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Computational methods in preformulation study for pharmaceutical solid dosage forms of therapeutic proteins

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Abstract:
Design and delivery of protein-based biopharmaceuticals needs detailed planning and strict monitoring of intermediate processing steps, storage conditions and container-closure system to ensure a stable, elegant and biopharmaceutically acceptable dosage form. Selection of manufacturing process variables and conditions along with packaging specifications can be achieved through properly designed preformulation study protocol for the formulation. Thermodynamic stability and biological activity of therapeutic proteins depend on folding–unfolding and three-dimensional packing dynamics of amino acid network in the protein molecule. Lack of favourable environment may cause protein aggregation with loss in activity and even fatal immunological reaction. Although lyophilization can enhance the stability of protein-based formulations in the solid state, it can induce protein unfolding leading to thermodynamic instability. Formulation stabilizers such as preservatives can also result in aggregation of therapeutic proteins. Modern instrumental techniques in conjunction with computational tools enable rapid and accurate prediction of amino acid sequence, thermodynamic parameters associated with protein folding and detection of aggregation "hot-spots." Globular proteins pose a challenge during investigations on their aggregation propensity. Biobetter therapeutic monoclonal antibodies with enhanced stability, solubility and reduced immunogenic potential can be designed through mutation of aggregation-prone zones. The objective of the present review article is to focus on the various analytical methods and computational approaches used in the study of thermodynamic stability and aggregation tendency of therapeutic proteins, with an aim to develop optimal and marketable formulation. Knowledge of protein dynamics through application of computational tools will provide the essential inputs and relevant information for successful and meaningful completion of preformulation studies on solid dosage forms of therapeutic proteins.

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1 Introduction

Formulation development requires a balance between physical and chemical properties to achieve a robust formulation with the desired physical and bioequivalent results. Stages of formulation development include description of formulation platforms for preclinical studies, development of preclinical formulations, preformulation studies of active pharmaceutical ingredient (API) to support drug–product development and finally formulation development. Preformulation is a branch of pharmaceutical sciences that utilizes biopharmaceutical principles in the determination of physicochemical properties of a drug substance. Preformulation testing encompasses all studies enacted on a new drug compound in order to develop a stable, elegant and biopharmaceutically suitable dosage form and successful commercial product. Goals of preformulation studies are to choose the correct form of API, to evaluate its physical properties and generate a thorough understanding of the material's stability under various conditions, thereby leading to the optimal, marketable drug delivery system. Preformulation study is therefore considered an exploratory tool initiated early in the development of any pharmaceutical dosage form [1].

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2 Challenges to formulation development of therapeutic proteins

Significant strides in the field of biotechnology have opened up possibilities for large-scale production of therapeutic proteins which have immense therapeutic potential in the treatment of a range of diseases, starting from rheumatoid arthritis to cancers. Different therapeutic proteins available in the market include monoclonal antibodies, erythropoietins, interferons, growth factors, insulin, interleukins, tissue plasminogen activator, blood clotting factors and replacement enzymes. Monoclonal antibodies are highly preferable owing to their target specificity. Worldwide sales of monoclonal antibodies are expected to reach US$125 billion by 2020 [2]. However, complex nature of these protein macromolecules and their marginal stability even in solid state act as barriers to successful formulation development of therapeutic proteins. Other major problems associated with therapeutic proteins are their exogenous sources, variations in processing condition of the same molecule from one manufacturer to the other, inter-batch differences in the conditions and finally heterogeneity. Since the processing involves numerous steps, minor modification in a single step can be detrimental to the stability of the protein molecule [3, 4]. Preformulation studies therefore form an integral part of protein-based product development which will accelerate clinical manufacturing and investigational new drug filing process. A thorough understanding of physicochemical characteristics of the protein molecule is essential towards setting up the preformulation study protocol. Stability of protein molecules is affected by manufacturing variables, sterilization conditions and container-closure system. The two major issues that are of primary concern during development of solid dosage forms for therapeutic proteins include aggregation propensity at various stages of manufacturing and possible immunogenicity with fatal outcomes [5].

