor Delivery

Polymers can be formulated into many different physical structures for developing growth factor delivery system like scaffolds, hydrogels, particulate system and nanofibers [27, 28, 29]. GFs are encapsulated in polymer matrix either by absorption or by covalent immobilization. In culture GFs are released either by diffusion from the matrix or by degradation of the biomaterial. In most of the cases, these kind of system show burst release profile dependent on the degradation rate which is difficult to control. Researchers have made several modifications in the biomaterials to control the release pattern of GFs [24, 25, 26].

Particulate systems are distinct from other aforementioned delivery systems and have advantage over them because of their reduced size that range varies nm to µm and other unique properties are such as solubility, biodistribution, immunogenicity, and release characteristics. The very important advantage of particulate system is its smaller size that ensure the ability to target a specific tissue with minimal distribution to normal tissues [30, 31]. Hence, particulate delivery systems will open possibilities of addressing the failure of traditional therapeutics in regenerative medicine and suggest some modification to curb limitations.

1.5 Delivery of Growth Factors

A variety of growth factor proteins have been available in large quantities as a result of advances in biotechnology. Many Protein delivery system (PDS) such as Hydrogels, Scaffolds, Nanofibers, Polymeric Nanosphere/ Microspheres, Lipid nanoparticles (Liposomes) have been developed to deliver growth factors to a specific organ or tissue systems with controlled release of growth factors for days to months [27, 28, 29]. For delivery of growth factors generally we use biodegradable polymers such as poly lactic acid (PLA), poly glycolic acid (PGA), and poly lactic-co-glycolic acid (PLGA) because the release profile of loaded growth factor mainly depends on the degradation kinetics of coating polymer and it is easy to understand how we

can change the release profile by changing the composition of polymer [20-23]. We can change the degradation kinetics by changing the ratio of lactic acid and glycolic acid. It may not be true always because other excipient also affect the release kinetics but the polymer composition is always a dominant factor [32, 33]. An ideal delivery system should have reasonably high protein encapsulation efficiency, loading capacity, and sustained release of the loaded protein with retained bioactivity [27, 29].

1.6 Common approaches to develop delivery systems for GFs

1. **Double Emulsion technique**
   - ➢ Water in Oil in Water (W-O-W)
   - ➢ Water in Oil in Oil (W-O-O)
   - ➢ Solis in Oil in Oil (SGNPS-O-O)
   - ➢ Solid in Oil in Water (W-O-W)
2. **Spray drying and Spray freeze-drying technique**
3. **Ultrasonic Atomization technique**
4. **Electrospray technique**
5. **Microfluidic technique**
6. **Pore-closing and Thermoreversible-Gel technique**
7. **Microfabrication technique**

Double emulsion technique consists solvent diffusion or solvent evaporation mechanism. In this method we use two immiscible solvent are homogenized for making emulsion and this emulsion again disperse in another immiscible solvent [34, 36, 57]. Solvent evaporation takes place in W-O-W while diffusion takes place in W-O-O (Fig-1.2).
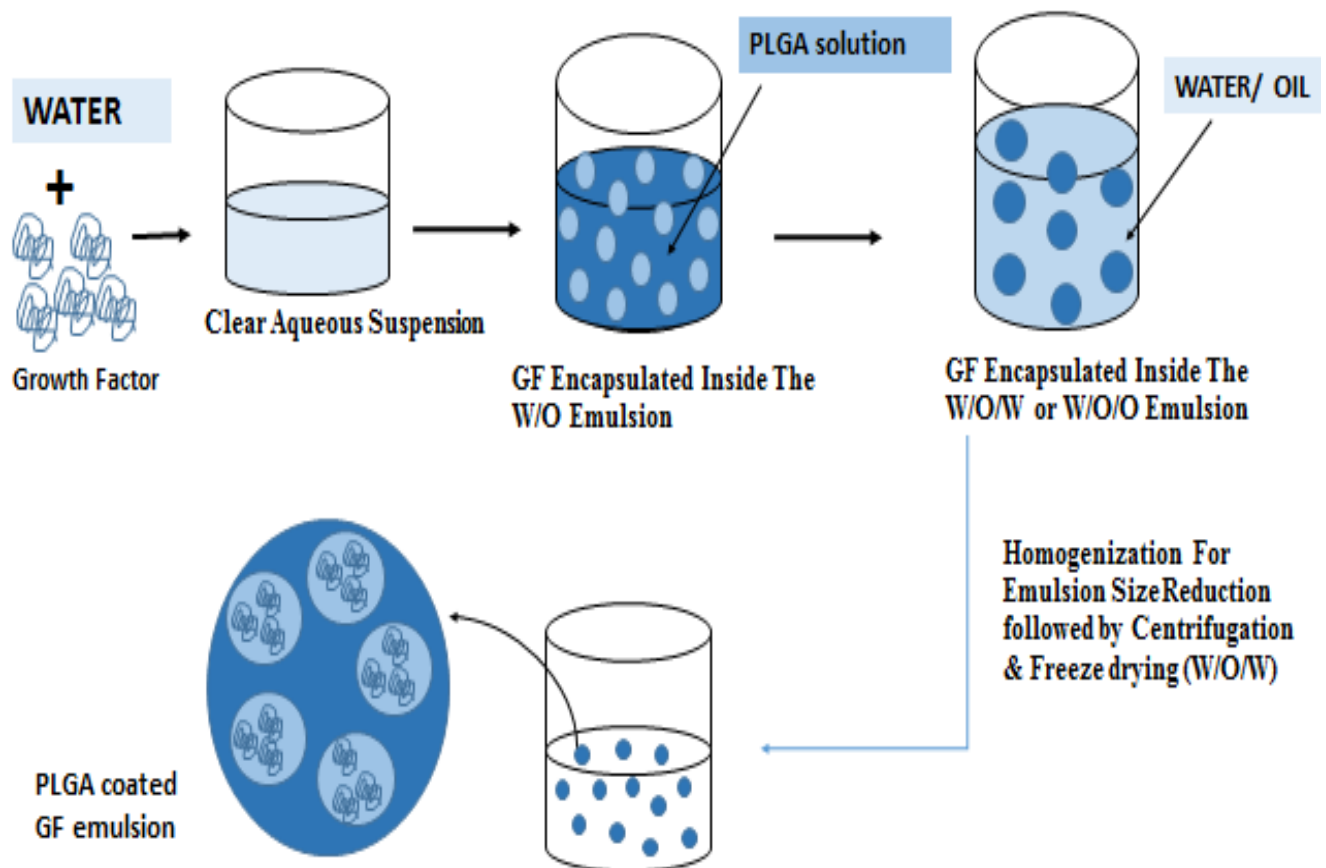
Fig.1.2- Double emulsion technique for PLGA microparticles. For primary emulsion DCM was used, for secondary emulsion 2.5 % PVA solution (W-O-W) and Light liquid paraffin oil (W-O-O) used.

Chapter 2

Literature Review

In 1999 Yi-Yan Yang et al. investigated the important parameters to fabricate PDLLA (Poly (DL-lactic acid)), PDLLGA (Poly (DL-lactic-co-glycolic acid) 65: 35 and blends of PDLLGA 65: 35 and PEG microspheres having BSA by double-emulsion (water-in-oil-in-water) solvent extraction/evaporation method. Release profiles of microspheres were carried at 22^oC in order to develop controlled release delivery system for marine fishes. They have investigated various factors that affects the size of microspheres, encapsulation efficiency, morphology of microsphere, release profile, and BSA distribution within microspheres. These factors include preparation temperature, solvent evaporation rate, ratio of oil phase to internal water phase, and polymer concentration. Microspheres were developed at a low volume ratio of oil phase to internal water phase and a low polymer concentration. Microspheres had a large surface area, a low bulk density, resulting in a high initial burst and a fast release of BSA. Temperature majorly affects solvent extraction/evaporation and mechanism of phase-inversion. It was reported that microspheres were fabricated at 4 and 38^0C yield the highest encapsulation efficiency (52.0%) and lowest initial BSA release (18.8%), while microspheres produced at 22^0C showed the low encapsulation efficiency and high initial burst. In this experiment they showed that encapsulation efficiency, initial burst and release profile of microspheres can be controlled by changing preparation conditions such as temperature, removal rate of solvent, and volume ratio of oil to internal water [35].

In 2008 Asep Bayu Dani Nandiyanto et al. developed spherical mesoporous silica particles having pore size and outer particle diameter in the nanometer range by water/oil phase using organic templates method. In this method simultaneous hydrolytic condensation of tetraorthosilicate forms silica and polymerization of styrene forms polystyrene. In this method an amino acid catalyst, octane hydrophobic-supporting reaction component, and cetyltrimethylammonium bromide surfactant were used. After removal of the organic components by calcinations, we get mesoporous silica particles. Unlike other common

mesoporous methodology, by this method we get particle with small pore size (4–15 nm) and particle diameter (20–80 nm). By changing the styrene concentration we can control pore size and changing in the concentration of the hydrophobic molecules can control outer diameter of the particle. Mesoporous particles with controllable pore size (4–15 nm) and outer diameter (20–80 nm) of Hiroshima Mesoporous Material (HMM) were successfully prepared in a water/oil phase using an organic template method. The adsorption properties of the prepared porous silica particle were too good in comparison with previous non-porous silica particles [37, 38].

In 2008 DONG Xiao Qing et al. prepared recombinant human epidermal growth factor (rhEGF) loaded poly-lactic-co-glycolic acid (PLGA) microspheres by w/o/w extraction–evaporation technique. Transmission electron microscopy (TEM) and particle size distribution by laser particle analyzer used for the characterization of microspheres morphology. Release profile, the proliferation and therapeutic effects of rhEGF-loaded PLGA microspheres were all studied. Those microspheres had a narrow size distribution and a high GF encapsulation efficiency (85.6%). In Experiment those RhEGF-loaded microspheres showed better growth rate of fibroblasts and wound healing in comparison to pure rhEGF. Sustained-release microspheres encapsulating rhEGF in the polymer, showed nontoxicity, sustained-release profile and higher activity of rhEGF [39].

In 2010 Yuejie Chu et al. prepared nanoparticles for delivery of recombinant human epidermal growth factor (rhEGF) using poly (lactic-co-glycolic acid) by double-emulsion method. Transmission electron microscope (TEM) and laser analyzer with zeta potential were used for the morphology of the nanoparticles and particle size distribution. Enzyme-linked immunosorbent assays (ELISA) was performed to determine the rhEGF encapsulation efficiency and release profile. The average particle size of rhEGF nanoparticles was around 193.5nm (diameter), and the particle size distribution was uniform. The encapsulation efficiency was 85.6% and rhEGF showed release lasted within 24 hours. Compared with other delivery modality the rhEGF nanoparticles showed the highest proliferation rate of fibroblast, and fastest healing rate. The result of this experiment showed that controlled release of rhEGF encapsulated in the nanoparticles enhanced rhEGF effects for cell proliferation, shorten the wound healing time and a controlled release of rhEGF for up to 24 hours without disturbing its biological activity [40].

