4. Summary and conclusions

The three dimensional structures of proteins are not only important for its specific functionality but also necessary for other supportive functions like feedback mechanism, transport, and solubility in physiological environments. Development of stable protein formulations may require more resources and efforts than that of conventional small molecule pharmaceuticals. Proteins typically have more stability issues as a result of their complexity and delicate structural flexibility. A great number of researches on protein stability has been done and information on that are readily available in the literature however not much promising results and outcomes have been reported because ultimately, it ideally requires retaining the native structure of protein. According to FDA, a pharmaceutical product is considered as stable as long as it deteriorates by no more than 10 % in 2 years. However in case of proteins the term stability needs to be defined with more accuracy and precision. Most of the time, the activity of peptides depends on the primary and possibly secondary structures, whereas proteins possess an additional tertiary and sometimes even a quaternary structure, that allows the protein chains to fold and adopt a three-dimensional conformation. So maintaining these fundamental physicochemical, conformational and the three dimensional structure of protein is necessary for its successful formulation development.

For protein formulation development the carrier based system often offers many advantages of controlled release, targeted release, pulsative or responsive release depending upon the type of carrier system used. However retaining protein stability during formulation development and in the final formulation is one the biggest hurdle to be overcome by pharmaceutical/ biotechnological industries. Methodology that involves excipients and various steps protecting therapeutic proteins to be delivered is absolute necessities for the protein formulation development. As it is very well known that the solid protein formulation offers more stable form of protein a solid in oil in water (S/O/W) emulsification method was selected for carrier based formulation development of proteins using FDA approved poly (D, L-lactide-co-glycolide) (PLGA) encapsulating olymer. This method requires a number of steps from initial lyophilization with an xcipient to the primary and secondary emulsification steps. The initial lyophilization vas done to get the solid form of protein particles which could kinetically trap the rotein to be encapsulated. This kinetic trapping of protein would protect the whole hree dimensional structure of protein from denaturation and aggregation at later stage

due to the use of organic solvents at interfaces etc. These factors were considered for the selection of excipients. Polyols like PEG are known for the interaction with many proteins but understanding the molecular level phenomenon that occurs between the protein and PEG was desirable to unlock the specific interactions needed for the protein stabilization. Initial lyophilization step was optimized using PEG for kinetic trapping of BSA (bovine serum albumin) which was taken as a model protein. The selection of a particular molecular weight PEG was performed by a specific protocol which forms the kinetically trapped solid protein particles inside of PEG molecules in co-lyophilized form. The different molar ratios (1:0.5, 1:0.75, 1:1 and 1:1.5) of BSA: PEG of different molecular weight (PEG 8000, PEG 6000 and PEG 3350) were selected for the study. The released BSA from solid protein particles prepared at different ratios of BSA: PEG was analyzed by circular dichroism (CD) spectroscopy. It was found that the BSA released from BSA: PEG molar ratio of 1:0.75 showed the maximum secondary structure conformational stability for each PEG sample (PEG 8000, PEG 6000 and PEG 3350). As PEG 8000 provided maximum conformational stability to BSA, it was selected for further studies. The effect of bioprocessing steps such as lyophilization and sonication on BSA was investigated by taking a mixture of BSA and PEG at different molar ratios (1:0.25, 1.0.5, 1:0.75, 1:1, 1:2, 1:3 and 1:4). Change in the ellipticity at 208 nm using CD-spectroscopy was used for visualizing varying α helical contents of released BSA. The released BSA from BSA:PEG molar ratios of 1:0.75 (38.29 % α-helical content with maximum MRE value of 15103.06) exhibited maximum conformational stability. To understand the molecular level interaction responsible for this conformational stability the flexible body docking studies of PEG on the BSA molecule was performed. The docking study revealed PEG molecules interacting with the hydrophobic core residues of the BSA. While calorimetric studies (ITC) for PEG-BSA interaction revealed the exothermic process, the DSC study showed highest thermal stability (T_m value 72°C) with additional peak showing the strong ligand binding to the protein (BSA) released from BSA:PEG molar ratio of 1:0.75. To further explore the mechanism of PEG binding to the BSA, the released BSA was analyzed by fluorescence spectroscopy (tryptophan quenching of released BSA) and the released BSA from BSA:PEG molar ratio of 1:0.75 showed the compaction of BSA. The binding studies with dye 8-anilino-1-naphthalene sulfonate (ANS) unveiled displacement of PEG from the hydrophobic core residues of the BSA at BSA:PEG molar ratio of 1:0.75. This

abilization of protein was further proved by the urea based denaturation study ghlighting the importance of hydrophobic core residues as even at higher ɔncentration of urea (6.25 molar) the released BSA from BSA: PEG molar ratio of :0.75 showed maximum chemical stability.

