PROTEIN DRUG STABILITY: A FORMULATION CHALLENGE

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Abstract | The increasing use of recombinantly expressed therapeutic proteins in the pharmaceutical industry has highlighted issues such as their stability during long-term storage and means of efficacious delivery that avoid adverse immunogenic side effects. Controlled chemical modifications, such as substitutions, acylation and PEGylation, have fulfilled some but not all of their promises, while hydrogels and lipid-based formulations could well be developed into generic delivery systems. Strategies to curb the aggregation and misfolding of proteins during storage are likely to benefit from the recent surge of interest in protein fibrillation. This might in turn lead to generally accepted guidelines and tests to avoid unforeseen adverse effects in drug delivery.

During the past two decades, recombinant DNA technology has led to a significant increase in the number of approved biotechnology medicines and a shift away from the production of biologically active materials (biologics) on the basis of animal or human material, and towards cloning and fermentation. A recent survey lists 324 biotechnology medicines, either in human clinical trials or under review by regulatory agencies. These drugs cover nearly 150 diseases, including cancer, infectious diseases, autoimmune diseases and AIDS/HIV[1]. A substantial number of these substances are proteins. This development will expand the list of medicines and open the way for more and better treatments to the benefit of patients.

This general trend accords with the vision of several analysts, including IBM Business Consulting Services[2]. According to this view, many of the new medicines will be based on the recombinant DNA expression of proteins rather than organic chemistry, because biologics in general are expected to be less toxic and to behave more predictably *in vivo*. Biotechnological medicines could therefore potentially reach the market faster than chemical entities developed through traditional methods. On the other hand, the decoding of the human genomic and proteomic maps is anticipated to lead to discoveries in molecular medicine that could revitalize the development of small-molecular synthetic drugs[3,4]. In many respects, the efficacy and safety requirements of biologics are similar to the requirements for small, chemically synthesized drugs. However, owing to the biological origin and macromolecular structure of biologics, there is particular focus on contamination of biologics with other biological impurities, such as viruses, as well as conformational changes introduced either during production of the bulk substance or the final formulation. Well-documented and validated biological, physical and chemical methods are important tools for securing the quality and safety of biologics.

Several challenges confront pharmaceutical scientists involved in the development of biotechnological medicines, such as proteins. The successful formulation of proteins depends on a thorough understanding of their physico-chemical and biological characteristics, including chemical and physical stability, immunogenicity and pharmacokinetic properties. The therapeutic activity of proteins is highly dependent on their conformational structure. However, the protein structure is flexible and sensitive to external conditions, which means that production, formulation and handling of proteins needs special attention in optimizing efficacy and safety, including minimized immune responses. The primary focus of this review is on the challenges related to quality and safety issues that arise in the development of protein-based medicines.

The chemical and physical stability of proteins can be compromised by external factors such as pH, temperature

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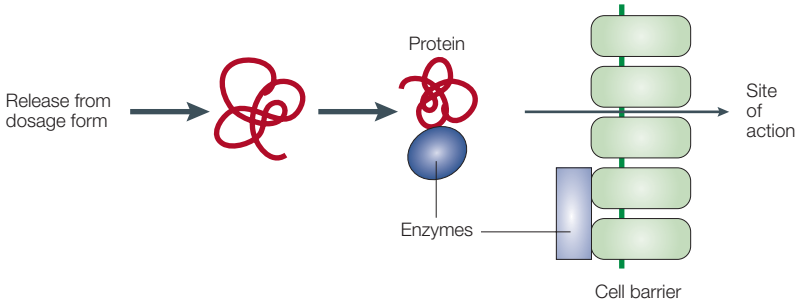

Figure 1 | **Barriers to biomacromolecular drug transport.** The major obstacles for efficient transport to the site of action are enzymatic degradation and biological membranes.

and surface interaction, as well as by contaminants and impurities for excipients, and so on[5,6]. Several recent reviews discuss the importance of protein stability in formulation development and the immunogenicity of proteins from a pharmaceutical perspective[5,7]. In addition, several recent reviews focus on conditions leading to undesirable changes in the chemical structure of proteins[8,9].

Delivery challenges

Oral administration of medicines is the preferred and most widely used route of administration. However, this route is generally not feasible for the delivery of macromolecules such as proteins. The inherent instability of proteins in the gastro-intestinal tract, as well as the low permeability across biological membranes due to the high molecular mass and polar surface characteristics of proteins, implies that proteins for systemic treatment should be administered PARENTERALLY; however, efforts are being made to improve bioavailability through alternative routes of administration, for instance, by the nasal or pulmonary route[10,11]. Nevertheless, bioavailability through the various non-parenteral routes of administration is low and normally insufficient for an effective systemic effect. The obstacles for efficient delivery to the site of action can broadly be categorized as either various enzymatic barriers that the protein encounters in moving from the administration site to the site of action, or physical barriers to effective transport, such as epi- and endothelial cell linings (FIG. 1).

