Stability of Proteins in Aqueous Solution and Solid State

S. JACOB, A. A. SHIRWAIKAR*, K. K. SRINIVASAN, J. ALEX, S. L. PRABU, R. MAHALAXMI AND RAVI KUMAR

Department of Pharmaceutics and Department of Pharmaceutical Chemistry, Manipal College of Pharmaceutical Sciences, MAHE, Manipal-576104, India.

In the last two decades, proteins and peptides have become an important class of potent therapeutic drugs. However, their susceptibility to chemical and physical degradation presents a challenge to formulation scientists, for the development of stable pharmaceutical preparations. This is in part, due to the unique physicochemical and biological properties of the proteins and peptide drugs. The insight into the fundamental understanding of the mechanism, by which protein stabilizes, will help in the formulation of protein based pharmaceuticals.

Biotechnology has established itself, as a mainstay in pharmaceutical research and development, and new protein based pharmaceuticals will enter the market at an increasing pace, during the next decade of the new millennium. Recombinant DNA and monoclonal antibody technologies are providing exciting opportunities for new pharmaceutical development and new approaches to the diagnosis, treatment, and prevention of diseases. Molecular biology has now given us the tools to expand the range of peptide and protein based drugs, to combat poorly controlled diseases. Such drugs include synthetic vaccines, that promise to offer protection against carcinogen and toxicants. There are already dozens of protein products on the market, and hundreds more in preclinical and clinical development[1]. These include Insulin oral bioadhesive polysaccharide chitosan nanoaprticles[2], Poly phosphoryl choline and β lacto globulin oral biodegradable poly (DL-lactide-co-glycolide) micro particles[3], Bovine serum albumin oral micro beads of metal ion cross linked carboxy methyl guar gum[4], Recombinant human granulocyte colony stimulating factor oral gastro intestinal muco adhesive patch system[5]. Leuprolide nasal/pulmonary polyhedral and spherical niosomes[6], C-reactive protein parenteral resealed erythrocytes[7]. These protein-based products will present unique challenges because of intrinsic instability, multifaceted metabolic properties, and limited GI absorption. The problems will include variable tissue penetration (because of the size of the molecule), and toxicity related to the stimulation of the immune or allergic reaction[8]. While there has been rapid progress in molecular biology, this has not been matched by progress in formulation and development of peptide and protein drug delivery system. This is due in part, to the lack of appreciation of the unique demands imposed by the physicochemical and biological properties of the protein and peptide drugs on routes of delivery, as well as on the delivery system design and formulation. These properties include molecular size, short plasma half life, requirement for specialized mechanism for transport across biological membranes, susceptibility to breakdown in both physical and biological environment, tendency to undergo self association, and complex feed back control mechanisms. As proteins and peptides continue to enter the pharmaceutical market, their stability becomes a pressing issue for the pharmaceutical scientists. Proteins are only marginally stable, and highly susceptible to degradation, both chemical and physical[9-11].

Chemical instability refers to the formation or destruction of covalent bonds, within a polypeptide or protein molecule. These changes alter the primary structure of the protein, and impact higher level of its structure. The common causes for chemical instability are deamidation, oxidation, and cystine destruction/disulfide exchange[12]. Physical instabilities include aggregation and precipitation, adsorption to surface, and protein unfolding[13-16]. Chemical instabilities such as deamidation and disulphide bond cleavage, may also lead to physical instabilities, and vice

***For correspondence**

E-mail: arunshirwaikar@yahoo.co.in

versa. This review examines the reasons for physical and chemical instabilities, how to overcome them in formulation, and how to detect them. It is important to remember that every protein is unique, both physically and chemically, and therefore exhibits unique stability behaviour. An insight into the fundamental understanding of the mechanism by which protein stabilizes, will help in the formulation of protein based pharmaceuticals.

