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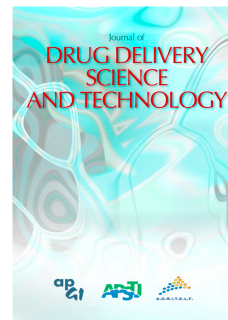

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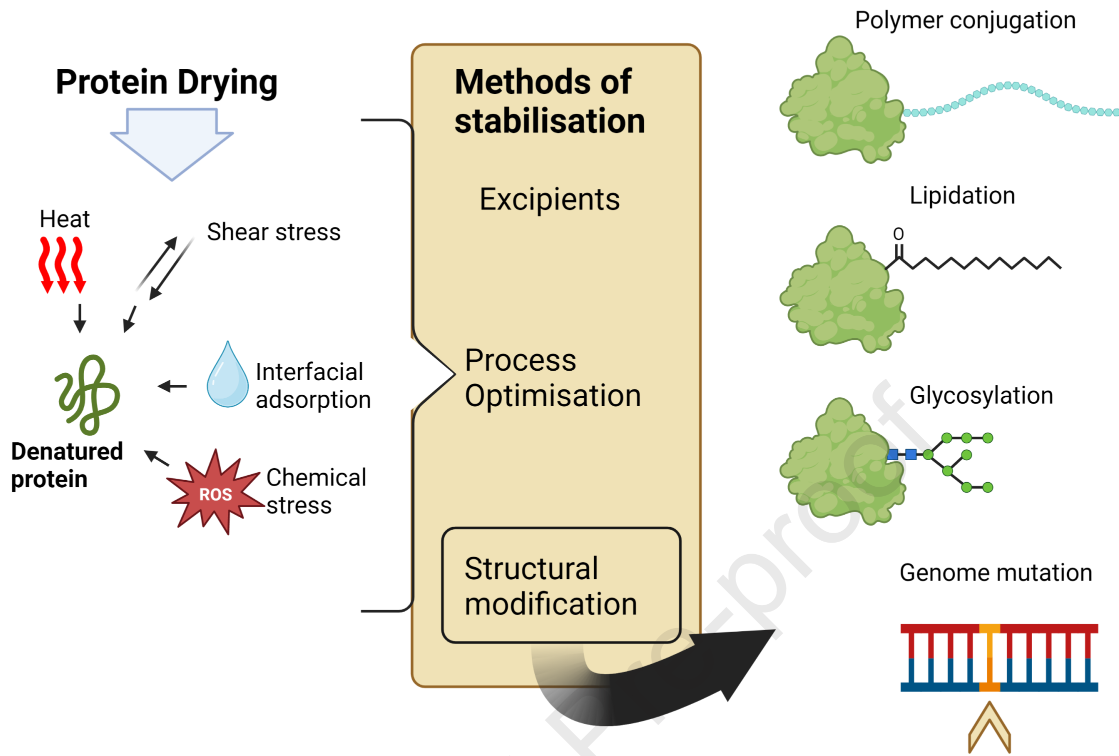

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Structural modifications for the conversion of proteins and peptides into stable dried powder formulations: A review.

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Abstract

The drying of biomolecules into powdered formulations has become the main form of long-term product stabilisation, allowing for the delivery of safe and efficient medicines. Stability of proteins and peptides during the drying process is paramount for product quality. Drying macromolecules with an appropriate excipient is often sufficient for product stabilisation, however there are limitations imposed on excipient use, particularly in the production of high-value biopharmaceuticals. Innovative approaches for the enhancement of protein stability during dehydration need to be further explored. In this review, we provide a brief discussion of the available drying methods and current stabilisation techniques available for proteins and peptides and review the current impact and limitations of excipient use. Alongside, we take a detailed look at the impact of post-translational modifications (PTMs) and structural mutations in drying stability of biomolecules. The structural modifications mentioned in this paper are discussed in light of published work on their impact on protein and peptide stability during commonly experienced stresses, particularly those which relate to drying processes, such as chemical, thermal and freeze-thaw. The aim of this review is to direct a research focus towards upstream modifications of proteins and peptides as a viable stabilisation approach during harsh drying processes.

Keywords:

Biological formulations; Protein and peptide stability; Protein engineering; Post-translational modifications; Polymer conjugation; Structural modifications

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35 # 1. Introduction

36 In 2020, the biopharmaceutical industry was placed at the forefront of innovation, breaking
37 records in the speed and efficiency of the development and manufacture of vaccines and
38 novel monoclonal antibodies for the treatment of SARS-CoV-2 [1, 2]. The turn of the decade
39 has highlighted the need for manufacturing affordable, yet highly potent, safe medicines, and
40 for their distribution in response to a growing market. Furthermore, the sensitive nature of
41 proteins limits the introduction of new manufacturing techniques, as preservation of the
42 secondary and tertiary structures are vital in ensuring safety and efficacy of biological
43 medicines. Therefore, drying methods which utilise harsh conditions must be tightly
44 controlled or revised to minimise stresses on protein molecules [3-5]. Drying is an effective
45 way of long-term protein stabilisation, improving storage stability by reducing mobility and
46 impeding certain degradation pathways [6]. Various methods have been utilised and proposed
47 for the drying of biomolecules, with freeze-drying and spray drying widely used in the food,
48 textile and biopharmaceutical industries [7]. Other widely researched methods include spray-
49 freeze drying, supercritical fluid drying, continuous freeze drying and electrospinning, as well
50 some emerging technologies such as Microglassification™ and Particle Replication in Non-
51 Wetting Templates (PRINT®) [6, 8-10].
52 All drying methods are associated with a certain level of protein degradation, which depends
53 on the protein model systems and excipients being used. This degradation is largely a result
54 of processing physical stresses and water removal. Nevertheless, each drying technique offers
55 unique potential in biomolecule formulation. Freeze-drying is the mildest technique of
56 protein drying, however the process is slow and does not offer particle engineering solutions.
57 Other techniques such as spray drying, spray-freeze drying and supercritical fluid technology
58 offer wide applications in production of dry protein inhalables (DPIs), implementation of
59 continuous processes and production of biomolecule particles with enhanced product
60 characteristics (e.g solubility, flowability and particle size). Further development of these
61 techniques towards production of bio-related products has been limited by the sensitivity of
62 biomolecules under a combination of stresses present, such as thermal, shear and chemical
63 stress [5, 6, 11, 12].
64 The usage of excipients is a long-used stabilisation approach for biomolecules during drying
65 and during subsequent storage and handling. The use of appropriate excipients, in many
66 cases, completely protects the protein molecules from degradation and slows deterioration
67 during storage [6]. The use of excipients however requires detailed evaluation for each
68 formulation. In applications where particle engineering is essential, excipients may influence
69 particle size and morphology [13]. Certain excipients are unsuitable for use due to
70 interactions in formulation, or lung-related toxicity when discussing the application of these
71 techniques in DPIs [14]. To combat these issues, directed studies of biomolecule stability
72 during harsh drying processes, without the use of stabilisers, are needed. The second section
73 of this review will provide a general overview of the stresses present in drying techniques and
74 the methods used to ameliorate them. The limitations of excipients are also considered.
75 Many biomolecules experience significant loss of activity after dehydration without
76 excipients [15-17]. The stability enhancement of native peptides and polypeptides by
77 structural modification is an alternative form of product stabilisation. Many reviews in the
78 field of bioengineering and conjugation chemistry have addressed and discussed the effect of
79 protein/peptide engineering and bioconjugation approaches on biomolecule stability [18-21].

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80 In the field of industrial enzymes, protein engineering is routinely used for the production of
81 thermostable molecules which are regularly dried using spray and freeze-drying [22].
82 However, to the best of our knowledge, the use of protein engineering and bioconjugation has
83 not been reviewed in the literature particularly for solid-state stabilisation. This review offers
84 a comprehensive rationale for the use of protein engineering and bioconjugation as solid-state
85 stabilisation approaches, by discussing their effects on stresses experienced during and after
86 drying. The impact of protein modifications on subsequent storage and handling stresses,
87 such as aggregation, oxidation and freeze-thaw are also considered. Structural modifications
88 of proteins and peptides provide an alternative to excipient use, where it is needed and
89 feasible, such as in the production of DPIs, supercritical fluid drying and other particle
90 engineering applications. These modifications may also be employed when the amount/type
91 of excipient in the formulation must be minimised.

92 ## 2. Stability of biomolecules during drying

93

94 ### 2.1 Stresses in dry protein formulations and current methods of stabilisation

95 *2.1.1 Freeze drying*

96 All processes which involve the removal of moisture possess some level of associated stress
97 which impacts biomolecule stability, due to the importance of moisture in biomolecular
98 structures [23]. Due to the lack of thermal denaturation, freeze drying is the main choice for
99 drying biomolecules, however stresses during processing can directly impact product
100 stability, and in the case of biopharmaceuticals, immunogenicity. During initial freezing the
101 protein may experience an array of stresses, including ice crystallisation, ice-water interface
102 adhesion and cold denaturation [24]. Various studies have identified critical points of
103 denaturation, such as ice crystal adsorption during initial freezing [25] and over-dehydration
104 during secondary drying [26] as the main sources of product denaturation. Currently, various
105 cryoprotectants are utilised to prevent damage caused by ice formation. They do so by
106 increasing the level of solutes in the feed, hence truncating the generation of ice crystals.
107 Non-toxic polymers and sugars are most often availed of, such as glycerol, PVP and PEG
108 [27]. Dehydration stress is minimised by the addition of sugars, such as trehalose and
109 sucrose, which protect the biomolecules according to the water replacement theory [28].

110 *2.1.2 Spray drying*

111 Unlike freeze drying, spray drying applies high temperatures to feed solutions containing
112 biomolecules during atomisation and drying. The temperature of the hot gas at the nozzle
113 (T_{in}) has been shown to exceed 120 ºC for efficient water removal, yet some sensitive
114 proteins may experience significant denaturation at temperatures as low as 60 ºC. For
115 example, IgG antibodies, unequivocally the largest class of biopharmaceuticals, experience
116 substantial irreversible denaturation at 65 °C [29]. Sample unfolding purely due to high T_{in}
117 (inlet temperature) however is usually minimal. Protein molecules experience this heat for
118 approximately 1 second, depending on flow and gas rate parameters, and are additionally
119 protected by moisture at the droplet surface at the moment of particle formation [30].
120 Although routinely lower than T_{in}, the temperature of gas at the dryer outlet (T_{out}) has been
121 reported as the main source of protein thermal degradation [31, 32]. The higher levels of
122 denaturation can be attributed to longer residence times of the dried particles at raised outlet
123 temperatures (typically a few seconds) [33].