In 2010 Yanfang Yang et al. prepared porous poly (L-lactide-co-glycolide) (PLGA)/b-tricalcium phosphate scaffolds containing dexamethasone (Dex) and bovine serum albumin (BSA) by spray-drying and PLGA microspheres containing Dex or BSA were prepared by double emulsion/ solvent evaporation method. Release studies of microspheres prepared from PLGA in 3:1 molar ratio of L-lactide/glycolide and 89.5 kDa prolonged release profiles comparison with particles those prepared from PLGA in 1:1 L-lactide/glycolide molar ratio and 30.5 kDa. Addition of poly ethylene glycol (PEO) with PLGA could improve the encapsulation efficiency and reduce the release rate [41].

In 2010 Oks and Karal-Yilmaz et al. synthesized Poly (lactic-co-glycolic acid; PLGA), microparticles encapsulating the human recombinant vascular endothelial growth factor (rhVEGF) to achieve rhVEGF sustained release pattern by a water-in-oil-in-water (w/o/w) double emulsion/solvent evaporation method. The microparticles were having diameters of 10–60 mm and the encapsulation efficiency was between 46% and 60%. The microparticles were characterized by particle size distribution, environmental scanning electron microscopy (ESEM), light microscopy, encapsulation efficiency and their degradation rate. Mw (Mw), composition and crystallinity of copolymer are the factors that affects the degradation rate and release profile of PLGA microparticles. The mass loss (%) during PLGA microparticles degradation study shows 2% after 7 days, 8% after 14 days and significant mass loss begins approximately after 21 days which reaches up to 92% in 35 days. The microparticles showed slower rate of degradation up to 21 days and after this degradation rate increases over the 25 days [42].

In 2013 Garazi Gainza et al. developed rhEGF-loaded PLGA-Alginate microspheres by modified w/o/w double emulsion/solvent evaporation method. Different formulations were evaluated for the optimization of MSs properties by adding sodium chloride (NaCl) to the surfactant solution and adding alginate as a second polymer. The characterization of the prepared MSs showed that incorporation of alginate with PLGA increased the encapsulation efficiency. Results showed that the addition of NaCl increases the EE and also make the particle surface smooth and even. After loading of 1% rhEGF in to PLGA-Alginate MSs, the particles were sterilized by gamma radiations for in vivo studies. This experiment showed the

advantages of using NaCl and alginate incorporation during the microsphere preparation for achieving a greater EE and sustained release profile [43].

In 2011 J. Giri et al. prepared sugar glass nanoparticle for encapsulating Horse reddish Peroxidase (HRP) by inverse emulsion method. In this method they made inverse emulsion of HRP with trehalose in AOT- Isooctane solution. This emulsion was flash freezed in Liq. Nitrogen (-198^0 C) to get sugar matrix encapsulated HRP. The sugar glass nanoparticles so produced showed marked effects in conserving the activity of protein against several process related stresses (Fig-**5.2**). This technique shows sustained as well as prolonged release of protein [44].

In 2015 Peng Zhai et al. encapsulated bovine serum albumin (BSA) in to Poly (lactic-co-glycolic acid) (PLGA) microspheres and PLGA/alginate composite microspheres by a novel double emulsion and solvent evaporation method. It was found that the addition of alginate with PLGA and the using surfactant in microsphere preparation has increased the encapsulation efficiency and reduced the initial burst release of BSA. Confocal laser scanning microcopy (CLSM) showed evenly distribution of PLGA, alginate, and BSA throughout the depths of microspheres and no core/shell structure was observed. By scanning electron microscopy (SEM) images it can be concluded that PLGA microspheres degrade more quickly than PLGA/alginate composite microspheres. In this experiment anti-laminin antibody was loaded in to PLGA/alginate microsphere, to know about the preservation of the activity and result showed that activity was more preserved in PLGA/alginate microsphere in comparison to PLGA microsphere [45].

In 2015 Xiaojun Zhou et al. synthesized Bone morphogenic protein (BMP-2) peptide encapsulated in mesoporous silica nanoparticles (MSNs). First BMP-2 peptide was covalently grafted on the surface of MSNs via an amino-silane linker, and then dexamethasone (DEX) was loaded into the channel of MSNs to construct nanoparticulate osteogenic delivery system. Potency of MSNs were tested with bone mesenchymal stem cells (BMSCs) in vivo resulting that the functionalized MSNs have better cyto-compatibility and cellular uptake efficiency than that of bare MSNs. The in vitro results also showed that MSNs promoted osteogenic differentiation and proliferation of BMSCs in terms of the alkaline phosphatase (ALP) activity, calcium deposition, and expression of bone-related proteins and the osteogenic differentiation and proliferation of BMSCs can be further enhanced by addition of DEX into MSNs [47].

Table- 2.1 Different protein delivery Systems (PDS)

S/N	PDS system & Polymer composition	Protein encapsulated	Size of particle	Reference
1	Mesoporous Silica nano particle with DEX	BMP-2 peptide of 73-79 residue	20-80nm	47
2	PLGA-Alginate microsphere	rhEGF	14.95µm	48, 49
3	Solid lipid nanoparticles (SLN)	Coenzyme Q10	200nm-4µm	50, 51
4	Chitosan oligosaccharide-heparin Np	Stomatal cell derived factor-1α & vascular endothelial GF	96.2-210.5 nm	52
5	Nano and micro chondroitin sulphate particles	Transforming growth factor-b1 and Tumor necrosis factor-a	324.1 ± 8.5 and 73.2 ± 4.4 nm, 4.3 ± 0.93 µm	53
6	Chitosan and poly (ethylene oxide) multifunction nano fibre	VEGF with plate derived GF	153 ± 36 nm	54
7	Low molecular weight heparin(LMWH)/protamine (P) nano/micro particles		100 nm–3 µm	55
8	Cyclodextrin-PELA fibre	bFGF		56
9	Oil-in-Oil Emulsions		20-100nm	57
10	Collagen microsphere and fibrilized collagen microsphere	collagen	2.2-440 µm	58

2.1 Challenges in delivery of growth factor from PDS

Though we have developed many advanced PDS but still there are some common problems with their release profile i.e. Burst release, Activity loss, Encapsulation efficiency, Duration of release, Particle size, Protein loading capacity, bioactivity of the released protein and extent of release. The protein release profiles from various PDS can be classified on the basis of the (i) Magnitude of burst release, (ii) Extent of protein release, and (iii) The protein release kinetics followed by the burst release [44, 59].

Clinically successful long-term biodegradable PDS based on micro/nano particles requires improvement in the drug loading efficiency, control of the initial burst release, and the ability to control the protein release kinetics and another side they undergo several process and storage stresses i.e. elevated temperatures, exposure to liquid and solid hydrophobic interfaces, and vigorous mechanical agitation etc [44, 59,].

We have developed many approaches to overcome a single problem but none of them was able to resolve all problems. For example solid in oil in water emulsion (SGnPs-O-W) was developed which is able to avoid hydrophobic interface interaction in comparison to water in oil in water emulsion system (w-o-w) but this system has reduced protein loading capacity, and burst release. For making SGnPs-O-W we have to freeze dry the protein for loading that tends to yield larger protein particle which provide poor dispersion in final delivery product [44].

Other approaches such as spray-drying, spray-freeze drying, or freeze drying with polyethylene glycol (PEG) have been used to generate smaller stabilizer-bearing protein particles but the drawback is a large amount of protein denatures during encapsulation process [59].

More recently researchers developed new PDS by precipitating protein in organic solvent but it is not good for storage, and not validated for therapeutic protein till now. Thus, the field of drug delivery systems for regenerative medicine still has huge scope to discover new approaches and new smart solutions to overcome these limitations with the support of materials science [60].

So the challenge was to develop a PDS that would able to avoid the hydrophobic interface interaction, increase the loading capacity, provide the burst free sustained release and increase storage and process durability. To achieve all phenomena in a single system we have developed new PDS Sugar Glass Nanoparticles (SGnPs) in which we are encapsulating the growth factors inside the SGnPs (Fig.5.2) [44].

Chapter 3

Objectives

A. **Preparation and characterization of GF encapsulated micro/nano particles by conventional emulsion and novel SGnPs methods.**

B. **Study the encapsulation efficiency of protein into polymer particle.**

C. **Study the release profile of protein from polymeric SGnPs.**

D. **Determination of activity loss during process.**

Chapter 4

Materials

1. Poly(D-lactic–co glycolic acid) PLGA, Lactide: glycolide-50:50 (RG502H) , Mw- 7000-17000 Da, Inherent viscosity (dl/g) - 0.16-0.24, End group- Free carboxylic acid

2. Poly Vinyl Alcohol (PVA) high Mw, Average Mw **-**88,000 - 97,000 Da, Hydrolyzed - 87-89%

3. Organic Solvent- Dicloromethane (Pure) HPLC grade, Mw- 84.93 Da

4. Modal Protein- a) Bovine serum albumin (BSA), Lyophilised powder-96% (Agarose gel electrophoresis), Mw- 66000 Da, FITC BSA

5. Surfactant- Span-80 viscosity- 1200-2000 mPa.s, Tween-20

6. Model Enzyme- Horse reddish peroxidase (HRP), Mw- 44000 Da

7. O-phenylenediamine dihydrochloride (OPD) Extrapure , Mw-181.06 Da

8. Hydrogenperoxyde

9. Trizma Hydrochloride (TRIS) 99.0%, Mw- 157.60 Da

10. D(+)Trehalose dehydrate, Mw-378.33 Da

11. Docusate Sodium (AOT)-USP, Mw- 444.56

12. Isooctane (HPLC grade)

13. Light paraffin oil

14. N-Hexane

15. BCA Assay kit

Chapter 5

Experiment and Methodology

5.1 Preparation and characterization of GF encapsulated micro/nano particles by conventional emulsion and novel SGnPs methods

5.1.1 Optimization process of conventional method

Here we used two conventional methods for developing novel GF delivery vehicle. First we optimized W-O-W and W-O-O double emulsion technique. For this we set some parameters to be optimized; are Polymer concentration, Polymer-Protein ratio, Organic-Aqueous phase ratio, Continuous phase volume and concentration, Homogenization speed, particle size, loading capacity, encapsulation efficiency.