The pharmaceutical and PHARMACOKINETIC properties of proteins can be optimized by different approaches — for example, by mutagenesis, chemical modification or by designing specific drug-delivery systems. However, most protein-based medicines today are still formulated as suspensions or aqueous solutions either in a ready-to-use form or as a lyophilized product for reconstitution.

Language to consider since we may argue that claims to a "solution" are not lyophilized compositions.

PARENTERAL
A substance that is introduced into the body anyway except by mouth.

PHARMACOKINETIC
The study of the absorption, distribution, metabolism, excretion and interactions of a drug.

PEGylation
The covalent binding of polyethylene glycol (PEG) to a protein.

Substitution and chemical modification

Proteins for therapeutic use can be chemically modified in several ways — for example, by mutating one or more amino acids (that is, creating a protein analogue) or by acylation or PEGylation. These modifications can be used to optimize the pharmacokinetic properties of the protein, but care must always be taken not to reduce their biological efficacy.

Protein analogues. The development of rapid-acting monomeric insulin analogues provides an excellent example of the analogue approach[12]. The basic idea behind this development was that the transport rate from the subcutaneous injection site of administration across the biological membrane to the systemic circulation would be increased if the self-association characteristics of insulin were shifted from hexameric insulin to di- or monomeric insulin (FIG. 2). By mutating amino acids involved in self-association, this approach was proven to be feasible. Monomeric insulin analogues with a more rapid time-action profile than human insulin are now on the market (for example, insulin lispro (Eli Lilly) and insulin aspart (NovoRapid; Novo Nordisk)).

Recombinant human interleukin-2 (IL-2) is another example in which the therapeutic effect is improved through molecular design. The commercially available non-glycosylated IL-2 analogue aldesleukin (Proleukin; Chiron) is a des-alanyl-1 analogue of human IL-2 in which cysteine in position 125 has been replaced by serine. The experimental IL-2 analogue BAY 50-4798 (Bayer) is another example of a genetically engineered protein with potentially improved therapeutic effect.

Acylation. Chemical attachment of fatty acids to exposed residues on the protein surface can in some cases increase the affinity of the protein to serum albumin sufficiently to increase its circulation time in the blood[13,14]. The effect of acylation is naturally greater for smaller proteins and peptides because of the greater relative increase in hydrophobicity. For example, the acylated insulin analogue [Lys.sup.B29]-tetradecanoyl des (B30) human insulin, insulin detemir (Novo Nordisk A/S) is long-acting and has recently been approved by the regulatory agencies in some countries. The principle of acylation has also been applied successfully to other proteins — for example, glucagon-like peptide 1 (GLP1) (REF. 15) and interferon-α[16], as well as for peptides such as desmopressin[17]. Whereas the acylation of insulin and GLP1 is site-specific, the acylation of interferon is less specific. This potential heterogeneity of acylated interferon could eventually prove to be a problem both from an efficacy and a safety point of view.

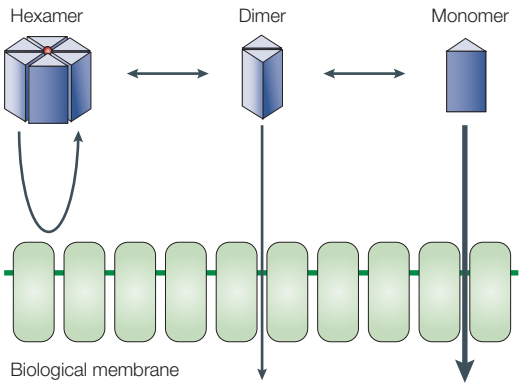

Figure 2 | **Transport of insulin across biological membranes.** Insulin monomer has the highest flux.

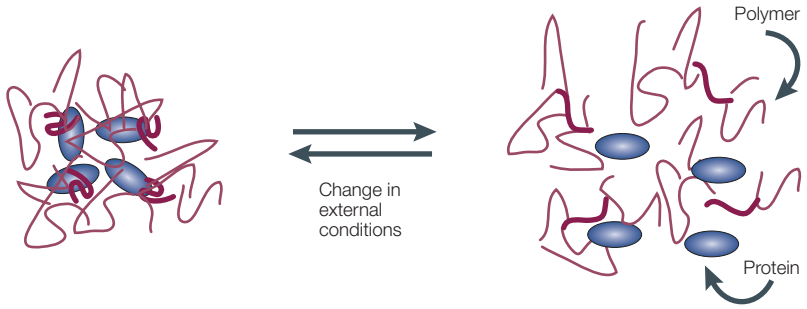

Figure 3 | **Schematic illustration of protein release from a polymer sensitive to external stimuli, such as change in pH, ionic strength and temperature.** Swelling of the polymer releases the entrapped protein.