124 The molecules also experience a high level of mechanical stress that is not imposed during
125 freeze drying. Shear forces inflicted on amphiphilic proteins during atomisation expose
126 hydrophobic residues, and while most proteins are tolerant to high shear stresses,
127 instantaneous absorption to the air-liquid interface of small droplets causes extensive
128 aggregation of the molecules [34] . In fact, aggregation is often reported as the main source of
129 protein degradation [35, 36]. Aggregation can cause loss of activity of the protein and poses a
130 high risk of immunogenicity when administered as a medicine.
131 Current methods used for minimizing aggregation and thermal denaturation include the use
132 of excipients and process optimisation. Similarly to freeze drying, saccharides such as
133 sucrose and trehalose can inhibit degradation pathways during spray drying. Sugars are well-
134 established stabilisers, also shown to prevent denaturation of β-galactosidase [37], lysozyme
135 [38], and biopharmaceuticals, such as antibodies [39-41] and therapeutic peptides [42-44].
136 Additionally, amino acids such as arginine and glycine can be used as stabilisers [45], as well
137 as surfactants such as PVP and PEG. These additives provide protein stabilisation in three
138 ways: 1) by competing with the protein molecules for space at the air-liquid interface, thereby
139 minimising protein aggregation, 2) by shielding hydrophobic residues with the addition of
140 amino acids, and 3) replacing water and hence diminishing dehydration stresses.

141 *2.1.3 Spray-freeze drying (SFD)*
142 The effects of shear stress and air-liquid interface adsorption are still present in spray-freeze
143 drying due to the atomisation step and pose issues concerning aggregation and loss of
144 activity. Consequent freezing of the atomised droplet into liquid Nitrogen, or during CO_2-
145 assisted spray-freezing, also allow for cold denaturation and denaturation by ice
146 crystallisation [6, 46]. Two papers reported in the literature conducted on the stability of
147 lysozyme showed that the atomisation step during SFD lead to high rates of aggregation and
148 loss of enzymatic activity, although interfacially-associated aggregation is more prevalent in
149 the spray drying of lysozyme when compared to spray-freeze drying [47, 48]. Air-liquid
150 interface adsorption during SFD was found to be comparatively more detrimental than ice-
151 liquid interfaces present during freeze drying, as demonstrated by Webb et al., with the use of
152 recombinant human interferon [49]. As with spray drying, the use of sugar excipients causes a
153 reduction in the rates of protein adsorption and unfolding, with similar mechanisms of
154 stabilisation (i.e. reduction of molecular mobility, competition for space at the droplet
155 interface). Trehalose and cyclodextrins show promise as stabilising agents, as demonstrated
156 in the production of Immunoglobulin G (IgG) antibodies [50]. The use of amino acids, in
157 particular hydrophobic residues such as leucine, phenylalanine and glycine, have also been
158 shown to ameliorate the formation of IgG aggregates in SFD [51]. Trehalose acts dually as a
159 surfactant and lyoprotectant of IgGs during spray-freeze drying (SFD), shielding the protein
160 from denaturation due to both shear and freezing stresses. Formulation optimisation during
161 SFD is of utmost importance, as it requires the combination of both lyoprotectants and
162 stabilisers for atomisation.

163 *2.1.4 Supercritical fluid drying (SCFD)*
164 Supercritical fluid drying (SCFD) methods do not typically employ high temperatures and
165 therefore provide the opportunity to avoid thermal degradation of biomolecules. In their study
166 on insulin microparticles, Amidi et al. used SCFD to produce insulin powders suitable for
167 pulmonary delivery, with N-Trimethyl chitosan (TMC) and dextran as carriers, which
168 displayed low levels of denaturation or aggregation [52]. Similarly, lysozyme has been

169 demonstrated to retain its molecular integrity when sprayed with supercritical CO_2 under
170 optimised process parameters [53]. The stresses which the biomolecules are exposed to are
171 relatively low compared to other techniques, limited to shear stress during atomisation (in the
172 case of SASD) and dehydration stress from rapid water removal. Aggregation rates of
173 proteins dried by supercritical methods are generally reported as low, with the first insulin
174 successfully formulated with only 7% aggregation, and lysozyme formulated by SAA
175 showing no evidence of aggregation [54] [55]. However, aggregation and activity loss still
176 pose an issue for some proteins, and requires the addition of sugar excipients such as
177 trehalose and sucrose [56]. In the case of lactate dehydrogenase (LDH), a relatively labile
178 protein, the addition of both a sugar (sucrose) and a surfactant (Tween 20) to the aqueous
179 LDH formulation substantially preserved its enzymatic activity after supercritical CO_2-
180 assisted aerosolization [57]. This is most likely a result of water molecules replacement and
181 protection from dehydration stresses. Proteins dried under certain SCFD methods are also
182 subject to denaturation sources which are not present in the other three methods (namely
183 freeze-drying, spray drying and spray-freeze drying). For traditional small molecule APIs
184 (Active Pharmaceutical Ingredients), the selection of appropriate organic solvents is
185 straightforward, where methanol, ethanol and DMSO are often used. The presence of any
186 organic solvents can, however, affect protein structures, even at small amounts [58]. Water is
187 used as a solvent in SASD, where possible, yet in some cases dissolution of CO_2 in water
188 may cause significant pH drops, disrupting hydrogen bonds within proteins' structure,
189 ultimately leading to unfolding. Buffers can be used to minimise this phenomenon, as
190 reported by Sellers et al. where buffer salts such as potassium phosphate, acetic acid and Tris-
191 HCl were used to raise the pH, minimizing loss of enzymatic activity of lactate
192 dehydrogenase [57].

193 *2.1.5 Other novel drying technologies*

194 Research has been conducted on the drying of biomolecules using novel techniques which
195 circumvent the current economic challenges associated with traditional freeze-drying.
196 Electrospinning, or electrospraying, is a relatively novel drying technique used to produce
197 monodisperse particles through the application of an electrical current to a liquid feed. A large
198 portion of the research into electrospraying of biomolecules has been concentrated on
199 encapsulation of the molecule in a polymeric carrier [59]. In drying the model protein,
200 lysozyme, electrospraying was comparatively less detrimental to biological activity than
201 spray drying, with shear stress being the only significant stress recognised by the authors
202 [59]. Electrospinning of the therapeutic mAb infliximab in a carbohydrate emulsion showed
203 no impact on the binding affinity, and negligible effect on aggregation (<0.01%) [60]. Similar
204 effects have also been published on bevacizumab [61]. Both studies focused on drying the
205 antibodies in excipient mixtures, and therefore effects of individual stresses on protein
206 stability could be observed.

207 PRINT® Technology or Particle Replication in Non-Wetting Templates, is a particle
208 engineering technique using film drying, followed by 'micro-moulding' which has been
209 recognised in the production of dry, inhalable protein particles [62]. The technique utilises a
210 lyophilisation-based drying method, followed by treatment with a heated mould. The
211 technique uses a combination of freezing and shear stress due to the use of rollers during
212 moulding, however studies with lysozyme showed no impact on bioactivity [62], while
213 insulin and albumin showed no sign of aggregation with this technique [63]. Again, stability
214 of protein particles were not the focus of these studies.

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215 Further drying techniques such as microglassification™ and continuous freeze-drying have
216 shown little reports of protein formulation. The application of a drying solvent (long-chained
217 alcohol) in the microglassification™ technique assumingly has little impact on the
218 aggregation of Bovine Serum Albumin (BSA) (+2%) [64]. The glassification of other
219 enzymes (e.g. lysozyme, chymotrypsin, catalase and horseradish peroxidase) showed that the
220 aggregation induced by the solvent technique was gone after reconstitution, however the
221 enzymatic activity was reduced to between 93%-36% depending on the solvent used [65].
222 Continuous freeze drying techniques show promise in circumventing current batch freeze
223 drying issues, however those techniques have only been reported in the literature to dry one
224 particular therapeutic protein, namely an IVIG polyclonal antibody [66]. Without the use of
225 excipients, the technique inflicted 15-20% damage to the protein secondary structure, the
226 same as batch freeze-drying.

227

228 ## 2.2 Limitations of excipient use

229 Excipients used for the drying of biomolecules are widely accepted to be inert constituents of
230 formulations, with many non-toxic sugar, polyol, and polysorbate stabilisers approved by the
231 FDA for use in parenteral formulations [28, 67]. Their use must still be evaluated individually
232 for each formulation, particularly in solid state. Interactions between sugars, other
233 formulation excipients, and the protein itself, have been shown to cause crystallisation and,
234 hence, potential detrimental pH shifts and phase separation of protein in the solid state [68].
235 Such effects have been well documented in the freeze drying of IgGs. In a study performed
236 by Connolly et al., both trehalose and mannitol crystallised under faster drying conditions and
237 enhanced protein aggregation up to 3%, while secondary structure perturbations were also
238 observed by FTIR (Fourier-Transform Infrared Spectroscopy), contrary to sucrose which did
239 not crystallise [69]. Sorbitol, a commonly used sugar alcohol, is also reported to increase
240 protein aggregation by phase separation in lyophilates [70, 71]. Storage stability of protein
241 solids may also be affected by sugar crystallisation. Pouya et al. demonstrated this
242 phenomena in spray-freeze drying, where a mannitol stabiliser was used in the creation of an
243 IgG powder formulation, ultimately resulting in high aggregation rates (>19%) [50]. Sugars
244 with a low glass transition temperature have been shown to have an increased propensity
245 towards crystallisation, and in the case of a freeze-dried formulation of insulin and dextran,
246 can cause higher rates of degradation than that of insulin stored without stabilisers [72].

247 Polysorbates such as Tween 20 and Tween 80 are widely used in biopharmaceuticals, and are
248 included in more than 70% of the currently manufactured mAb formulations [73]. The
249 tendency of polysorbates to auto-oxidise in storage calls for re-evaluation of these excipients
250 as stabilisers for solid-state proteins. Studies reported on IgG formulations exemplified
251 increased degradation of polysorbates during storage at 25 °C and 45 °C, and an increase in
252 antibody fragmentation and aggregation as a result of toxic peroxide release [73] [74].
253 Surfactants have also been linked to enhancing photo-oxidation of other compounds, severely
254 degrading photo-sensitive biologics such as Interleukin-2 in the solid state [75]. Storage of
255 powders formulated with polysorbates still require cold chain, or alternatively require
256 addition of amino acids to prevent peroxide build-up [74]. Due to the presence of several
257 aggregation pathways, the choice of excipients for each formulation is often a trial-and-error
258 process, and kinetics of drying stabilisation are poorly understood [68, 76]. Routinely, one
259 excipient is added to counteract the negative effects of another. In an investigation of amino

260 acid behaviour during freeze drying of lactate dehydrogenase (LDH), the crystallisation of L-
261 arginine during freezing was counteracted with addition of L-phenylalanine [77].
262 Aggregation-causing mannitol crystallisation during freeze drying of LDH has also been
263 counteracted with the use of Polysorbate 80 [78]. Addition of partner excipients may also be
264 necessary to enhance the effects of another, as in the case of PEG and small sugars including
265 glucose and sucrose.

