Protein therapeutics in the solid state

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ABSTRACT

When formulated as liquid dosage forms, therapeutic proteins often show instability during handling as a result of chemical degradation. Solid formulations are frequently required to maintain protein stability during storage, transport and upon administration. Herein we highlight current strategies that have been developed to formulate pharmaceutical proteins in the solid form. An overview of the physical instabilities which can arise with proteins is first described. The mechanism, challenges and applications of the key solidification techniques of crystallisation, freeze-drying and particle forming technologies are subsequently discussed. Examples of current commercial products are provided to give an insight into the medical applications of this science: these include NPH-insulin crystal suspensions, freeze-dried monoclonal antibodies and leuproride polylactide-co-glycolide microparticles. Finally, future perspectives in solid state protein formulation are described.

INTRODUCTION

Pharmaceutical proteins form a major class of medicines including therapeutic enzymes, peptide hormones, cytokines, monoclonal antibodies (mAbs) and vaccines. They are used to treat and prevent a wide range of diseases. New expression systems and more efficient downstream processing have in recent years significantly improved the manufacture of therapeutic proteins. Protein instability often contributes to variations in the manufactured product, and is a major concern in the final dosage form. Retaining the native configuration in protein formulations is of vital importance in maintaining their function. Loss or change in structure may result in the loss of dose reproducibility and may increase the propensity for deleterious side effects (*e.g.* increased immunogenicity).

In this review, we consider the range of platforms which have been used to manufacture therapeutic proteins in stable solid-state dosage forms. First, some background information on the physical instability of therapeutic proteins is provided. A range of solidification techniques including crystallisation, freeze-drying and particle forming technologies are then discussed in terms of their underlying principles, limitations, product stability and applications. Examples of commercial products containing proteins solidified by each method are given. Future opportunities and challenges in stabilising proteins in the solid state are also considered.

1. POTENTIAL PHYSICAL INSTABILITIES IN PROTEIN THERAPEUTICS

Biologically active proteins will have a unique native three-dimensional flexible structure, typically known at the tertiary or folded structure, that allows them to undergo specific interactions with other molecules in biochemical reactions [1-2]. Protein tertiary structure is maintained by non-covalent interactions, where generally the more insoluble amino acid residues are less solvent accessible and much of the thermodynamic stability of the folded structure is maintained by the hydrophobic effects that are possible in the folded state [3–5]. The non-covalency of the tertiary structure means that changes in the chemical (*e.g.* pH or ionic strength) or physical (*e.g.* agitation or shear) environments can lead to loss of tertiary structure (*e.g.* misfolding, aggregation, denaturation) [1] .

Under physiological conditions, protein folding is a complex post-translational process that has evolved for proteins to attain their biologically active form. Often proteins also need to be secreted from the intracellular environment. While the information encoded in the amino acid sequence of the polypeptide chain [2] dictates the most stable tertiary structure of a protein, during the folding process there are many possibilities for misfolding. Such misfolded structures may occupy thermodynamic energy states that inhibit formation of the correctly folded protein. Many relevant proteins of therapeutic interest tend to be potent and to be physiologically available only in small amounts. Maintaining the tertiary structure is therefore of critical importance to be able to utilise protein therapeutics effectively.

When pharmaceutical proteins are placed in a non-physiological environment or exposed to physicochemical stress during handling or storage, the tertiary structure can alter to adapt to the changing environment [1,6]. These structural changes can result in formulations containing combinations of structural variants or intermediate species; these will have different physical properties including solubility, surface activity or propensity to aggregate [7]. Changes in protein conformation may result in the formation of insoluble precipitates, or lead to a denatured form [1,2,8] (see Figure 1). Subtle changes in the tertiary structure of a protein can lead to the formation of intermediate conformational states that have an increased tendency to interact with

other protein molecules to form aggregates. The aggregated molecules may be capable of de-aggregating to a monomeric intermediate form but more often undergo further conformational changes to form irreversible aggregates [1,9,10]. Considerable effort by pharmaceutical companies and regulators is focused on characterising protein aggregates and minimising the processes that result in their formation.

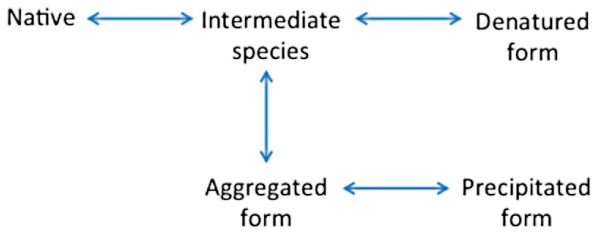

Figure 1: A schematic illustration of the different physical forms a protein can adopt.

Denaturation can occur as the result of a loss of tertiary and then secondary structure. As with misfolding and aggregation, denaturation will also result in the loss of protein function. While the chemical composition [10] of the protein remains the same, its function will be compromised so that providing a reproducible dose is not possible. All these processes may potentially result in the protein becoming immunologically more toxic. This is a situation that must be avoided, especially with replacement therapeutic proteins that are used to supplement an endogenous protein (*e.g.* erythropoietin). Loss of tertiary stucture can be caused by many different conditions, such as an increase in temperature, change in pH, the addition of organic solvents [7,10], freezing and thawing [11] or in the presence of certain chemicals such as urea or guanidinium hydrochloride [9-10].

1.1 PROTEIN AGGREGATION

Generally therapeutic protein species such as dimers, oligomers or multimers (as opposed to a single unit monomeric form) are referred to as “aggregates” [14]. Aggregation often results from non-covalent interactions between normally solvent inaccessible residues. Bond formation (*e.g.* intermolecular disulfide bond formation) between protein molecules can also drive aggregation. Reversible aggregates are

frequently formed when the protein concentration is above its equilibrium solubility [15]. Under such conditions proteins tend to form insoluble aggregates, which can sometimes be returned to their native monomeric form when the concentration is decreased below the solubility limit [1,14]. Irreversible aggregates generally form as a result of non-covalent interactions (such as hydrogen bonding, hydrophobic or electrostatic interactions) or covalent bonding through disulfide bridges or dityrosine formation [6,14,16]. Protein aggregation can cause an immune response to be stimulated *in vivo* [14-15]. The deposition of proteins as amyloids fibrils is the underlying pathogenesis for more than 20 degenerative diseases including Parkinson's and Alzheimer's [19]. Understanding and controlling protein aggregation is thus a major challenge for the biopharmaceutical industry.

2. STRATEGIES TO IMPROVE PROTEIN STABILITY IN THE SOLID STATE

Therapeutic proteins in liquid formulations are susceptible to loss of tertiary structure during shipping and storage [20]. The presence of water can also facilitate chemical degradation reactions which change the covalent structure of the protein [21]. Hydrolytic processes including deamidation, oxidation and aspartate isomerisation may lead to aggregation, denaturation, and loss of activity [10,22]. Ideally, the development of stable liquid formulations would be the optimum solution to these problems. However, this is very frequently not possible. In such cases, the removal of water to give a solid protein formulation can improve protein stability during storage [23–25], and can also significantly lower the rate of hydrolytic reactions. Much effort is focused at removing the need for a cold chain, so that protein therapeutics can better withstand storage at ambient conditions: solid formulations can also be advantageous here. The following sections discuss the various strategies that have been considered to stabilise the protein in the solid state including crystallisation, freeze-drying, and particle forming technologies. These solid state formulations will require reconstitution with water prior to administration, which can be disadvantageous particularly where high injected volumes are required. However, the use of solid-state formulations, particularly freeze dried formulations has led to a number of commercially available medicines, and to very significant improvements in the health of many individuals.

2.1 PROTEIN CRYSTALLISATION

Proteins are generally amorphous materials, meaning that in the solid state there is no regular arrangement of the macromolecular units. There is thus no **lattice enthalpy**, and as a result proteins in the solid state are normally highly prone to interact with denaturants [26]. In contrast, crystalline solid state forms of macromolecules are resilient to chemical denaturation because of their lattice enthalpy: the existence of a regular arrangement of molecules and intermolecular bonds in the solid state enhances stability [27]. The development of crystalline forms of therapeutic proteins is thus a potential route to increase their stability. In addition, crystallisation may remove the requirement for further downsteam purification, therefore minimising production costs [28]. The crystal phase diagram (Figure 2) is a useful tool to understand crystal formation and to design a crystallisation process [29]. Typically, a crystallisation process will be undertaken by adding an amorphous solid state protein to a solvent. The protein solutes diffuse from the solid to the bulk solution, and when the solution concentration reaches a certain point nuclei are generated. These act as "seeds" for further crystallisation. The number of nuclei will increase in number when the concentration of protein approaches the supersolubility curve (see Figure 2). The protein concentration is spontaneously decreased upon nucleation, which shifts the system from the supersaturated region to the metastable zone (II) where nuclei 'growth' occurs. Crystal formation proceeds as further protein molecules are deposited on the seeds, eventually yielding visible crystals [26]. Amorphous precipitation (IV) may occur beyond the nucleation zone (III) [30]. In order for a successful crystallisation process to occur, all this must take place without the protein tertiary structure being altered.

Key term

Lattice enthalpy: A measure of the strength of the intermolecular bonds between macromolecules in the solid state.