Table 5.1- Optimization of W-O-W double emulsion technique

S/N	BSA Con. (w/v) %	PLGA Con. (w/v) %	Homogenization speed for primary emulsion (RPM) and Time	PVA Con. (w/v)%	Homogenization speed for Secondary emulsion (RPM)	Avg. Particle size range (μm)	Encapsulation efficiency (%)
1	5	2.5	12000, 20 Sec	2.0	12000, 5Min.	1.0-2.0	30.71
2	10	2.5	20000, 20 Sec	2.0	12000, 5Min	1.5-2.5	22.97
3	2.5	2.5	12000, 1 Min	2.5	12000, 5 Min	1.0-2.0	18.01
4	2.5	2.5	20000, 1 Min	2.5	12000, 5 Min	1.0-1.5	55.88

5.1.2 W-O-W emulsion technique

2.5% mL PVA solution was prepared. 2.5 mg of BSA was dissolved in 200µL of deionized water to make a clear suspension. This suspension was added dropwise in to solution of 50mg PLGA in 2mL DCM. Homogenize this mixture at 20K for 1 minute to get homogenous, milky and stable primary emulsion. This primary emulsion was added dropwise in 50mL of PVA solution with continuous homogenization at 12K for 5 minute to make double emulsion. This system was kept for continuous stirring for 4.5 hours at 600 rpm to evaporate DCM. After evaporation of DCM we get solid PLGA particle suspended in PVA solution. Centrifuge it at 8K rpm for 15 minutes to get solid particle. Wash these particles with deionized water 3 times. These particle were freeze at -80∘C for 3 hours and then immediate kept for lyophilization for 72 hours. Then particle were preserved at 4∘C for further study [34, 36].

5.1.3 W-O-O emulsion technique

For W-O-O emulsion technique primary emulsion was made by same procedure like W-O-W for secondary emulsion we used 10mL of light liquid paraffin with 100µL of tween-20 instead of PVA solution. We add primary emulsion in dropwise manner with continuous homogenization at 20k for 5 minutes. We get PLGA particle suspended in paraffin. Keep suspension for continuous stirring for 3 hours. To make particle solid and hard add 5mL of n-hexane with interval of 30 minutes. Centrifuge it at 8000rpm for 15 minutes and wash it 3 times with n-hexane. Preserve particles at 4∘C for further process [47, 61].

5.2 Preparation of Sugar glass nanoparticles (SGnPs)

SGnPs are formed from inverse micelles of AOT [sodium 1, 4-bis (2-ethylhexoxy)-1,4-dioxobutane- 2-sulfonate] in isooctane. AOT was dissolved in 12 mL of isooctane in a 25 mL centrifuged tube to produce a 0.3 to 0.4 mol L^{-1} solution. Aqueous phase of 0.4 to 0.8 mL containing protein and other excipients was then added. The mixture was vortexed for 30 s to 2 min to obtain a clear suspension. The aqueous phase contained protein with other protein-specific excipients (e.g., Trehalose sugar, Tween-20, TRIS-HCl). The inverse micelle suspension was flash-frozen by slowly injecting it into a 50 mL vial containing liquid nitrogen

(N2). The vial with the frozen nanoparticles and isooctane was then lyophilized to evaporate isooctane and water (Fig.5.1). After lyophilization, the nanoparticles were washed in isooctane by resuspending and subsequently centrifuging them at 400 g for 10 min. This washing process was repeated 4 to 5 times and finally the SGnPs dispersion (Fig.5.2) was stored in isooctane under desiccation at –20 ° C or –80 ° C for future use [44].
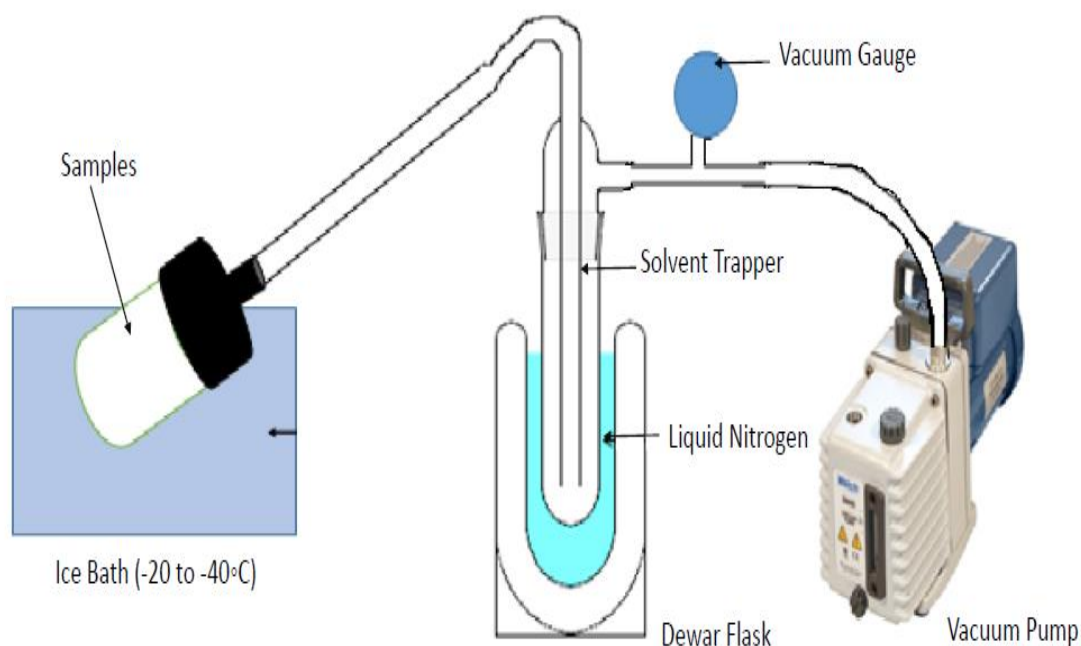

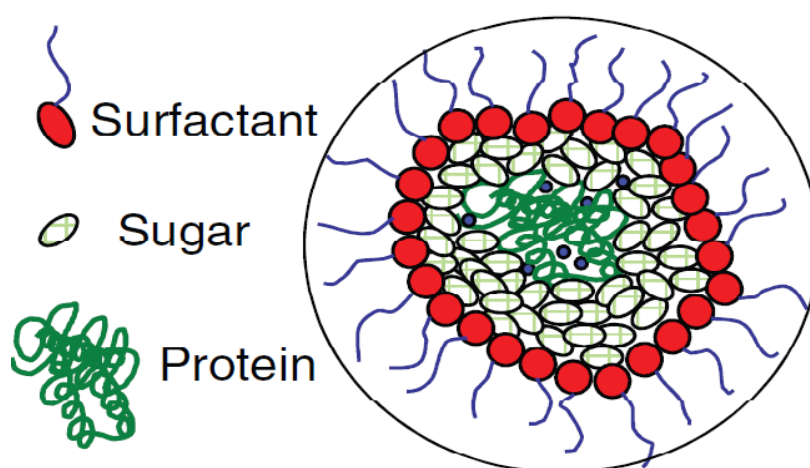**Fig.5.1** Solvent trapping system for sugar glass nanoparticle system

Fig.5.2- Sugar glass nanoparticle: protein laden in sugar matrix that is coated by surfactant

5.3 Encapsulation of SGnPs

These SGnPs are encapsulated in to PLGA polymer by aforementioned double emulsion technique to make a proper delivery vehicle for GFs (Fig.5.3).
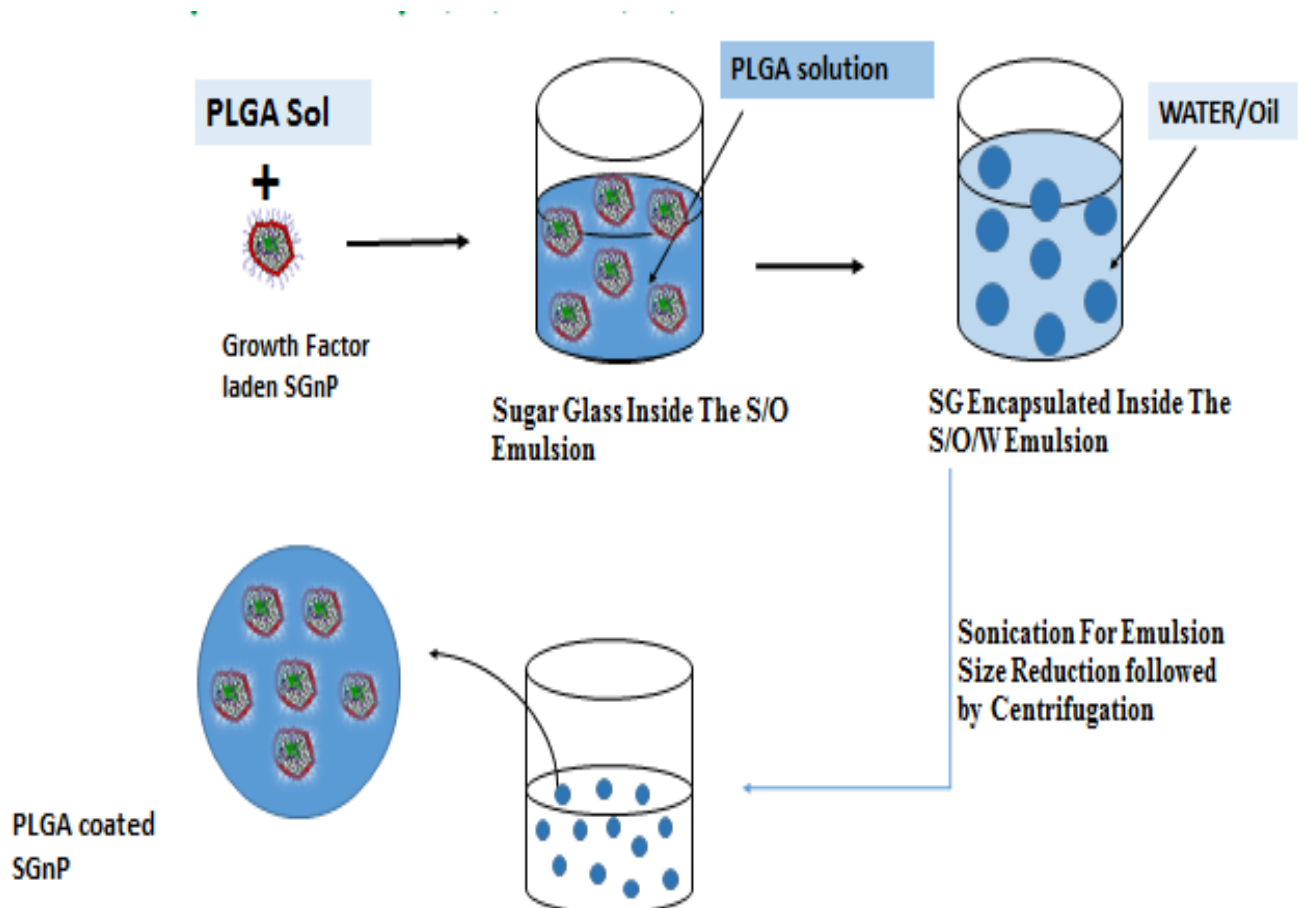

Fig.5.3 Novel SGnPs technique for develop delivery vehicle for GFs. Schematic presentation of

SGnPs distribution across the polymer nanoparticle. For primary emulsion DCM was used, for secondary emulsion 2.5 % PVA solution (W-O-W) and Light liquid paraffin oil (W-O-O) used.