266 Commonly used additives which aid in drying stability have potential hazards associated,
267 which contribute to patient hesitancy and medicinal side-effects, although the number of
268 adverse effects caused by excipients post-clinical trials is low. Degradation effects of
269 stabilisers in the solid state is a particular cause of concern, as it is heavily influenced by
270 storage time and environmental conditions. Polysorbate 80 is listed as potentially causing
271 renal dysfunction, hypotension, and metabolic acidosis (acidification due to renal issues)
272 [79]. In one study of 230 formulated biologics, polysorbates have been reported in 6 cases of
273 anaphylactic shock and skin irritations, while overloading of sugars have been reported in 10
274 cases of renal complications [80]. In inhalable formulations the choice of excipients is,
275 overall, limited as many commonly used stabilisers such as amino acids and surfactants may
276 cause injury during lung deposition. The knowledge surrounding excipient safety of spray
277 dried inhalables is limited, and must be vastly studied due to increased toxicity associated
278 with pulmonary administration [81]. Similarly, use of lyoprotectants during freeze and spray-
279 freeze drying must be undertaken with great care when manufacturing parenterals, as
280 increased cytotoxicity is a concern [82].

281

282 Excipient effect on powder morphology, flowability and overall quality must also be
283 considered in the development of dried protein formulations. During freeze-drying the
284 volume and type of excipient affect cake appearance and reconstitution times Surfactants may
285 cause cake deterioration in the form of 'fogging' or dispersion of dried product on freeze-
286 dried vials [83], yet also promote lower reconstitution times due to increased porosity [84].
287 Sugars may participate in Maillard reactions with certain proteins, but are also used as
288 bulking agents to preserve cake integrity [85].
289 For inhalable powder formulations produced by spray drying or spray-freeze drying,
290 excipients have shown a major impact on the aerodynamic suitability of the powders.
291 Excipients are instrumental in determining mass transfer properties within a droplet and
292 therefore have a direct correlation to particle size and morphology. For example, sugars and
293 polyols display high diffusion rates upon heating and tend to encapsulate the biologic in a
294 small, dense particle rather than in a hollow, low-density particle that is needed for dry
295 powder inhalables [86]. For spray-dried powders, particle morphology is usually improved by
296 the presence of excipients. 'Naked' spray-dried proteins display as collapsed, wrinkled
297 particles, and addition of sugars and surfactants often improves their sphericity, to varying
298 extents. Figure 1, adapted from Chen et al. [13], shows the effect of trehalose, mannitol and
299 leucine on spray dried BSA morphology by SEM (Scanning Electron Microscopy). Although
300 excipient-free BSA (Figure 1A) is shrunken, trehalose and mannitol do not improve this
301 feature. In fact, particles formulated with mannitol show an even more irregular structure, a
302 phenomenon the authors declared to be an effect of mannitol crystallisation. The amino acid
303 leucine provided to the particles a more acceptable sphericity. This highlights the mixed
304 effect of excipients for the production of homogenous proteins with a defined morphology

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305 and size. Careful selection of excipients is necessary to balance the effect of product
306 interaction, stability, associated toxicity (in the case of biopharmaceuticals) and powder
307 consistency.

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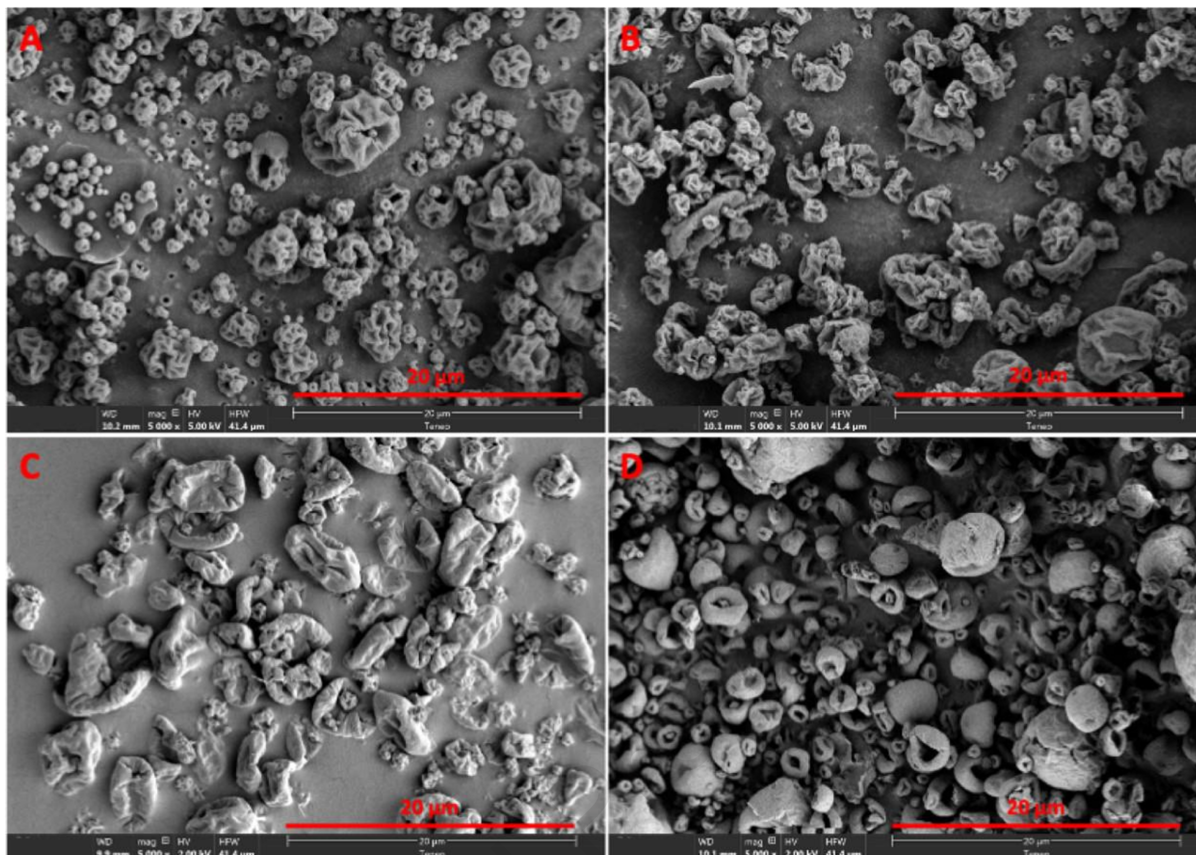

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327 **Fig. 1–** Scanning Electron Microscopy (SEM) images of spray dried bovine serum albumin
328 (BSA) with/without excipients: A) BSA without excipients, B) BSA with trehalose, C) BSA
329 with mannitol, and D) BSA with leucine. Reproduced with permission [13].

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343 Due to many restrictions posed on excipient use, risk of toxicity and product deterioration,
344 the popularity of screening of novel excipients and creating low excipient-based formulations
345 has increased over the years. High-concentration protein formulations allow to minimise the
346 variety and amount of excipients during freeze drying, as the proteins act as their own
347 bulking agents for the retention of cake uniformity [68]. While many novel excipients are
348 under development, the current approval journey is long and the increased risk of failure and
349 toxicity alienates investors [87]. Consequently, other avenues should be explored in the
350 research of solid-state formulation of biopharmaceuticals. In section 3 we discuss structural
351 modifications during upstream processing as an alternative means of solid-state protein
352 stabilisation. Stabilisation of the native proteins or peptides by these methods may minimise
353 or alter the excipient composition needed for the formulation.

354 # 3. Structural modifications in enhancing drying stability

355

356 ## 3.1 Protein engineering

357 The modification of a protein's primary structure is well documented and practiced for
358 stability enhancement in solution [7, 20]. Many studies in the literature report enhancement
359 of a native protein's stability to thermal or chemical denaturation using a variety of methods.
360 A single amino acid change in a protein or peptide may signal a rise in enzymatic melting
361 temperature (T_m), therefore conferring a higher thermal stability [88]. Genetic engineering of
362 thermostable mutants is achieved by rational site-directed mutagenesis or directed evolution,
363 and has been utilised extensively in the past in the textile and detergent industries, albeit not
364 for the direct purpose of stabilising during solid formation [7, 89]. Directed evolution has
365 become a highly efficient method of producing stable mutants, without previous knowledge
366 of the protein's sequence. It harnesses the natural evolution of bacterial genomes to screen
367 beneficial amino acid changes. The first thermostable mutant created by directed evolution
368 was achieved by six generations of random mutagenesis and gained a >14 ºC increase in its
369 T_m, with subsequent mutants achieving a T_m rise of >30 ºC [90, 91]. In the debut work on
370 directed evolution, a protease subtilin A was randomly engineered to withstand treatment
371 with the organic solvent DMF and showed an almost identical activity in 60% DMF as the
372 native enzyme in aqueous solution. Indeed, Nobel-prize winner Frances Arnold has shown
373 directed evolution to be superior to other methods in identifying stable mutants of proteins
374 without rigorous analysis of the protein sequence [92]. More recently, directed evolution has
375 been used increase the T_m of a poly(ethylene terephthalate) (PET) polymerase to 82.5ºC,
376 increasing its breadth of application in plastic recycling [93]. Similar success was observed
377 with a fungal xylanase, which showed a 420-fold increase in its half-life at 70 ºC, using a
378 more rational design of directed evolution based on computational modelling of the mutants
379 [94]. The simplified process of directed evolution is portrayed in Figure 2 below.