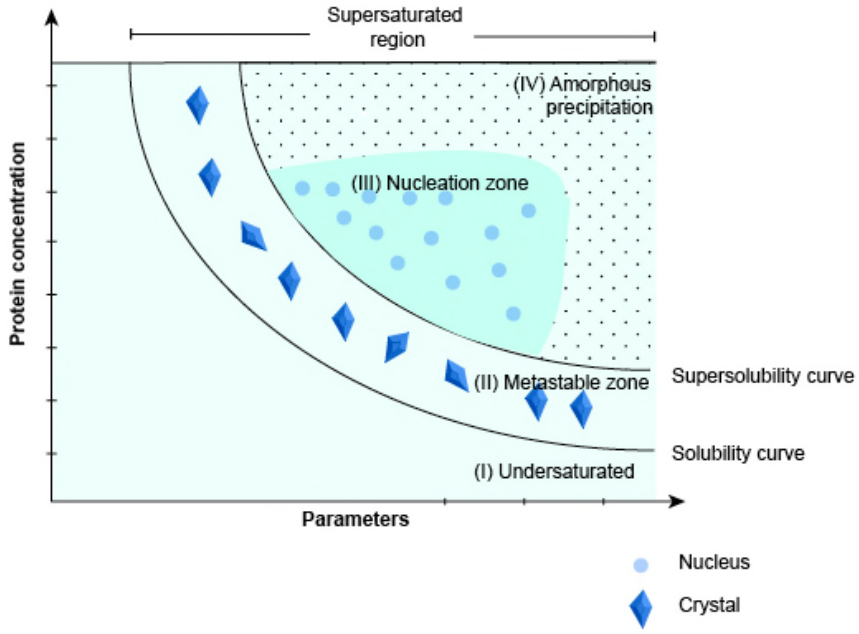

Figure 2: A schematic crystal phase diagram illustrating the (I) undersaturated region, (II) metastable zone, (III) nucleation zone and (IV) amorphous precipitation region. Adapted from Ref. [29].

A range of parameters (*e.g.* pH, temperature, metal ions, precipitants) can be adjusted to facilitate crystallisation. Additives may also be employed to drive the process forward. For instance, insulin is negatively charged at physiological pH, and forms a complex with divalent ions in the pancreas. This suggests that such ions can be used to aid the formation of protein crystals [31,32]. The addition of phenolic ligands or high chloride concentrations also assist crystallisation [33,34]. This highlights a role for phenolic derivatives (*e.g.* m-cresol and methyl paraben) in insulin preparations beyond simply acting as preservatives. Rhombohedral insulin crystals, for instance, were obtained with 0.25 % aqueous phenol at pH 5.5, while monoclinic crystals were formed with 1% phenol at pH 6.49 [35,36]. These results demonstrate the importance of the crystallisation conditions (*e.g.* pH and phenol ligand concentration) on the interaction of the insulin molecules and the resultant crystal habit.

Insulin is rather unique in being a small protein which forms stable hexamers, but is unstable as fibrils. However, other proteins have also successfully been crystallised. Spherical crystals of recombinant human interferon-α-2b (rhIFN) were prepared by adding zinc acetate to a solution of the protein; see Figure 3(a). The extent of crystal recovery is proportional to the zinc acetate : rhIFN ratio used in processing [37]. X-

ray diffraction data clearly demonstrated distinct Bragg reflections when rhIFN was reacted with a 12 : 1 mol ratio of zinc acetate, confirming that crystals had been formed: this is depicted in Figure 3(b) and can be compared with the amorphous halo seen in the diffraction pattern of the native protein. In another study, Hebel *et al.* demonstrated the successful crystallisation of antibody fragments (FabC225) in a stirred tank [38]. For a more detailed treatment of the crystallisation of bioactive protiens the interested reader is directed to a recent review [28].

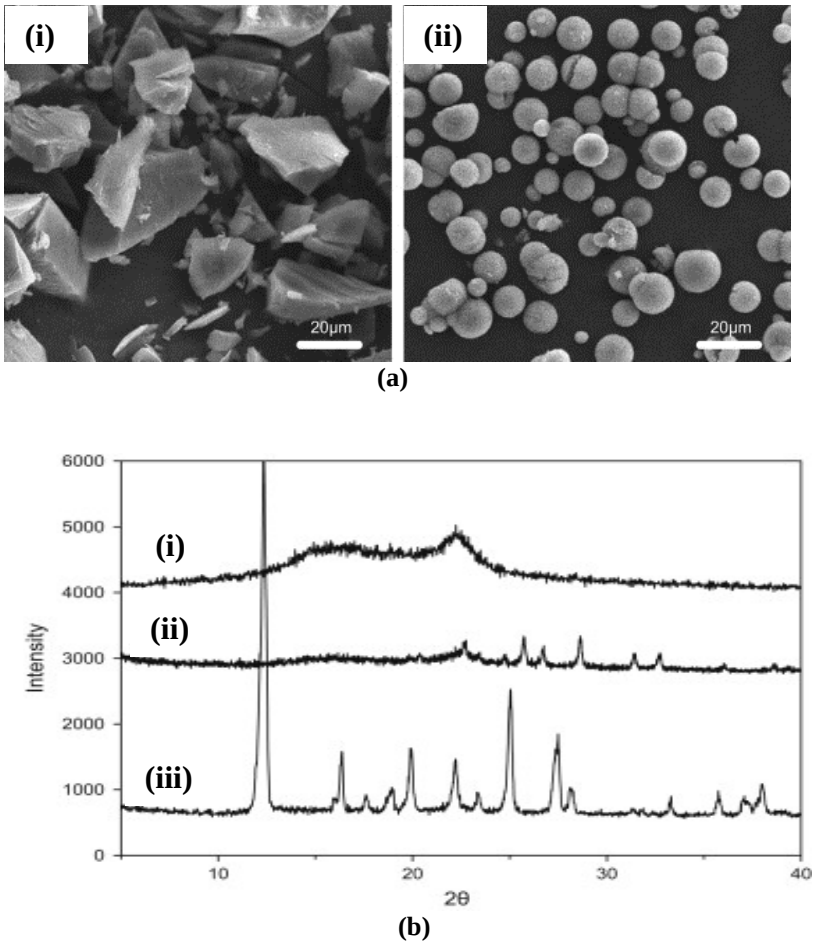

Figure 3: Characterisation data for spherical rhIFN crystals formed after reaction with Zn acetate. **(a)** Scanning electron micrographs of i) native rhIFN and ii) rhIFN crystals, and **(b)** X-ray diffraction patterns of i) native rhIFN, ii) rhIFN crystals and iii) zinc acetate. Reproduced with permission from ref. [37]. Copyright Shenyang Pharmaceutical University 2013.

For small molecule drugs, nucleation is considered to be the rate limiting step in crystallisation. However, the mechanism of protein crystal formation is rather more complex, and has not to date been fully elucidated. This stems from the increased size and complexity of macromolecules, which means they are less likely to pack together

as long-range ordered systems. The size, length and flexibility of amino acids also influence lattice formation [39]. In addition, the translational and rotational diffusion of protein molecules in solution is crucial for nucleation. The former can bring two macromolecules into intimate contact [40], which is explained by the Smoluchowski equation (Equation 1) :

$$k_D = 8\pi Dr \qquad \text{(Equation 1)}$$

In Equation 1, k_D is the diffusion rate constant for a second order bimolecular reaction, D is the translational diffusivity and r is the radius of the molecules colliding. The rate of protein diffusion is slow (much less than that for small molecules). It is clear from this that steric hindrance influences molecular diffusion, and is likely to complicate protein lattice formation.

This diffusion process can be simulated for proteins using Brownian dynamics, under the assumption that two spherical proteins have to reorient the complementary parts of their structures ("patches") in the correct configuration prior to association [41]. Nanev has devised a patch model to describe protein nucleation, depicted in Figure 4 [40]. In brief, the surface patches through which two protein molecules can interact are considered to be symmetrically distributed on spherical protein. The complementary patches on two molecules must be paired in order to form clusters of molecules. The model suggested that the pattern of patches on a protein's surface is likely to have a significant impact on the nucleation process [42]. The energy barrier to nucleation and the homogeneity of the nuclei forming are also important to consider [43]. In practical terms therefore, to achieve successful protein crystallisation we need to understand their structural biology, and extent of purity. Those technologies have been reviewed elsewhere [29] and may be applied to formulate crystalline proteins. However, manufacturing issues such as batch to batch reproducibility and aseptic manufacture also need to be addressed to achieve large scale production [28,38].

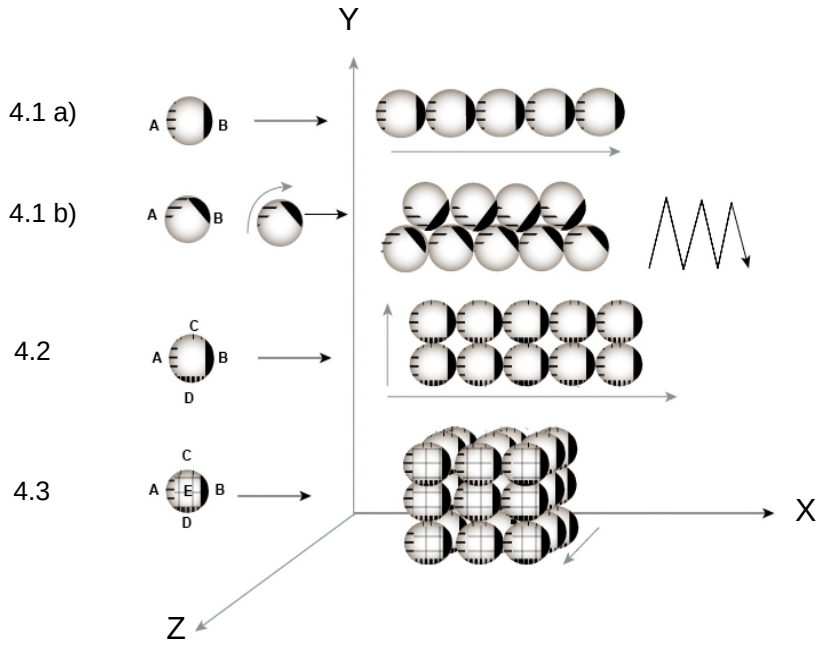

Figure 4: An illustration of the contact patch model for protein nucleation (not to scale). **4.1a)** a linear protein cluster is formed by pairing two complementary patches (A and B) in one direction. **4.1b)** The spherical protein has to reorient to match patches A and B prior to association, giving rise to the growth of one dimensional in "zigzag" clusters. **4.2** The protein shown here is more complex, with additional patches C and D which can interact to form a 2D-cluster. **4.3** A three-dimensional aggregate is formed when complementary patches are paired in all directions: A and B, C and D, E and F (patch F is behind E). Adapted from refs. [43,44].