In the context of protein structure, the term stability can be defined as the tendency to maintain a native (biologically active) conformation. Native proteins are only marginally stable. Change in free energy (ΔG) separating the folded and unfolded states in typical proteins under physiological conditions, is in the range of 20-65 KJ/mol[17]. A given polypeptide chain can theoretically assume countless different conformations; as a result the unfolded state of protein is characterized by high degree of conformational entropy. This entropy and hydrogen bonding interaction of many groups in the polypeptide chain, tend to maintain the unfolded state. The chemical interactions that counteract these effects and stabilize the folded native conformation, include strong bonds like peptide bonds and disulfide bonds, and weak (non covalent) interactions; hydrogen bonds, hydrophobic, and ionic interactions. To break a single covalent bond 200-400 KJ/mol are required, whereas weak interactions can be disrupted by a mere 4-30 KJ/mol. Individual covalent bonds that contribute to the native conformation of proteins such as disulphide bonds linking separate parts of a single polypeptide chain, are clearly much stronger than individual weak interaction, yet it is a weak interaction that predominates as a stabilizing force in the protein structure, because they are so numerous .In general, the protein conformation with lowest free energy, is the one with the maximum number of weak interactions. It is not only many weak interactions taking place between different molecules (Intermolecular interaction), but also they are taking place within a single molecule (Intramolecular interaction). In general, intramolecular interactions are much more favorable energetically, than intermolecular interactions.

The stability of protein is not simply the sum of free energies of formation of the many weak interactions within it. Every hydrogen bonding group in a folded polypeptide chain is hydrogen bonded to water prior to folding, and for every hydrogen bond formed in a protein, a hydrogen bond (of similar strength) between the same group and water is broken. The energy required to break the hydrogen bonds to water, must be subtracted from the energy gained from the formation of the new hydrogen bonds between atoms in the folded protein, in the calculation of the net thermodynamic contribution of hydrogen bonding to the folding. The net stability contributed by a given weak interaction, or the difference in free energies of the folded and unfolded states may be close to zero. Hydrophobic interactions are clearly important in stabilizing a protein conformation; it is important to realize that the strength of a hydrophobic interaction is not due to a high intrinsic attraction between nonpolar groups, but rather to the properties of the water solvent in which the nonpolar groups are dissolved. A nonpolar residue dissolved in water, induces in the water solvent, a solvation shell, in which water molecules are highly ordered. When two nonpolar groups come together on the folding of a polypeptide chain, the surface area exposed to the solvent is reduced, and a part of the highly ordered water in the solvation shell is released to bulk solvent. Accordingly, the entropy of water is increased. The increase in entropy is a thermodynamically favorable process, and is the driving force causing nonpolar moieties to come together in aqueous solvent. The interior of a protein, is generally a densely packed core of hydrophobic aminoacid side chains. Proteins in aqueous solutions, swell and enclose water. Protein solutions are colloidal emulsoids or micelles, because they are charged, and each molecule has an envelope of water around it. Protein denaturation occurs when a polypeptide loses its higher level of structure, and often results in loss of biological activity.

The conformation (shape) of a protein is determined by interaction between a polypeptide and its aqueous environment, in which the polypeptide attains a stable three-dimensional structure. The number and kind of amino acid contribute the primary structure of polypeptide, and its sequence. The covalent peptide linkage is the only type of bonding involved at this level. A quaternary structure is the arrangement of two or more polypeptide chains to form a functional protein molecule. There is a significant relationship between the conformational stability and chemical integrity of each molecule.

Denaturation:

Perturbation of secondary structure or tertiary structure can lead to exposure of previously burried amino acid, facilitating its chemical reactivity; thereby leading to loss of its native or original characteristics. This is called