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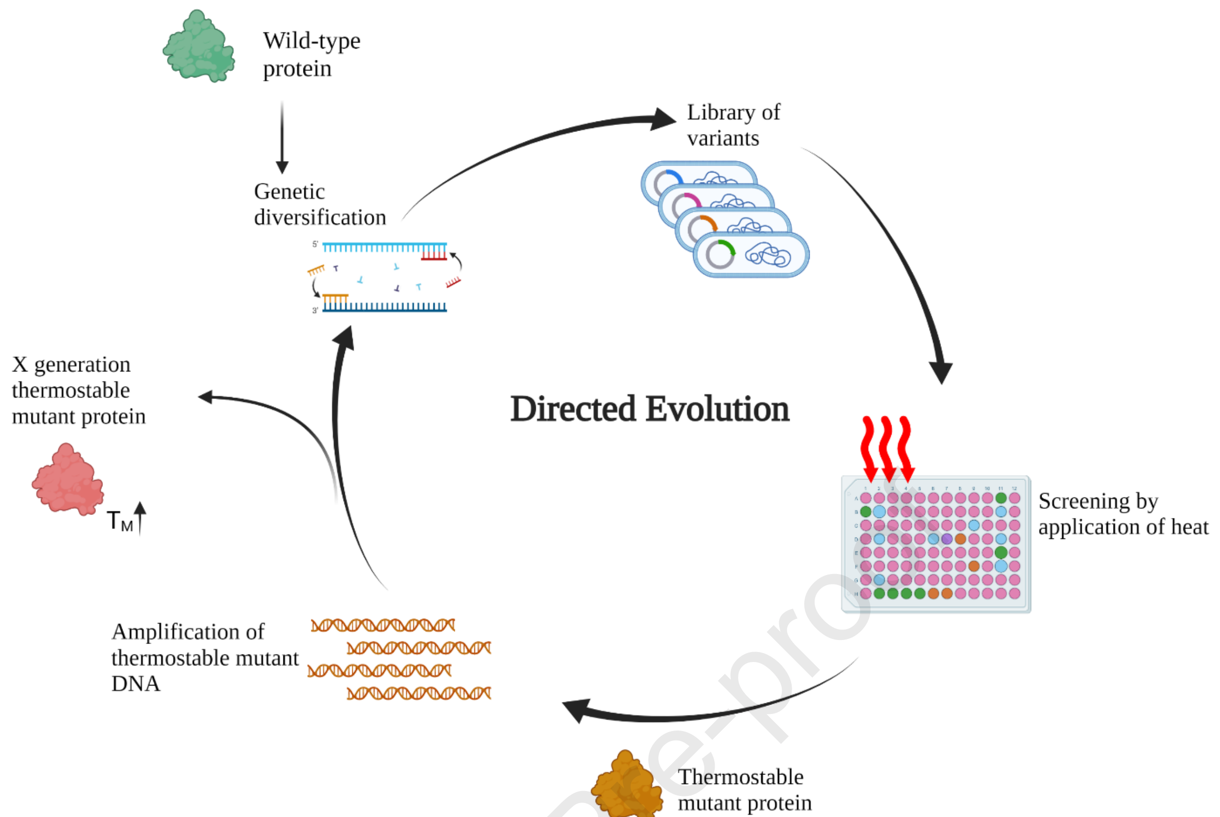

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382 **Fig. 2** - Process of directed evolution for the creation of thermostable protein mutants.
383 Induction of a high ΔT_m may take many generations. Created with BioRender.com.
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399 Site-directed mutagenesis is the most popular protein engineering technique, however it
400 requires a knowledge of the protein sequence and host genome, with single amino acid
401 mutations having a limited impact on the protein's thermal stability. Structure-guided design
402 of two amino acid sites of a bacterial pullulanase by site-directed mutagenesis increased the
403 enzymatic half-life 4.3 fold, a small feat when compared to mutants created by directed
404 evolution [95]. Bacterial lysozyme mutants with a single Glycine to Alanine substitution
405 showed an increased entropic stability compared to native lysozyme, through the stabilisation
406 of structural a-helices [96]. The site-directed mutagenesis of proteins for the purpose of
407 stabilisation has also been performed on a cellobiohydrolase enzyme from *Hypocrea jecorina*
408 enhancing its T_m by 10.4 °C, however this increase was only achieved after 18 residue
409 substitutions in regions distant from the active site [97]. The Free Energy Perturbation
410 modelling technique (FEP), first developed by W. Zwanzig, has been in recent years used for
411 the prediction of stabilising point mutations by calculating the difference in Gibb's Free
412 Energy of residue changes [98-100]. Another recent computational modelling technique
413 termed HoTMuSiC has made the modelling of protein modifications more accessible. The
414 technique utilised the resolved structure of the protein and its T_m in predicting thermal
415 stability changes of mutations, and it is currently available online [88]. In novel molecular
416 simulation methods including FEP+ and HoTMuSiC, accurate modelling of the thermal
417 stability of proteins is possible and provides additional opportunities for the engineering of
418 proteins for manufacturing purposes, without cumbersome trial-and-error methods.

419
420 Mutagenesis and directed evolution for stability are routinely conducted on enzymes for
421 process applications in biotechnology and pharmaceutics. Often, the mutations are introduced
422 to increase the breadth of application of the product i.e. increasing bioavailability and optimal
423 working temperature [101]. Many biopharmaceuticals on the market are mutated to conserve
424 stability, as in the case of Proleukin® (Interleukin-2) and Betaseron® (IFNβ-1b) where Cys
425 and Ser substitutions are introduced to minimise storage oxidation [102, 103]. Extensive
426 mutation of the Human Growth Hormone (HGH) peptide core, through computational design,
427 leads to a 16°C increase in its T_m and an extended shelf-life [104]. A similar approach was
428 taken to increase the thermal stability of Granulocyte-colony stimulating factor (G-CSF) up
429 to 13°C [105]. Truncation mutations of aggregation-prone, hydrophobic moieties
430 substantially improves the storage stability of keratinocyte growth factor (KGF), a peptide
431 preventing digestive tract inflammation in chemotherapy patients [106]. Creation of
432 biobetters for enhanced *in vitro* properties, particularly monoclonal antibodies (mABs), by
433 mutant creation and rational sequence screening is a growing field of research [107, 108]. A
434 list of commercially available engineered proteins and peptides is shown in Table 1. The
435 formulation of these pharmaceuticals into dried powders is attractive in the field of inhalation
436 therapy, due to improved patient compliance, proteolytic stability and therapeutic efficacy
437 [109, 110]. Primary structure modification of proteins has yet to be directly addressed for the
438 implementation of drying technologies which utilise heat, such as hot-melt extrusion and
439 spray drying. A recent study of interleukin 8 and its variants compared the spray drying and
440 freeze-drying of IL8, one of its monomeric mutants and an IL8-HAS (Human Serum
441 Albumin) fusion protein. From the results, it can be deducted that the smaller, monomeric
442 mutant of IL8 behaved favourably during spray drying, retaining more native helices than
443 native IL8. This study, however, focuses on the effect of the drying methods on the proteins
444 separately, and does not address the effect of the mutation [111]. Additionally, the mutation
445 of proteins for higher organic solvent resistance may direct research of supercritical fluid

dried proteins, which has not been addressed in the literature. With more robust computational techniques growing in popularity, it is feasible to apply this stabilisation method towards research advancements in the area of solid-state proteins.

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496 **Table 1**. Engineered biopharmaceuticals on the market for the purpose of *in vitro* stability
497 enhancement.
498

Biopharmaceutical	*Engineering approach*	*Original protein*	*Effect of mutation on stability*	*Ref.*
Proleukin®	Mutagenesis of Cys/Ser residues	Interleukin-2	Decreased oxidation propensity	[102]
Betaseron®	Mutagenesis of Cys/Ser residues	Interferon IFNβ-1b	Decreased oxidation propensity	[103]
Filgrastim/Lenograstim	Computational screening	Granulocyte-colony stimulating factor (G-CSF)	Increase in T_m +13°C	[105]
Humatrope/Genotropin/ Norditropin/Omnitrope/ Nutropin	Computational screening	Human Growth Hormone (HGH)	Increase in T_m +16°C, increased storage stability at room temperature	[104]
Trastuzumab	Site-directed mutagenesis	Anti-HER2 mAB	Increased degradation resistance by proteases	[112]
Palifermin	Truncation mutation	keratinocyte growth factor (KGF)	Decreased aggregation propensity	[106]
ReFacto®	Truncation mutation	Recombinant factor VIII	Improved stability during manufacturing	[113]
Humalog® / NovoLog®	Site-directed mutagenesis	Insulin	Decreased aggregation propensity	[114]

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526 As the thermodynamic interactions which lead to protein thermal stability are elaborate, it is
527 often difficult to pinpoint a single reside or bond as having a stabilising effect. In cell-wide
528 analysis of *Thermus thermophilus, Escherichia coli* and yeast protein thermostabilities,
529 specific amino acid enrichment, peptide length and secondary structure were shown to play
530 key roles in propagating stable proteins by lowering system entropy and increasing bonding
531 [115]. Although the exact sources of stabilisation for thermostable proteins are difficult to
532 explore, distinct structural differences between thermophilic proteins and their mesophilic
533 counterparts have been theorised to confer their unique properties. For example, modelling of
534 the aldehyde dehydrogenase (ALDH) enzyme of *T. thermophilus* revealed a C-terminal
535 extension of ~11 residues, not possessed by ALDH protein from alternative sources. Its
536 presence allows for the creation of a network of hydrogen bonds and disulfide bridges
537 between the four monomers of the protein's tertiary structure. A deletion of the residue
538 extension yielded an active, yet thermally instable protein, leading the authors to conclude
539 that the extension of the oligomerisation domain plays a vital role in stabilisation of the
540 thermophilic protein [116]. Terminal extensions can also be found in archaeal thermophilic
541 proteins, specifically in several Acetyl-CoA synthetases with stabilised, high-order,
542 oligomeric states [117]. In the archaea *Halobacterium salinarum*, an N-terminal extension of
543 negatively charged amino acids in the Ferredoxin protein increases the resistance to high salt
544 and high temperature environments [118]. Certain amino acids in thermophilic proteins may
545 confer more thermal stability than others, as demonstrated by Singer et al. Purine-rich
546 residues like glutamic acid, valine and isoleucine are observed more frequently in
547 thermophilic proteins, while others like glutamine and histidine are less observed, owing to a
548 plethora of reasons including increased deamidation rates and reduced G-C content [119].
549 Thermoenzymes are widely used in industry, and many have been dried with great success
550 for use in detergents, biocatalysis and food manufacture [7, 120]. The use of thermoenzymes
551 is not utilised in biopharmaceutical production as many sources are of bacterial, archaeal or
552 fungal origin. Studying the mechanisms of thermal stability using these enzymes however
553 forms a rationale for thermostable biomedicine design [121].
554

555 3.2 Natural Post Translational Modifications (PTMs)

556 A therapeutic protein's efficacy and stability is largely reliant upon its amino acid sequence
557 and final conformation. However, a large influence also lies within the modifications that the
558 protein undergoes once synthesis in the ribosome is complete. These steps, termed Post-
559 Translational Modifications (PTMs), are heavily liable for the maturation of the protein and
560 defining its role in the organism. Glycosylation, phosphorylation, ubiquitination, deamidation
561 and methylation are amongst the most notable PTMs of wild-type proteins, playing major
562 roles in determining protein-protein interactions, target binding and activity regulation [122].
563 Functionally, PTMs have a major influence over biopharmaceutical stability during
564 manufacture, storage and upon administration. Modulation of biopharmaceutical PTMs has
565 been shown to improve thermal and chemical stability, proteolytic degradation, and pH
566 denaturation, and therefore may hold an application in solid-state stabilisation.
567

568 *3.2.1 Glycosylation*

569
570 Glycosylation is an important native feature of antibody stability and target affinity and has
571 therefore been a focus for the biopharmaceutical industry. Under forced deactivation studies
572 of various immunoglobulins, deglycosylation of the antibody -CH_2 domain results in a

573 decrease of the melting point (T_m) by 6-8 °C, as well as increased aggregation propensity
574 [123-126]. Other glycoproteins have also been shown to retain more activity under elevated
575 temperatures, and under storage conditions in comparison to their deglycosylated
576 counterparts, a summary of which can be found in Table 2 [127-129]. In many proteases,
577 deglycosylation causes loss of activity, and increased susceptibility to proteolysis [130]. The
578 retention of glycans in biomanufacturing is vital, therefore, to consistently produce stable
579 protein products.