Although it is hypothesised that the generation of crystalline protein materials can enhance stability [27], there are other complicating factors that may counteract this effect. Huus *et al.* investigated the thermal stability of insulin complexes at 37 ºC [45]. Their results demonstrated that a zinc-insulin complex with one Zn^{2+} per hexamer (0.1 mM Zn^{2+} to 0.6 mM insulin) can prevent insulin from B3 deamidation if the Zn coordinates at a specific site. However, Zn^{2+} itself may cause the Asn^{B3} residues to become more electrophilic and subject to processes such as dimerization. This issue, along with that of hexamer fragmentation, is alleviated when the hexamer is stabilised with phenol. Differential scanning calorimetry data from the same study indicated that anionic ligands could reinforce zinc affinity for the protein complex.

Commercial application: NPH-insulin crystals

Insulin is a peptide hormone secreted by the β-cells of the pancreas, and is vital for the regulation of blood sugar levels. Artificial insulin formulations comprise one of the most successful biopharmaceutical products developed to date, and the development of insulin replacement products has been hugely successful in the management of both type I and II diabetes.

Native insulin contains 51 amino acids arranged into two polypeptide chains: the A-chain contains 21 residues and the B-chain 30 residues. The chains are linked with three disulfide bonds, two inter-chain (A7-B7 and A20-B19) and one intra-chain (A6-A11). The structure of insulin is given in Figure 5. Genetic engineering has been heavily exploited to modify the insulin sequence in order to tailor the pharmacokinetic profile of the molecule, as shown in Table 1 [46,47].

Table 1: Genetically engineered insulin therapies approved in the clinic. Adapted from refs. [46,47].

Product	Structure modification	Duration of action	Manufacturer
Lispro	B28-B29 proline-lysine inversion	Short-acting	Eli Lilly
Aspart	B28 aspartic acid substitution	Short-acting	Novo Nordisk
Levemir	B30 depletion and B29 myristoyl lysine	Long-acting	Novo Nordisk
Glulisine	B3 lysine, B29 glutamic acid substitution	Rapid-acting	Aventis Pharmaceuticals
Lantus	A21 glycine substitution and B chain elongation by two arginines	Long-acting	Aventis Pharmaceuticals
Degludec	B30 depletion and B29 acylation with hexadecandioic acid via γ-L-glutamic linker	Long-acting	Novo Nordisk

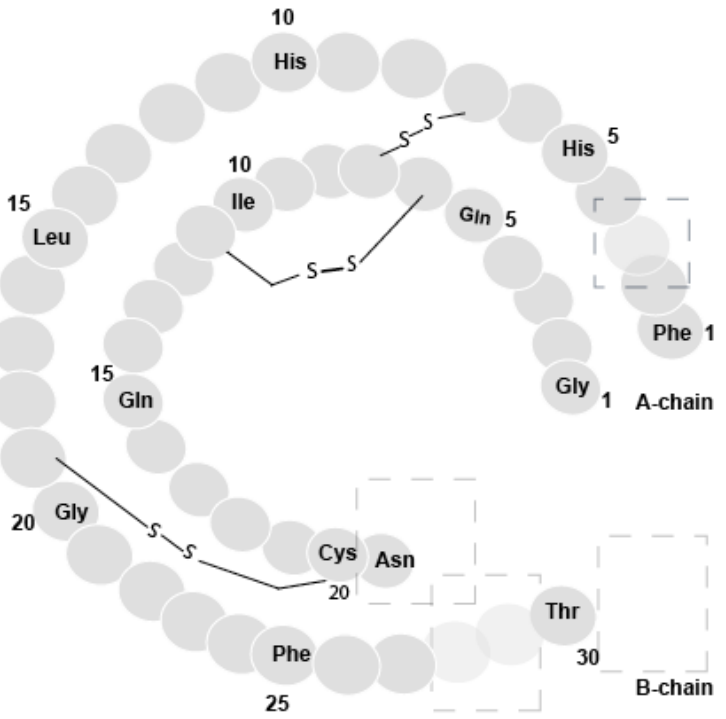

Figure 5: The native human insulin peptide sequence. The dashed boxes illustrate the segments of the sequence which have been genetically engineered in commercially-available products.

Of all the insulin products on the market one, neutral protamine hagedorn (NPH) insulin, is prepared by crystallisation. This is based on work by Krayenbuhl and Rosenberg in the 1930-40s. These authors successfully co-crystalised an insulin-zinc solution with protamine at neutral pH using the **isophane ratio** [48]. Protamine is a 4 kDa basic peptide predominantly comprising Arg residues. It is isolated from the sperm of chum salmon [49]. Tetragonal insulin crystals containing two zinc ions, the insulin hexamer and one protamine in the unit cell were formed at pH 7.3 [50]. It was thought that the hydrophobic interaction of protamine with the insulin Asn^{B3} residue possibly stabilises the N-terminus of the B-chain, although Norrman *et al.* ascertained that the effect stems from the fact that protamine neutralises the overall charge of insulin, rather than specific binding [51].

NPH formulations are known as premixed insulin suspensions. The marketed product contains a mixture of isophane insulin (*i.e.* the insulin : proteamine complex) and soluble insulin. A range of formulations exists containing the two components at various ratios. The aim of the medicine is both to deliver intermediate acting insulin and act as a sustained release formulation to extend insulin release and absorption. The duration of action of the NPH insulin is twice as long as regular insulin [52].

Upon subcutaneous injection, zinc and other ligands (*e.g.* phenol derivatives and anions) from the formulation are transported into solution as a result of the dilution effect [53]. This followed by insulin diffusion from the formulation into solution. The loss of zinc and other ligands destabilizes the insulin : protamine complex, thus breaking the hexamer into dimers and monomers.

The principles underlying the function of the NPH medicine have been further exploited in insulin degludec, a new ultra-long acting basal insulin. With this system, the rapid loss of phenol upon injection contributes to the association of multiple hexamers [47]. The complex then breaks into dimers and monomers when zinc slowly diffuses into the solution. As a result, insulin degludec has a plasma half-life of 25 hours [54].

Key term

Isophane ratio : The molar ratio of protamine and insulin forming a complex with no free protamine or insulin in the supernatant.

2.2 FREEZE-DRYING

Freeze-drying is used widely in the pharmaceutical industry for preparing parenteral dosage forms. Protein formulations are often freeze dried since it is frequently not possible to prepare stable liquid formulations. Though freeze-drying is expensive, it has several advantages in that it does not require a terminal sterilisation step, maintains a particle-free state much more easily than other methods, can be relatively easily scaled up, and has high recovery yields [55]. Although liquid dosage forms of proteins in ready-to-use syringes are desirable because they avoid the need for reconstitution and are more easy to administer to the patient, proteins in such formulations are susceptible to hydrolysis-mediated loss of tertiary structure during storage [20]. This issue may be overcome by the removal of water through freeze-drying. However, some residual water is essential for the maintenance of a protein's tertiary structure [56] and thus it is critical to optimise the formulation of each protein with excipients that cause minimal disruption of its native structure on freeze-drying.

Great care must also be taken to consider the phase interfaces which arise during the process, and on which proteins may unfold.

Freeze-drying comprises three steps: freezing, primary drying, and secondary drying. As shown in Figure 6, the freezing cycle (I-II) cools the product until it completely solidifies and both adsorbed and bound water crystallise. During crystallisation, the temperature may rise as a result of the latent heat associated with this process [57]. The rate of cooling affects the size and type of ice crystals formed, which can in turn affect the primary and secondary drying rates. If the rate of cooling is rapid, smaller ice crystals are formed, while slow rates of cooling yield large crystals.

Subsequently, primary drying (Figure 6, II-III) removes the frozen or unbound water through the process of sublimation, in which ice is directly transformed to water vapour under vacuum. The frozen specimen is placed upon a shelf in a temperature-controlled chamber, and water vapour sublimes out of the solid state. The vapour is subsequently solidified on a condenser maintained at a lower temperature than the shelf. This sublimation process results in the formation of pores in the solid product, the size of which are dependent on the size of the initial ice crystals. Smaller pores cause higher resistance to water vapor flow, thus decreasing the sublimation rate, increasing the primary drying time, and rendering the process less efficient. This phenomenon is also referred to as dried layer resistance [55,58–61].

The driving force for sublimation, ΔG_s, is provided by the vapour pressure difference between the ice front in the solid product and the surface of the condenser on which sublimed water is collected. Vapour pressure is inversely proportional to temperature, and hence the temperature difference between the ice front and the condenser surface is key to successful primary drying. The drying chamber pressure is always maintained below the saturation vapour pressure of ice at the operating temperature [62]. The fill depth and geometry of the vial used for drying can also affect the product temperature and primary drying time (they affect the surface area of the formulation being dried) [62], as do the manner in which it is mounted in the drying chamber [63]. Primary drying is the longest step in the freeze-drying of biopharmaceutical products, and is therefore economically important. A higher shelf

temperature for primary drying results in a faster process, favoured for economic reasons [60]. Hence, it is important to optimise the shelf temperature for primary drying to ensure a process which is both economical and efficacious.

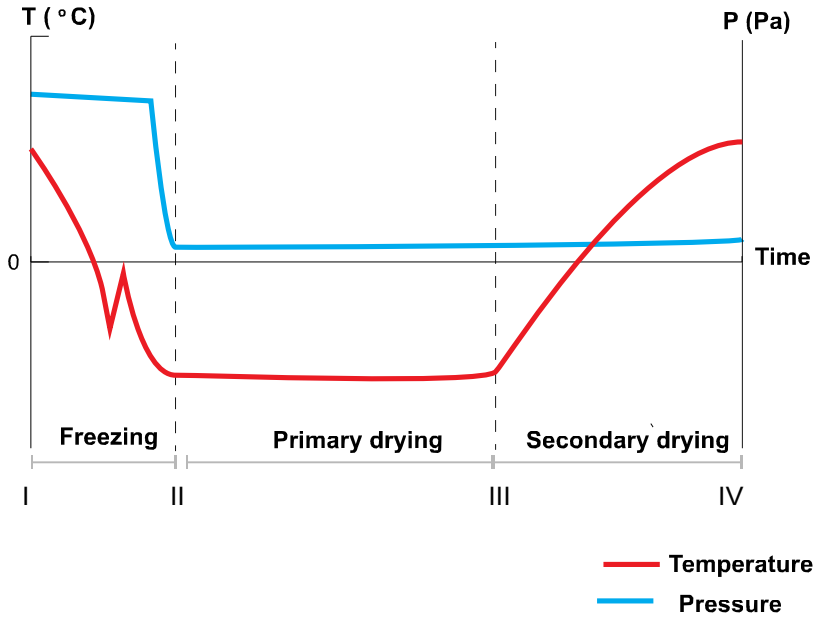

Figure 6: A schematic diagram illustrating the three cycles in freeze-drying processes: (I-II) freezing, (II-III) primary drying, (III-IV) secondary drying. Adapted from ref. [57].