PEGylation. In general, PEGylation reduces the plasma clearance rate by reducing the metabolic degradation and receptor-mediated uptake of the protein from the systemic circulation. PEGylation also improves the safety profile of the protein by shielding antigenic and immunogenic EPITOPES[18]. However, it is important to realize, both from an efficacy and safety point of view, that PEG polymers consist of a mixture of polymers with different molecular mass; in addition, larger proteins will have several sites that are accessible to PEGylation. Owing to this potential product heterogeneity, the clinical and pharmaceutical documentation required for drug approval could be even more demanding than for non-modified proteins. PEG-interferon-α is a commercial example in which the pharmacokinetic profile is improved by PEGylation, which enables less frequent administration and results in improved efficacy with a similar side-effect profile[19]. On the other hand, mono-PEGylated epidermal growth factor (EGF) with PEG 3400 at Lys28 and Lys48 was significantly less active than an EGF isomer PEGylated at the amino terminus in an *in vitro* assay for mitogenic activity[20]. Several excellent reviews are available on these specific types of derivative. A recent issue of *Advanced Drug Delivery Reviews* is devoted to PEGylation[21].

Drug-delivery systems. Entrapment and encapsulation are the most widely used formulation principles adopted for protein-delivery systems. Polymeric drug-delivery systems, such as hydrogels, nanocapsules and microspheres, and lipid-based drug-delivery systems such as liposomes and solid lipid nanoparticles, are all examples of protein-delivery systems (FIG. 3).

Hydrogels are crosslinked hydrophilic polymers that form three-dimensional networks, which can either be synthesized to degrade at a certain rate or to respond to several physiological stimuli present in the body, such as pH, ionic strength and temperature. Both principles are used for the controlled release of proteins[22–25]. These hydrogels can be formulated either as microspheres or as *in situ* forming delivery systems. The *in situ* forming systems could have several advantages over conventional microspheres, such as ease of administration and less complicated and potentially harmful manufacturing conditions appropriate for sensitive drug molecules such as proteins[26].

Examples of lipid-based formulations with the potential for serving as delivery systems for macromolecules such as proteins include liposomes, solid lipid nanospheres and water-in-oil emulsions[27–29]. The incorporation and release of proteins from liposomes, for instance, can be controlled by the physico-chemical characteristics of the protein — that is, the hydrophobic surface area, which can be modified, for example, by acylation[30] or by constructing lipid bilayers that are sensitive to triggered degradation by enzymes such as secretory phospholipase A2 that are present in inflammatory tissues and certain cancerous fluids[31]. Representative examples of modifications and delivery systems are given in TABLE 1.

Immunogenicity of therapeutic proteins. The ability to produce highly purified proteins that are identical or nearly identical to endogenous human proteins is often considered as the key to avoiding T- and B-lymphocyte reactivity during treatment, because patients are expected to be immune tolerant to their own proteins. However, although the level and incidence of immune responses is low, most of these proteins have been shown to be immunogenic, and in some cases have even led to serious safety problems and inhibition of the therapeutic effect due to neutralizing antibodies. An example is antibody formation after long-term treatment with recombinant interferons in patients with multiple sclerosis, which indicates that the presence of neutralizing antibodies against interferons reduces the clinical effect of the drug[32,33]. Recombinant human erythropoietin is another example in which the neutralizing antibodies react against erythropoietin and cause pure red-cell aplasia[34]. It has been suggested that the reason for anti-erythropoietin antibody production seen in patients might be related to the production process of the hormone[35]. A formulation change introduced in the late-1990s has also been proposed as a plausible reason for the induction of antibodies that neutralize the endogenous erythropoietin. A recent study indicates that this formulation contains micelle-associated erythropoietin, which could be a risk factor for the development of antibodies[36]. The difficulties in detecting structural changes in proteins in more complex pharmaceutical formulations is a major challenge for formulation scientists responsible for the development and quality of biopharmaceutical medicines[37].

In general, it is the protein structure that can trigger an immune response during treatment. Proteins are complex and flexible molecules, and even a small change at a particular site can result in a major change in the overall structure (TABLE 2). For instance, recombinant mutated allergens with a modified surface topography, but that retain an α-carbon-backbone folding pattern, have been shown to retain the capacity to induce allergen-specific immune responses, but with different anaphylactic potential[38]. This shows that although the surface structures positioned outside the mutated regions are retained, the introduction of mutations that change both the topography and the charge

EPITOPE
An alternative term for an antigenic determinant. These are particular chemical groups on a molecule that elicit a specific immune response.