5.3.1 Study of encapsulation efficiency of protein in polymer particles

For the encapsulation efficiency we have also optimize the process that include optimization of polymeric particle digestion, incubation period for protein estimation. For optimizing the process we made triplicate batch of PLGA particle of different loading 1%, 5%, 10% and 20%. Protein encapsulation efficiency was done by BCA Assay kit. This assay kit contains three reagent; A, B, C. For estimation of the protein we made a mixture of A, B and C (BCA solution) in the ratio of 25:24:1 respectively [63]. This BCA assay kit shows protein amount in range of 20 µg/mL to 100 µg/mL. Before calculating encapsulated amount of protein in polymeric particles we mad standard curve of protein (20-100 µg/mL) with BCA assay kit (Fig.5.4). We digested 5.0 mg PLGA polymeric particles in DCM and add 2 mL of PBS (pH-7.4) in 8 mL culture vial in triplicate manner from each batch. We kept it in incubator-shaker at 37^0C for overnight (16 Hrs.).

5.4 Encapsulation Efficiency (EE): - Part of loaded protein encapsulated into nanoparticle. Indirectly it tells the wastage amount of protein during encapsulation. It is also represented in percentage.

$$\%EE = \frac{\text{Weight of encapsulated protein}}{\text{weight of total protein used for encapsulation}} \times 100$$
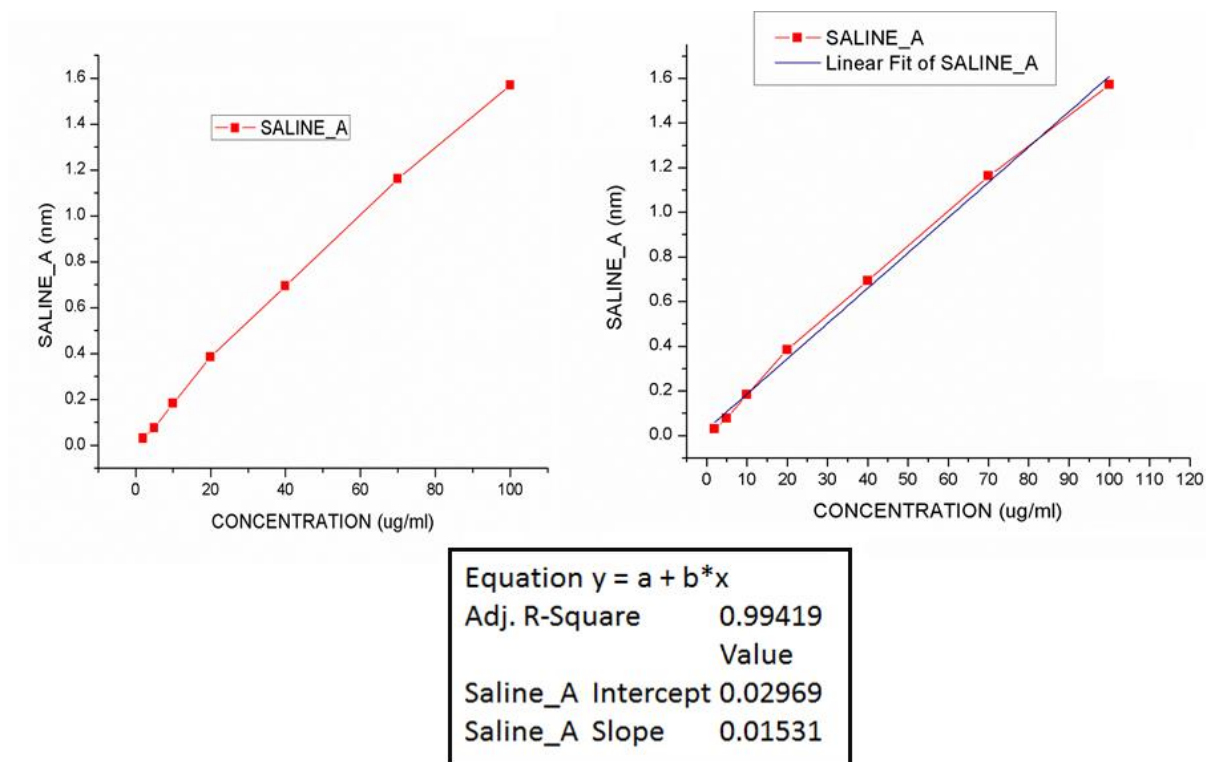
Equation y = a + b*x
Adj. R-Square   0.99419
                       Value
Saline_A Intercept 0.02969
Saline_A Slope   0.01531

Fig. 5.4- Standard curve of BSA by using BCA kit

After incubation supernatant part (Aqueous phase) was carefully separated and diluted it 5 times to bring it with in the range of assay kit. From each vial 150 µL of diluted part was loaded in to 96 well plate in triplicate manner and then add 150 µL of BCA solution was added. Well plate was covered carefully with paraffin membrane and kept for incubation at 37 ^{0}C for 2 hours. After incubation the protein was estimated by micro plate assay reader.

5.5 Study of release profile of protein from polymeric microparticles

We did comparatively release profile study of polymeric particle made by conventional method as well as by novel method. Microspheres were observed over a period of 30 days in PBS at 37 °C. The rate of degradation of PLGA microspheres depend on some factors such as Mw of polymer, water permeability, porosity, additives, environmental pH. The degradation mechanism of PLGA polymer is water penetration into microspheres, the swelling and erosion of PLGA microspheres thus subsequent hydrolysis of PLGA chains, which prompts bulk erosion of PLGA microspheres. We kept 10 mg of different loading (5 and 20%) PLGA particles in eppendorf tube having 1.2 mL of PBS for release study. The sample was collected on 0.17. 0.417, 1, 2, 3, 5, 7, 14, 20, 30 days and BSA estimation was done by BCA assay kit.

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Release study of SGnPs loaded PLGA particle prepared by novel approaches are done in same manner as we did for conventional approach.

5.6 Characterization of polymeric particles

Particle size distribution was analyzed by microscopy (Carl Ziess) and DLS (Beckman Coulter). Surface morphology study was done by Scanning electron microscopy (SEM). To know protein distribution in to polymeric matrix we used fluorescein conjugated BSA (FTIC) to make particles by conventional and as well as novel method. After formation of particle we examined these under fluorescent microscope at 512 nm (Fig.6.13 & 6.14). Each and every step of this experiment carried in dark to make sure the fluorescent effect proper. Each assay is done in triplicate manner.

5.7   Determination of activity loss during process

For determining the activity loss during process we used HRP as model enzyme which shows its activity towards OPD substrate in colorimetric assay at 435 nm [62]. That parameter was also optimized. For getting optimized time of incubation we did BCA assay and OPD assay. BCA assay showed release of total HRP over the time while OPD assay shows total activity of released protein over the time. For the comparison we made HRP standard curve with BCA and OPD (Fig.5.5 & 5.6). For this we took 10mg of HRP encapsulated PLGA particles and kept in 5mL TRIS-HCl (pH 5.4) at room temperature. Each hour we collected sample and estimated the activity. This experiment was carried out in triplicate.
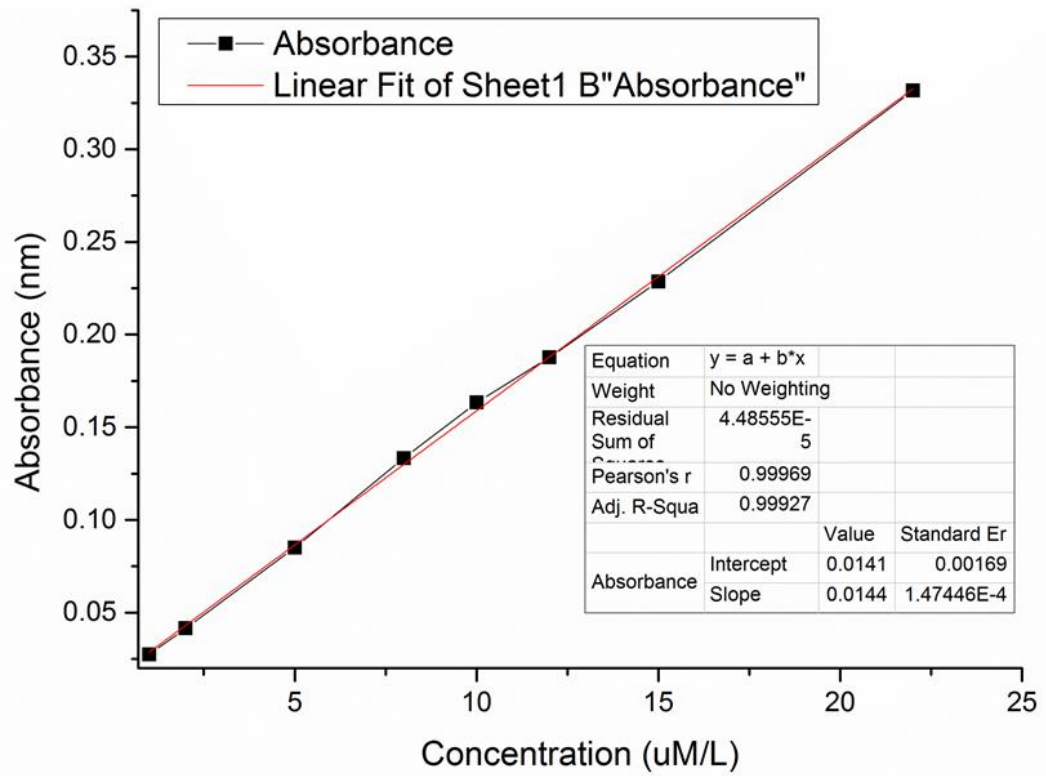
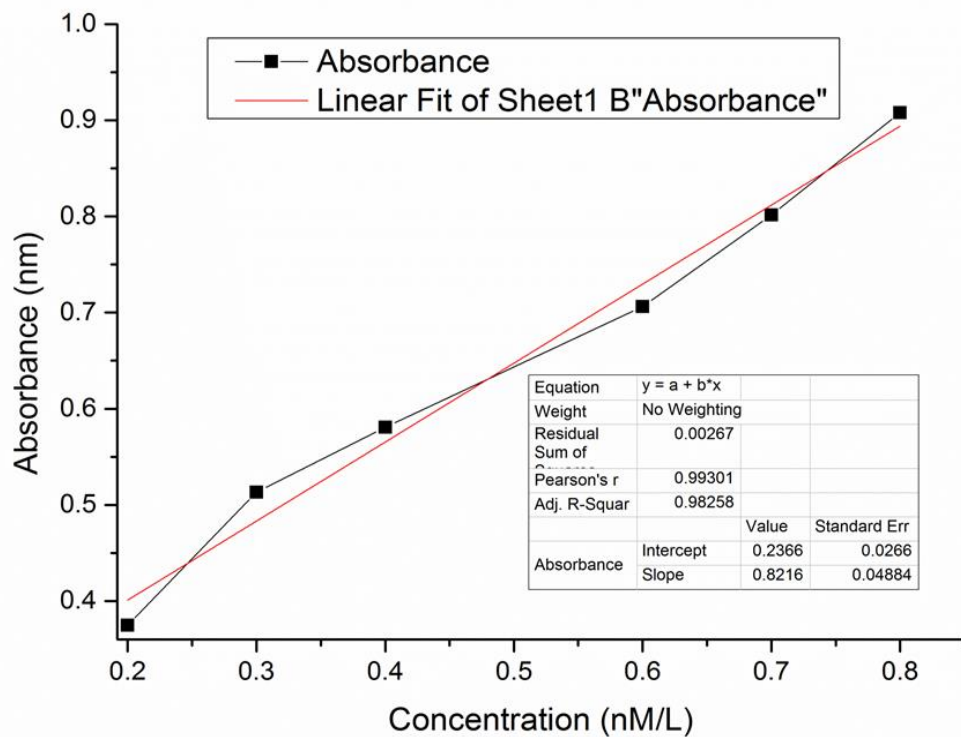**Fig.5.5** HRP concentration Standard curve by BCA assay kit