denaturation. Denaturation can be caused by destabilizing agents such as Excipients (reducing sugars, antioxidants, surfactants, metal ions), heat, hydrolysis by strong acid or alkali, enzymatic action, exposure to urea or other substances, or exposure to ultra violet light. Excipients like reducing sugars can react with protein amino groups to form schiff's bases (Maillard reaction). The first phase of the Maillard reaction involves a condensation reaction between the carbonyl group of a reducing sugar, and an amino group to form a schiff base, and a molecule of water[18]. Lyophilized human relaxin (Rlx) formulated with glucose was observed to degrade via the Maillard reaction, to form adducts with glucose, as shown by LC/MS[19]. Antioxidants themselves, should not be added to the protein formulation, since they may contain reducing agents that will destroy disulphide bonds. Several surfactants like Tween 20 and Tween 80 and polaxamer, can cause oxidation of aminoacid due to residual peroxides present in these materials. Many chemical reactions involving polypeptides and proteins are catalyzed by metal ions such as Zn^{2+}, Cd^{2+}, Pb^{2+}. The importance of temperature, moisture, hydrogen ion activity, and excipient in determining the stability of proteins, is widely reported and accepted[20-22]. The exposure of proteins and peptide formulations to elevated temperature, generally decreases chemical stability by accelerating all chemical degradation reactions. At high temperature (80-100°), aspargine and glutamine are susceptible to deamidation, Aspartate-Xaa peptide bonds are susceptible to hydrolysis, disulphide bonds rupture, and Xaa-pro peptide bonds undergo cis-trans isomerisation (where Xaa is any amino acid). High temperature can result in physical degradation due to irreversible denaturation. This often results from the destruction of disulphide bonds in cystine via β-elimination. This results in a change in native protein conformation, and thereby aggregation e.g streptokinase[23]. Residual moisture can be responsible for protein instability in the solid state. As a result, many polypeptide drugs are formulated as lyophilized or freeze-dried products, to prolong their shelf life[24-26]. The covalent aggregation of bovine serum albumin in the solid state greatly depends on water content[27]. Maillard reaction usually has maximum rates in low-moisture range[28]. Insulin[29], casein, and blood plasma proteins[30] in the dry state, show maximum degradation at relative humidity, in range 40-80%. This may be due to molecular mobility and reactant concentration. Water can affect the reaction pathways. The formation of the cyclic imide intermediate during deamidation of the asparginine-hexapeptide at pH 5, was favoured over direct hydrolysis as water content increased[31]. pH also has strong influence on aggregation rate.

Proteins and peptides are often formulated with excipients such as polyalcohols and polymers, to protect them during freeze-drying and storage. Polymers are also used to form a matrix, for controlled release. Excipients such as heparin, and anionic polymers, decreased the rate of covalent aggregation in recombinant human keratinocyte growth factor (rhKGF), at elevated temperatures[32]. Polyhydric alcohols like mannitol, sorbitol, and non reducing sugars like dextrose, sucrose, and trehalose, are the most commonly used excipients in lyophilized protein and peptide formulations. The covalent dimerization of human insulin was markedly decreased by incorporation into a glassy matrix of trehalose[33]. Rapid covalent modifications of lyophilized human relaxin was observed in the presence of glucose. Polymeric excipients can influence the reactivity of peptides and proteins through direct chemical interactions, or by altering the physical state (glassy vs. rubbery). The stability of carbonic anhydrase and atriopeptin III (APIII), encapsulated in poly- DL-lactide-co-glycolide was studied[34-35]. Degradation of PLGA microspheres produced water soluble oligomers, which decreased the pH from 7 to 3, over a month. Carboxylic end group of lactic and glycolic acid fragments may react with carbonyl group of the amide linkage, and thus have a direct catalytic effect on the chemical stability of proteins in solids. Many successful formulations with PLGA, have been prepared[36-38]. The thermodynamic stability of the native proteins is characterized by negative free energy at both high (45-100°), and low temperature (less than 5°). Partially unfolded proteins are reactive species that form aggregates. pH determines the type and total charge on the protein, thereby affecting electrostatic interactions. Increasing the acidity or basicity of the solution can increase the charged groups on a protein. Increase in charge repulsion within the protein destabilizes the folded protein conformation, because the charge density on the folded protein is greater than on the unfolded protein. Thus, a pH –induced unfolding leads to a state of lower electrostatic free energy[39]. Salt bridges (or ion pairing) can sometimes stabilize the proteins. When protein possesses both positive and negative charges (e.g., pH close to pI value), anisotropic charge distribution leads to dipoles.In this case, repulsive protein-protein interactions could make assembly processes such as aggregation energetically favourable[40,41]. The

deamidation rate of the Asn-hexapeptide increases, as the solution pH prior to freeze-drying increases from 5 to 8[31]. The deamidation of lyophilized human insulin is also quite sensitive to pH[33,42]. To avoid the drastic shifts in pH, the weight ratio of buffer to other solutes should remain low[25].