Table 1 | **Modified proteins and protein-delivery systems approved for marketing**

Product (company)	Drug	Modification/delivery system	Administration route
Proleukin (Chiron)	Aldesleukin	Analogue	Intravenous
Humalog (Eli Lilly)	Insulin lispro	Analogue	Subcutaneous
NovoRapid (Novo Nordisk)	Insulin aspart	Analogue	Subcutaneous
Neulasta (Amgen)	PEGinterferon α-2a	Mono-pegylated	Subcutaneous
Pegasys (Roche)	Pegfilgrastim	Mono-pegylated	Subcutaneous
Somavert (Pharmacia)	Pegvisomat	Multi (4–6)-pegylated	Subcutaneous
Levemir (Novo Nordisk)	Insulin detemir	Mono-acylated	Subcutaneous
Nutropin Depot (Genentech)	Human growth hormone	PLGA microspheres	Subcutaneous
InductOs (Wyeth (MDT))	Bone morphogenic protein 2	Absorbable collagen sponge	Implanatable medical device

PLGA, poly(lactide-co-glycolic acid)

distribution can be sufficient to affect the immunogenicity of the protein (FIG. 4). However, it is normally difficult to relate a particular change in protein structure to a change in immunogenicity.

Two is a crowd: coping with aggregation

During processing and formulation of the drug product, the protein is exposed to conditions that could have significant effects on its chemical and physical stability, and lead to aggregation and ultimately precipitation[9,39–41]. It is therefore important to understand the circumstances by which protein stability is compromised. The main factors are shear/shaking, temperature, pH and protein concentration (see REF. 39 for a detailed overview). Shear forces encountered by vortexing can partition proteins to the air–water interface, which encourages partial unfolding on exposure to the more hydrophobic air phase[41,42]. This will be even more pronounced if the second phase involves an organic solvent such as chloroform[43]. Elevated temperatures[44], changes in pH or intermediate denaturant conditions[45] can also favour the formation of such states. Small, single-domain proteins usually require extreme conditions to unfold, but for large, multidomain proteins, a few 'weak links' unravelling under relatively gentle conditions can be sufficient to initiate aggregation. Partially unfolded states are much more susceptible to aggregation than the native or unfolded state, due to the exposure of contiguous hydrophobic regions that are buried in the native state or absent in the denatured state[40,46].

Although aggregation is fundamentally bi-molecular, the dependence on concentration will reflect the mechanism of the process. If unfolding of the native state is rate limiting, aggregation kinetics can be pseudo-first order[47,48], whereas there is an inverse dependence for processes involving air–water interfaces[49]. Initial dimer formation is seen with colony stimulating factor[50], whereas lysozyme aggregation follows higher-order kinetics[51]. Aggregation often shows high activation barriers, which make the process slow, irreversible and kinetically controlled. This means that the handling history of a sample can have a disproportionate role in the outcome. Irreversible aggregation, caused by disulphide-shuffling or stable hydrophobic association, can have a direct impact on drug potency, immunogenicity[47] and the unfolded protein response[52,53]. Reversible aggregation, however, can also be problematic during drug administration, if dissociation is slow on the physiological time scale. Slow dissociation kinetics can be a consequence of crowding effects in the body, which favour the aggregated state even after dilution[54,55].

The protein chain is chemically complex and can assume an astronomical number of possible conformations, the relative stabilities of which are very sensitive to pH, ionic strength and temperature. This creates a large number of 'conformational end stations' — including both those that are monomeric and oligomeric — that depend on environmental conditions (FIG. 5). The aggregates are not just featureless heaps; it has been known for a long time that aggregation is generally quite specific[56], involving well-defined oligomerization interfaces. Probably the most important — and certainly most well-characterized — aggregation state is the amyloid fibril, associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and many others. Curiously, fibrillation is exploited in a wide range of organisms, ranging from biofilm in *Escherichia coli*[57] to melanosome formation in humans[58]. It has been suggested that all proteins can form fibrils with the same structural characteristics, namely a cross-β structure and parallel β-helices[59], irrespective of the native structure and primary sequence[60]. This presents a potential hazard during production and/or storage. Fibrillation is particularly encouraged under moderately destabilizing conditions, such as pH below 3 or above 10, temperatures above 40° C and/or intermediate denaturant concentrations, which allow the protein better access to other conformations while retaining some structure.

Table 2 | **Potentially immunogenic protein modifications**

Modification	Effect
Engineered modifications	
Amino-acid sequence	Human versus analogues and non-human proteins
Chemical modification	Acylation, PEGylation
Pharmaceutical formulation	Lyophilization, micro-encapuslation
Unwanted modifications during processing, production and storage	
Chemical degradation	Deamidation, oxidation
Physical degradation	Denaturation, aggregation, fibrillation, misfolding

PEG, polyethylene glycol.