Fig.5.6 HRP activity standard curve with OPD substrate

Chapter 6

Result and Discussion

6.1 Optimization of W-O-W and W-O-O double emulsion method

2.5 % BSA solution in DIW, 2.5 % PLGA solution in DCM, aqueous-organic phase ratio-0.1, primary emulsion at 20K rpm for 1 minute, secondary emulsion 12K for 5 minute, 50mL of 2.5% PVA gives highest encapsulation efficiency (55.88%) and average particle size (1.0-1.5µm) and for W-O-O 10mL of liquid light paraffin, 100µL SPAN-80 was optimized for maximum encapsulation efficiency.

6.2 Morphological study of particle

6.2.1 Particle size distribution

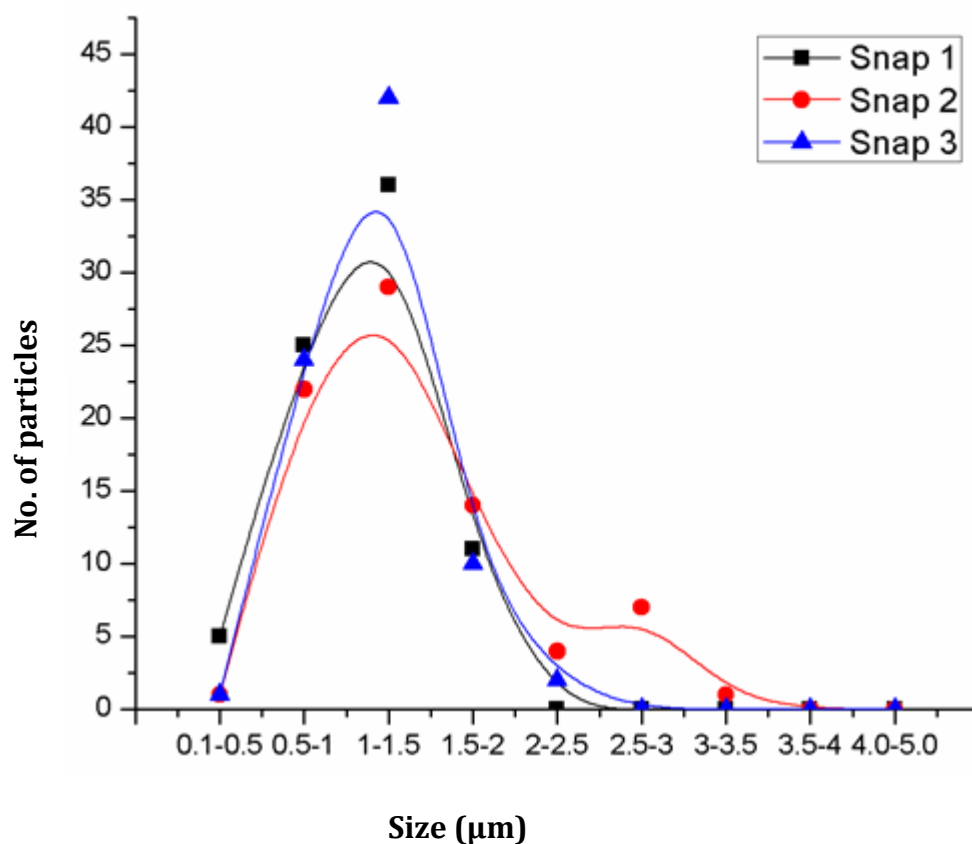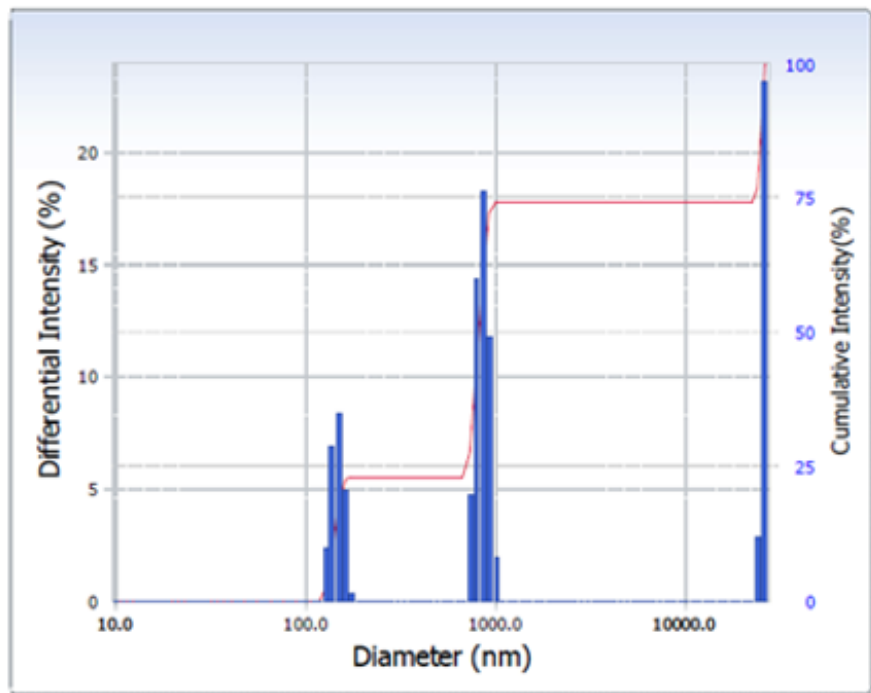By the optical microscopy and DLS showed the particle size 1.0-1.5 µm (Fig.6.1-6.2).

Fig. 6.1 Particle size distribution by optical microscopy

Diameter   (d)   : 1163.5   (nm)

Polydispersity Index (P.I.) : 0.433

Fig. 6.2   Average diameter of PLGA particles by DLS
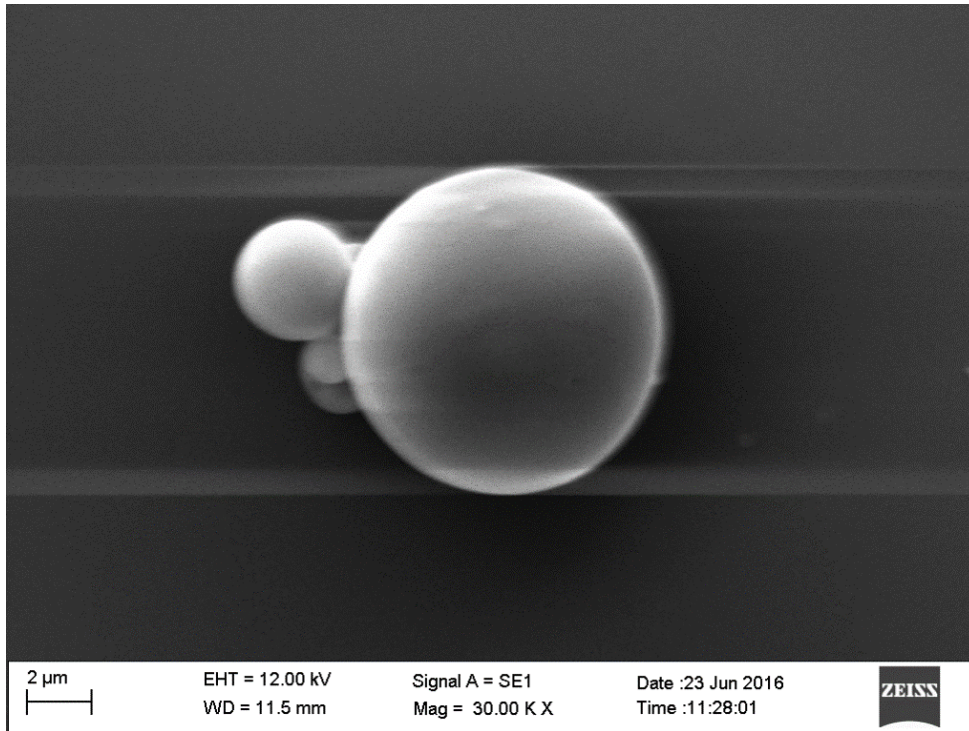
6.2.2 Surface morphology

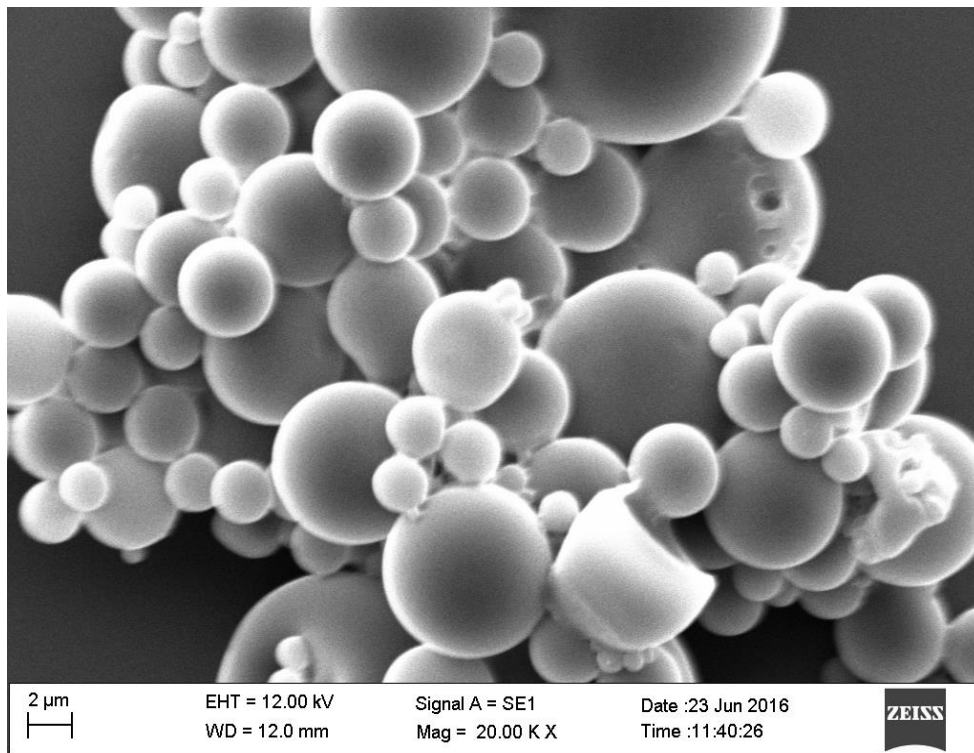**Fig.6.3** SEM image of PLGA microparticle by conventional W-O-W emulsion technique.