he protein therapeutics can be administered by carrier based system like microspheres/ anoparticles of biodegradable polymers (PLGA, PLA), polymeric vesicles like olymerosomes and micelles etc. The protocol for microspheres based formulation evelopment encapsulating BSA as a model protein was optimized. The microspheres ere prepared by using carboxy methyl cellulose (CMC):Tween20 as surfactant blend in ıner aqueous phase. The selection of a particular ratio of CMC:Tween 20 was ptimized by preparing four kinds of microspheres containing different ratios of ꞉MC:Tween 20 (1:1, 2:1, 3:1, and 4:1). The selection of specific ratio of surfactant lend was done on the basis of particle size analysis (DLS) of prepared microspheres nd conformational stability analysis (CD-spectroscopy) of released BSA. The nicrospheres prepared with 3:1 ratio of CMC:Tween 20 have found to be best among all ecause it showed the narrow size distribution and also smaller size with single opulation [3:1- (BC-30 nm-60μ), (BS- 5μ)]. Also the released BSA from this ratio (3:1 atio of CMC:Tween 20) showed the maximum conformational stability. This ratio of ꞉MC:Tween 20 was selected for further studies. Then optimization of PLGA oncentration with respect to concentration of BSA was carried out with four different 3SA: PLGA ratio (1:5, 1:10, 1:15 and 1:20) and BSA released from microspheres was ınalyzed for conformational stability (confirming the small size and low PDI of the orepared microspheres). The microspheres prepared with BSA: PLGA weight ratio of 1:10 showed narrow size distribution [without PEG (BC) - 4.3 μ, with PEG (BS) - 3.9 μ]. This BSA: PLGA weight ratio of 1:10 showed the maximum conformational stability of released BSA and was even highest among the samples prepared with PEG. This weight ratio of BSA:PLGA was selected for the further studies. The selected surfactant mixture of CMC:Tween 20 was further explored by analyzing the stability of loaded BSA on varying the type of surfactant mixture. The study on three kinds of surfactant mixture (CMC:Tween 20, CMC:Tween 80 Tween 20:Tween 80) with PEG and without PEG were carried out by preparing six kinds of formulation. Solid in oil in water (S/O/W) emulsification method was employed to prepare the microspheres. The loading of BSA In PLGA based microspheres was found to be 69 % in all the six formulations.

The *in-vitro* release behavior of BSA, from all the six formulations showed the controlled nature of release. The comparative analysis of particle size of all the six formulations was performed. It was found that the microsphere prepared with CMC:Tween 20 as a surfactant blend in inner aqueous phase along with PEG at primary lyophilization step showed the particle size of acceptable range (5μ) with single population. The SEM analysis of prepared microspheres showed spherical confinement geometry. The stability analysis of released BSA by SDS-PAGE showed intactness in decreasing order of CMC:Tween 20> CMC:Tween 80> Tween20:Tween 80. The comparative analysis of stability of released BSA from all the six prepared microspheres showed interfacial phenomenon responsible for stabilizing the loaded BSA. The CD-spectra of released BSA from microspheres prepared with PEG with single surfactant mixture showed the increase in ellipticity of released BSA when compare to BSA released from microspheres prepared without PEG. The prepared microspheres also showed surfactant blends in the following increasing order of ellipticity CMC:Tween 20> CMC:Tween 80> Tween 20:Tween 80. The fluorescence emission wavelength of maximum intensity (λ_{max}, nm) of all the 6 formulations compared for tertiary structure analysis which showed compactness (328 nm) in case of released BSA from microspheres prepared with CMC:Tween 20 and PEG. The size exclusion chromatography (SEC-HPLC), Native-PAGE and Thioflavin-T assay studies proved no aggregates formation of released BSA from any of the prepared microspheres. The calorimetric study of released BSA and prepared microspheres showed interfacial process to be exothermic in all microsphere formulation. The thermodynamic interaction studies between BSA and PEG (affinity capillary electrophoresis) were found to be enthalpy driven and the temperature of melting (T_m) and enthalpies (ΔH) data indicated that these were higher for BSA released from microspheres prepared with PEG than those without PEG. To explore the effect of type of surfactants blend on the thermal stability of released BSA was monitored by analyzing the change in secondary structural contents. The thermal-CD of released BSA from 12 different types of microspheres prepared with single (Na-CMC, Tween 20 and Tween 80) and double surfactants systems (CMC:Tween 20, CMC:Tween 80 Tween 20:Tween 80) were performed. The analysis of thermodynamic graph with respect to change in temperature (heating) showed little effect of temperature on the secondary structural contents (ellipticity) in released BSA from microspheres prepared with surfactant blend as compared to those

with the single surfactant system. The microspheres containing PEG showed kinetic trapping and restriction of rotational and tensional motions of BSA by excluding the volume of water around the protein due to macromolecular crowding by PEG. This would have led to the maintenance of hydrodynamic radius and compactions of BSA which further caused the protections of various linkages and interactive bonds inside the conformational structure of BSA. On conclusion, based on our observations and understanding on the role of PEG in increasing the secondary structure content, compactness and thermal stability of the protein (BSA), spherical confinement geometry of microspheres and the role of surfactants in the stability of this protein (BSA), the following two mechanisms could be proposed to be operating at the interface, responsible for making the protein more compact and structurally integrated: 1) Macromolecular crowding due to PEG and molecular confinement of spherical particles at the interface., 2) Electrostatic repulsion forces between the particles due to (a) chosen surfactants and their particular ratio, (b) surfactant orientation on the particle surface and (c) interfacial increase in Gibbs free energy (ΔG).