Certain solutes (e.g., sugars, polyols, ammonium sulphate) stabilize the native state of protein, whereas other solutes e.g., urea and guanidine hydrochloride, act as protein denaturants[43,44]. Protein stabilizers preferentially excluded from the surface of the protein molecule, and the degree of exclusion, is proportional to its solvent exposed surface area[45,46]. When the co-solutes are depleted and water-enriched in that domain, they can be interpreted as negative binding. During unfolding, protein surface area increases, and there is greater degree of preferential exclusion and larger negative binding. By LeChatelier's principle, the system will adjust itself to minimize this unfavorable effect. The net effect of greater negative binding to the unfolded state is to favour the unfolded state (native state). Thus, the protein states with reduced surface area that exhibit lower preferential exclusion are favored over more solvent-exposed states. As a result, free energy of unfolding is increased in presence of preferential excluded salts[47]. Lower concentration of salts reduces the electrostatic interactions, due to charge shielding. Higher concentration of electrolytes can decrease the thermodynamic stability of the native conformation, and an increase in the equilibrium solubility[48]. Electrolytes have complex effects on protein physical stability. They will change the conformational stability, equilibrium solubility (salting in and salting out), and formation of aggregates[49-52]. The preservatives and surfactants are sometimes essential in protein formulation for prevention of microbial growth, and to prevent aggregation and adsorption. However, preservatives can induce aggregation of protein in aqueous solution e.g., phenol induces aggregation of human growth hormone[53]. The differential binding of surfactants to native and unfolded states of protein, influences the protein's conformational stability. Surfactants bind strongly to the native state, and increase free energy of denaturation. e.g., human growth hormone[54]. The solid state characteristics of proteins and peptides influence the physical bulk state characteristics of the formulation. For small molecule drugs in the solid state, the crystalline drug is generally less prone to degradation, than the amorphous form. However, crystalline state may not be always more stable for protein and peptide formulations[43,55,56]. Lyophilized human growth hormone (hGH) formulated in a partially amorphous excipient system (glycine: mannitol), was less susceptible to chemical degradation and aggregation, than hGH formulated in either totally amorphous (dextran) or crystalline (mannitol) system[57].

CHEMICAL INSTABILITY

Chemical instabilities are due to deamidation,oxidation, and cystine destruction/disulfide exchange.

Deamidation:

Deamidation of asparginine residues (glutamine residues to lesser extent) to aspartate or isoaspartate via succinimide intermediates (positive to negative charge), occurs in many proteins and peptides, and is a major cause of spontaneous degradation and loss of aminoacid sequence homogeneity. This occurs in conditions of neutral to basic pH. The susceptibility depends on the sequence and conformation[58]. Clarke[59] noted that conformation and reactivity in deamidation depends on the juxtaposition of the nucleophilic NH center of the Asn-residue. The more difficult this juxtaposition, the slower the deamidation.Two of the common forms of the secondary structure (α-helices and β-forms) tend to stabilize Asn residues against deamidation. Stabilization probably results atleast in part, from conformational structures.

It is detected by charge, molecular weight, and formation of succinimide residues. Deamidation can make protein prone to proteases and denaturation. This can affect the *in vivo* half-life, activity, and conformation of protein, and also increase the immunogenicity of certain protein[60]. Human insulin has also been observed to undergo deamidation in the solid state via a mechanism similar to that in solution[61]. In insulin formulation lyophilized from acidic solutions (pH3-5), the rate determining first step involves intermolecular nucleophilic attack of the C-terminal $AsnA_{21}$ carboxylic acid onto the side chain amide carbonyl, to release ammonium, and to form reactive cyclic anhydride intermediate which can further react with various nucleophiles. The deamidation of polyanion-stabilized acidic fibroblast growth factor (aFGF;FGF-1) can be induced by prolonged storage under accelerated conditions of elevated pH and temperature. Methods include peptides maps, capillary electrophoresis, isoelectric focusing, and enzyme catalyzed radio labeling of the isoaspartyl sites. Formulation approaches include

lowering of pH (desialylation can occur, therefore optimization essential), compatibility studies in presence of various buffers, because deamidation is also affected by buffer composition.