Fibrillation is generally modelled as a nucleation-dependent process, in which the nuclei accumulate during the lag phase without any bulk conformational changes, followed by the rapid accumulation of fibrillated protein as the nuclei are extended[61]. The lag phase is typically very long, mainly because the higher-order equilibria leading to nuclei are unfavourable at low protein concentrations. This makes the process very sensitive to protein concentration; for sickle-cell haemoglobin fibrillation, concentration dependencies of up to 50 have been predicted and observed[62]. Simulating aggregation *in silico*[63,64] or setting up mathematical models[65] can be useful to predict and prevent problems in protein production, but it remains challenging to incorporate all the environmental factors that impinge on the process. Aggregation is fundamentally a deviation from the 'productive' folding pathway, and it has been proposed that the individual energy landscapes or funnels describing protein folding should really be represented as 'double funnels', the second of which represents intermolecular interactions of partially folded states (FIG. 6)[66].

Intermolecular β-strand formation can be a powerful driving force for aggregation, and the propensity to form β-strands generally correlates positively with aggregation, in contrast to that of α-helices[67]. However, it has recently been suggested that α-helices can contribute to aggregation through the formation of coiled-coil contacts[68], although this is unlikely to be a general mechanism. It is now possible to predict absolute aggregation rates and the effect of different mutations on aggregation using simple physico-chemical approaches[69–71], such as changes in secondary structure propensities, hydrophobicity, patterns of alternating hydrophobic–hydrophilic residues, pH, ionic strength, concentration, and so on. For example, the sequential location of hydrophobic amino acids controls fibrillation of the amyloid β (Aβ) peptide[72] and insulin[73]. Aggregation propensities can even be predicted at the level of individual residues to pinpoint aggregation hot spots[69]. Key 'gatekeeper' residues are sometimes involved in stabilizing/destabilizing such interactions, so a few mutations can easily tip the balance towards the monomeric form[74,75]. However, it is characteristic of the complexity of the reactions leading to aggregation that a simple parameter such as stirring has yet to be incorporated into these models.

The lag time is probably the most important parameter from a formulation point of view. Unfortunately, it is very difficult to predict, not only because of the stochastic nature of the nucleation processes that can give rise to a lag phase, but also because of the complexity of the mechanism involved. It is common to assume that lag times represent a nucleation process[61], but this is not always the case[76]. Lag phases can also be observed even with a supply of preformed fibril seeds[76] (J.S. Pedersen and D.E.O., unpublished observations). In such cases, fibrillation is more likely to be promoted by small fibrils fragmenting to smaller entities that can capture more monomers before they fragment again, leading to the exponential growth of monomer-ensnaring fibrils[77,78]. Here, the lag phase does not have a simple physical interpretation, but simply reflects that the amount of amyloid is too low for detection in the lag phase. For polyglutamine sequences involved in Huntington's disease, the nucleus is actually a monomer[79], revealing that the rate-limiting step is not the assembly of an oligomeric species but a conformational change occurring at the monomer level.

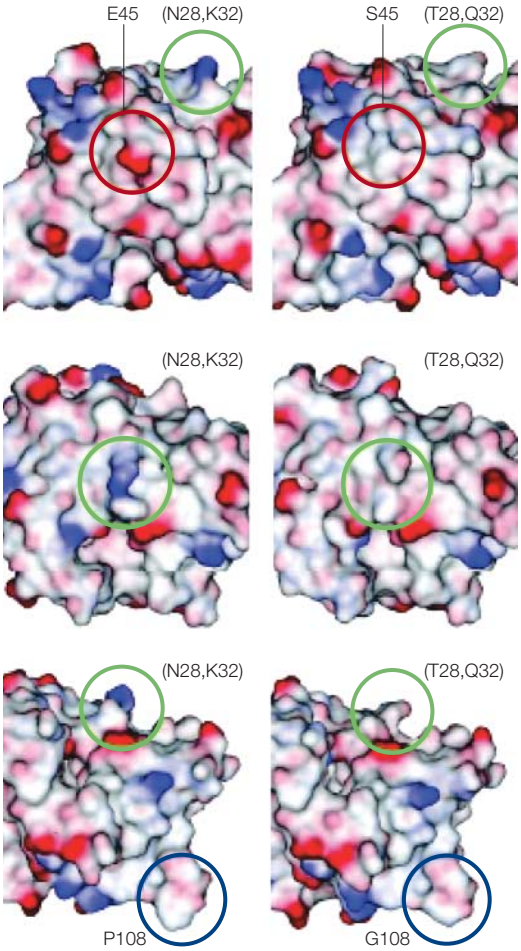

Figure 4 | **Protein structure and immunogenicity.** Comparison of molecular surfaces coloured according to electrostatic potential. Introduction of amino-acid mutations change both the topography and the polarity locally, whereas surface structures outside the mutation sites are retained. Adapted, with permission, from REF. 38 © (2004) American Association of Immunologists.