In secondary drying (Figure 6, III-IV), the water remaining after primary drying is removed by diffusion. Secondary drying is typically carried out at a temperature much higher than primary drying, but for a much shorter period of time [60]. At the end of primary drying, typically 10 – 20 % residual water remains. Secondary drying reduces the moisture levels to 1 – 2 % or less. The idea underlying this process is that reducing the water content further will improve the storage stability of proteins [55,60]. However, caution must be applied because in secondary drying, while attempting to remove bound water, it is possible that hydrogen bond imbalances can be introduced and/or the native protein conformation disturbed. This can in turn result in reversible or irreversible folding [25].

The freezing rate can also affect protein structure. It has been reported that rapid freezing resulted in an increase in insoluble aggregate formation and a decrease in monomer content for the human growth hormone (hGH). This was believed to be a result of surface denaturation occurring at interfaces between the protein and very small ice crystals [64]. Freezing a protein solution generates an ice-water interface,

and proteins can get adsorbed onto this causing disrupting of their native folding state. The concomitant loss of secondary and tertiary structure can subsequently result in surface-induced denaturation [65]. A number of proteins (*e.g.* lactose dehydrogenase, lysozyme) are cold-labile, and undergo unfolding on exposure to sub-ambient temperatures. The tertiary structure of such molecules may result in denaturation independent of the ice/water interface.

Slow freezing has the potential to increase the damage to proteins because as the water crystallises, there is an increased chance that phase separation may occur. The latter is a kinetic process and slow freezing provides sufficient time to allow it to occur [59,66]. Hence, the freezing rate is critical and a moderate cooling rate of 1 ºC / min is recommended if the formulation is prone to phase separation [60]. This rate leads to a moderate ice surface area, while providing reasonably fast freezing. The effects of cooling rate are summarised in Table 2.

Table 2: The effect of cooling rate on freeze- drying (Adapted from [62]).

Cooling rate	Ice Nucleation rate	Number of ice crystals	Size of ice crystals	Ice sublimation time
Low	Low	Small	Large	Shorter
High	High	Large	Small	Longer

Excipients can be added to protect the protein from the freezing and drying stresses imposed during lyophilisation. The most commonly used stabilising excipients are sugars, polymers and surfactants. Inspiration is taken from nature here: most **anhydrobiotic organisms** have adapted a similar strategy of survival, by synthesising and accumulating trehalose and sucrose [67]. Such sugars can replace the monolayer of water usually hydrogen bonded to the surface of protein molecules, thereby helping to preserve the native structure [63]. Disaccharides are thus widely used in protecting a protein from freezing and drying stresses. A number of theories have been put forward to explain the excipient-mediated stabilization of proteins in the solid state including the roles of glass formation, water replacement, and hydrogen bonding between excipients and proteins [58,68]. Spectroscopic studies have shown that water replacement by excipients during dehydration better preserves the tertiary structure of proteins in the solid state and during the freeze drying process [69–71]. When the

protein is trapped in the amorphous state, the viscosity of the matrix is very high, and physical and chemical processes are essentially halted [58,71,72].

Key term:

Anydrobiotic organism : A microbe, animal or plant with a water content of less than 1%.

A range of excipients including albumin [73–75], dextran [73,76–78], polyvinyl pyrrolidone (PVP) [75] and polyethylene glycol (PEG) [79–81] have been used as stabilising excipients in freeze-drying. These are selected by virtue of their ability to introduce steric hindrance to protein-protein interactions, and increase solution viscosity (and glass transition temperature) such that protein structural movement is limited [58]. Alternatively, they may simply act as bulking agents to increase the pharmaceutical elegance of the product [82] and ensure rapid, controlled and reproducible reconstitution. Protein denaturation, aggregation or instability due to partial unfolding can be caused by their adsorption at the surfaces presented during freeze-drying [58,82]. Surfactants (*e.g.* polysorbate 20 or 80) [83,84] can ameliorate these issues, and thus are often used in freeze-dried formulations. Buffers added to the formulation must be carefully selected such that they do not crystallise during freezing: the selective crystallisation of buffer salts can cause pH changes, leading to degradation and instability of proteins [85–87].

Commercial application: Monoclonal Antibodies (mAbs)

IgG-based monoclonal antibodies (mAbs) are a major class of biological medicine with over 25 monoclonal antibodies registered for clinical use, and many more in clinical trials. There are also antibody fragments, Fc fusions and antibody drug conjugates that have been approved, and again many are in clinical development. This is because of their specific action, and the reduced immunogenicity which can now be achieved with the advent of completely human mAbs [88]. As mAbs and related proteins are susceptible to degradation by aggregation and related particle formation [89,90], lyophilisation is an appropriate formulation strategy when the solution stability of the protein is low [20]. The addition of sugars (*e.g.* trehalose, raffinose) during the freeze drying process can help to protect the native structure of

the antibody [91]. Currently there are a number of monoclonal antibody-based products on the market (Table 3). These have been developed to provide a stable antibody therapeutic on reconstitution with an aqueous-based medium. The resultant medicine can be administrated as a subcutaneous (SC) injection, intravenous (IV) infusion or intramuscular (IM) injection, with roughly equal numbers of products using each route.

Table 3: A list of commercially available freeze-dried therapeutic monoclonal antibodies.

Name	mAbs	Company	Year	Indication	Route of administration	Strength	Excipients
Herceptin (Trastuzumab)	Humanised IgG1k	Genentech	1998	Metastatic breast cancer	IV infusion	150 mg/vial, 21 mg/ml after reconstitution	136.2 mg Trehalose, 3.36 mg L-histidine HCl, 2.16 mg L-histidine, 0.6 mg Polysorbate 20 to be reconstituted in 7.2 ml of sterile water for injection (SWFI), pH 6.0
Raptiva (Efalizumab)	Humanised IgG1k	Xoma and Genentech	2003 (withdrawn in 2009)	Psoriasis	SC injection	150 mg/vial, 100 mg/ml after reconstitution	123.2 mg Sucrose, 6.8 mg L-histidine HCl, 4.3 mg L-histidine, 3 mg Polysorbate 20, reconstitute with 1.3 ml SWFI, pH 6.2
Remicade (Infliximab)	Chimeric IgG1k	Centocor	1998	Rheumatoid arthritis and Crohn's disease	IV infusion	100 mg/vial, 10 mg/ml after reconstitution	500 mg Sucrose, 2.2 mg Monobasic sodium phosphate, 6.1 mg dibasic sodium phosphate, 0.5 mg Polysorbate 80, reconstitute with 10 ml SWFI, pH 7.2
Synagis (Palivizumab)	Humanised IgG1k	MedImmune	1998	Prevention for Respiratory Syncytial Virus	IM injection	50 and 100 mg/vial, 100 mg/ml after reconstitution	For 50 mg vial – 40.5 mg Mannitol (5.6 % w/v), 5.2 mg Histidine (47mM), 0.16 mg Glycine (3.0 mM), reconstitute with 0.5 ml SWFI, pH 6.0
Xolair (Omalizumab)	Humanised IgG1k	Genentech, Novartis	2003	Asthma	SC injection	202.5 mg/vial, 150 mg/1.2 ml after reconstitution	145.5 mg sucrose, 2.8 mg L-histidine HCl, 1.8 mg L-histidine, 0.5 mg Polysorbate 20, reconstitute with 1.4 ml SWFI, pH 6.0
Simulect (Basiliximab)	Chimeric IgG1k	Novartis	1998	Prevention of organ transplant rejection	IV infusion	10 or 20 mg/vial, 4 mg/ml after reconstitution	For 10 mg vial – 10 mg Sucrose, 40 mg Mannitol, 20 mg Glycine, 3.61 mg monobasic potassium phosphate, 0.5 mg disodium hydrogen phosphate, 0.8 mg NaCl, reconstitute with 2.5ml SWFI

2.3 PARTICLE TECHNOLOGY

Particle formulation is another approach that has attracted much attention for the solidification of proteins in an effort to improve their stability and ease of administration. It is hypothesised that this approach can capture protein molecules within a polymer matrix, thus reducing molecular mobility [92]. Many strategies have been applied to manufacture protein particles, each with its own limitations. These include spray drying and emulsion preparations. Several products have made it into the clinic using these strategies.

2.3.1 SPRAY DRYING

Spray drying is a conventional method commonly used in the food and pharmaceutical industries to produce dry powders. This technology allows the large scale production of biotherapeutics with high batch-to-batch reproducibility. Typically a spray-drying system consists of an atomiser, drying chamber and cyclone recovery unit, as illustrated in Figure 7 [93]. In brief, the drug solution is atomised through small nozzles to generate fine droplets. Subsequently, hot dry air or an inert gas (*e.g.* nitrogen) is blown over the formulation in the drying chamber. This rapidly evaporates the solvent from the atomized droplets, yielding dry particles which are separated by cyclone recovery and ultimately collected in a collecting tube [93]. Often the particles obtained are in the size range of 1-5 μm, but smaller particles can be obtained using different collecting tubes and spray drying conditions. Various parameters including the inlet temperature (T_{inlet}), feeding rate, air flow rate, and humidity can be controlled to alter the properties of the spray-dried particles. Particularly when working with proteins, it is crucially important to understand and optimise these factors (especially the temperatures applied). Limitations of the technique include the fact that product recovery is often very challenging in early pre-clinical work necessitating working with large amounts of material, which may not be possible. Much spray drying is done using mixed aqueous solutions with for example ethanol or acetone; these can cause protein degradation, and thus care is needed. Surfactants are also often employed to enhance the process, and again may compromise the integrity of protein actives.