Oxidation:

Methionine, cystine, (more common) tryptophan, tyrosine residues, are all susceptible to oxidation. Air, residual peroxide content, or intense fluorescent light, can convert thioether to sulfoxide, and then sulfone. A major chemical decomposition pathway for human growth hormone (hGH) in the solid state is methionine oxidation at Met_{14}, to form the sulfoxide[62]. It is important to prevent it during the modification of the process[63]. Both oxygen content and light exposure, affect the oxidation rate[64]. Change in pH, ionic strength, and solvent polarity, can change both rate and extent of methionine oxidation. This oxidative modification can be variable e.g. human leptin[65]. Peptide maps are convenient for detecting methionine oxidation, and MS.RP- HPLC is used to separate the oxidized forms. Oxidation occurs in a number of proteins favored by factors like temperature, pH etc. Formulation approaches include addition of anti oxidants, (sodium thiosulphate, catalase, or platinum), and adjustment of environmental conditions (pH, or temperature). Cystine oxidation can be prevented by keeping low pH. Tryptophan oxidation occurs in leutinizing hormone releasing factor, somatostatin, and ACTH[66,67]. Methionine oxidation occurs in a number of polypeptides, and proteins, like antistatin[68], granulocyte colony stimulating factor (G-CSF)[69] antithrombin[70], epidermal growth factor (EGF)[71], and adrenocorticotropin hormone (ACTH)[72]. Cystine residues that contain thiol groups can be oxidized to form disulphide bonds. These bonds occur naturally in polypeptides and proteins, either intra or intermolecularly. It can be formed either by direct oxidation of cystine or thio-disulphide interchange, often catalyzed by metal ions, the relative stability of reduced cystine being dependent on the redox potential of the protein environment. Acidic pH can decrease the oxidative reactivity[73]. Certain conditions such as reducing potential, heat and alkaline pH can break disulphide bonds, i.e.,b elimination. Disulphide scrambling has been documented in human insulin, like growth factor (IGF)[74]. Atypical disulphide bonds are also formed in acidic fibroblast growth factor. (aFGF). Atypical disulphide bond can be detected by Sodium dodecyl sulphite polyacrylamide gel electrophoresis (SDS-PAGE), and Elleman's reagent by estimating free thiols[75]. Methods used to study the reaction between free thiols and disulphide bonds in more detail, include various proteolytic digestions, peptide mapping, partial reductions, and assignment of disulphide by N-terminal sequencing, and matrix assisted desorption ionization (MALDI) MS.

Formulation approaches include, maintaining acidic pH, and avoiding potential reducing agents (like anti-oxidant excipients), lyophilization, substituting non critical cystine residues with other residues to reduce the potential instability of free thiols in presence of disulphide e.g. human interferon (IF-N) beta analogue[76]. Other less common bond cleavage that occurs in protein and peptide includes hydrolysis of aspartic acid residues under acidic condition e.g., human epidermal growth factor at pH 3 and 45°, and Diketopeptide formation at higher pH[77]. Presence of protease enzyme can result in the cleavage of recombinant protein. Protease inhibitors can minimize this to a certain extent. Disulphide aggregation can also occur via free amino, carbonyl, carboxyl or hydroxyl functional groups, with ester or amide linkages e.g., insulin, relaxin[78-80].

