Expert Opinion

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Recent trends in stabilising peptides and proteins in pharmaceutical formulation – considerations in the choice of excipients

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In the area of peptide and protein pharmaceuticals, both the physical and chemical stability of biopharmaceuticals are critical and need to be optimised when formulating a drug product, in order to optimise the outcome after processing and storage. This review focuses on the effects on the stability from various types of excipient and the choices that have to be made during formulation of drug products containing peptides or proteins. It is illustrated, through examples, how the choice of one excipient over another can affect the stability of a protein drug formulation, along with other problems linked to this choice. The excipients used in pharmaceutical preparations are limited and from an academic point of view there is a clear requirement for new excipients.

Keywords: excipients, peptide and protein pharmaceuticals, production, stability

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1. Introduction

In the area of peptide and protein pharmaceuticals, both the physical and the chemical stability of biopharmaceuticals are critical and need to be optimised during formulation in order to optimise the outcome after processing and storage. Maintaining the three-dimensional structural integrity of the therapeutic proteins is essential for preserving biological activity, sustaining the release from a controlled release formulation and avoiding undesirable immunological reactions [1-4]. Hence, the major challenge in the development of pharmaceutical formulations is to avoid unwanted changes in the structure, and also to understand the process and formulation parameters that affect stability [5-7]. For successful formulation and delivery of peptides and proteins, it is crucial that the formulation scientist has a thorough knowledge of several factors: how to optimise the physical and chemical stability of the active ingredient; how, rationally, to include specific excipients in the formulation; how to obtain the optimum conditions for stability; how to prevent stability issues during up-scaling; and, finally, how to design a formulation that is suitable for the route of administration, that is, one that allows the absorption barriers to be overcome. The choice of excipients is often based on previous experience, and on which excipients have been approved by the authorities [8,9]. Excipients are chosen: to ensure that a certain function can be obtained, for example, controlled release; to ensure a successful end point for a preparation process, for example, allowing a dry powder to be obtained; to ensure that a liquid formulation remains at a constant pH value; or to stabilise the protein against a certain production-induced effect, for example, adsorption. However,

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Table 1. Examples of excipients and their main function in peptide and protein pharmaceutical formulations.

Formulation effect	Excipient type	Example
Anti-adsorption	Surfactants	Poloxamer [1,57] Polysorbate 20 and 80 [1,34]
	Polymers	Dextran [11,37] Poly(ethyleneglycol)-*b*-poly (L-histidine) [59] PEG [1,11,105]
	Other proteins	BSA and HSA [32]
Oxidation protection	Antioxidants	Ascorbic acid [1,26-27] Ectoine [24,106] Glutathione [1] Monothioglycerol [25] Morin [107] Poly(ethylenimine) [24] Propyl gallate [2,3] Vitamin E [2,3]
	Chelating agents	Citric acid [2,3] EDTA [23,27] Hexaphosphate [1] Thioglycolic acid [2,3]
pH	Buffer salts	Phosphate, bicarbonate, sulphate, nitrate, acetate, chloride, pyruvate [2,29]
	Antacids	$Mg(OH)_2$[31] $ZnCO_3$[56]
Stabilisers	Amino acids	Alanine [1,11] Arginine [1,13,18] Aspartic acid [1] Glycine [1,11] Histidine [108] Lysine [1] Proline [1,11,14]
	Sugars	Glucose [15] Sucrose [1,11,31,15,45,109,110] Trehalose [1,11,16,45,43]
	Polyols	Glycerol [1,11,109,75,111] Mannitol [1,11,45] Sorbitol [1,11,24,109,111]
	Salts	Potassium phosphate [1,11] Sodium sulphate [1,11,82]
	Chelating agents	EDTA [1,23,27] Hexaphosphate [1]
	Ligands	Phenol [22] Zinc [21]
	Polymers	Cyclodextrin [1,19,112,113] Dextran [1,11,114] PEG [1,11,38] PVP [1,11,110]
Tonicity	Salts	NaCl and many other salts [80,86]
	Other	Glycerol [75]

BSA: Bovine serum albumin; HSA: Human serum albumin; PEG: Polyethylene glycol; PVP: Poly(vinyl pyrrolidone).

what may be useful for one protein can have detrimental effects on another. So, there is room and a need for guides to lead to the right choice of excipients. Excipients are added to formulations for several reasons, and some of them may have more than one effect or purpose for being part of the formulation (**Table 1**). In the following sections, these effects are addressed as a specific stabilising effect, as a measure against destabilising factors that arise as a result of the preparation process and from a functional point of view.