In cases in which seeding has been shown to accelerate the process, such as for the Aβ peptide[80], it might provide some relief to the biotechnological community to know that spurious fibrillar contaminants of another protein need not wreak havoc. The seeding process is very specific and demands very close sequence[81] and enantiomer[82] correspondence between the soluble monomer and the fibril; even point mutations can completely abolish seeding ability[83]. Nevertheless, it should also be noted that fibrillating proteins can form fibrils that differ significantly at the molecular level, simply by varying growth conditions such as stirring/aggregation[84], temperature or ionic strength (J.S. Pedersen and D.E.O., unpublished observations), and these differences can be propagated from one generation to the next. This polymorphism is

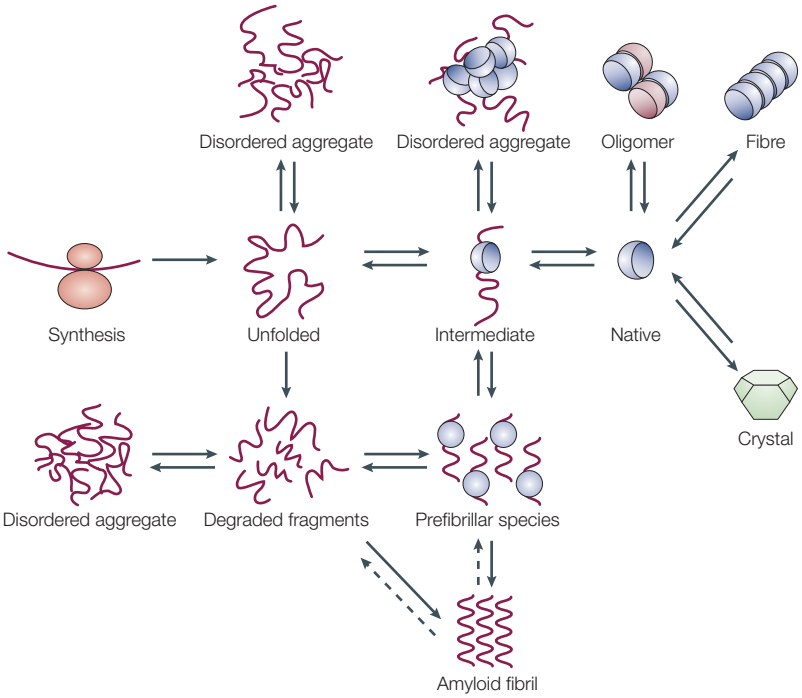

Figure 5 | **The many conformational choices for a polypeptide chain.** Adapted, with permission, from REF. 138 © (2003) Macmillan Magazines Ltd.

to be expected, simply because there has not been evolutionary pressure to evolve one particular kind of fibril, and fibrillogenic proteins are typically flexible, giving access to many conformations. Calorimetric[85] and structural[86] studies also indicate that fibrils are more porous than conventional globular proteins.

Peptides are more of a liability than proteins because of their greater flexibility and access to fibrillogenic conformations. The peptide hormone glucagon, which does not fibrillate in the body, can nevertheless fibrillate fairly easily if mishandled by, for example, prolonged storage at 2.5 mg per ml or higher at 37° C[87]. It is possible to select for less aggregation-prone peptide variants by fusing the peptide in question to green fluorescent protein (GFP), which does not show fluorescence when it accumulates in inclusion bodies[88] and thereby indicates loss of biological structure and function.

Formulating longevity: additives and storage

Recent work has revealed a strong correlation between different types of kinetic stability, as evaluated by unfolding in denaturant conditions and detergents, as well as by proteolysis resistance[89], and has found oligomeric β-sheet proteins to be particularly robust. These proteins are more rigid than their α-helix or mixed α/β counterparts. Analogously, proteins become both rigid and kinetically stable in very high concentrations of organic solvent[90,91], probably because the lack of bulk water acting as a lubricant reduces the energetic driving force for partial and global unfolding. Kinetic stability can be increased by introducing hydrophobic mutations, disulphide bonds, salt bridges and metal ions at the protein surface to stabilize and rigidify regions involved in local unfolding[92].