Deglycolisation:

In glycoproteins, sugars are attached either to the amide nitrogen atom in the side chain of asparagines (termed N-linkage), or to the oxygen atom in the side chain of serine or threonine (termed O-linkage). An asparagine residue can accept an oligosaccharide only, if the residue is part of an Asn-X-Thr sequence, where X can be any residue. Thus, a potential glycolisation site can be detected within aminoacid sequences. There are a number of glycosylated proteins that have sugar and sialic acid molecules covalently linked to peptide structure. e.g., IFN-beta has greater stability to aggregation than corresponding protein produced by bacterial fermentation, in the non-glycosylated form.[81] Desialylation can occur at acidic pH on storage. Differing sialic acid content has shown to be responsible for variability in the biological activity of highly purified pituitary lutinizing hormone isoforms[82]. The modification of human insulin by the covalent attachment of monosaccharide moieties to insulin amino groups altered the aggregation and self association behavior, and improved both the pharmaceutical stability and biological response[83].

Change in glycocylation can be detected by various gel methods including flurophore – assisted carbohydrate electrophoresis (FACE) and MS. Change in sialic acid content can be detected by measurement of free sialic

acid. Oligosaccaride structure can be analyzed by normal phase HPLC combined with MS, and high resolution of normal phase, by high pH anion exchange chromatography combined with MS.

Photodegradation of proteins:
Side chains of tyrosine, phenylalanine, and tryptophan, as well as peptide bonds in proteins, absorb ultraviolet light. Both ionizing and non ionizing radiation can cause protein inactivation. It can directly interact with amino acid, or indirectly via various sensitizing agents like oxygen. Photodegredation products in an aerated, neutral pH include S-S bond fission, conversion of tyrosine to DOPA, etc. It is also important to take into account, potential damage to the protein during analysis using circular dichorism (CD), UV or fluorescent measurements, where incident radiation is being used. UV spectroscopy can be used to study changes in secondary and tertiary structures of proteins. As protein is denatured, differences are observed in the absorption characteristics of the peptide bonds due to the disruption of the exciton system.

PHYSICAL INSTABILITY

Physical instability includes the reactions that do not involve the formation or destruction of covalent bonds. It results in aggregation and precipitation of proteins. Environmental stress like pH and temperature can cause structural modification or conformational changes, which can be detected by standard spectroscopic techniques like circular dichorism, and fluorescent and infrared spectroscopy.

Aggregation and precipitation:
Certain proteins undergo self-association resulting in the formation of multimers, and in the extreme cases, aggregates and precipitates. The most common mechanism of protein aggregation is believed to involve protein denaturation and non-covalent association, via hydrophobic interfaces. Aggregation and gelling occurs in insulin infusion therapy[84]. Denaturation is usually induced at gas-liquid, liquid-liquid interface, as during micro encapsulation process[85], or container liquid interfaces. pH variation, solvents, salts, and excipients, can also contribute to this. Agitation and freeze-thawing induced aggregation of recombinant human factor XIII (rFXIII), is due to interfacial adsorption and denaturation at the air-liquid and ice-liquid interfaces. The aggregation pathway proceeds through soluble aggregates to formation of insoluble aggregates, regardless of the denaturing stimuli. $CaCl_2$ and sugars can stabilize human deoxyribonucleic (rhDNAse) against thermal denaturation, but divalent cations, urea and guanidine hydrochloride destabilize the protein[86]. Evidence that unfolding preceded the formation of the aggregate, was provided by far UV-CD[87].

Other non-covalent mechanisms of aggregation include ionic complexation, salting out, charge neutrality close to the isoelectric pH, and results in limiting solubility of the molecule. Process development activities like spray drying and nebulization[88-91], and freeze-thaw cycling, which involve any of the above factors, can induce denaturation, and if formed it should be reversible.