This review elucidates some of the key processes and stability issues in the development of protein pharmaceuticals. It stresses the importance of careful choice of excipients by exemplifying specific excipients utilised to stabilise proteins in the various process steps and to obtain the desired function of the pharmaceutical preparation. The influence this choice might have on the stability of the protein is also addressed.

2. Excipients added to optimise the physical and chemical stability of the protein in a pharmaceutical preparation

In the choice of excipients, both physical and chemical stability have to be optimised. Excipients are often used to slow down or prevent the physical destabilisation processes (**Figure 1**).

There are specific mechanisms of solvent-induced stabilisation of proteins, which are specifically related to the excipients in the formulation. Stabilisation is achieved by strengthening of the protein-stabilising forces, by destabilisation of the denatured state, or by direct binding of excipients to the protein (**Table 2**) [3].

The structure of water surrounding the folded protein is extremely important in maintaining the structure of the protein, and excipients are typically added to replace missing interactions (i.e., resulting from drying) or to increase the interactions (i.e., to stabilise) [10]. In the presence of a stabilising excipient, the protein preferentially hydrates and the excipient is preferentially excluded, that is, more water molecules are found on the surface of the protein than in the bulk, and such a process is believed to stabilise the protein [3,11,12]. Some typical excipients that have this hydration approach are amino acids and sugars [13-17]. Prevention of the direct interaction between proteins can also stabilise proteins, as this interaction leads most often to aggregation [13,18]. Arginine has previously been reported to bind strongly to some proteins and to be excluded from the surface of others. However, recent reports indicate that arginine is less likely to bind to the surface of proteins, and this interaction minimises the interaction between proteins, which thereby stabilises the protein against aggregation [13]. Some excipients may compromise protein stability by stabilising the unfolded state. An example is cyclodextrins, where solution studies of lysozyme have shown that addition of hydrophilic cyclodextrins yields a decrease in the thermal unfolding temperature (Tm) of lysozyme, suggesting a destabilisation of the native