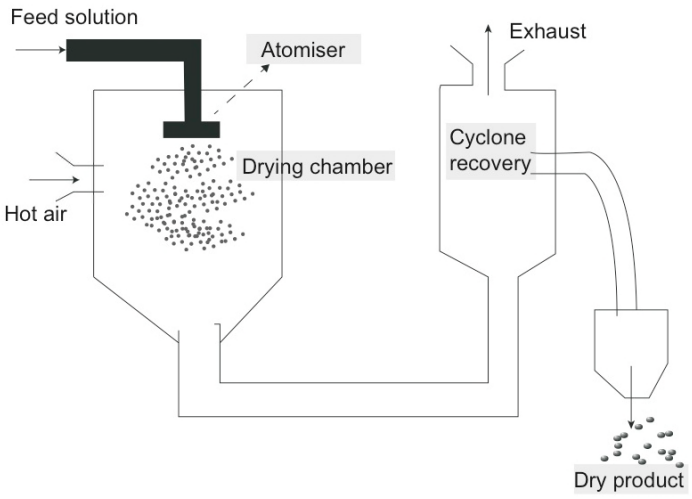

Figure 7: The apparatus used in spray drying.

Some processing parameters (*e.g.* heat, or the air-water interface) can have profound effects on the in-process stability of proteins. Prinn *et al.* devised a statistical model to determine the effect of processing parameters on spray-dried BSA particles prepared on the lab scale [94]. In another study, the aggregation and deamidation of insulin was found to rise significantly when the outlet temperature exceeded 120 ºC [95]. Heat degradation can be mitigated if the contact time between the sprayed droplets and hot air is as short as possible [96], but aggregation induced at the air-water interface appears to be more pronounced under such conditions.

Mumenthaler *et al.* reported that spray-dried human growth hormone (hGH) exhibited more aggregation when the atomisation rate was increased, ultimately yielding approximately 67 % of soluble aggregates from the total spray dried particles [84]. Because of the surface-active properties of proteins, they tend to adsorb onto boundaries, as previously described in in Section 2.2. Therefore, surfactants are often added to prevent adsorption and aggregation [93]. It has been reported that the formation of both insoluble and soluble aggregates of hGH was reduced 10-fold after adding 0.1% (w/v) Tween 20 [84] during spray drying. This is consistent with the results obtained by Abdul-Fattah *et al.* [97], showing that the total protein surface accumulation was significantly decreased when Tween 20 was added during the spray-drying of Met-hGH. Phospholipids exert the same effect with albumin, but care must be taken when using ionic surfactants because they may alter the protein structure [98].

Dehydration in any drying method may perturb the native state of proteins, and so (as in freeze-drying) sugars (*e.g.* di-and trisaccharides) and polyols are often added in spray dried formulations to minimise destabilisation. By adding 10 – 20 % (w/w) mannitol, the formation of soluble aggregates of a recombinant humanised anti-IgE monoclonal antibody (rhuMabE25) during spray-drying was halved. The storage stability at 5 ºC and 30 ºC also seemed to be improved compared to non-mannitol formulations [99]. However, mannitol may undergo crystallisation during long term storage, and potentially transforms into different polymorphic forms depending on the temperature used in the drying process [100]. To avoid the possible inter-batch variability which might result, sodium phosphate can be used to inhibit crystallisation in spray-drying [99]. The presence of amino acid residues in proteins means that the Maillard reaction can be a concern, particularly when they are heated. Hence, non-reducing saccharides (*e.g.* trehalose and sucrose) are also often used in spray dried formulations. This have the additional benefits of acting as glass forming agents, limiting the molecular mobility of proteins which can contribute to their unfolding [101], and also possibly stabilising the protein through the water replacement mechanism [102,103]. Combinations of trehalose with other excipients (*e.g.* mannitol, amino acids) have been applied to improve the physical stability of protein particles [104].

Theoretically, low dynamic motion is believed to be responsible for the long term stability of proteins embedded in a glassy matrix [101] (and indeed those which are freeze dried). With low molecular weight excipients, spray-dried hGH showed greater physical stability relative to analogous formulations prepared with high molecular weight excipients. This is because lower molecular weight additives have higher true densities, and true density is inversely proportional to the polymer's free volume. A lower free volume leads to a longer **relaxation time** (τ), contributing to resultant higher stability [97]. However, the interplay between a large number of factors including process parameters and stabilisers needs be taken into account to determine the ultimate stability of a spray dried protein formulation.

As protein adsorption is critical in spray-drying, the surface analysis of dry particles (using *e.g.* specific surface analysis (SSA), or total protein surface accumulation) can be useful to provide some insight into the stability of a spray dried formulation.

Abdul-Fattah *et al.* conducted a study to examine the effect of spray-drying on the stability of IgG1 (Medi-522) [105]. FTIR spectra showed that spray-drying can more effectively preserve a native-like structure of the protein than other methods, regardless of the formulation used. However, they found that IgG-sucrose formulations (1:4 and 4:1) with a surfactant added were rather unstable at both 40 ºC and 50 ºC. This is an unexpected result, as spray dried particles are generally expected to be more stable if surfactants are added.

Key term:

Relaxation time: The period required for a macromolecule to rearrange itself to occupy the free volume (*i.e.* the unfilled space) in a formulation.

Spray dry proteins have been intensely investigated for pulmonary administration, resulting in one commercially available product. The process is capable of producing '**inhalable' micron sized-particles** if the processing parameters are appropriately controlled. A high atomising flow rate and low protein concentration in the formulation gives rise to particles with mass median aerodynamic diameters (MMAD) in the micron range [94,95]. The nozzle can also be modified to improve particle size. For example, ultrasonic or two-fluid nozzles are found to generate more uniform sized particles [106,107]. The additives discussed earlier also assist particle formation and enhance aerosolisation properties, in addition to their roles in protein stabilisation.

Healy *et al.* have explored the use of spray drying to prepare spray-dried sugar/protein composites. Using an optimised solvent ratio of 80:20 methanol : butyl acetate, spray-dried trehalose was found to form porous particles with low MMAD (3.14 ± 0.62 μm), and lower bulk and tap densities than similar non-porous formulations (see Figure 8) [108]. The model enzyme lysozyme could be incorporated into the spray-dried particles by simply making a mixed solution of the protein and sugar in 80:20 methanol : butyl acetate. Its specific biological activity was, within experimental error, identical to the lysozyme prior to processing (ranging from 93.5 ± 4.4 % to 103.9 ± 4.4 %). This study thus offers a composite system with ideal properties for protein delivery *via* the pulmonary route. In another study, dipalmitoylphosphatidylcholine (DPPC) could be combined with albumin to yield

porous particles by spray-drying from an ethanol-water system [109]. L-leucine, an amino acid often added to improve powder flowability, can also be applied to encapsulate proteins [104].

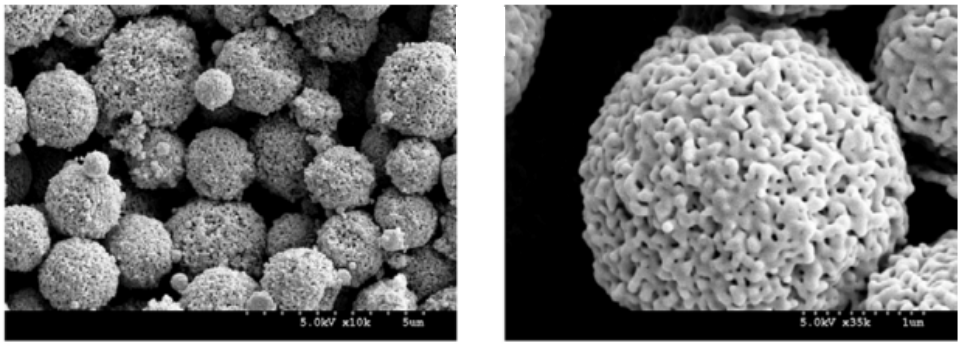

Figure 8: Scanning electron microscopy images of spray-dried trehalose prepared using MeOH : butyl acetate (80:20). Reproduced with permission from ref. [108]. Copyright Elsevier 2011.

Key term:

Inhalable micron sized particles : Formulations with particles whose size ranges from 1 – 10 μm.

Commercial application: Insulin

Only one spray-dried protein therapeutic has completed clinical trials and reached the marketplace. This is Exubera®, an insulin inhaler developed by Pfizer. The formulation developed allowed insulin stabilisation at room temperature and showed excellent product performance in term of dose reproducibility and efficacy [110]. However, this product unfortunately had to be withdrawn from the market because of poor sales [111]. More recently, MannKind Corporation has developed a technology platform (Technosphere®) to deliver a range of proteins *via* the pulmonary route [112]. The technology uses a novel excipient fumaryl diketopiperazine (FDKP) to form microparticles in this technology. An ultra-rapid insulin formulation, Afrezza®, has been developed from this pipeline and is in late stage clinical trials.

2.3.2 EMULSION METHODS

Emulsion methods have been extensively investigated in pharmaceutical research for preparing polymeric particles (often known as microspheres) both for small-molecule active ingredients (APIs) and therapeutic proteins. The process can be undertaken with a wide range of polymers, and is relatively easy to up-scale (although sterilisation can be challenging). Emulsion fabrication generally involves two steps: emulsification and solvent removal. Initially, a stable single or double emulsion is required to obtain protein - polymer oil droplets dispersed in a continuous aqueous phase. This can subsequently be emulsified by adding an emulsifier or through agitation (see Figure 9). It should be noted that these processes have significant potential to damage a protein through shear forces, the presence of a non-aqueous co-solvent, *etc*: hence, very careful control of processing parameters is required, together with robust testing of a protein's efficacy post-emulsification.