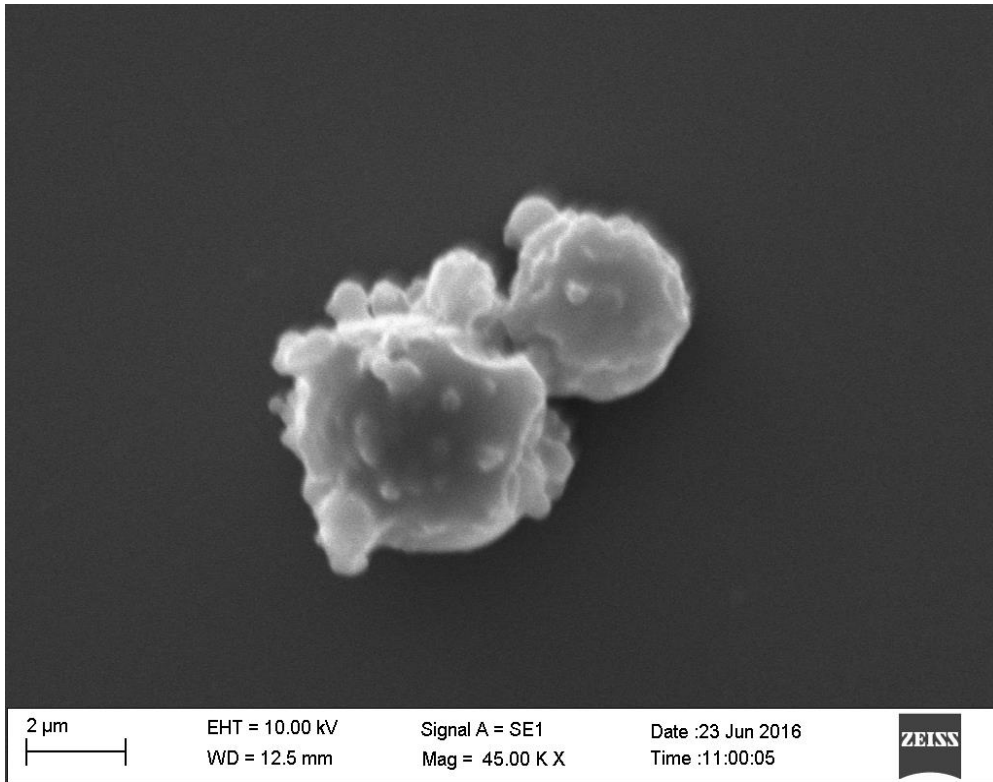**Fig.6.4** SEM image of PLGA microparticle by novel SGnPs-O-W emulsion technique.

Fig.6.5 SEM image of PLGA microparticle by conventional W-O-O emulsion technique.
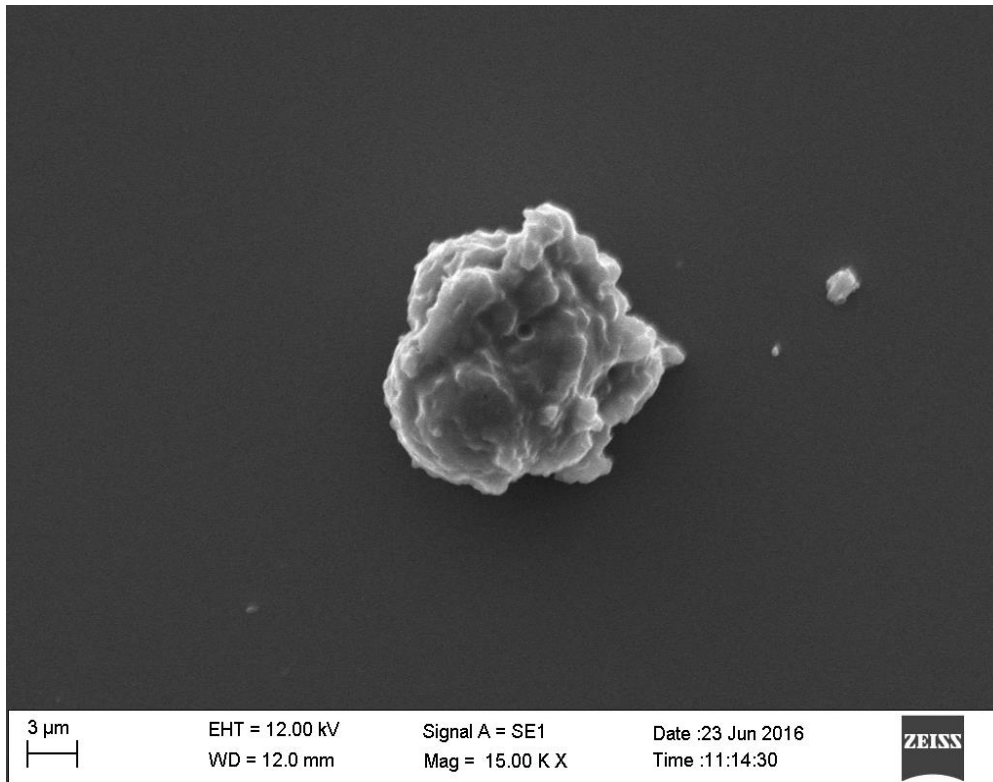

Fig.6.6 SEM image of PLGA microparticle by novel SGnPs-O-O emulsion technique.

SEM result show that the W-O-W emulsion gives smooth, regular surface and no pores or pits on the surface while W-O-O emulsion produced irregular surface having many pores on surface. This could be the reason for higher burst release of protein from the latter particles. After studying all four images it can be concluded that the presence of SGnPs in polymeric solution doesn't change the surface property. Surface morphology are same for W-O-W and SGnPs-O-W, W-O-O and SGnPs-O-O. (Fig.6.3-6.6).

6.3 Encapsulation efficiency of polymeric particle

It was found that at very low loading (1%) show 100% EE not depends on preparation method, it means very low amount of protein can be completely entrapped by PLGA. As we increase the loading amount it shows lesser loading capacity it means up to a certain amount PLGA can entrap the protein not more than that but it can be increased by changing the particle preparation method (Table-6.1). W-O-O emulsion technique shows more encapsulation efficiency in comparison to W-O-W because of protein distribution (Fig.6.13 & 6.14). Novel method (SGnPs-O-W) shows 100% EE which make it more useful tool to deliver growth factors.

Table-6.1 Encapsulation efficiency of different loading and methods

Method		1%	5%	10%	20%
Conventional Approach	W-O-W	100	40.3	25	7
	W-O-O	100	50.59	35	28
Novel Approach	SGNPS-O-W	100	100	-	-
	SGNPS-O-O	100	50	-	-

6.4 Study of release profile

Fig.6.7 Release study of 5% BSA loaded PLGA particle prepared by conventional W-O-O emulsion method.

Fig.6.8 Release study of 5% BSA loaded PLGA particle prepared by conventional W-O-W emulsion method.

Fig.6.9 Release study of 20 % BSA loaded PLGA particle prepared by conventional W-O-O emulsion method.
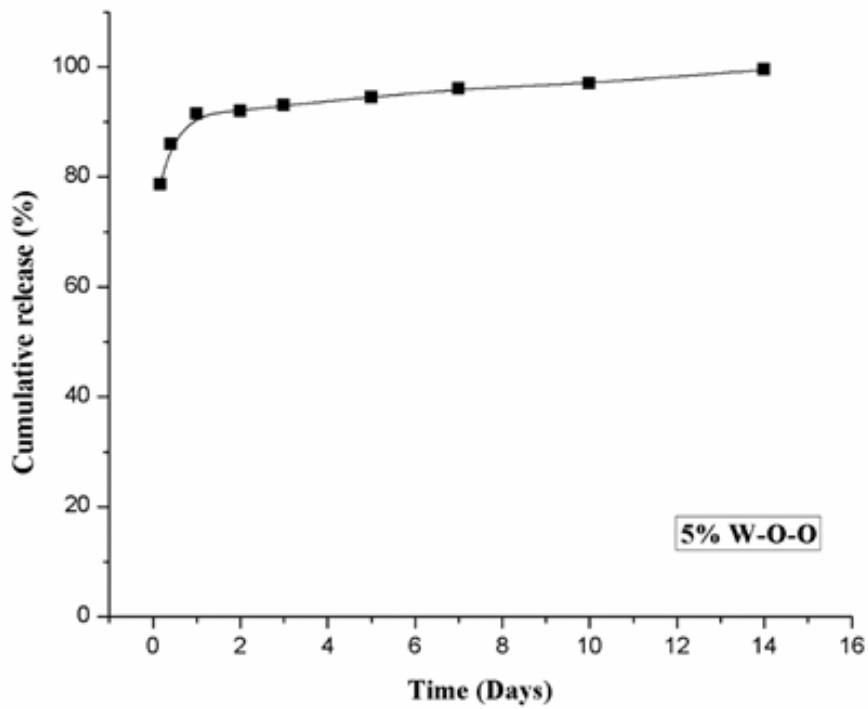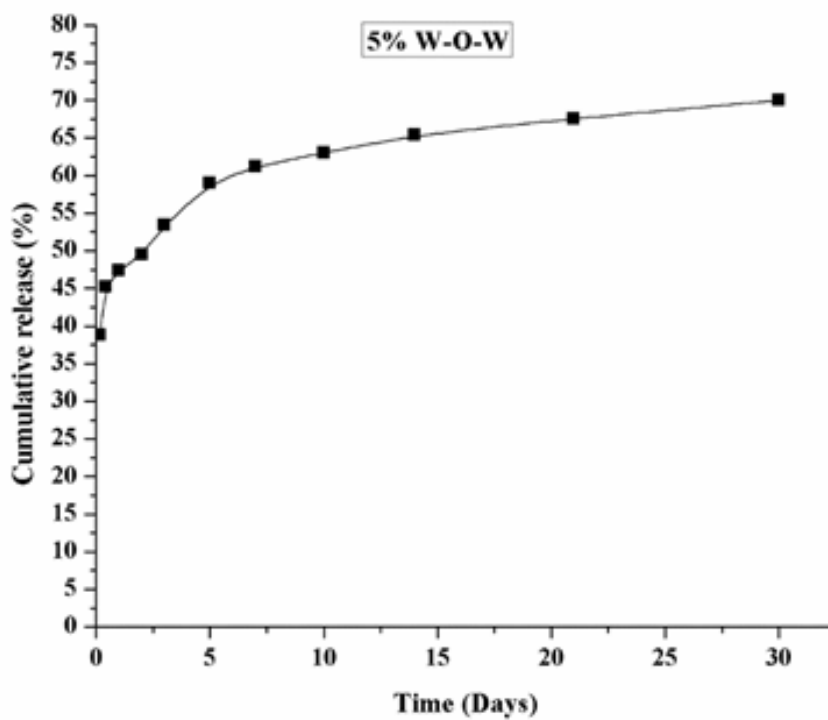