Formulation and filling can induce denaturation by exposing protein to gas-liquid interfaces. Non ionic surfactants are often added to protein formulations, to prevent aggregation and adsorption to surfaces. Surfactants such as Tween 80, Pluronic F-68, and Brij 35, have been shown to induce aggregation of human growth hormone (hGH)[92], but they do not stabilize against thermal stress, as shown in DSC studies. Higher concentration can destabilize the molecule. Formulation with sugars is also known to stabilize a number of proteins against aggregation[93, 94]. Soluble aggregates can be detected by HP-SEC (High Performance Size Exclusion Chromatography), found in many proteins like hGH, insulin, interferon-2 (lL-2), anti trypsin-α1,IFN-γ, basic fibroblast growth factor and IFN-β[95]. In general, low levels of soluble aggregates in pharmaceutical products can be tolerated as long as the product remains stable, and soluble aggregates do not progress to insoluble forms[96]. Increase in soluble aggregates can result in change in immunogenicity of the therapeutic protein. Aggregates in certain cases can be visualized e.g., insulin and IL-1β[97, 98]. Insoluble aggregates can be detected by FTIR, Raman, and electron spin resonance spectroscopy, or light scattering techniques (UV absorption).

Protein aggregation is arguably the most common and troubling manifestation of protein instability, encountered in almost all stages of protein drug development. Protein aggregation, along with other physical and/or chemical instabilities of proteins, remains to be one of the major road barriers, hindering rapid commercialization of potential protein drug candidates. Although a variety of methods have been used/designed to prevent/inhibit protein aggregation, the end results are often

unsatisfactory for many proteins. The limited success is partly due to our lack of a clear understanding of the protein aggregation process. This article intends to discuss protein aggregation and its related mechanisms, methods characterizing protein aggregation, factors affecting protein aggregation, and possible venues in aggregation prevention/inhibition in various stages of protein drug development[99].

Pompa, *et al*[100] have reported the folding properties of the protein azurin, deposited onto SiO_2 surfaces, and subsequently dehydrated. The molecular films have been maintained at ambient conditions through several days, and the ageing effects have been investigated by fluorescence spectroscopy. The experimental results show a modest initial conformational rearrangement, followed by long-term stability. Interestingly, upon rehydration of the biomolecular films at the end of the investigated period (approximately one month), azurin returns to exhibit a native-like conformation. This study indicates a rather surprising resilience of proteins to ambient conditions, and sheds a somewhat unexpected positive light on reliability in biomolecular electronics.

A study was conducted to examine the stability of bovine serum albumin (BSA) in poly (dl-lactic acid-co-glycolic acid) (PLGA) microspheres upon addition of a new excipient, poly(ethylene glycol)-poly (l-histidine) diblock copolymer (PEG-PH). Poly (l-histidine) component can form an ionic complex with BSA under acidic conditions, within a narrow pH range. To optimize the ionic complexation conditions for BSA with PEG-PH, the resulting complex sizes were monitored using the Zetasizer. PLGA microspheres containing BSA as a model protein were prepared by the w/o/w double emulsion method. BSA stability in aqueous solutions, and after release from PLGA microspheres, was determined using circular dichroism (CD) spectroscopy for secondary structure analyses, and fluorescence measurements for tertiary structure analyses. The release profile of BSA from the microspheres was monitored using UV spectrophotometry. The rate of PLGA degradation was monitored by gel permeation chromatography. The pH profile within the microspheres was further evaluated by confocal microscopy, using a pH-sensitive dye. Approximately, 19 PEG-PH molecules and one BSA molecule coalesced to form an ionic complex around a pH range of 5.0-6.0. Plain BSA/PLGA and BSA/PEG-PH/PLGA microspheres had a mean size of 27-35 mum. PLGA microspheres with a BSA loading efficiency >80% were prepared using the double emulsion method. PEG-PH significantly improved the stability of BSA, both in aqueous solutions and in PLGA microspheres. The release profiles of BSA from different formulations of PLGA microspheres were significantly different. PEG-PH effectively buffered the local acidity inside the microspheres, and improved BSA release kinetics by reducing initial burst release and extending continuous release over a period of time, when encapsulated as an ionic complex. PLGA degradation rate was found to be delayed by PEG-PH. There was clear evidence that PEG-PH played multiple roles when complexed with BSA, and incorporated into PLGA microspheres. PEG-PH is an effective excipient for preserving the structural stability of BSA in aqueous solution, and in BSA/PLGA microspheres formulation[101].