580 The stabilisation effect of glycans to protein structure, their lack of toxicity and negligible
581 effects on protein activity has fuelled researchers to examine the possibility of exploiting this
582 mechanism in enhancing protein stability. The process of glycosylation is a naturally
583 occurring PTM in eukaryotic systems, and selectively in some prokaryotic systems, but can
584 be artificially replicated both *in vivo* (by introducing glycosylation sites in the genome
585 sequence of an organism which possesses glycosylation cell machinery) and *in vitro* by
586 enzymatic and chemical glycoengineering techniques [131, 132]. Glycosylation of antibodies
587 may also be modulated by optimising the host cell line, culture conditions and culture
588 medium [133]. In recent work, whole glycoforms have been synthesised by chemical ligation
589 procedures for the precise attachment of glycans [134]. The relevance of glycosylation
590 position and glycan composition on protein stability and activity has made glycoengineering
591 a relatively challenging field. For example, calorimetric studies performed by differential
592 scanning calorimetry (DSC) of IgG glycoforms have shown that T_m (melting temperature)
593 decreased with mannose-terminated glycans, and IgG aggregation increased by galactose-
594 terminated glycans. Other glycosylation patterns retained IgG thermodynamic stability [135].
595 However, removal of glycans may also increase activity in proteins, as with the case of
596 insulin protease plasma kallikrein (KLKB1) where removal of sialic acid increased cleavage
597 activity of the protein [136]. The phenomenon of destabilisation by glycans in solution has
598 been annotated to the reduction of accessibility of protein-protein interactions [137]. The
599 stabilisation effect of some glycan patterns are accredited to modulation of protein enthalpy
600 of unfolding, prevention of aggregate formation by limiting protein-protein interactions at
601 interfacial boundaries and decreasing the entropy of the protein [21]. A thermodynamic
602 analysis of the Gibb's Free Energy profile of protein glycosylation is shown in Figure 3. The
603 glycosylation of a native protein may decrease the stability of the denatured state and hence
604 decrease protein enthalpy in the folded state [138].

605 Minimal research has been undertaken so far in monitoring the effect of glycosylation in
606 drying stability. A recent publication by Liu et al. assessed the structural integrity of
607 glycosylated ovalbumin after spray drying and microwave freeze drying (MFD) by glycation
608 with lactose [139]. Glycosylation has also been shown to improve the stability of a protein
609 (soy protein isolate) in the food industry during treatment with freeze-thaw [140]. Mancini et
610 al. performed covalent conjugation of trehalose to an artificially added thiol group on
611 lysozyme, which increased the protein's stability during freeze drying [141]. Addition of the
612 sugar as a mere excipient did not protect the activity of lysozyme to the same extent.
613 Furthermore, the glycoprotein retained 80% of its activity after heat treatment to 90 °C for 1
614 hour, a 60% increase from its wild-type format. Glycosylation by point mutation of a
615 bacterial lipase improved its stability in the presence of organic solvents such as DMSO, a
616 relevant finding which could be taken into account for the introduction of supercritical fluid
617 drying for protein solid forms [142]. Site-directed N-linked glycosylation of *Rhizomucor*
618 *miehei* lipase modulated the peptide's resistance to 60% methanol, increasing its application

619 in biodiesel production [143].
620 Regarding increasing the thermostability of proteins by glycosylation, a large amount of
621 research has been dedicated to the improvement of industrial enzyme applications by
622 modulating glycan patterns. The technique can be employed to engineer glycoproteins with
623 increased thermal drying and solid-state stability. Cellulases and cellobiohydrolases from
624 many bacterial and fungal sources have been modified to produce beneficial glycan patterns
625 by recombinant expression, mutagenesis or by enzymatic glycosylation and deglycosylation
626 for improved cellulase activity and thermostability [144]. Many other enzymes have been
627 recently glycoengineered for increased thermostability, with applications in a number of
628 industries, including cutinases [145], phytases [146, 147], glucanases [148] and lipases [149].
629 A list of recent reports on the modification of enzymes and other proteins by glycosylation
630 can be found in Table 2. Increasing the thermostability of proteins by glycosylation holds
631 potential in increasing their stability during solid state production and storage, particularly by
632 thermal drying techniques such as hot melt extrusion and spray drying. A more directed
633 research approach is vital in uncovering the stabilisation effects of glycans on drying
634 stability, one which is sure to expand with the growth of the glycoprotein industry.

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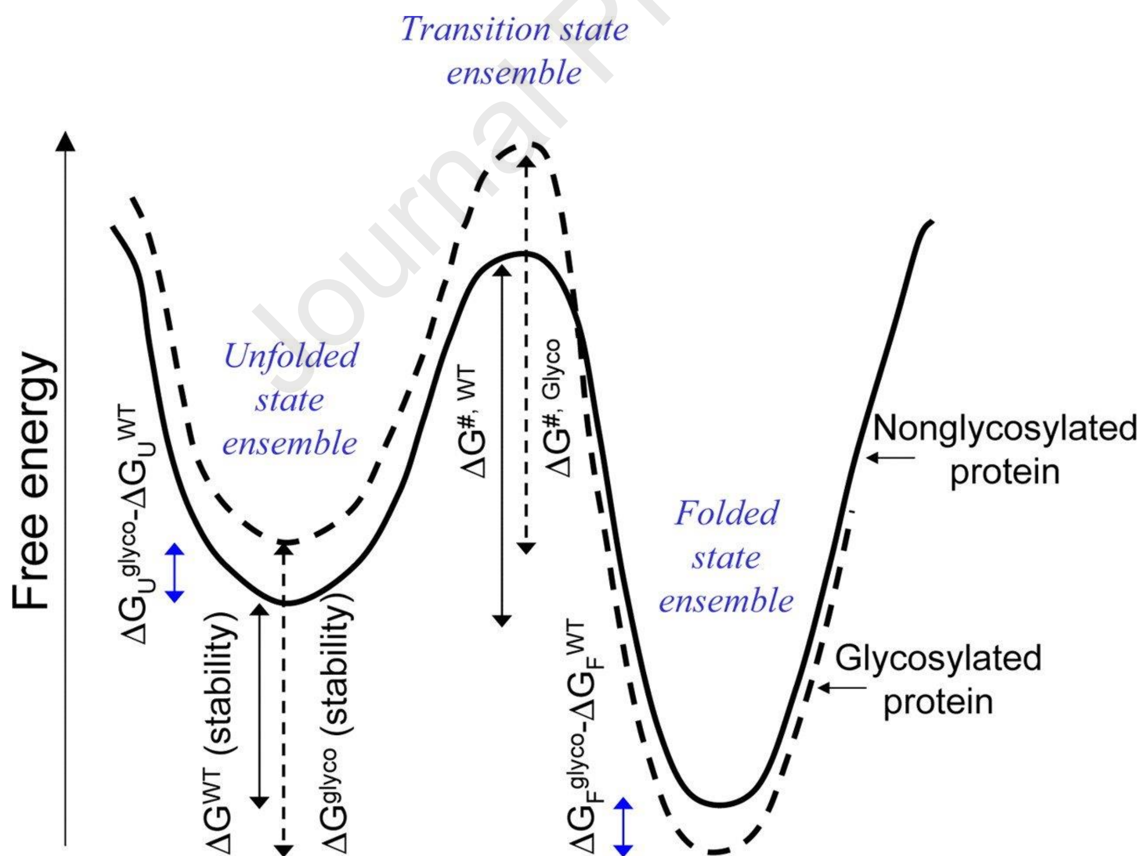

638

639 **Fig. 3** - Stabilising effect of glycosylation (glyco) of a wild-type (WT) protein on the Gibb's
640 Free Energy states (ΔG) of folding (ΔG_F) and unfolding(ΔG_U) Adapted with permission from
641 [138] Copyright (2008) National Academy of Sciences, U.S.A.

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656 *3.2.2 Oxidation, Lipidation and other PTMs (Post-Translational Modifications)*
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658 Many other PTMs (Post-Translational Modifications) such as methylation, oxidation,
659 acetylation and phosphorylation are viewed as key players in degradation pathways and have
660 not been recognised as having any potential in enhancing solid-state stability. Oxidation of
661 therapeutics is a known source of product degeneration and a common PTM for eukaryotic
662 proteins. Few reports however have delved into the enhancement of biopharmaceutical
663 stability by oxidation of the Methionine residue, with certain conformations having a positive
664 impact on protein stability. Methionine oxidation of the human Calcitonin peptide slowed the
665 formation of aggregates under 37 °C storage and low pH conditions, however the exact
666 reason for this stabilisation mechanism was never identified. The authors noted the
667 importance of evaluating PTMs on a case-to-case basis [150]. A similar phenomenon was
668 observed in the methionine oxidation of Human Serum Albumin (HSA), where the
669 conformational change due to oxidation increased interactions between the tertiary structure
670 of HSA, decreasing the formation of fibrils after treatment at high temperatures [151].