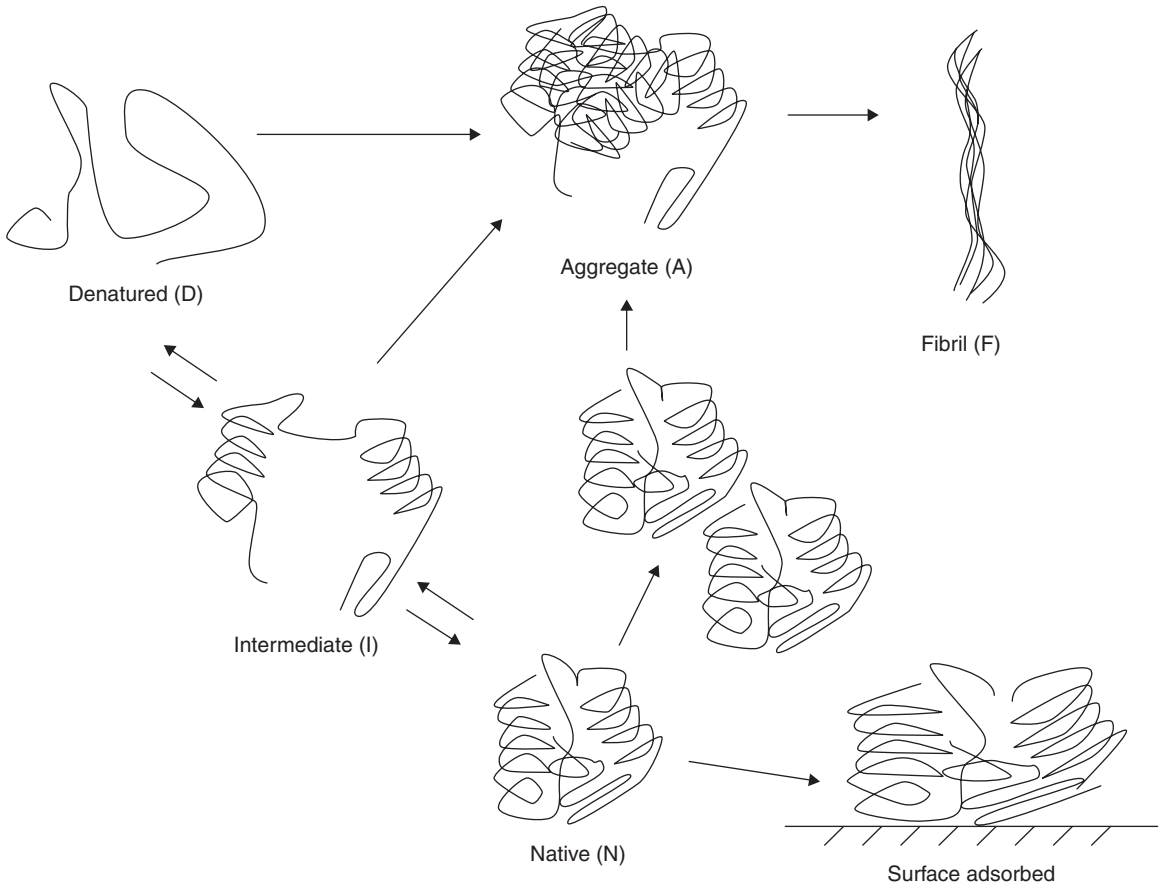

Figure 1. The protein aggregation process. It is an equilibrium between the folded native (N) and the disordered, unfolded or denatured (D). An intermediate step, the formation of possible intermediates (I), can be present between the transformation from N to D. The aggregate (A) formed may occur from irreversible changes to the unfolded species, protein–protein interaction, and can result in formations of fibrils (F). Surface adsorption of proteins could also lead to denaturation, aggregation, or fibrillation.

lysozyme or a stabilisation of the unfolded state of lysozyme [19]. Dendrimers can both stabilise (i.e., prevent fibrillation) or destabilise proteins [20]. The destabilising effect depends on the generation of the dendrimer, and is mediated by electrostatic interactions [20]. Stabilisation can also be brought about by binding of specific ligands, for example metal ions in specific binding sites [21], or phenol-like ligands that induce the formation of insulin hexamers [22]. The most important stabilising effect is whether the protein is stabilised either by preferential binding or by hydration. In this regard the effect on the specific protein in question needs to be addressed.

The chemical destabilisation of proteins that prevails most is oxidation [23-25]. Chemical stability can be minimised by appropriate choice of preparation procedures, storage temperature, vials, or by the addition of antioxidant [25]. In a study of the oxidation of relaxin, ascorbic acid was added as an antioxidant; however, the presence of Cu ions together with ascorbic acid accelerated rather than prevented oxidation [23]. Other studies recommend that a combination of ascorbate and catalysing metal ions be avoided [26,27]. In the case of relaxin, the antioxidant did not stabilise the protein unless a chelating agent, EDTA, was co-added. This combination effectively prohibited oxidation [23]. The antioxidant monothioglycerol has been shown to destabilise gastrin/GGK-2 receptor targeting peptide, and in this formulation study antioxidants were not included in the final formulation [25]. In a study of the oxidation of lactate dehydrogenase with several different approaches to minimising oxidation, polyethyleneimine and EDTA turned out to be the best combination [24]. The effect from antioxidants seems to be rather dependent on the presence and type of metal ions present, as they catalyse oxidation, so in many cases chelating agents also need to be added [23,24]. Therefore, the most efficient antioxidants are those able to interact directly with the oxidising agent and thereby prevent the formation of free radicals [24].

2.1 Excipients added to avoid changes in pH

In liquid formulations, optimising the pH – in essence optimising the hydrolytic stability – is a major issue. It is important to study the stability, especially in the range

Table 2. Recent examples of stabilising excipients used in peptide and protein formulations and their mechanism of stabilisation.