Wang, *et al*[102] presented a coarse-grained approach for modeling the thermodynamic stability of single-domain globular proteins in concentrated aqueous solutions. Our treatment derives effective protein-protein interactions from basic structural and energetic characteristics of the native and denatured states. These characteristics, along with the intrinsic (i.e., infinite dilution) thermodynamics of folding, are calculated from elementary sequence information, using a heteropolymer collapse theory. We integrate this information into Reactive Canonical Monte Carlo simulations, to investigate the connections between protein sequence hydrophobicity, protein-protein interactions, protein concentration, and the thermodynamic stability of the native state. The model predicts that sequence hydrophobicity can decide how protein concentration impacts native-state stability in solution. In particular, low hydrophobicity proteins are primarily stabilized by increases in protein concentration, whereas high hydrophobicity proteins exhibit richer nonmonotonic behavior. These trends appear qualitatively consistent with the available experimental data. Although factors such as pH, salt concentration, and protein charge are also important for protein stability, our analysis suggests that some of the nontrivial experimental trends may be driven by a competition between destabilizing hydrophobic protein-protein attractions, and entropie crowding effects.

Developing recombinant protein pharmaceuticals has proved to be very challenging, because of both the complexity of protein production and purification, and the limited physical and chemical stability of proteins. To overcome the instability barrier, proteins often have to be made into solid forms to achieve an acceptable shelf life as pharmaceutical products. The most commonly used method for preparing solid protein pharmaceuticals, is

lyophilization (freeze-drying). Unfortunately, the lyophilization process generates both freezing and drying stresses, which can denature proteins to various degrees. Even after successful lyophilization with a protein stabilizer (s), proteins in solid state may still have limited long-term storage stability. In the past two decades, numerous studies have been conducted in the area of protein lyophilization technology, and instability/ stabilization during lyophilization and long-term storage. Many critical issues have been identified. To have an up-to-date perspective of the lyophilization process, and more importantly, its application in formulating solid protein pharmaceuticals, this article reviews the recent investigations and achievements in these exciting areas, especially in the past 10 years. Four interrelated topics are discussed: lyophilization and its denaturation stresses, cryo- and lyo-protection of proteins by excipients, design of a robust lyophilization cycle, and with emphasis, instability, stabilization, and formulation of solid protein pharmaceuticals[103].

Adsorption:

The interaction of proteins with the surface of their storage containers is potentially a significant problem. The amphiphilic nature of the protein molecule results in their adsorption to a wide variety of surfaces and also both their loss and destabilization[104-109]. Adsorption of protein on surfaces is an important phenomenon, which should be considered while formulating and selecting container and closure for pharmaceutical products. This is extremely important in low dose drugs. Adsorption to a surface is problematic in parenteral administration. In these types of situations, adsorption of an inert protein like serum albumin to saturate the container surface, or compounds that reduce surface interactions such as surfactants, carbohydrates or aminoacids, can be employed to reduce this problem[110-111]. In formulation, surfactant addition can reduce adsorption losses e.g., Tween 80 and Pluronic F68 have been shown to reduce the adsorption of calcitonin to a glass surface[112]. X-ray and neutron reflection are used to study the adsorption of protein at liquid-gas and solid-liquid interfaces, and parameters like adsorbed amount, total thickness of the adsorbed layer, pH, and excipients[113-114].

CONCLUSIONS

The susceptibility to chemical degradation in solution, presents a challenge in the development of stable protein pharmaceuticals. As a result, many polypeptide drugs are formulated as lyophilized or freeze-dried products to prolong their shelf life. Recently PEGylation technology has been designed to improve the safety and efficacy of protein pharmaceuticals. Advanced PEGylation, including site-specific PEGylation, can improve drug performance by optimizing pharmacokinetics, increasing bioavailability, and decreasing immunogenicity and dosing frequency. In all cases, for a successful protein formulation, one should consider the clinical indication, pharmacokinetics, toxicity and physicochemical stability of the drug product in the specific delivery system.

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