Assessment of physical stability of a protein-based therapeutic is a challenging task as it is linked to the primary sequence of amino acids in the protein. Secondary structure of the protein or its conformation results from intra- and intermolecular hydrogen bonds resulting in the formation of a typical geometric shape. The conformations that usually occur are $\alpha-$helix, $\beta-$sheets, turns, bends and irregular random coil [6, 7]. Stability and biological activity of proteins are closely linked to their global flexibility, fluctuations and other dynamic processes. Flexibility which is essential for its activity poses a challenge to the manufacturers as it compromises on the stability. Although the unfolded state of the protein is unstable, the folded state is only marginally stable and any alteration in manufacturing conditions or in the neighbouring environment may cause various types of instability problems for the protein molecule such as aggregation, degradation by oxidation, hydrolysis, deamidation and lastly, inactivation. Thermodynamic stability of proteins is thus a complex balance between folded and unfolded conformations. Protein stability is greatly affected by those factors which disturb the delicate balance between the stabilizing and destabilizing forces. Protein dynamics should be studied in detail in order to develop and optimize manufacturing conditions for protein-based drug delivery systems. During manufacture and storage, proteins are vulnerable towards aggregation, leading to degradation, denaturation and resultant loss in biological activity. Rational design of protein-based biopharmaceuticals and self-assembled protein-inspired supramolecular aggregates requires a thorough understanding of the protein structure, thermodynamic events and factors responsible for switching of conformation. Information about the thermodynamics of the process of unfolding enables redesigning of proteins, enzymes and antibodies with better solubility and stability through modification of amino acid sequence [8].

3 Aggregation of therapeutic proteins

Protein aggregation is a natural phenomenon because it results in the formation of non-covalent bonds which are very similar to those present in native, stabilized structure [9]. Aggregation-induced degradation with antibodies may evoke fatal immunological reaction. Elevated temperature during storage or mechanical vibration during transportation can contribute to aggregation [10]. Various other physical stress factors have been found equally responsible to cause aggregation and include pH, ionic strength, presence of metal ions and surface adsorption [11]. Although stability of protein-based dosage forms can be enhanced by the process of lyophilization or freeze-drying, the dried powder needs to be reconstituted with suitable vehicle prior to administration. However, aggregation may also occur in the lyophilized proteins. Lyophilization process variables and composition of the solid determine the extent of aggregation which is greatly affected by the nature of the protein [12]. Stringent processing conditions during lyophilization may result in thermodynamic instability and reversible unfolding of the protein. If no irreversible damages occur to the protein during storage or reconstitution, then refolding takes place as soon as product is reconstituted, and there is no negative impact on the pharmaceutical stability of the product [13]. Aggregation in proteins can also be induced by the antimicrobial preservatives added to multiple-dose protein formulations. Therefore, suitable biophysical computational tools

and instrumental analytical techniques are essential for prediction of aggregation propensity and profiling and for studying the factors affecting alteration in conformation [14].

Local interactions (e. g. hydrogen bonding, steric interactions) are responsible for the formation of α- and β-sheets, loop structure which govern packing dynamics of the molecule to a specific 3D-structure and thus dictate the specific biological function exhibited by the protein. These interactions are constituted by networks of amino acid residues. These networks are assumed to represent a subset of all potential interactions of residues. Literature surveys have revealed the availability of different structural and computational methods to provide insight into amino acid networks. The methods which can provide valuable information even in the absence of any data on protein structure include multiple sequence alignments (MSAs) and algorithms like statistical coupling analysis (SCA), mutual information (MI), McLachlan-based substitution correlation (McBASC) and observed minus expected square (OMES). These computational methods will guide the scientists in the design of engineered proteins with enhanced biological function or biobetters [15]. Physicochemical properties of amino acids, presence of aromatic side chains and charged residues are determinants for aggregation tendency of protein molecules [16]. There are various approaches for correlating solid-state protein stability with inherent protein characteristics such as glass transition temperature, moisture content and free volume measurement with the help of instrumental techniques relying on bulk properties or population averaged properties. However, meaningful correlation cannot be established always because protein instability is highly dependent on the nature of the functional groups [12].

3.1 Instrumental methods of analysis

Different instrumental methods can be utilized for the determination of protein structure and quantification of thermodynamic parameters controlling the mechanisms of protein folding-unfolding. They include differential scanning calorimetry (DSC), X-ray diffraction (XRD) crystallography, circular dichroism (CD) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and infrared (IR) spectroscopy. DSC is used to measure protein's change in molar heat capacity, enthalpy, entropy and transition midpoint to account for transition of 50 % of protein molecules. DSC study of very dry proteins in solid state indicates that heat of denaturation and heat capacity change on denaturation are very close to the values for solution. This does not mean that proteins in dry state exist in native state but indicates that tertiary structure is present. Transition during unfolding can be reversible, two state and highly cooperative, which can be computed from DSC. The method is capable of estimating the shape of folding free energy surface when fitted to a specific model. Accuracy of prediction depends on the proper selection of model. Model independent estimates of folding barrier height can be obtained with Bayesian probabilistic approach. Ultrafast folding proteins are those where conformational transition occurs in sub-millisecond timescales. Modern analytical tools in conjunction with newer computational methods help in characterization of such ultrafast folding proteins exhibiting non-cooperative folding with no sharp demarcation between thermodynamic states having different energy landscapes. Ultrafast kinetic perturbation method employs laser pulses as triggers to stimulate very rapid shift in folding–unfolding thermodynamic equilibrium under the effect of changes in temperature, pH, pressure or chemical potential. Relaxation rate can be determined by spectroscopic monitoring of the shift and microscopic rates of interconversion between the species can be estimated by analysing with a suitable kinetic model. In molecular dynamic (MD) simulation technique, potential energy of the protein is calculated as a function of atomic coordinates. For the purpose, a simulation box is constructed using all atoms present in the protein molecule together with the neighbouring solvent molecules, numerical integration of Newton's equation of motion is performed where position, force and velocities of each individual atom are defined over a very short time span followed by analysis and establishment of correlation between molecular structure and terabytes of data obtained from simulated trajectories. Atomistic MD simulation helps in deriving mechanistic information. Combining NMR dynamics experiment with DSC also facilitates the characterization of ultrafast folding proteins. These proteins can be used in the design of high-performance biosensors [[8, 13, 17–19][20]].