Stabilising excipient	Protein	Mechanism of stabilisation	Ref.
Amino acids	BSA, lysozyme, α-chymotrypsinogen, human antibody, cellular retinoic acid-binding protein	Preferential hydration Preferential exclusion Decrease protein–protein interactions Increase solubility (reduce viscosity*)	[13] [14,108]
Polymers	BSA, yeast hexokinase, recombinant human insulin	Competitive adsorption Steric exclusion Preferential exclusion Preferential hydration	[38] [59] [105]
Polyols	β-lactoglobulin, Invertase (EC 3.2.1.26)	Preferential exclusion Accumulation in hydrophobic regions	[111] [115]
Salts	Apoflavodoxin, amyloidogenic light chain protein	Preferential binding Interaction with protein bound water	[80,82]
Sugars	Ribonuclease-A, BSA	Preferential hydration Preferential exclusion Reduction of mobility resulting from increased viscosity	[15-17,43]
Surfactants	hGH, Albutropin, recombinant haemoglobin	Competitive adsorption	[34,57,116]

*Desirable in highly concentrated formulations.

BSA: Bovine serum albumin; hGh: Recombinant human growth hormone.

pH 3 – 10, early on in the formulation process. The means to maintain the pH in liquid solutions is to add an appropriate buffer system. The buffer system may also affect the overall stability of the formulation, for example the rate of deamidation appears to be faster in phosphate and bicarbonate buffers than in sulphate, nitrate, acetate, chloride and pyruvate buffers [2]. However, the actual pH value appears to be the major controlling variable in deamidation reaction reactions [28]. The choice of pH, however, is not always optional as other stability or solubility issues may arise at the pH value where the deamidation rate is lowest. Aggregation rates are also influenced by the choice of buffer, for example, for interferon-τ it was shown that the rate increased in the order phosphate > Tris > histidine buffer [29]. An obvious choice also lies in the actual purpose of pH in the formulation, which is determined partly by the administration pathway, but also in the optimum stability of the protein.

During the process of preparing the formulation, for example during the drying process (i.e., that water is removed), changes in the microclimate pH can occur, owing to the changes in the proteins environment. In the drying process, one component will stay in solution for a longer period (selective freezing) than others, which can lead to a pH shift of more than three units [30]. Usually, specific excipients for avoiding these pH changes are not added, but instead very low concentrations of buffer, or no buffer, should be used if possible. Smaller pH changes are also encountered during temperature changes such as lyophilisation, as pH is dependent on temperature. The microclimate pH also changes during degradation of PLGA particles, and for that reason antacid excipients are sometimes added to the formulation. Addition of $Mg(OH)_2$ is shown to protect efficiently the protein against degradation resulting from changes in pH [31].

2.2 Excipients added to avoid structural changes caused by adsorption

Adsorption to interfaces can be avoided by a careful choice of excipient and interfaces during processing or by design of the preparation method, for example by avoiding unnecessary agitation and minimising the surplus air in the vials, that is, exposure to interface. Excipients used should ideally be more surface-active than the protein itself. Mainly ionic or non-ionic surfactants or other proteins are used to coat or adsorb competitively to the inner surface of the containers, or adsorb to the surfaces created in the preparation of the delivery system [32]. For example, poloxamer (Pluronic F-68) and polyoxyethyleneglycol dodecyl ether (Brij 35) are able to prevent interfacial-induced aggregation of human growth hormone (hGH) below their critical micellar concentration (CMC), whereas polysorbate 80 has to be above CMC to do the same [33]. For another protein, Albutropin, polysorbate is able to protect against agitation-induced aggregation below its CMC [34], and it is able to inhibit the aggregation of hGH in ratios of polysorbate/protein > 4 [35]. At solid surfaces, low concentrations of non-ionic surfactants are often sufficient to prevent adsorption [36]. Other proteins, in particular human serum albumin (HSA), have also been used to prevent adsorption because of its high interfacial

activity. However, adjusting the concentration, type and combinations of non-protein excipients can substitute the use of HSA in formulations [32]. Polymers or dextran [37] can also be used to protect against surface adsorption, where large PEGs are reported to have a stabilising effect on proteins, whereas small PEGs seem to induce slight unfolding [38,39]. The chosen concentration and type of surfactant depends on the effect that needs to be avoided, but typically it is just above the CMC value, where a monolayer of the surfactant is present at the interface [34].