Although the oil-in-water method (o/w) or double emulsion (w/o/w) is most widely employed, it often results in low encapsulation efficacy as proteins can diffuse from the oil phase to the continuous aqueous phase [113]. Additionally, proteins are prone to aggregation in these approaches because of the existence of an aqueous/organic interface, and exposure to the organic solvent. The shear forces present during emulsification also cause proteins to unfold and to aggregate. Modified emulsion systems in which solid proteins in are dispersed in an organic solvent, such as solid-in-oil-in-water (s/o/w) or solid-in-oil-in-oil (s/o/o) approaches, can ameliorate the aggregation and initial burst release which are often observed in the w/o/w method [114].

In the second step of an emulsification process, the solvent is removed and the droplets are allowed to solidify. Solvent transfer is a critical factor in this step because it can affect the morphology, entrapment efficacy and release profile of the particles. Evaporation by heating is commonly used to eliminate solvent from the emulsion (great care must be taken to avoid compromising the protein during heating). Rosca *et al.* conducted a mechanistic study to examine particle formation by solvent evaporation from both single and double emulsions of poly(lactic-co-glycolic acid) (PLGA) [115]. It was observed that a thin layer of the inner oil phase forms around

the surface of the particles upon solvent removal, which is linked to burst release. In the case of a double emulsion, the inner droplets may aggregate and form holes, again contributing to burst release.

Other than using heat, the solvent in an emulsion can also be removed by using other techniques such as supercritical fluid (SCF) technology or an electric field: these methods have recently been reviewed elsewhere [116]. SCFs can be used as drying media or antisolvents, and expose proteins to relatively low stress conditions [117]. Solvent evaporation using an electric field can also avoid the use of heat. Both these approaches therefore offer advantages over traditional heating approaches when working with protein formulations. Following solvent removal, the protein particles are collected and sterilised.

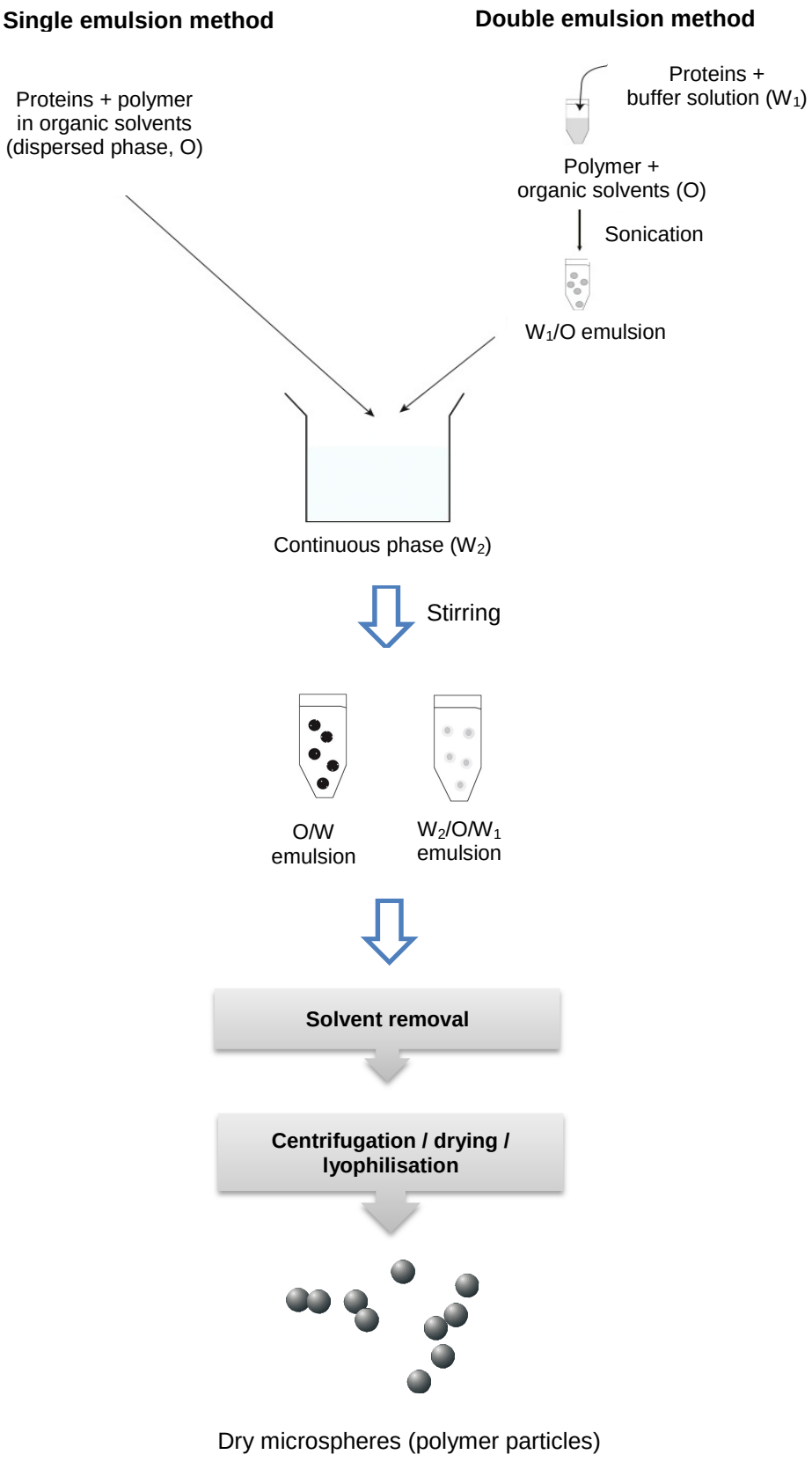

Figure 9: A flow diagram depicting polymer/protein particles (or microspheres) prepared by the emulsion method.

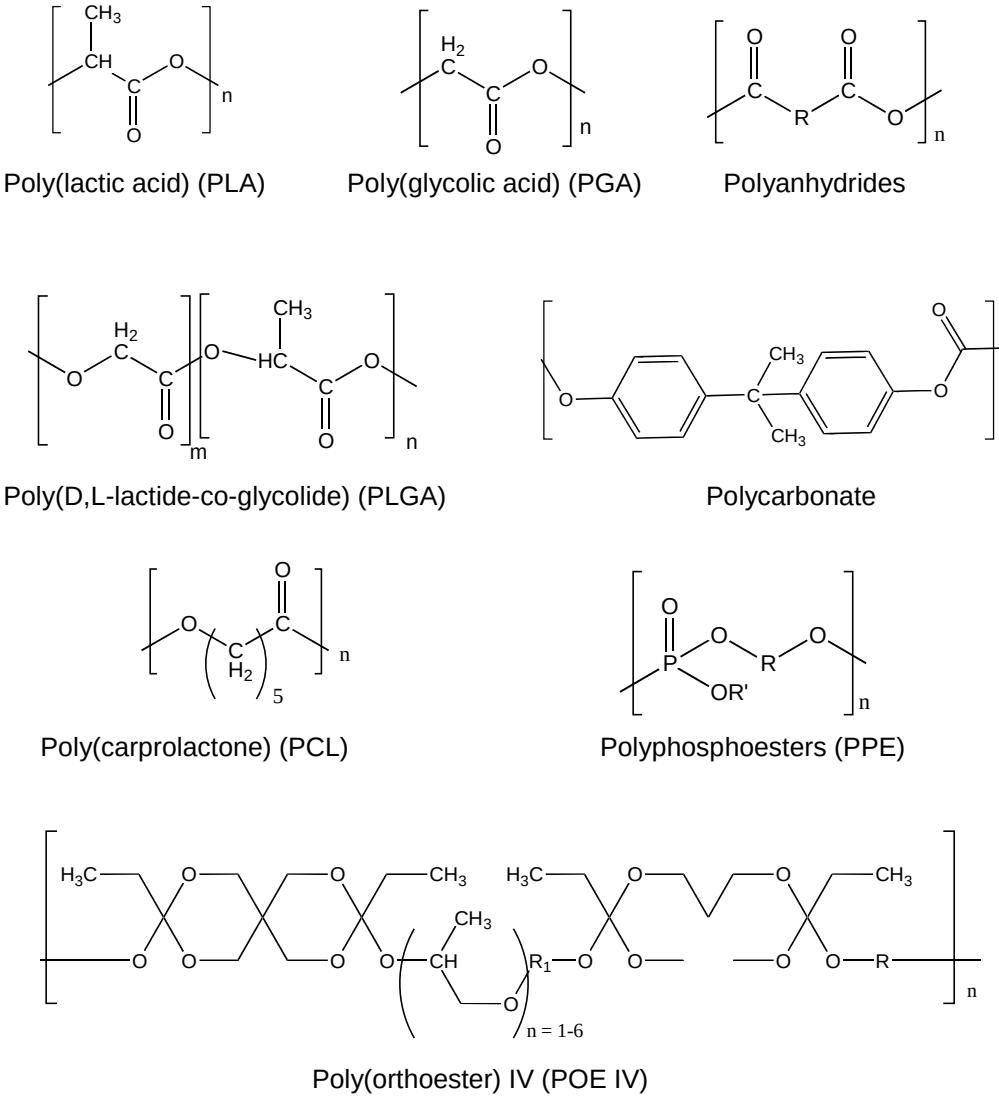

Figure 10: Commercial synthetic polymers used for microsphere formulation.

Polymer selection is another perspective which has a significant influence on the properties of particles prepared through the emulsion route. In addition, some polymers, such as PLGA, may be incompatible with a particular protein of interest. In general, biodegradable polymers are most appropriate for micro sized carrier formulations, aiding their biocompatibility. A range of both synthetic polymers and natural polymers have been explored for protein microsphere preparation. Natural polymers display many advantages such as being processable under mild conditions, ideal for generating protein formulations, and biocompatibility. However, challenges with scalability have limited their utilisation [118]. Natural polymers which have been explored include sodium alginate [119], pullulan [120], hyarulonic acid [121] and chondroitin sulfate [122].