671 Lipidation is a vital post-translational modification in eukaryotic cells and plays a role in
672 protein interaction, stability and function. It has been vastly studied for its role in human
673 disease and enzymatic propagation/inhibition of fatty acyltransferases, and fatty acid
674 synthases is a focus of study in oncology [152]. When used in the modification of
675 biotherapeutics, synthetic lipidation may increase bioavailability, intracellular delivery and
676 drug stability. Synthetic lipidation is undertaken using solid-phase synthesis, by anchoring
677 the peptide/protein on a polymeric support and covalent attachment of a lipid, or a lipid and a
678 reactive linker molecule, to the desired amino acid. The lipidated amino acid may also be
679 synthesised separately and introduced to the anchored biomolecule [153]. Enzymatic
680 approaches using fatty acid transferases and synthases have also been employed [154].

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681 Although lipidated biopharmaceuticals are mainly explored to improve serum half-life and
682 immunogenicity, they also have been reported to improve stability. For example the
683 myristoylation of insulin increases the peptide's Gibbs Free Energy of unfolding (G_u) by 30%
684 in chemical-mediated denaturation [155]. In some studies of the collagen protein, the
685 stabilising effect of lipidation was attributed to newly formed Van der Waals forces and
686 increased rates of refolding (see Table 2), however most fatty-acid mechanisms of
687 stabilisation are unknown [156]. Attachment of fatty acid polymers has also been used to
688 stabilise proteins and peptides under acidic pH [157] and chemical stresses [158].
689 The addition of an acetyl-CoA functional group in the form of acetylation has significant
690 impact on enzymatic activity and cell metabolism, however its role in stability is unknown. In
691 an experimental study published by Geng et al., the acetylation of a lysine residue on a
692 Hypoxia-inducible factor 1 α (HIF) transcriptional protein prevented protein degradation by
693 proteolysis and ubiquitylation [159].
694 Deamidation is a common degradation pathway in storage of proteins and
695 biopharmaceuticals, however it has been recently exploited in production of novel
696 succinimidyl intermediates of a glutamine amidotransferase with extreme thermostability.
697 The enzymatic intermediate did not denature at temperatures >100˚C and at high
698 concentrations of guanidine chloride. Introduction of an artificial deamidation site in the gene
699 encoding the enzyme was used to produce the succinimide, a major finding in the field of
700 protein stabilisation [160].

701 Table 2 provides information on the effect of post-translational modifications on the stability
702 of various proteins. The variability in the stabilisation highlights the importance of
703 characterisation of individual PTMs and monitoring their effects on protein integrity.
704 Harvesting the stabilisation effects of naturally occurring PTMs for drying, which can be
705 induced during recombinant production and chemically or enzymatically *in vitro*, requires a
706 vast expansion of the current knowledge into their individual stabilisation mechanisms.

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707 **Table 2** – Post-translational modifications (PTMs) of proteins for enhanced stability against stresses commonly experienced by proteins and
708 peptides during drying. ΔT_{50}; Change in the temperature at which 50% of enzyme in the solution is active; $t_{1/2}$; Half-life, T_m; Melting
709 temperature.

Modification	*Protein/peptide*	*Method of modification*	*Effect on stability*	*Reference*
Glycosylation	Phytase	Directed evolution	Increased thermostability ($\Delta t_{1/2}$ = + 22.75min at 100˚C) Increased stability at gastric pH	[146, 147, 161]
	Glucanase	Site-directed mutagenesis	Increased thermostability, improved catalytic activity	[148, 162]
	Lipase	Site-directed mutagenesis	Increased resistance to methanol, improved catalytic activity	[143]
	Cellulase	Recombinant expression Site-directed mutagenesis Enzymatic glycosylation	Increased thermostability, improved catalytic activity, protect from protease degradation	[144]
	Lipase	Recombinant expression	Increased thermostability, improved catalytic activity	[149]
	Cutinase	Recombinant expression	Increased thermostability, decreased aggregation propensity	[145, 163]
	IgG1	Enzymatic glycosylation Optimised expression Gene knockout Site-directed mutagenesis	Decreased thermostability (ΔT_m= - 6˚C), increased aggregation propensity (mannose/galactose terminated glycans), Increased thermostability for glycans with fucose core Decreased aggregation propensity, increased storage stability	[133, 135, 164, 165]
	Cytochrome c	Chemical conjugation of amine-reactive sugars	Increased thermostability, increased chemical stability	[166]
	α-Glucosidase	Site-directed mutagenesis	Increased thermostability (ΔT_{50} = +7.7˚C)	[167]
	Chymotrypsin	Chemical conjugation of reactive sugars	Increased thermostability, increased chemical stability, decreased aggregation propensity	[168]

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	RNAse (Human)	Site-directed mutagenesis	Increased thermostability, increased proteolytic stability, decreased catalytic activity	[169]
	Erythropoietin	Full glycoform synthesis	Increased storage stability	[134]
	Cystatin	Site-directed mutagenesis Recombinant expression	Increased cryopreservation	[170]
Lipidation	Insulin	Chemical synthesis	30% increase in free energy of unfolding due to chemical denaturation	[155]
	Collagen	Chemical synthesis	Increased T_m by 27˚C, promotes refolding.	[171]
	Hormone PYY3-36	Chemical Synthesis	Higher retention of α-helices under acidic pH and elevated temperatures (70˚C)	[157]
	Hisactophilin	Site-directed mutagenesis	Increased Free energy of unfolding (3.15 to 1.13 kcal·mol^{-1})	[172]
Oxidation	Human Serum Albumin	Chemical reaction with hydrogen peroxide	Decreased aggregation propensity	[173]
Deamidation	Glutaminase	Site-directed mutagenesis for formation of intermediate	No loss of native structure at 100˚C and enhanced chemical stability	[160]

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711 ## 3.3 Polymeric bioconjugates

712 The area of bioconjugation has gained traction in improving drug delivery systems as an
713 alternative to nanocarriers [174]. The use of polymer and lipid conjugates flaunts advantages
714 over traditional biotherapeutics including enhanced specificity, dissolution rates, stability and
715 safety profiles [175]. Herein we aim to discuss the impact of bioconjugation on the thermal,
716 chemical, mechanical and storage stability as related to the formation of protein powders,
717 which has been scarcely discussed in the literature. Chemical conjugation of polymers to
718 proteins or peptides is usually made possible using a linker capable of reacting with a
719 naturally occurring amino acid in the protein backbone. Polymerisation mechanisms, such as
720 reversible deactivation radical polymerisation (RDRP) and ring opening metathesis
721 polymerisation (ROMP), are traditionally utilised for growth of polymers at mild conditions.
722 Of these, RDRP is most used in research due to its simplicity and includes various sub-
723 techniques such as reversible addition–fragmentation chain transfer polymerisation (RAFT)
724 and atom transfer radical polymerisation (ATRP). These methods mainly differ in the choice
725 of chemical reaction utilised to grow the polymer backbone. The route of synthesis may also
726 differ between "grafting to" - where a fully synthesised polymer is attached to the
727 biomolecule, "grafting from" - where a chain transfer agent is utilised as a linker and initiates
728 polymer growth from the peptide backbone, and "grafting through" - where the protein acts
729 as a pendant group to the polymer chain [176].

730 ### *3.3.1 Use of Polyethylene glycol (PEG) and its derivatives*

731 The synthetic conjugation of polymers to macromolecules for the augmentation of
732 biotherapeutic activity and stability was introduced by Abuchowski et al. in 1977 by the
733 process of PEGylation [177]. Those authors demonstrated that the covalent attachment of
734 PEG to BSA improved its solubility and *in vivo* half-life by omitting immunogenic detection.
735 Since its invention, PEGylation of biomolecules has become an established method of
736 biotherapeutic enhancement, with 17 FDA approved PEGylated biomolecules on the market
737 in 2021 [178]. PEG derivatives are the polymers of choice for the biopharmaceutical industry
738 due to their lack of toxicity and immunogenicity, as well as their solubility in both aqueous
739 and organic solvents.
740 The effect of PEG conjugation on biomolecule stability is well addressed in the literature, and
741 indeed has been utilised to structurally strengthen biomolecules for formulation by drying.
742 Heller et al. showed an increase in the resistance of haemoglobin to interfacial stress during
743 cryoconcentration by PEGylation with a 5000 kDa PEG molecule [179]. The increase in
744 resistance was attributed to favourable interactions between the PEGylated haemoglobin with
745 the sugar excipient dextran. Similarly, freeze drying of a PEGylated hormone inhibitor
746 protein showed favourable interactions with sucrose, decreasing dissolution rates of the
747 protein powder, however a stabilising effect was not reported [180]. PEG (5000 kDa)
748 improved the retention of secondary structure after freeze drying (and purification) in the
749 case of the peptide glucagon [181]. Using temperatures as high as 95 °C, mPEG-NHS
750 (methoxy-PEG N-hydroxysuccinimide) protected lysozyme from aggregation during melt
751 processing, an encapsulation technique which exerts a high level of heat and mechanical
752 stress [182]. Although spray drying exerts similar stresses, no publications are available in
753 the literature on PEGylation stabilising proteins in spray drying.
754 The enhancement of thermal, chemical and mechanical stability of many proteins by
755 PEGylation is, however, reported in the literature. PEGylation of antibodies has been shown
756 to increase the T_m by various degrees, depending on the structure and length of the PEG

757 moiety and its position of conjugation [183-186]. These studies have also shown increased
758 colloidal stabilities (aggregation propensity) and chemical stabilities to denaturants such as
759 Guanidine Hydrochloride. Mono-PEGylation has been used to stabilise other biotherapeutics
760 such as Human Growth Hormone (hGH). The peptide's T_m was increased up to 4 °C by
761 PEGylation of the N-terminus and showed increased rates of secondary structure refolding
762 under heating and cooling conditions [187]. The aggregation-prone drug, interferon-β, was
763 conjugated to activated PEG molecules ranging from 12-40 kDa, reducing the formation of
764 precipitates during storage, with an increase of 2 °C in T_m [188]. Conjugation of PEG is an
765 attractive method for biotherapeutics with low solubility or stability, and is appropriate for
766 sustained drug delivery. PEGylation of protein drugs intended for inhalation possesses
767 secondary advantages. PEGylated peptides show decreased rates of clearance by mucosal
768 proteases after administration via the intranasal route [189-191]. PEG has also been shown to
769 aid in controlled drug release through the pulmonary route due to increased retention times in
770 the pleura, without impacting aerosol performance [192]. The PEGylation of therapeutic
771 proteins has been recognised in its advantages for the pulmonary delivery of treatments for
772 lung infections, cancers, and lung related diseases, such as Cystic Fibrosis (CF) [193, 194].
773 The PEGylation of biotherapeutics therefore may have a dual advantage for solid-state
774 formulation and stability during the drying process and efficacy post-administration.