2.3 Excipients added to optimise protein stability during changes in water content

In the solid state, proteins are frequently more stable, so formulations of powder for reconstitution are often chosen to increase stability. Two frequently used drying methods are freeze-drying (lyophilisation) and spray-drying [2,11,40]; common for both processes are the removal of water (solvent) from the liquid formulation. The effect of the removal of water and the use of excipients to substitute the hydrogen bonds are major issues when using these techniques. Often stabilising excipients are added to stabilise proteins during the stress induced by a freeze-drying process. The stabilising excipients can have cryoprotectant and/or lyoprotectant effects on the protein [38,41,42]. Commonly used excipients are sugars or polyols, which have an effect during both the freezing and the drying processes. Furthermore, several other excipients can act as protectants, for example, surfactants, amino acids, non-aqueous solvents, and other peptides (**Table 3**) [3].

The freeze-drying of proteins yields a dried powder containing the protein in a glassy phase, often including amorphous excipients and residual water. The residual moisture will depend on the solid state properties of the system, that is, amorphous versus crystalline, in combination with the chosen process conditions. On addition of trehalose under freeze-drying of bovine serum albumin (BSA), moisture-induced aggregation was considerably reduced [43] and the thermal stability also increased, thought to be caused by a reduction in the exposure of the protein's hydrophobic groups to water [16]. It has been shown that a ratio of mannitol–sucrose of at least 3:1 is desirable to ensure physical stability of the freeze-dried cake [44]. In a study of freeze-dried albumin-based nanoparticles, sucrose, trehalose and mannitol at levels > 2% (w/w) reduced particle growth during storage and increased the glass transition temperature (T_g) of the formulations. It was also shown that sucrose and trehalose are superior to mannitol [45], possibly owing to the formation of an amorphous glass. For a freeze-dried formulation of an IgG antibody, the use of sorbitol as an extra stabiliser, in addition to sucrose or trehalose, was investigated. Whereas no benefit was observed for the trehalose–sorbitol mixtures, the stability was increased for the sucrose–sorbitol mixture [46]. Furthermore, for spray-dried lysozyme a mannitol–trehalose mixture showed better long-term stability compared with trehalose [47]. Considering the need for both crystalline and amorphous structures in the dried product, it can be concluded that mixtures such as mannitol–sucrose or mannitol–trehalose may be beneficial.

3. Excipients added to optimise the function of the product

In the formulation and design of advanced drug delivery systems of proteins, the choices of excipients may be based mainly on obtaining the desired function and the required release profile.

3.1 Excipients added to optimise a drug delivery system

In microspheres and nanospheres for protein delivery, certain excipients are often needed to stabilise the therapeutic protein during production [48-50]. In some of the delivery systems, effects from the excipients used to design the particles can also have adverse effects on the protein stability [51]. Poly(lactic-*co*-glycolic acid) (PLGA) particles are perhaps the most successful and also well-studied controlled release formulations for peptides [52]. The main obstacles for using PLGA particles as protein delivery systems have been the exposure to interfaces during production and the formation of lactic and glycolic acid under during storage and release [53]. Both can damage a therapeutical protein [54,55]. The release of acids has been counteracted by including antacid excipients in the PLGA particles, for example $Mg(OH)_2$ [31] or $ZnCO_3$ [56], where the appropriate choice depends on possible specific interaction with the protein. The addition of these basic salts can, however, lead to increased deamidation [28]. The potential detrimental exposure to interfaces during production of particles can be limited by the addition of surfactants [1,11,34,37,57] or polymers, for example PEG [39,58]. New excipients have also been reported, for example a polycationic PEG-polyHis, which decreases the aggregation of insulin at interfaces during encapsulation into PLGA particles by forming complexes with insulin [59]. PEG-polyHis probably acts by stabilising the protein in a similar manner to a surfactant at a pH where complexes would not normally form. Another approach that can be used for some proteins is the preparation of, for example, dextran-encapsulated particles from an aqueous–aqueous system rather than an aqueous–organic system, thereby decreasing the unfavourable exposure to the interface [37]. Materials other than PLGA are also used to form delivery systems other than solid particles, for example, hydrogels of gelatines [60,61], alternative polymers [62], or complexes with dendrimers [20,63]. Gelatines can be used in several different protein drug delivery systems, for example in gelatine hydrogels for the delivery of tissue growth factor [60] and nanoparticles for vascular growth factor delivery [61]. The use of recombinant human gelatines circumvents the quality and immunogenicity issues related to this raw product [64]. The gelatines appear to interact with the partially unfolded protein and thereby inhibit aggregation [64].