Valuable information about protein structure dynamics can be extracted from XRD studies on the protein crystal. However, traditional crystallographic method removes sharp Bragg reflections or report on correlations in charge density variations and hence fail to produce a clear image of protein motion. Diffractograms from multiple crystal orientations are integrated into a 3D dataset where statistical averaging of the signal is done. To overcome this problem, a new 3D modelling and data extraction technique has been introduced in the investigation of protein crystal structure which produces a sharp image. In this method, previously discarded data from diffuse XRD have been processed using computer modelling [21, 22].

Despite advantages of the new technique, XRD analysis requires a single purified well-ordered crystal of the protein molecule which becomes difficult to obtain most of the times. Moreover, the technique is expensive and time consuming. CD spectrophotometry helps in identification of secondary and tertiary structures on the basis of distinct chiral positioning of the amide chromophores and the local environment of aromatic chro-

mophores. Accuracy level of CD spectral data becomes lower if the secondary structure is rich in β-sheets or comprises a mixture of α-helices and β-sheets [14]. Multidimensional NMR spectroscopy is advantageous, but it is time-consuming when one attempts to obtain high resolution of the structure and it requires large amount of sample. The method is not suitable for proteins with short half-life or low stability at room temperature. Fourier-transformed infrared (FTIR) spectroscopy generates quick response and can provide high-resolution results for small soluble proteins, lyophilized proteins as well as large membrane proteins and low sample weight is needed for the characterization. Moreover, FTIR spectroscopy helps in the selection of suitable cryoprotectant excipients to be used as stabilizers during lyophilization. Amide groups in proteins are groups with IR-active vibrations. Change in the position of amide band I accounts for alteration in the secondary structure and is used as an index of different conformations in the protein molecule. However, amide band I position is determined in an aqueous environment and spectral subtraction needs to be done for elimination of water absorption. Therefore, a novel method of drop coat deposition (DCD) for the protein sample preparation, coupled with confocal Raman spectroscopy, has been employed for structure determination of bovine serum albumin and ovalbumin. Peak fitting parameters obtained from ovalbumin can form the basis for the estimation of secondary structure of unknown immunoglobulin-based monoclonal antibodies and fusion drugs. The novel technique can predict secondary structure of any type with high accuracy in a short time and negating the need for spectral processing of raw data [6, 7]. Recently, few studies have been undertaken to determine the secondary structure composition of therapeutic proteins through second-derivative of amide I region of FTIR spectra via principal component analysis, spectral correlation coefficient, and area of overlap. These approaches could compare and detect minor changes in higher-order structure induced by small changes in solution condition such as temperature, pH, salt concentration and type. Another more sensitive technique, known as soft independent modelling of class analogy (SIMCA), performs chemometric analysis to determine the class or group of observations. The technique is very helpful in the case of multiple samples from each condition. Lyophilization has been found to induce varying degree of changes in protein conformation, such as appearance of new bands and disappearance of solution bands. The spectral correlation coefficient values may vary between 0.5 and greater than 0.9. Bandwidths increase and band positions may shift, indicating disorder in configuration. Loss in water due to freeze-drying may result in the formation of β-sheets [13, 23, 24]. Aggregation during freeze-drying can be remarkably prevented by rapid freezing of the entire solution at the same time which will minimize thermal transitions and glass transitions [4]. The low solubility and non-crystalline nature of protein aggregates create hindrance to their high-resolution characterization by XRD and NMR spectroscopy. Low-resolution data obtained from various instrumental methods like fibre diffraction, electron microscopy, hydrogen–deuterium exchange and electron paramagnetic resonance spectroscopy can be used for deciphering the conformation to a limited degree. Computational approaches can extract valuable information from low-resolution data. These tools are validated using MD simulations [25].