The use of synthetic polymers thus appears to be a more viable option. Many types of polymer have been commercialized, as depicted in Figure 10. Polyesters (*e.g.* POEs, PLGA and PCL) are the most commonly used for biomedical applications; they degrade in *in vivo* by undergoing ester hydrolysis into monomers [123]. Varying the monomer ratio or modification of the polymer backbone can modulate the release rate of the drug encapsulated in the matrix. For instance, the POE IV matrix erodes on the surface with no resultant change in local pH, enabling controlled drug release [124].

In terms of stability, some studies have examined the structural conformation of bioactive proteins in encapsulated biodegradable polymers. FTIR spectra indicate that the characteristic amide I and II bands for insulin extracted from PLGA microparticles are relatively close to those of native insulin [125]. DSC thermograms show that the melting point of insulin increases from 91.08 ºC to 104.4 ºC upon encapsulation, thus indicating an enhancement of stability in the microcapsule formulation. Recombinant hGH released from PLGA microparticles after 28 days remained active and was able to stimulate Nb2 cell proliferation [126].

Aggregation during *in vitro* study is a major challenge encountered when working with biodegradable particles. The protein is vulnerable to aggregation if there is an interface present; hydrophobic/hydrophilic interfaces between protein and polymer may also result in aggregation. Taluja *et al.* deduced that the presence of an acidic environment during dissolution is a major cause of aggregation for several protein drugs, especially in polyester matrices [113]. Many strategies, such as changing the parameters used in emulsion preparation or adding stabilisers, have been proposed to improve protein stability on storage and in physiological media [124,127].

Commercial application: Lupron Depot® LH-RH analogue-sustained release injectable microspheres

The use of polymeric carriers for peptide formulations has attracted much attention because the drug release can be prolonged, which can eliminate the need for frequent dosing. PLGA is the most widely explored biodegradable polymer and is clinically approved by the Food and Drug Administration (FDA). There are four PLGA-based peptide formulations currently available, detailed in Table 4. It should be noted that in

the case of such small peptides there is no real tertiary structure, and thus formulation into microspheres is less problematic than is the case with larger proteins. Considering the latter, only Nutropin Depot®**,** a 1 month sustained release formulation of the recombinant human growth hormone (rhGH) contains a bioactive protein incorporated into microspheres. In addition LB03002, a once-weekly rhGH microsphere medicine formulated using sodium hyaluronate, has completed phase 3 clinical trials [121,128].

Lupron Depot® contains leuprolide acetate, a synthetic nonapeptide analogue of luteinizing hormone releasing hormone (LH-RH)) shown in Figure 11. It was developed by Takeda using poly(lactic acid) (PLA) or PLGA microparticles as the delivery platform. It has been approved to treat prostate cancer, endometriosis and precocious puberty. The treatment for such diseases usually requires frequent injections, and to improve patient comfort and convenience monthly and three-monthly release formulations were developed using the w/o/w technique [129]. The differences between the monthly and three-month formulations lie in the drug loading, type of polymer and preparation procedure, and are illustrated in Figure 12.

The study of *in vivo* release from both formulations showed that PLA microspheres exhibited continuous release over 3 months. In contrast, depots based on PLGA release over a shorter period of 1 month. In both preparations, the matrix initially exhibits protein diffusion from the surface, followed by further release mediated by polymer erosion. Miller *et al.* pointed out that the latter phase is key to controlled drug release, and the inclusion of glycolic monomers increases the rate of erosion [130]. Okada has suggested that the long hydrocarbon chains of the polymers (PLGA or PLA), in which the terminal anionic groups interact with the peptide serve as a diffusion barrier for the protein, therefore retarding the release [129]. This explains why PLGA (75:25) (lactic:glycolic) and PLA were chosen to develop once-a-month and three-monthly formulations respectively. Currently, there are eight commercial products developed using this platform; they are available in a ready-to-use two part compartmentalized syringe in which reconstituted microspheres are separated from the dispersing solution until immediately before use [124].

Table 4: A list of commercially available peptide formulations based on injectable PLGA microspheres. Adapted from refs. [123,124].

Trade name (Drug)	Product	Polymer	Indication	Methods	Company
Lupron Depot ® (Leuprolide acetate)	Lupron Depot, 7.5 mg (1-month)	PLGA	Palliative treatment of advanced prostatic cancer	Emulsion	Takeda
	Lupron Depot, 3.75 mg (1-month)				
	Lupron Depot, 11.25 mg (3-month)	PLA	Endometriosis		
	Lupron Depot, 22.5 mg (3-month)		Palliative treatment of advanced prostatic cancer		
	Lupron Depot, 30 mg (4-month)				
	Lupron Depot, 45 mg (6-month)		Precocious puberty in children		
	Lupron Depot PED, 7.5 mg (1-month)	PLGA			
	Lupron Depot PED, 11.25 mg, 15 mg (3-month)	PLA			
Sandostatin ® LAR ® (Octreotide acetate)	Sandostatin LAR Depot (1 month)	PLGA	Acromegaly, Carcinoid tumors, VIPomas	Emulsion	Novartis
Trelstar™ Depot (Triptorelin pamoate)	Trelstar LA 3.75 mg (1-month)	PLGA	Palliative treatment of advanced prostatic cancer	Spray-drying	Watson Pharma
Bydureon (Exenatide)	Bydureon (weekly)	PLGA	Type II diabetes	Emulsion	Amyllin/ Eli Lilly/ Alkermes

Figure 11: The amino acid sequence of leuprolide.

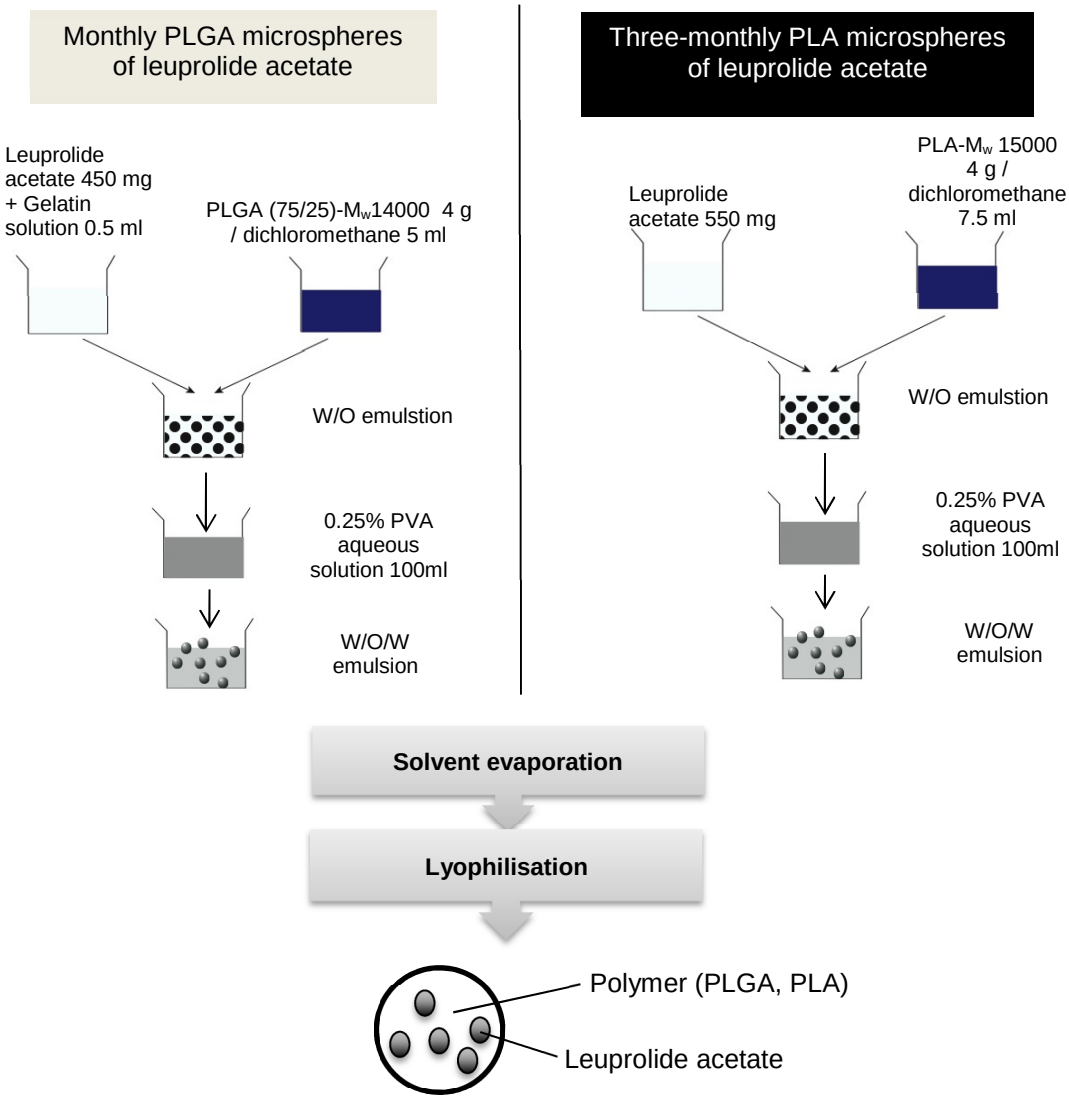

Figure 12: A schematic illustrating the preparation of the once-a-month and three-monthly Lupron Depot® formulations using the emulsion method. Adapted from ref. [129].

3. FUTURE PERSPECTIVE

3.1 PROTEIN STABILISATION IN NANOFIBRES

The previous sections have highlighted the solidification techniques that are currently used to manufacture protein therapeutics. It appears from the trend of publications in the literature and the number of available commercial products that protein encapsulation in a polymer-based solid is growing in popularity relative to crystallization approaches. The former provides additional advantages including scalability, ready to use dosage forms and versatile drug administration. Advances in nanomaterials engineering are attracting growing interest in the biomedical research field, and could offer new opportunities to enhance the stability of biotherapeutics. However, the difficulty of controlling bottom-up processes and the high manufacturing costs involved present hurdles to industrial application.