Because of the grave consequences of protein fibrillation in neurodegenerative diseases, the prevention of aggregation *in vivo* is a subject of intense research[93]. Many of the medically relevant fibrillating proteins have been subjected to extensive screening procedures, resulting in promising small-molecule leads for α-synuclein[94] and tau[95], for example. Fragments of the fibrillating peptide, which can bind to the growing edges of the fibrils by hydrogen bonding but prevent further elongation due to their small size (or by the inclusion of 'β-breaker' residues such as proline), also have an effect[96]. A combination of these two approaches has led to the use of small molecules with hydrogen-bonding patterns complementary to the exposed β-strand, but not to new incoming β-strands[97]. An even more ingenious 'Trojan horse' approach has been to couple the fibril-binding dye to a peptide, which, when in the cell, binds to the FK506-binding protein (FKBP) chaperone, thereby increasing its steric bulk and blocking further fibrillation[98]. Inhibiting aggregation under application-relevant conditions *in vitro* is obviously also very important. Mutagenesis to reduce aggregation is problematic because it can entail extensive clinical trials to document lack of other adverse effects. A more direct approach, therefore, is to alter the formulation conditions of the protein, but this is also challenging. Many therapeutic proteins are required in high doses, administered as part of frequent-dosing regimens, which means that it is important to keep them stably dissolved for extended periods of time at concentrations of tens of mg per ml. Determing the solubility of a protein is a complex task, because of the propensity of proteins to interact with themselves, surfaces and solutes. Of the many techniques used for concentrating proteins, none are without problems[99], and most are only appropriate at certain concentrations of protein.

At present, the prevention of aggregation remains largely empirical, due to a lack of insight into the molecular details of the aggregation process (see REF. 39 for an excellent overview of different approaches). One popular approach is to stabilize the protein and thereby reduce access to partially folded conformations favouring aggregation by hydrophobic contacts, for example. A typical strategy is to add sugars or salts to a protein solution. These solutes are thought to be preferentially excluded from the surface of the protein, therefore favouring a compact state[100,101]. However, because aggregation buries even more surface area per protein molecule, over-stabilization can ultimately lead to aggregation. Other stabilizers include polyols, PEGs and other polymers that sterically hinder protein–protein interactions and limit diffusion. Free amino acids are also often used; arginine is particularly good at preventing aggregation during the refolding of proteins from inclusion bodies[102]. A more sophisticated approach is to add 50 mM each of arginine and glutamate, which leads to a marked increase in the long-term stability of the sample, and also prevents aggregation, precipitation and proteolysis[103]. It has been proposed that the basis for this remarkable effect lies in the capacity of the pair to neutralize opposite charges (which would otherwise lead to intermolecular association) combined with the ability to cover adjoining hydrophobic areas through aliphatic tails[103].

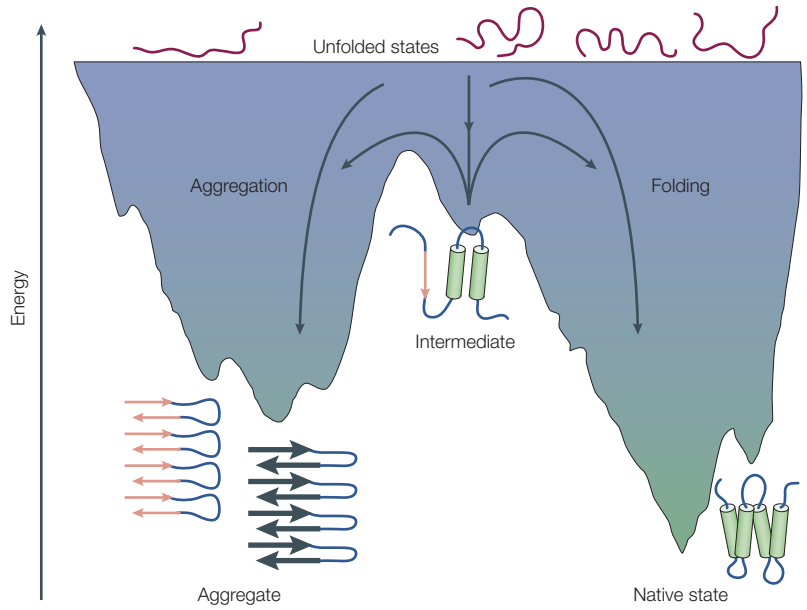

Figure 6 | **The double funnel of folding and aggregation.** Folding is a journey through conformational space, whose architecture is sensitive to environmental conditions, including protein concentration. Whether the protein chooses the folding or aggregation funnel will depend on these conditions. Note that the aggregation funnel is less jagged than the native funnel, emphasizing that there is space for more 'conformational largesse' due to the lower restrictions on aggregate conformations. Adapted, with permission, from REF. 66 © (2004) Elsevier Science.

Detergents and other amphiphiles. Non-ionic detergents are often used to reduce the effect of shear, as they outcompete proteins at the air–water interface[104], but this can sometimes be counterbalanced by accelerated aggregation under long-term motionless shelf storage[105]. They are sometimes[106], but not always[107], able to prevent aggregation induced by heat. Ionic surfactants are generally denaturing, but reversible unfolding has been used to prevent aggregation of heat-denatured RNase[108]. They tend to stabilize α-helical conformations that do not form intermolecular contacts with the same alacrity as β-strands. However, just as the water–chloroform interface can be a powerful stimulant of aggregation[43], recent work indicates that anionic surfaces encourage protein aggregation and fibrillation — for example, through negatively charged phospholipid vesicles[109,110], anionic surfactants[111] and carboxylate-modified polystyrene beads[112]. The phospholipid environment encountered *in vivo* is chemically diverse, providing an opportunity for both hydrophobic interactions in the transmembrane region as well as polar and electrostatic contacts through the head group of lipids in the membrane. It has been suggested that the lipid surface acts to concentrate and align protein molecules, possibly through clusters of cationic residues on the protein. Another mechanism could be for the negative surface to stabilize a fibrillogenic monomer conformation, which through intermolecular interactions forms the nucleus for subsequent fibrillation[112].