3.2 Computational approaches in study of aggregation

Computational algorithm methods have been developed where spatial aggregation propensity (SAP) has been employed for prediction of aggregation-prone areas in monoclonal antibodies, utilizing MD simulation approach. Information on the structure and amino acid sequence forms the basis, which ultimately facilitates the identification of exposed hydrophobic patches/motifs or aggregation-prone zones [10, 14, 26]. Several antimicrobial phenolic preservatives (m-cresol, phenol, benzyl alcohol, phenoxyethanol and chlorobutanol) have been found to induce destabilization, protein unfolding and aggregation to a different extent in protein-based multidose preparations [27]. Antimicrobial preservative, benzyl alcohol, has been found to induce aggregation in IFN-α-2a, when investigated by multiple aggregation propensity programs. Benzyl alcohol may perturb the predicted "hot-spots" in the protein molecule, disrupt the contacts between the critical residues, may initiate local unfolding event or may cause swap of domain. The SAP approach is highly accurate although time consuming. However, the expenditure in full antibody atomistic simulation is quite high. There are cheaper and more viable options such as antibody fragment (Fab, Fc) simulations, implicit solvent models or direct computations from a static structure. The last method is very fast but compromises on the level of accuracy. But it is highly suitable in high-throughput screening of therapeutic protein candidates. Identification of the most stable and soluble analogue of mAbs is possible through adoption of SAP. Aggregation "hot-spots" in the IFN-α-2a can be blocked through mutations which can prevent preservative-induced aggregation [28]. Fibroblast growth factor 21 controls hormone levels and may prove highly beneficial in the management of metabolic disorders like diabetes, obesity, cardiovascular disease, etc. However, multiple-dose formulations of the hormone regulator for parenteral delivery contain a phenolic preservative which induces the aggregation of the protein and lower solubility. With an aim to reduce the aggregation tendency without compromising its therapeutic utility, a variant of the molecule has been developed [29]. Similar approach has also been adopted for the design of biobetter therapeutic mAb and becavizumab. Stabilized mAb was produced through single-point

mutations of aggregation-prone zones and design of engineered glycosylated protein where the carbohydrate moiety has been used to mask the "hot-spots" [30]. However, difficulties arise during prediction of aggregation in folded globular proteins because aggregation hot-spots are not exposed or they are buried inside the hydrophobic core and may be discontinuous. They may be involved in cooperative non-covalent interactions sustaining secondary and tertiary structure of the protein. Aggregation propensity of globular proteins can be studied by AGGRESCAN 3D Dynamic mode and extracted information can be utilized to engineer protein variants or mutants with increased solubility and stability. It demonstrates higher accuracy in comparison to first-generation sequence-based programs [9].

4 Computational tools in assessment of immunogenicity of therapeutic proteins

Therapeutic antibodies, exogenous enzymes, signalling peptides of well-defined structure and function can be immunogenic, if they are of non-human origin. Non-immunogenic or de-immunized variant of the antibody, immunotolerant variant of the enzyme with unaltered binding specificity, affinity and stability can be developed by grafting of key functional residues from an exogenous therapeutic antibody onto human antibody framework. Grafting can be done on a trial-and-error basis or it can be done on a rational basis with a prior knowledge of structure–function relationship. An important obstacle to grafting is the absence of common modular structure in these therapeutic proteins and also lack of homologous human counterpart. Computational methods have been used on a small subpopulation of peptides (T-cell epitopes) for identification of mutations to minimize MHC II binding, responsible for eliciting immune response. Epitope databases like Immune Epitope Database (IEDB) and the proprietary T Cell Epitope Database™ (TCED™) have also been used for the purpose. Improved computational algorithms will target amino acid sequence for prediction of immunogenicity of the entire protein and will ultimately reveal the best structure with reduced immunogenicity potential. Such dynamic programming-based algorithms will enable mutations at the most promiscuous amino acids that are elements of multiple overlapping immunogenic peptides and resulting in the elimination of over six epitopes per mutation [31, 32]. Although several predictive models have been employed till date, the success rate in accurate prediction is not very encouraging. Low success rate is attributed to lack of sufficient knowledge relevant to the underlying mechanisms for immunogenicity of therapeutic proteins [33].

5 Conclusion

Owing to their large, complex and conformationally heterogeneous structures, therapeutic proteins are vulnerable to different instability problems induced by various physicochemical and mechanical stress factors during the various processing steps starting from the first process in multi-step manufacturing to administration. Propensity of the molecules towards aggregation needs attention as it can have deleterious effect on biological activity and safety. Therefore, preformulation studies need to be conducted prior to development of stable and efficacious solid dosage forms for the therapeutic proteins. Stability assessment is of primary concern in the preformulation study which utilizes different low-, intermediate- and high-resolution instrumental methods. Application of computational tools to low-resolution data helps in the prediction of secondary and tertiary structure of proteins with high level of accuracy. Data extracted from such analyses can be exploited in the redesigning and engineering of therapeutic proteins or biobetters with improved solubility and stability, which can be easily developed into a stable and effective formulation.

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