Table 3. Recent examples of commonly used cryoprotectants and lyoprotectants.

Excipient type	Example of relevant excipients	Ref.
Sugars and polyols	Trehalose, sucrose, lactose, mannitol, sorbitol	[17,110,117-120]
Polymers	Dextran, PVP, starch derivatives	[117,118,121]
Surfactants	Polysorbates 20 and 80	[122-124]

PVP: Poly(vinyl pyrrolidone).

Dendrimers used to prepare particulate delivery systems have an effect on the stability of proteins. They have been shown to destabilise proteins dependent on the type and structure of dendrimers, where the factor of importance is the branching and the hydrophobicity of the dendrimer, that is, that the destabilisation is dependent on the surface structure [20,63]. In the formation and design of particulate delivery systems for proteins, the main concerns should be the induced stability issues from the preparation, potential destabilising effects from the excipients, for example the degradation of PLGA, and obtaining release of an active protein.

3.2 Excipients added to optimise a dry powder formulation

A dry powder needs to have a certain appearance to be useful, for example, a freeze-dried cake needs an acceptable appearance, it should be rapidly dissolvable, and blow-out of the formulation must be prevented. Bulking agents such as sugars and polyols can be selected for this purpose, as they can also act as cryoprotectants and lyoprotectants [45]. When selecting an appropriate excipient, one should first consider the solid state properties. Mannitol will usually crystallise and thus lead to a cake with good structural stability. However, mannitol can crystallise into three different polymorphic forms (α, β and δ) with different stability and mannitol hemi-hydrate, which may release its crystal water during storage [65,66]. The solid state of mannitol depends on the freeze-drying conditions and the presence of other excipients [65,66]. Sucrose, on the other hand, usually remains amorphous on freeze-drying, which is desirable for protein stability [67,68], but it also increases the water content after primary drying and increases the danger for deliquescence and collapse of the final product. Salts for the adjustment of pH and tonicity are found quite frequently. Although these salts sound rather uncomplicated, one should be aware that during freezing pure solvent freezes first. This leads to an increase of the salt concentration in the remaining liquid phase (freeze concentration), thereby increasing the ionic strength. In the case of buffer systems, one component stays in solution for a longer period (selective freezing), which can lead to a pH shift of more than three units [30], which will obviously have an impact on the stability of the protein. Furthermore, both sodium chloride and sodium phosphate buffer salts have been found to reduce the crystallisation tendency of mannitol [69,70], leading to a more amorphous and thereby unstable system, or to induce the aggregation of IgG [71]. The amino acid glycine has also been shown potentially to inhibit the crystallisation of mannitol [70], which implies the possibility of using other amino acids, and thus also proteins, to influence the solid state of the excipients. Surfactants such as polysorbates 20 and 80 can influence the crystallization behaviour of excipients, as has been shown for a combination of polysorbate and mannitol where an increasing transformation from β- to δ- mannitol has been reported for increasing concentrations of polysorbate 80 [72-74]. Summarising, it can be stated that the number of functional excipients should be kept as low as possible to reduce instability resulting from solid state interactions.

3.3 Excipients added to optimise the protein stability in solutions and suspensions

In solution, the stability of peptides and proteins depends on the characteristics of the solvent (typically water), but also on the co-solvent, pH and the addition of salts [3,75]. Solutions are typically designed to give optimum stability and functionality of the pharmaceutical formulation. The optimum solubility of a protein involves a combination of several parameters, such as ionic strength, solution composition and pH [76], and a minimum solubility is often observed around the protein's pI value. Examples are known where the solubility of proteins can be increased up to 8.7 times by simultaneous addition of L-Arg and L-Glu in concentrations of 50 mM, and these concentrations also increase the long-term stability [77].

Suspensions are another way of altering the solubility and increasing stability, and they are most frequently used to obtain an alteration in release profile [78]. The chemical stability is increased in suspensions, but the physical stability does not always increase compared with a solution [78,79]. When formulations of insulin in solution and suspension, respectively, were compared, the solution formed fewer visible agglomerates after agitation than the suspension did [79]. A pharmaceutical formulation would most often be a solution rather than a suspension if the stability of the protein allowed it owing to more complicated processing and handling of suspension products.