Electrospinning (ES) is a facile 'top-down' approach to the production of nanostructured materials which has recently attracted increasing interest in the preparation of protein formulations. It allows the fabrication of a wide range of materials using synthetic polymers, carbohydrates, or proteins. In the simplest solution ES, a polymer and functional component (*e.g.* a small molecule drug or a protein) are dissolved in a volatile solvent, and electrical energy used to evaporate the solvent [131]. The polymer solution is ejected from a metal-tipped syringe at a controlled rate towards a metal collector plate. A high voltage is applied between the two, which causes rapid evaporation of the solvent and the production of polymer particles on the μm scale .This process occurs very rapidly, yielding one-dimensional solid fibres. The process can be conducted at room temperature, precluding thermally-induced protein denaturation. A proof of concept study indicated that proteins can be processed by ES and remain in the native state [132].

As for the other techniques discussed above, however, the interplay of material properties and processing parameters during ES is very important, and may complicate the use of ES in the large-scale manufacturing of protein formulations. Batch-to-batch or intra-batch inconsistency in the size and shape of the fibres produced is often observed, and can influence the functionality of the materials

produced. ES uses organic solvents, which may have an effect on protein folding, and polymer-protein interactions must also be understood. Ideally, water would be used for ES to prevent protein denaturation, but the use of aqueous based polymer solutions is generally not practical because of water's high surface tension, high boiling point and high dielectric constant. To achieve the effective evaporation of water in an ES process it is generally necessary to use heat, which of course introduces other protein degradation possibilities. These issues can be minimised to some extent by adding surfactants or using SCF as a solvent.

The most simple one-fluid electrospinning process is thus probably not suitable for the development of protein formulations. **Multicomponent electrospun fibres** are more promising, however, and have the potential to protect proteins from physical stress. A process known as coaxial ES can fabricate core-shell structured fibres by simultaneously co-spinning sheath and core polymer fluids. The apparatus is the same as used in single-fluid ES except that a concentric spinneret with one needle nested inside another (a "coaxial spinneret") is used. This is depicted in Figure 13. A protein solution is generally loaded in the core, and surrounded by an outer polymer layer. It has been shown that lysozyme remains in its native structural conformation after being encapsulated in the core of PCL-based core-shell fibres, and is slowly released over 13 days [133]. These results are depicted in Figure 14. The same study suggested that other stabilisers (*e.g.* PEG or sugars) may be incorporated to enhance protein stability. A similar core-shell structure can be also achieved through the ES of emulsions. Protein droplets dispersed in a polymer solution can be processed by ES, which typically results in fibres with a protein-based core and polymer shell [134]. Microfluidic technology can also be applied to produce composites which contain both fibres and protein encapsulated in microbubbles [135], as shown in Figure 15.

Key term:

Multicomponent fibres: Electrospun fibres containing more than one functional component.

Electrospun nanofibres are being increasingly explored in a variety of medical areas, such as in tissue engineering. This is attributed to the similarities in structure between

electrospun fibre products and the natural extracellular matrix (ECM); the latter plays an important role in cell adhesions. ES fibres have also been widely explored in the development of drug delivery systems for a range of different purposes including as wound-healing dressings, implants or controlled release carriers: these were reviewed recently [131]. ES protein formulations have featured in a number of studies: for instance, the successful sustained release of vascular endothelial growth factors (VEGF) from PLGA/dextran core-shell fibres suggest the potential to fabricate protein loaded scaffolds with activity lasting beyond 28 days [136]. This is highly attractive in the development of formulations for protein therapeutics with long-lasting action, required in areas such as regenerative medicine and hormone replacement therapy.

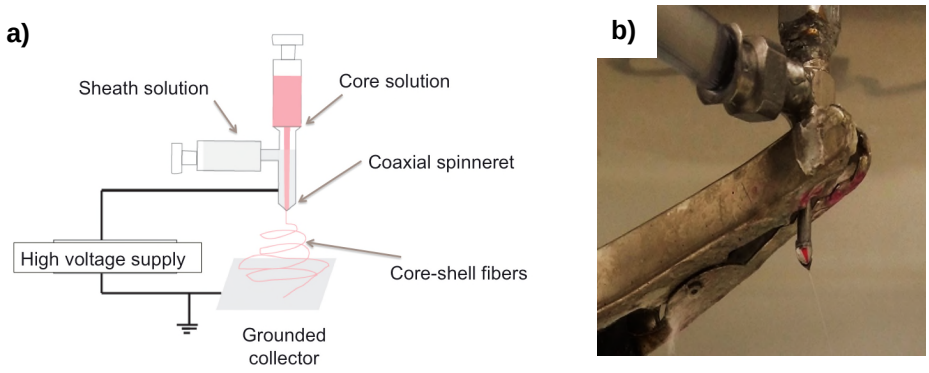

Figure 13: a) a schematic diagram of the coaxial electrospinning apparatus; **b)** a photo of the droplets ejected from the coaxial spinneret. The core fluid is pink, and the shell colourless.

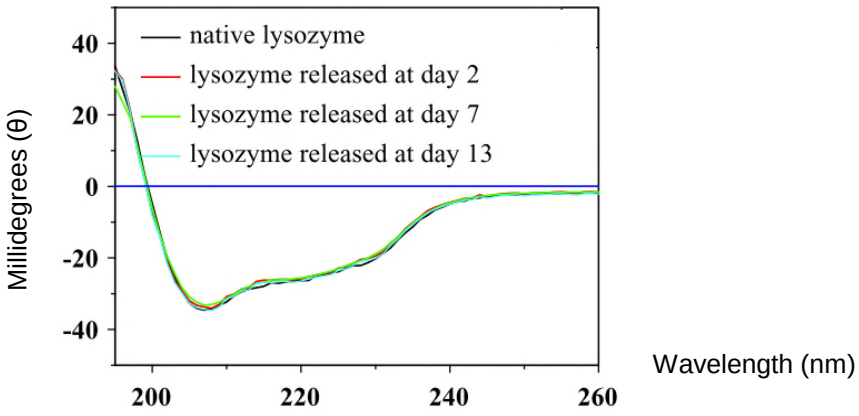

Figure 14: CD spectra of native lysozyme and the protein released from PCL core-shell fibres, showing that the structural integrity is retained throughout electrospinning and drug release. Reproduced with permission from ref. [133]. Copyright Elsevier 2005.

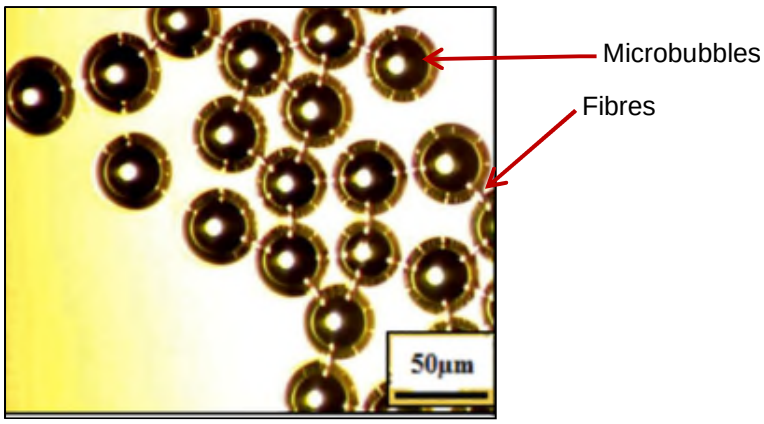

Figure 15: The product of combining electrospinning and microfluidics, showing the presence of microbubbles connected by fibres. Taken with permission from ref. [135]. Copyright Elsevier 2012.

3.2 OTHER TECHNOLOGIES

Protein therapeutics are rapidly evolving and becoming even more complex. Important classes of proteins such as monoclonal antibodies, cytokines and enzymes have become clinical mainstays, and in several cases, first line treatments. New classes of therapeutic proteins have achieved clinical validation including multifunctional proteins (*e.g.* bispecific antibodies) and immunoconjugates (*e.g.* antibody drug conjugates). Other types of multifunctional proteins and new product motifs are expect to continue to emerge. One example is antibody mimetics: these possess the binding attributes of monoclonal antibodies, but may also prove to be more stable

[137]. More stable therapeutic proteins and hybrid protein formats will drive the development of new dosage forms. The use of new excipients [138] that inhibit protein aggregation will also be critical to develop stable dosage forms of protein therapeutics.

There remains a need for solid-state forms of protein therapeutics that can be stored without a cold chain. When considering all the manufacturing steps to produce such a therapeutic, the costs involved with the processes used to formulate and fabricate the final dosage form are considerable. Ensuring that these processes are scalable, reproducible and cost effective are key goals that are being targeted early in development. Whilst the processes described in this review to make solid state forms of protein therapeutics will continue to evolve, computational modeling strategies are being increasingly employed, along with high throughput strategies, in efforts to identify optimal process parameters early in development.

4. CONCLUSIONS

The development of solid protein formulations has provided great benefit in enhancing their stability, resulting in a range of applications and marketed products. A range of methods have been explored to achieve this goal including crystallization, freeze-drying, and encapsulation into a solid (usually polymer-based) matrix by spray-drying or emulsion routes. In all cases, great care must be taken during the formulation process to ensure that processing parameters, or the interplay between multiple parameters, do not compromise the stability of the protein in the final formulation. In addition to the current routes, there exist exciting future possibilities for developing new protein/polymer composites, including electrospinning. However, for these techniques also a careful consideration of how the processing will influence the protein structure is required to ensure efficacious products are developed.

Executive summary

Stability of solid protein therapeutics

- The formulation of proteins in the solid state can preclude or retard the hydrolytic degradation which is commonly observed with liquid formulations during handling and storage.

Solidification techniques for proteins

- Crystallisation, freeze-drying and particle technology are used to manufacture solid protein products with different drug delivery applications. Care must be taken when realising the processes, however, in order not to inadvertently cause damage to the protein.
- These solid state formulations can yield controlled drug release resulting for instance in reduced dosing frequencies, as well as providing protein stability enhancement.

Multicomponent electrospun nanofibres for protein stabilization

- Co-axial electrospinning allows the fabrication of multicomponent nanoscale fibres, and has great potential in the development of solid state protein formulations, both for stability enhancement and release modulation.

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