775 Non-therapeutic proteins such as lysozyme have been extensively refined with different PEG
776 moieties as a model for stabilisation studies. Mono- and di- PEGylation of lysozyme can lead
777 to increased stability in high salt environments [195], in extreme pH values ($6<pH<10$) [196]
778 and at high temperatures (90 °C) [197]. The protease papain showed increased thermal
779 stability in storage at 40 °C after modification with PEG, interestingly by hindering the
780 access of the protein to autolytic activity [198]. This effect was also observed in Trypsin,
781 where PEG (5 kDa) maintained the activity of the enzyme 20% better than its native
782 counterpart when subjected to storage at accelerated autolytic conditions. Additionally, the
783 study also showed a significant rise in the denaturation temperature of the protein, with a
784 70% higher enzyme activity retention than the native protein at 50 °C [199]. Chemical
785 instabilities can also be positively modified with PEGylation. Lopez-Cruz et al. observed
786 increased enzymatic retention rates in PEGylated laccase treated with organic solvents such
787 as methanol, ethanol, acetonitrile and propanol by providing a 'blockage' for reactive amino-
788 acid side chains [200].

789 Although an abundance of literature on PEG bioconjugates points to the stabilising effect of
790 the polymer, the cost of this effect often comes at the expense of enzymatic activity.
791 Lysozyme activity is inversely correlated with increasing PEGylation, an effect exemplified
792 by da Silva Freitas and Abrahão-Neto [196]. A deleterious effect is also observed with
793 increasing molecular weight of PEGs (>10kDa) [195]. Both of these effects are visualised in
794 Figure 4. The steric hinderance generated by longer, more branched PEGs, causes an
795 inaccessibility of the substrate to the active site. Higher order of PEGylation (di- and tri-
796 PEGylation) may also cause steric hinderance.

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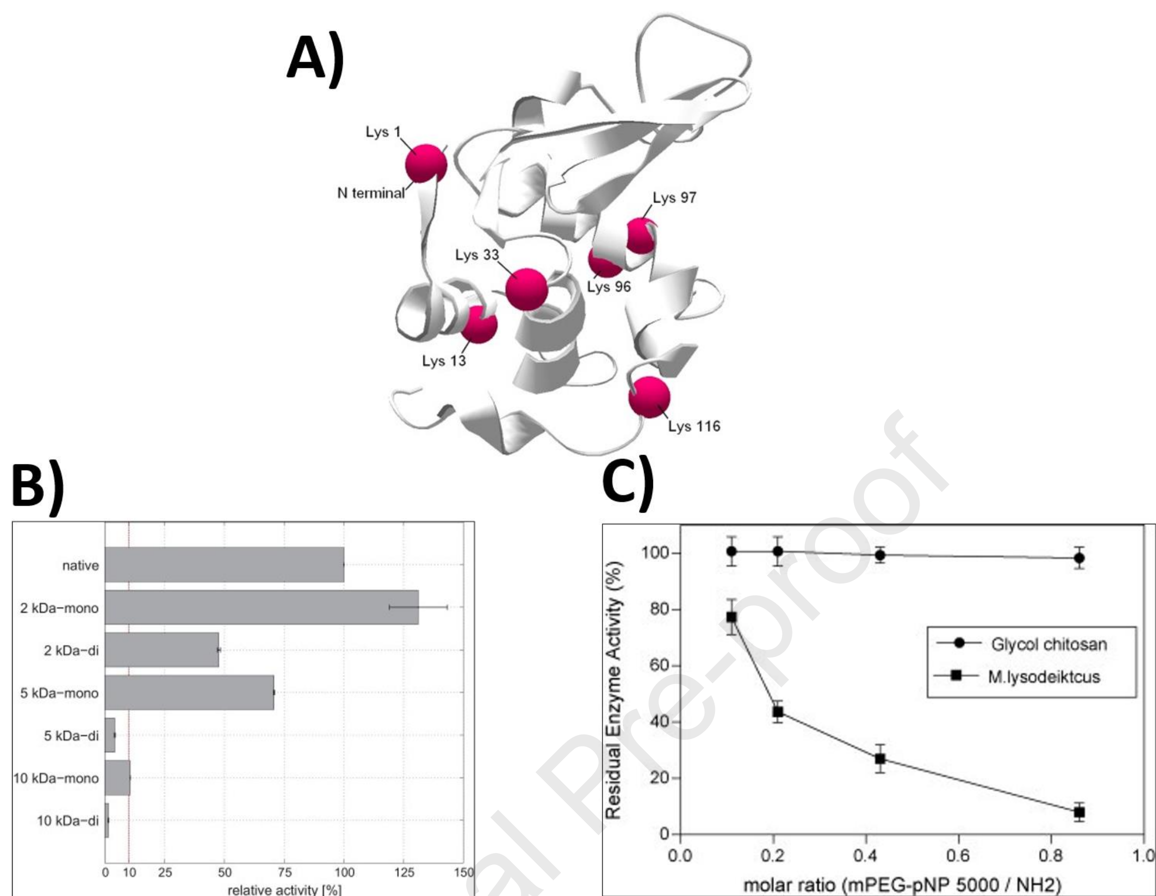

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799 **Fig. 4 -** Ribbon diagram of lysozyme with six native PEGylation sites present at Lys residues
800 (A). The effect of Methoxy-PEG-propionaldehyde (mPEG-aldehyde) molecular weight and
801 degree of PEGylation on lysozyme activity against *M.lysodeiktcus* (B). The effect of molar
802 ratio of methoxy-PEG-p-nitrophenyl carbonate (mPEG-pNP) (5kDa) on the activity of
803 lysozyme against *M.lysodeiktcus* and glycol chitosan (C). Reproduced with permission [201],
804 [195], [196].

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815 Moreover, PEGylation may have an opposite effect on stability, depending on the protein
816 system in which it is used. BSA was modified with PEG 5-60 kDA by Plesner et al., however
817 in this case thermal stability was negatively affected, with T_m decreases of up to 3 ºC in
818 comparison with native BSA [202]. As with glycosylation, and indeed any protein
819 modifications, PEGylation extent, position and method must be evaluated individually for
820 each protein system. Introduction of site-specific PEGylation and molecular modelling in
821 creating PEG conjugates is an attractive route of protein modulation and, as exemplified, may
822 have a positive impact on drying stresses.

823 *3.3.2 PAA, PVA and other synthetic polymers*

824 Although PEGylation is the most-well known form of protein-polymer post-translational
825 modification (PTM) stabilisation method, other synthetic polymers have also been explored
826 for this purpose, with success of the system reliant on the biocompatibility, safety, solubility,
827 and ease of adjunction of the polymer. Many synthetic polymers have been shown to
828 ameliorate protein stabilities. The large Poly(acrylid acid) (pAA) polymer of 450 kDA
829 conjugated to the therapeutic enzyme Haemoglobin decreased denaturation of the protein
830 under prolonged exposure to high temperatures, subsequently increasing its storage stability
831 at room temperature by 30% [203]. A set of various Polyphosphoesthers (PPE) covalently
832 bonded to myoglobin were capable of positively and negatively affecting protein stability,
833 depending on the level of hydrophobicity of the polymer. Soluble hydrophilic PEEs protected
834 myoglobin from thermally-induced precipitation and protease activity [204]. Hyaluronic acid,
835 a disaccharide polymer, has been utilised to retain the activity of Trypsin, RNAse A and
836 insulin after 24 hour incubation at 37 °C, while also displaying favourable biodegradability in
837 comparison with traditional PEG [205].

838 As with PEG, other polymers have a varied effect on stability, yet they offer potentially
839 different capabilities to PEG, such as pH and temperature responsiveness and modulated
840 hydrophobicity and charge. Zwitterionic polymers have been proposed in tackling the issue
841 of diminished activity commonly found in bioconjugates, while still increasing the protein's
842 stability. Zwitterionic polymers, like polyampholytes and polybetaines, are 'smart' polymers
843 with a net neutral charge capable of adapting electrolyte behaviour upon environment
844 changes [206]. Keefe and Jiang conjugated one such polybetaine (poly(carboxybetaine) to α-
845 chymotrypsin increasing the thermal stability of the protein significantly; residual activity
846 after heating to 50ºC was ~60% higher than in the native protein, and up to 30% higher than
847 the stability conveyed by PEG [207]. Thermoresponsive polymers, which change their
848 physical properties under varying levels of temperature, can also be used to dramatically alter
849 the thermal and chemical stabilities of a protein. Conjugation of one such novel polymer,
850 poly(N,N-diethylacrylamide-co-glycidyl methacrylate) (P(DEAAm-co-GMA), to α-
851 Chymotrypsin by Kasza et.al, resulted in significantly higher activity retention (~30%) under
852 two hour treatment at 45 °C and at denaturing pH > 7.5 [208]. Another group studied
853 chymotrypsin and thermoresponsive polymer poly(sulfobetaine methacrylamide)-block-
854 poly(N-isopropylacrylamide) (pSBAm-block-pNIPAm) and acquired similar results,
855 additionally showing the bioconjugate's resistance to proteases [209]. Mechanisms proposed
856 for the stabilisation of polypeptides by responsive polymers are depicted in Figure 5. The
857 effect is hypothesised to be due to the increased density of the conjugated polymers under
858 stressful temperature conditions: a formation of a dense 'shell' which blocks the active site
859 under these conditions, capable of reformation and retention of the original enzymatic
860 activity upon return to ambient temperature. The potential of these polymers to modulate

drug delivery has been recognised, however the application to drug manufacturing and formulation has not been explored.