Fig.6.10 Release study of 20% BSA loaded PLGA particle prepared by conventional W-O-W emulsion method.
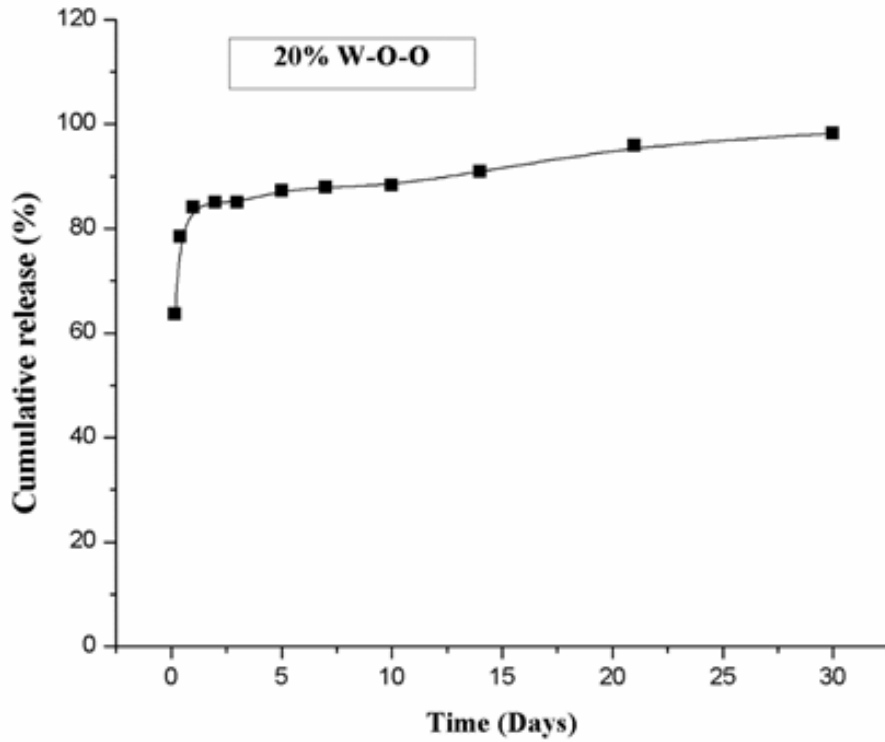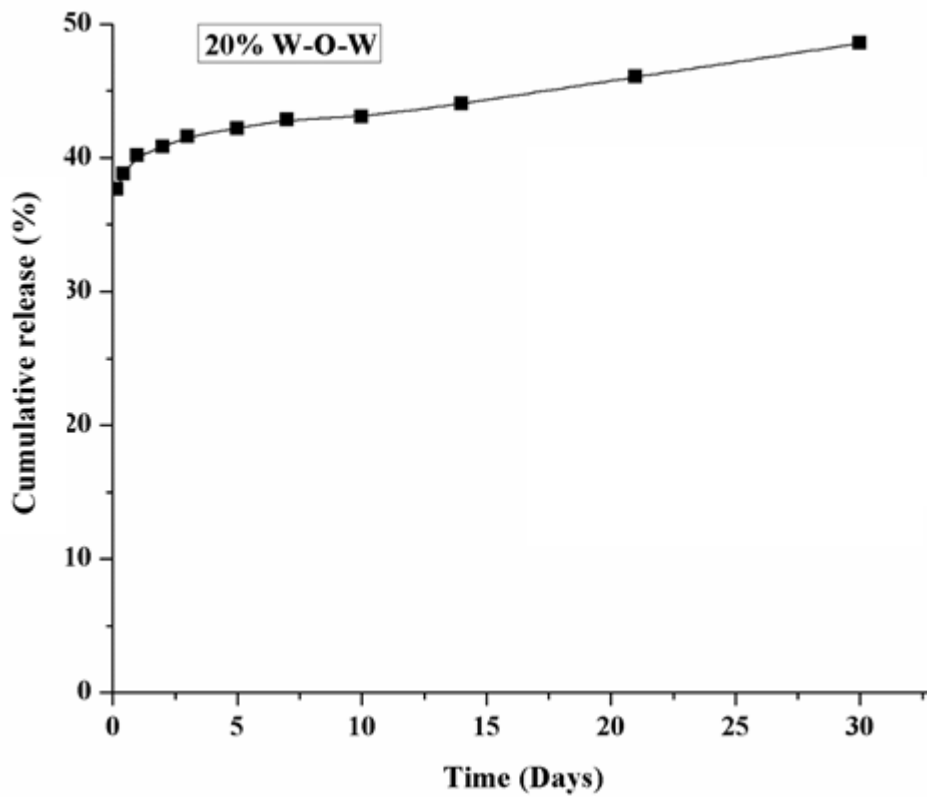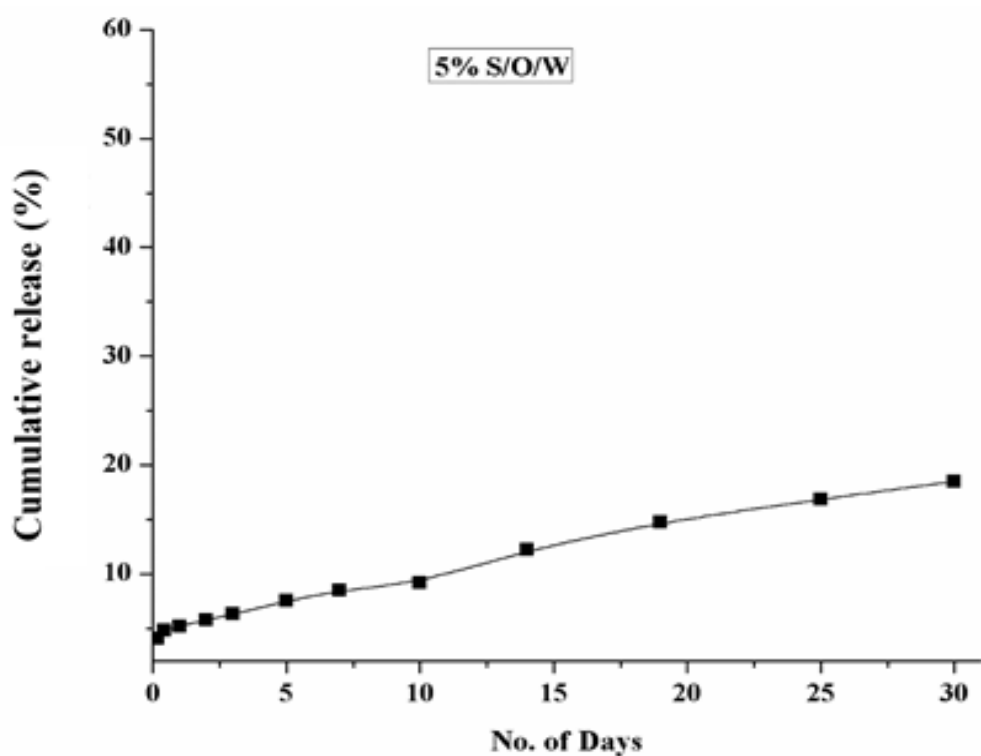
Fig.6.11 Release study of 5 % BSA loaded PLGA particle prepared by novel SGNPS-O-W emulsion method.
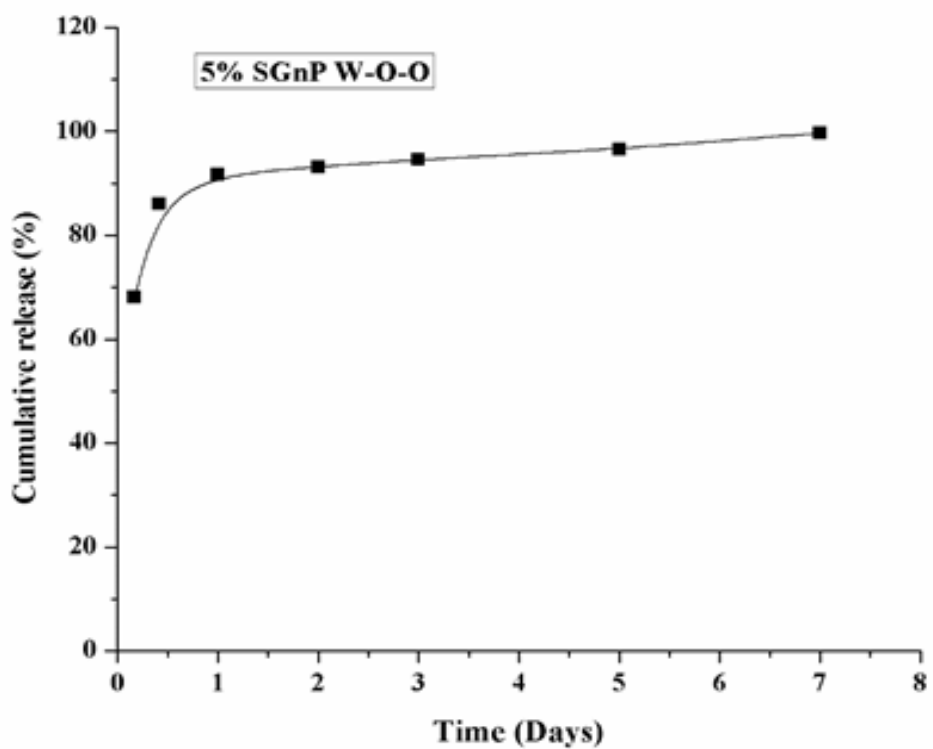

Fig.6.12 Release study of 5 % BSA loaded PLGA particle prepared by novel SGNPS-O-O emulsion method.