Encapsulation of therapeutic protein of importance like insulin is a challenge as it is to be delivered in structurally integrated form. As insulin molecule easily undergoes denaturation and aggregation when exposed to external environmental conditions during formulation, so designing a development strategy is very important for its delivery without compromising its stability. Insulin loaded nanoparticles using three kinds of surfactant systems were prepared for addressing the stability of loaded insulin on prepared nanoparticles. The insulin molecule easily undergoes aggregation at high pH value and on the other hand carboxy methyl cellulose (CMC) to be used as surfactant needs the high pH value for dissolution. Hence CMC was excluded from preparation of insulin loaded nanoparticles and instead, three surfactant systems Tween20:Tween80 3:1), small molecular weight poly vinyl alcohol (SMWPVA) and high molecular weight oly vinyl alcohol (HMWPVA) were selected. The released insulin from three anoparticles formulations was compared for structural integrity. These preparations howed different particle size distribution when analyzed by DLS and SEM. Nanoparticles prepared with Tween 20:Tween 80 mixture showed particle distribution in he range of 300 to 1600 nm (800 nm mean diameter) while nanoparticles prepared with MW-PVA and HMW-PVA were having particles in the range 800 nm to 8 μ (4.5 μ ean diameter), and 200 nm to 1200 nm (400 nm mean diameter), respectively.

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a leading molecular mechanism for prevention of aggregation of insulin during PLGA based nanoparticles formulation development.

Maintenance of protein stability is one of the main aspects of any formulation development however enhancing certain properties of protein of interest could also be important aspect of protein formulation development. Combining the advances of carrier based with enhancement/ retention of important properties of a protein would constitute a great achievement in the carrier based formulation development of any protein. In light of the above, the PLGA based insulin loaded nanoparticles of insulin were prepared taking advantage of microstructural features of surfactant (PVA) and temperature of formulation process [(S/O/W) emulsification method]. By this modified methodology and with given microstructural features of PVA the nanocrystalline form of insulin inside of PLGA nanoparticles was prepared. The nanocrystalline formulation offer several advantages like better handling, stability, and varied dissolution characteristics, the last property allow better control over bioavailability. The controlled release of insulin from PLGA nanoparticles offers other advantage of long term release and therapy. The PVA used in the present case possesses crystalline nature which imparted the crystallinity to the prepared nanoparticles as confirmed by the PXRD studies. The crystallinity of prepared nanoparticles was also confirmed by SEM (crystalline edges formation) and TEM based SEAD analysis (electron diffraction pattern). The microstructural feature analysis of lyophilized PVA indicated the porous structure formation with (1% w/v) PVA showed nanoscopic pore formation. This nanoscopic pore formations and crystalline features of PVA was thought to be responsible for nanocrystals formation of insulin inside nanoparticles. This was confirmed by the DSC based enthalpy of fusion and crystallinity index analysis of prepared nanoparticles by changing the PVA concentration (0.5 % to 4 % w/v) in inner aqueous phase. The crystalline surfaces and microstructural features of PVA might have generated the template for heterogeneous nucleation of the loaded insulin moieties. The effect of PEG used in the first step of lyophilization was analyzed. It was found that PEG caused the formation of loose aggregates of insulin (LAI) as confirmed by mass spectroscopy and TEM micrographs analysis (released insulin). These loose aggregates could have attached to the nanoporous template offered by the added PVA in inner aqueous phase (1 % w/ v). To confirm that the PVA molecule provided the template and interaction with LAI the insulin-PVA interaction studies was performed using Raman spectroscopy.

The Raman spectroscopy analysis of co-lyophilized insulin-PVA mixture showed the peaks to be associated with hydrogen bond formation which was further confirmed by the FTIR spectroscopy analysis. The nanocrystal nature of released insulin from PLGA nanoparticles was confirmed by releasing the insulin molecules in double distilled water and analyzing it by HRTEM based EDX analysis for the presence of zinc and by HRTEM based SEAD diffraction pattern of surface of nanocrystal. Finally, the results were combined to understand the molecular mechanism of nanocrystal formation inside of PLGA nanoparticle.

Another important fact on carrier based protein delivery is the availability of very few numbers of FDA approved biodegradable polymers (PLGA, PLA). Hence, synthesis of a di-block polymer from the already existing polymer PEG (hydrophilic portion) and PLA (hydrophobic portion) were explored. Stannous octoate as a catalyst was used to synthesize the di-block polymer using ring opening polymerization methodology. The synthesized block polymer was characterized with the help of FTIR and NMR spectroscopy. The FTIR and NMR based studies of heated block polymer revealed the hydrogen bond assisted crosslinked structure formation when heated at 180°C for 15 mins. The synthesized block polymer was explored for the unique property of crosslinking structure formation with the help of SEM, TEM and DLS based studies, while the analysis of viscosity of heated block polymer was performed by viscometer.

The study resulted in the development of a new formulation "Solid in oil in water (S/O/W) emulsification" methodology for biopharmaceuticals delivery along with synthesis of new block polymer.