Cyclodextrins. A class of substances that has been found to have significant potential in reducing aggregation is the cyclodextrins. These are circular polymers of typically five to seven glucose rings[113], although larger polymers also occur[114]. Cyclodextrins suppress aggregation of therapeutically relevant proteins, such as insulin[115–117] and growth hormone[118,119], as well as several other proteins[120,121]. This derives from their ability to bind to aromatic residues[122], which can lead to preferential stabilization of the unfolded state[123] and a reduction in folding rates[124]. The ability to suppress aggregation has also led to their use as simple chaperones for the refolding of proteins in combination with SDS[107,125]. The commonly used β-cyclodextrin is not approved for human consumption in an unmodified form because it is capable of extracting biomembrane components[126], and forms a highly insoluble complex with cholesterol, which could lead to toxic effects after prolonged exposure[127]. However, careful derivatization of β-cyclodextrin alters its selectivity towards biomembrane components and minimizes its toxicity[127]. Drug formulations containing hydroxypropyl-β-cyclodextrin (for the peptides leucine enkephelin and a neuromedin B-receptor antagonist) and sulphobutylether-β-cyclodextrin (for the small-molecule drugs ziprasidone (Geodon; Pfizer) and voriconazole (Vfend; Pfizer) have been approved for parenteral drug administration.

Lyophilization. Storing proteins in solution at low temperatures generally extends their shelf-life. Proteins can be denatured at low temperatures in the liquid phase, but, in general, cold denaturation is reversible, unlike most high-temperature denaturation, and so offers no practical problems[128]. Freezing obviously provides access to even lower temperatures, but repeated cycles of freeze–thawing can strongly stimulate aggregation by providing nucleation surfaces at the ice–water interfaces[105], as well as leading to pH changes and phase separation[129]. Another approach is therefore to restrict protein mobility by lyophilization or freeze-drying, for example. Freeze-drying is the separation of liquid water from a solution frozen to ice by vacuum sublimation, leaving the solutes in an anhydrous or almost anhydrous state. As described by Franks[130,131], the process is not just trial-and-error, but can be controlled by well-established physicochemical and engineering principles, taking into account parameters such as the composition and concentration of the product, the type of container, the equipment and process cycle (including primary and secondary drying). The method is not without challenges, because differential precipitation of buffers or other solvent compositions during freezing can lead to pH changes that irreversibly inactivate proteins. This can be counteracted by excipients, such as certain carbohydrates, soluble polymers, salts and volatile compounds, which form glasses during freezing, which in effect preserves liquid properties in the solid state and prevents unwanted crystallization and chemical side reactions. However, their effect on the glassing temperature must also be taken into account. Temperature and chamber pressure both affect sublimation rates and can be combined judiciously to avoid overheating and structural collapse of the lyophilized solute network. Assessing the stability of the freeze-dried product is not straightforward; accelerated storage

testing by product stressing can be misleading, because the Arrhenius kinetics used to extrapolate to low temperatures are not appropriate for temperatures around the glass transition[132]. It should also be noted that proteins can undergo reversible conformational changes in the lyophilized state that expose otherwise buried regions, making them susceptible to undesirable side reactions, such as disulphide bond shuffling in the presence of trace moisture. Examples include insulin[133] and β-galactosidase[134] (see also REF. 135 and references therein). Other crosslinking reactions include insulin transamidation[136] and dityrosine formation occurring under oxidative conditions or ultraviolet stress[137].

Conclusions

The successful development of protein-based medicines depends on an intimate understanding of their physico-chemical and biological characteristics, including chemical and physical stability, and their efficacy and safety profile. To get new, safe therapeutic proteins to the market more quickly, it is important to obtain a detailed understanding of what kind of protein modification might be acceptable from a safety and efficacy point of view early in the development process. However, it is equally important to understand the mechanisms by which the protein structure can be compromised during bulk processing and production of the final product. This includes strategies to reduce or prevent chemical degradation, denaturation, aggregation and other structural changes that could prove to be prohibitive for successful development of the drug. It is the joint responsibility of academia, the pharmaceutical industry and the regulatory authorities to establish the scientific background for the safe, fast testing and assessment of promising new biopharmaceuticals to the benefit of patients and society.

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Competing interests statement
The authors declare no competing financial interests.

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