3.4 Excipients added owing to the route of administration

To make the formulation acceptable for various routes of administration, certain excipients need to be added, and these may have detrimental effect on the stability. For example, if salts are added to adjust the tonicity this will influence both stability and solubility [3,80]. These effects can be dependent on pH, the type and concentration of salt, the nature of the interaction between salt and proteins, and on the amount

of charged residue in the protein [3,81]. Salts affect the electrostatics of the protein by specific ion-binding, by nonspecific (Debye–Hückel) electrostatic shielding [3], or by making water less available to the protein [82]. These shielding effects may not be the sole explanation for the effect on the electrostatics from changes in ionic strength [83]. At high salt concentrations electrostatic interaction can both decrease solubility as well as increase the strength of hydrophobic interactions [82,84]. If the ionic strength is increased from 0.04 to 0.11 by the addition of NaCl, the fibrillation of insulin increases, possibly by shielding the interactions between similarly charged groups [85]. Changes in pH and salt concentration can also alter the distribution between insulin hexamer and monomer, that is, at low pH and high salt insulin is mainly found as hexamer, whereas high pH and low salt concentration give mainly monomers [86].

In addition to the above-mentioned excipients and functions, preservation of formulations in multiple dose containers is also required. Therefore, antimicrobial preservatives such as phenol, benzyl alcohol, methylparabene and propylparaben are added. Apart from their formulation function, the excipients also interact specifically with the protein and can thereby cause alterations in its function and stability. For example, phenol-like ligands affect the stability and conformation of the insulin hexamer. For example, the addition of these ligands shifts the structure from a less stable to a more stable conformation of insulin [22]. Other antimicrobial preservatives, for example benzyl alcohol, can accelerate the aggregation and precipitation in aqueous solutions [87,88]. These excipients are not, however, the most important ones and can often be circumvented by using containers that do not allow for exposure to the surroundings, that is, not using multiple dose containers.

4. Regulatory issues and official guides to choices of excipients

For an excipient to be approved as a part of a formulation its inclusion has to be justified, the compatibility with the active ingredient shown, and the quality (or grade) will have to be either justified or shown to be sufficient to fulfil the requirements for the final product. Furthermore, the suggested amount of excipient must be shown to be sufficient for the intended function of the excipient [8]. No official lists are readily available to guide on the amount, types and use of excipient. Nevertheless, the Federal Drug Agency (FDA) has made a database and an *Inactive Ingredients Guide* from 1996 publicly available, in which the use of various excipients in registered products for the different delivery pathways is listed [89,90]. A similar list was available at one point on the European Medicines Agency (EMEA) site and can be found in Matthews [9].

The introduction of excipients to a formulation requires thorough studies of the interaction between the specific excipient and the active ingredient or other excipients and of how stability is influenced. This requires extensive knowledge, especially if a new substance is being used. As a consequence, there is a tendency among pharmaceutical companies to use well-known excipients as a starting point for new protein formulations.

The challenge in formulation design is that a combination of excipients that is most favourable for a particular protein can be less favourable for another. This may be owing to specific interactions between excipients and the active ingredient or to differences in the inherent stability of proteins. Thus, formulation design is not always straightforward, and the formulation scientist may have to look for substances other than the ones commonly used.

Several requirements must be fulfilled in order to introduce new substances as excipients in protein drug products. The excipient must be available from a reliable supplier and obtainable in the right quality with a controlled level of residual solvents, preferably of non-animal origin and produced according to GMP requirements. When new excipients are proposed, their prior use in either the food or the cosmetic industry for similar delivery pathways (e.g., dermal) may ease the application process [8]. Excipients that are controlled by pharmacopoeial monographs are often preferred. However, even though an excipient may be of pharmaceutical grade it may previously have been used only in formulations for routes of administration other than that desired for the product under development. Protein and peptide formulations are mostly formulated as injectables, and because of the poor stability of proteins the processing of the pharmaceutical product does not allow for terminal sterilisation. Instead, a sterile filtration step is usually included in the production along with intensive control of the active substance and excipients. Thus, further requirements for excipients to be used in protein injectables are, for example, a low bioburden and absence of endotoxins. This, however, involves the risk that pharmaceutical products end up being suboptimum (e.g., with regard to stability, pH or processing properties) compared with a situation where all substances were readily available, owing to a compromise between development cost and time on the one hand and product and process quality on the other.