After studying it was found that W-O-W shows less initial burst release compared to W-O-O emulsion technique. Reasons are discussed in morphological study (Chapter-6.2). After studying the release profile it was found that SGnPs-O-W shows sustained release for a long time. It released only 18 % of BSA in 30 days (Fig.6.11). It is because SGnPs are hydrophobic in nature so they don't release protein easily in PBS and also because of surface morphology of particles (Fig.6.14) which is regular smooth and without any pits or pores.

It showed very interesting release for SGnPs-O-O approach; unlike SGnPs-O-W it showed initial burst release and released all BSA within 7 days. (Fig.6.15). It is because continuous phase is oil and SGnPs are hydrophobic in nature, so SGnPs have tendency to migrate towards surface during preparation of secondary emulsion and also because of the surface morphology of particles prepared by oil in oil emulsion which creates pits and pores on surface and this is also a reason of low encapsulation efficiency in comparison to SGnPs-O-W.

6.5 Protein distribution
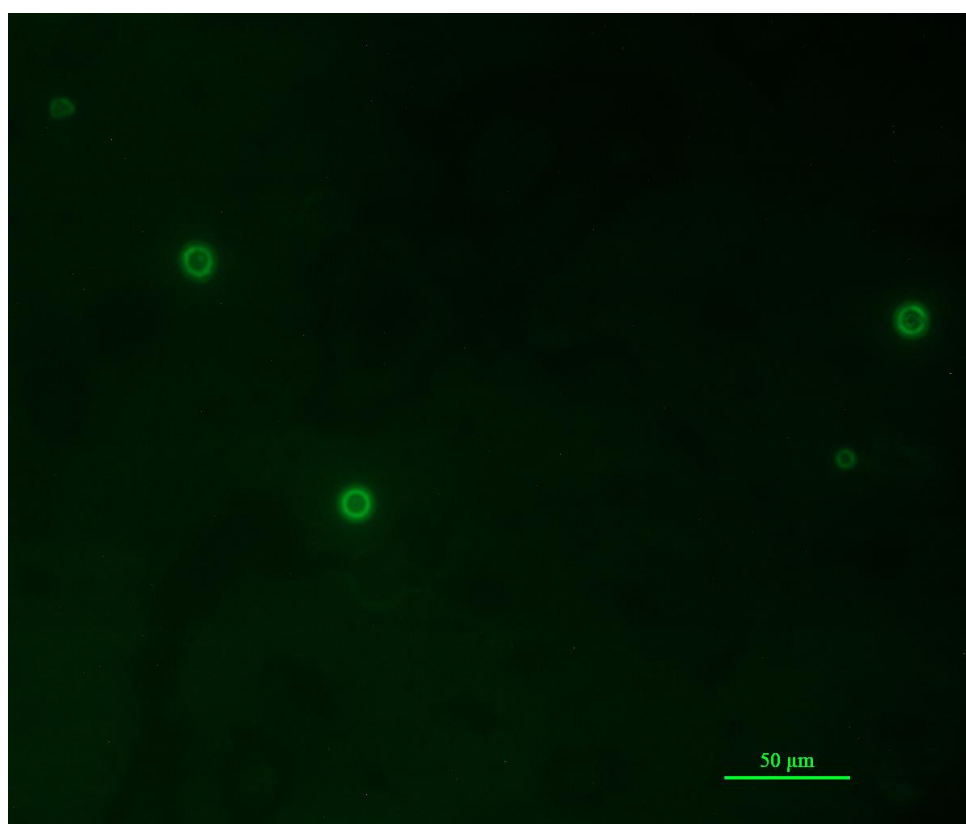
Fig.6.13 Fluorescent microscopic image of conventional W-O-W emulsion technique
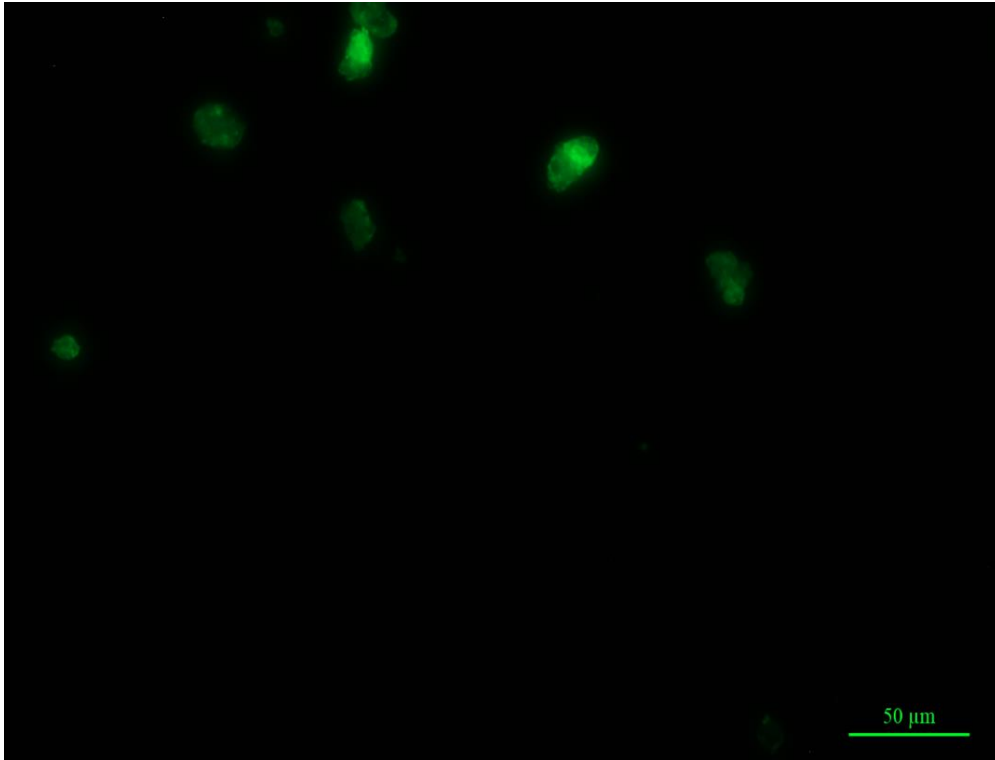
Fig.6.14 Fluorescent microscopic image of conventional W-O-O emulsion technique

After study of fluorescent images it was found that BSA accumulated in the peripheral part of particle, while in W-O-O emulsion technique BSA was evenly distributed over the surface of matrix (Fig.6.13 & 6.14) because these particles were washed with n-hexane which is nonpolar and it is possible that surface protein was not washed and that's may be a reason of initial burst release and also give a clue why W-O-O has more encapsulation efficiency.

6.6 Activity loss
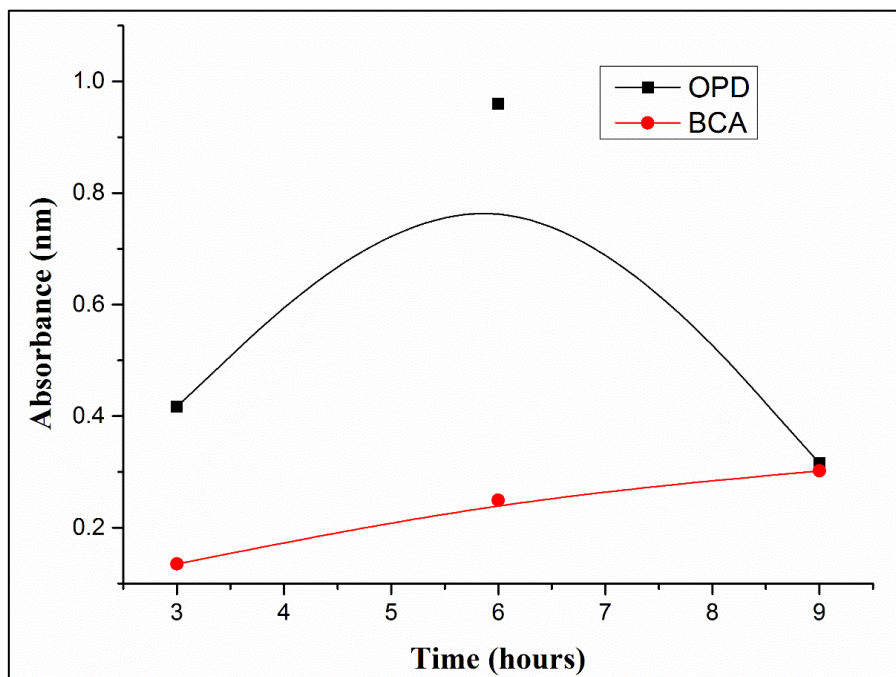
Fig.6.15 Optimization curve for HRP activity

To determine activity loss of released protein we used Horse reddish peroxidase (HRP) instead of BSA because BSA doesn't show any enzymatic activity. HRP shows activity with OPD substrate in the presence of hydrogenperoxide. It was found that HRP extracting from particle over the time shows bell shape curve for activity, up to 6 hours ate activity increases but after 6 hours HRP release increases but activity decreases so the optimum incubation period is 6 hours at room temperature (25^0 C) (Fig. 6.15).

Chapter 7

Conclusion and future work

We have prepared PLGA micropaticles by conventional (W-O-W & W-O-O) and novel method (SGnPs-O-W & SGnPs-O-O). Comparative studies has shown the following results:

Surface morphology of W-O-W is even, continuous and without any pores while W-O-O the surfaces have lot of pores that's why W-O-O shows more burst release and no effect of SGnPs on the surface of particles synthesized by conventional methods (Fig.5.15 - 5.18).

Novel method (SGnPs-O-W) is showing more protein encapsulation efficiency (100%) in comparison to conventional methods (W-O-W & W-O-O) so we can effectively encapsulate very less amount of GFs by this novel method (tab-5.2).

SGnPs-O-W is showing sustained release of protein because the hydrophobic nature of SGnPs (Fig.-5.9) and this technique could be a great approach to deliver GFs for long duration sustain release, but when we tried SGnPs-O-O; it showed only 50% encapsulation efficiency (Tab-5.2) and high burst release in comparison of other process (Fig.5.10). For less encapsulation efficiency possible reason may be the migration of SGnPs in to the continuous oil phase as we know like attracts like and the remaining SGnPs may be arranged on the surface of PLGA matrix and showing high burst release. In future we would like to optimize and change the SGnPs-O-O method so we can get more encapsulation efficiency and sustain release.

In near future this novel delivery system can be modified according to use or site of application. SGnPs can be electrospinned with polymer solution to get nanofiber scaffold, it can also be encapsulate in hydrogels, can be modified in to fibrous patches for dermal application, wound healing etc.

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