4.1 Safety, toxicity and immunogenicity of excipients

In drug formulation, the safety of excipients is as important as the safety of the active product ingredient. For well-known excipients that have been recognised and used for a long time, the question of safety is mainly one of quality control of the products received from suppliers. For new excipients, thorough documentation is required by the EMEA and FDA [91,92]. This is expensive as well as time-consuming and unfortunately is hampering the development of new excipients, but as the safety of the patients is paramount, this is a necessity. The concentration level of the excipients must be qualified with respect to safety before use in clinical trials. This is one of the main barriers to the use of new

excipients in pharmaceutical products, as safety studies are time-consuming and expensive. Furthermore, the risk of adverse events is considerably higher for injectables compared with non-invasive routes of administration, which reduces the number of available substances significantly [93]. Some suppliers of pharmaceutical excipients are able to deliver the preclinical data needed. However, it must still be considered whether the data are adequate with regard to route of administration and the intended frequency of use. Thus, unless patents or insufficient stability can justify alternative approaches, pharmaceutical companies will often limit themselves to well-known substances.

As the toxicity of a given compound will always be related to the dose, some excipients that are known generally to be 'non-toxic' are in fact toxic to humans in larger doses. Toxic effects of excipients have been reported after fast intravenous administration of drug formulations containing, for example, poly(ethyleneglycol) [94] and polysorbate [95]. Some cyclodextrins have nephrotoxic effects if administered parenterally [96]. The relationship between the unwanted immunogenicity of a biopharmaceutical and the excipients in the formulation has, perhaps, been slightly overlooked in the past. The insight into and especially the assessment of the unwanted immunogenicity of protein drugs are still expanding, and the notion of excipient-induced immunogenicity will be studied much more in the future. Very little research has been published on the subject. As an example, the addition of HSA as an excipient in a formulation of IFN-α_{2a} has been linked to aggregation and increased immunogenicity [97,98]. Mukovozov *et al*. gives an overview of the factors that contribute to the development of immunogenicity of recombinant human proteins [99].

5. Expert opinion

Excipients are crucial for making optimal protein and peptide pharmaceuticals. Their role and function in the formulation may be quite different, as has been elucidated above. Excipients that are essential for the stability or function of the formulation are usually more difficult to replace than example excipients, which are added to obtain appropriate tonicity of the product. In the design of new formulations, all effects should be considered for each protein, specific stabilisers be identified, and the mechanism of stabilisation for each protein should be elucidated. Some excipients may alter their stabilising effect over time. For example, the stabilising effect of polysorbate is shown to change during storage [72,100], owing to oxidation.

The best possible basis for effective formulation development is found through preformulation work. Basic knowledge of solubility, pI, chemical and physical stability as well as the most common degradation products, and how these are affected by solution characteristics is important input to the formulation scientist. When a preliminary target with regard to, for example, shelf-life, pH range and concentration of active ingredient, is set, excipients can be chosen based on their expected function, for example, to keep pH within in a certain range, to increase the solubility of the protein, or to increase physical stability. When a series of excipients and an appropriate concentration range have been chosen, formulation screening can be more or less automated, with chemical or physical stability at accelerated temperatures as target parameters. The concentrations of the selected excipients are typically optimised even further hereafter. A way to screen a large array of excipients is by constructing an empirical phase diagram [101-103], where the effect on protein structure and stability from several excipients in solution is evaluated and boundaries are established. It is considered a rapid and rational approach to protein formulation [101,102], and it is even compatible with high-throughput instrumentation that can reduce the time for a formulation study to days or weeks [101].

In view of the expectation that an increasing number of pharmaceuticals will be based on peptides and proteins in the future, there is a substantial need for the development and approval of new excipients to aid formulation development. The list and documented use of excipients for the formulation of peptide and protein drugs is limited to some extent and the search for new and improved excipients is rather inadequate. Some efforts are being made in this regard, for example in designing new stabilisers for enzymes based on glycosides from microorganisms [104]. However, much more focus will be needed really to add new and improved excipients to the list of useful excipients. As mentioned above, the approval process is expensive and time-consuming, therefore this challenge will most probably be met by chemical and pharmaceutical manufacturers in cooperation.

Declaration of interest

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