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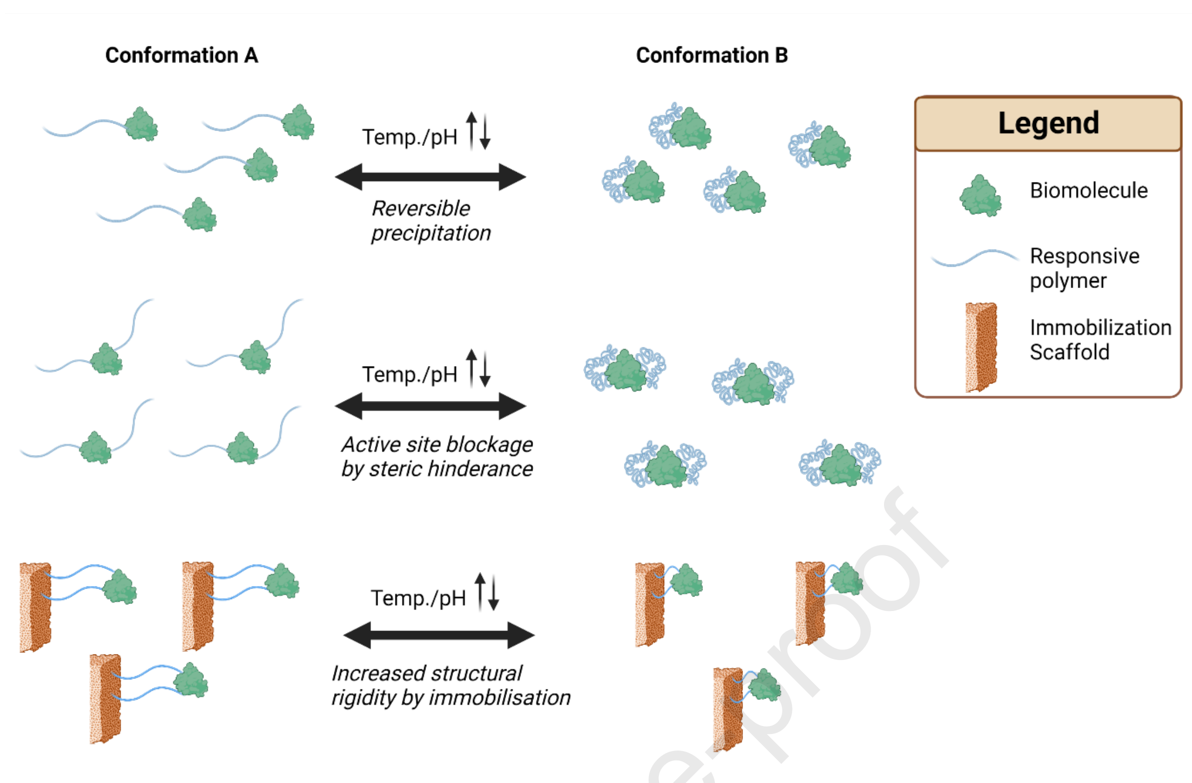

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888 **Fig. 5** - Proposed mechanisms of biomolecule stabilisation conveyed by responsive polymers
889 [209, 210]. Created with BioRender.com.

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906 To the best of our knowledge, the literature does not address the effect of polymer
907 conjugation on solid state formation of proteins. In one study reported in the literature, the
908 impact of Poly(vinyl alcohol) (PVA), a widely used water-soluble polymer, on the freeze-
909 thaw stability of Green Fluorescent Protein (GFP) was examined. The PVA-GFP conjugate
910 exhibited higher cryostability, measured by fluorescent emission of the protein after 15
911 freeze-thaw cycles. While native GFP lost all fluorescence after this treatment, GFP-PVA
912 retained >50% of its original emission [211]. In Table 3, we address the polymer-protein
913 conjugants with increased stability against commonly observed drying stresses.
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Table 3 - Polymer conjugants with increased stability against commonly observed drying stresses. CTA: Chain Transfer Agent

Biomolecule	*Polymer conjugated*	*Effect on stability*	*Reference*
Lysozyme	MethoxyPEG-aldehyde	Increased resistance to ionic strength	[195]
	Oligo-acrylamide-CTA (O-Am-CTA) (various functional groups)	Decrease in T_m Increased chemical stability to Guanidine Hydrochlorde	[212]
	Trehalose glycopolymers	Increased resistance to freeze drying cycles Increased activity retention at 90˚C	[141]
	Glycidyl methacrylate (GMA) PEGmethyl ether methacrylate (PEGMEMA)	Increased activity retention at 90˚C	[197]
	Poly(N,N-dimethylamino-2-ethyl methacrylate)	Increased activity retention at 90˚C	[213]
	MethoxyPEG- N-hydroxysuccinimide (PEG-NHS)	Decreased aggregation propensity under 95ºC during hot melt extrusion	[214]
	MethoxyPEG-p-nitrophenyl carbonate (5000 Da)	Increased activity retention in pH ranges 6<pH<10	[196]
	Poly(N-acryloylmorpholine) (PNAM), Poly(oligoethylene glycol methyl ether methacrylate) (POEGMA)	Native activity increased by 25% Decreased aggregation propensity under high ionic strengths	[215]
	Tragacanthin	Increased thermal stability (T_m +6.35ºC)	[216]
	Xanthan gum	Decreased aggregation propensity at acidic pH and at 60ºC	[217]
	PEG-β-cyclodextrin (conjugated to adamantane-appended lysozyme)	Increased activity retention at 70ºC for 6 hours (20-40%)	[218]
Chymotrypsin	Poly(sulfobetaine methyacrylamide)-block-poly(N-isopropylacrylamide) (pSBAm-block-pNIPAM).	50% more activity retention at 37ºC than native chymotrypsin Increased chemical stability to Guanidine hydrochloride	[209]
	Poly(N,N-diethylacrylamide-co-glycidyl methacrylate) (P(DEAAm-co-GMA)	Increased activity retention under 45ºC incubation Increased activity retention at pH >7.5	[208]
	Poly(carboxybetaine)	Increased activity retention at 50ºC	[207]
	MethoxyPEG- N-hydroxysuccinimide (PEG-NHS)	Increased T_m +6ºC	[219]
	Poly(ethylene glycol) methyl ether methacrylate (POEGMA)	Increased activity at pH 8 Increased stability at acidic pH	[220]
Bovine Serum Albumin	MethoxyPEG maleimido-propionamide	Decreased propensity towards aggregation T_{agg}+4-	[202]

		7ºC Decreased T_m -3ºC	
	methoxy-PEG succinimide	Increased stability in storage as a ly powder	[221]
	methoxy-PEG–succinimidyl carboxymethyl ester	Decreased aggregation propensity	[222]
Laccase	Cyanuric chloride-activated methoxy poly(ethylene glycol)	Increased stability in organic solvents	[200]
	O-[2-(6-oxocaproylamino)ethyl]-O′-methylPEG	3-fold increase in enzymatic activity	[223]
L-Asparaginase	Silk fibroin	Increased storage stability, Doubled activity retention at 50ºC and 60ºC	[224]
	poly(N-vinylpyffolidone-co-maleic anhydride) (P(VP-co-MA))	Increase in optimum enzymatic activity temperature (+10ºC)	[225]
Interferons	PEG- N-hydroxysuccinimide (PEG-NHS)	Decreased aggregation propensity	[188]
	Hydroxyethyl starch PEG (unspecified)	Increased glass transition temperature (T_g) in freeze-dried powders	[226]
	MethoxyPEG-aldehyde	Decreased aggregation propensity at 50ºC	[184]
Haemoglobin	PEG-vinyl sulfone	Increased resistance to interfacial related aggregation during freeze drying	[179]
	Poly(acrylid acid)	30% increased storage stability after 120h at 25ºC	[203]
Pegvisomant (growth hormone receptor antagonist)	PEG (unspecified)	PEGylation improves reconstitution of freeze dried powders	[180]
Human Growth Hormone	poly(ethylene glycol) Methyl ether methacrylate (PEGMA)	Increased physical stability at 37ºC (+60% activity retained after 40 hours)	[227]
	methoxy-PEG-propionaldehyde Methoxy-PEG-amine	Increased T_m (+4.1ºC)	[187]
	Methoxy-PEG- amine	Decreased degradation of peptide 3-fold at 37ºC for 3 weeks	[228]
	Methoxy-PEG-aldehyde	Increased stability of formulation during freeze drying Decreased storage stability	[229]
Myoglobin	Poly(phosphoesthers)	Decreased high-order aggregation propensity at 50ºC	[204]
	N-hydroxysuccinimide-PEG	Increased reversible denaturation	[230]
Green Fluorescent Protein	Poly(vinyl alcohol)	50% higher stability towards freeze-thaw cycles	[211]
	PEG methyl ether methacrylate (POEGMA)	310-fold increase in thermal stability at 90ºC	[231]

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Glucagon	Methoxy polyethylene succinimidyl propionate	Increased physical stability during freeze-drying	[181]
Antibody fragments	Maleimide-polyethylene glycol	Increased resistance to interfacial related aggregation	[186]
	Maleimide-polyethylene glycol	Increased T_m (+5.4ºC)	[232]
	PEG (unspecified)	Increased Tm (+5.7ºC)	[233]
	poly(N-isopropylacrylamide) (PNIPAAm)	Increased reversible precipitation at 37ºC	[234]

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959 The possibility for the introduction of point mutations or post-translational modifications
960 (PTMs) to therapeutic proteins is a novel concept for their directed stabilisation for drying. In
961 particular, this approach to stabilisation during protein drying is attractive in applications
962 where use of excipients is limited, for example in the creation of dried powder inhalables, or
963 where the protein product associates with the chosen excipients. This area, however, demands
964 directed research. Structural alterations to protein backbones may potentially affect their
965 efficacy and immunogenicity, with potential harmful effects to patients [3, 4]. It is widely
966 accepted that even a single residue modification may increase risks of aggregation,
967 fragmentation and deterioration throughout the manufacturing process, as well storage and

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968 administration [236-238]. Moreover, no studies have been conducted so far to analyse
969 particle properties of structurally modified protein powders. Such knowledge is necessary for
970 the validation of this stabilisation approach for the creation of drug delivery systems or
971 inhalable formulations. It is possible that structural modifications of proteins could
972 potentially cause adverse results on the integrity of the dried formulations. For instance,
973 involuntary glycosylation of milk proteins may cause increased rates of aggregation during
974 freeze-drying with sugar excipients [239]. A thorough understanding of these effects is still
975 lacking in the literature.

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977 ## 4. Conclusions and Perspectives

978 Drying of biologics has opened avenues for novel administration routes, enhanced efficacy,
979 and a more economically viable product, however protein stability during the drying process
980 remains a prevalent issue in formulation. This review offers a succinct overview of the
981 stresses present in conventional and novel drying techniques, and the most common ways to
982 stabilise them. We also present some of the limitations of excipient use in dry protein
983 formulations, considering their effect on formulation stability, process interactions and
984 particle properties, showcasing a need for exploiting other stabilisation avenues for protein
985 drying. Structural modifications which govern the stabilisation of proteins are widely studied
986 and reported, particularly in potency and bioavailability improvement. Insight into solid-state
987 stability imposed by structural modifications can offer new avenues in protein production and
988 formulation. The preservation of protein structure under multiple mechanical and
989 environmental stresses is widely accepted as a Critical Quality Attribute (CQA) in many
990 processing unit operations, none more so than drying. With the rapid growth of the
991 biopharmaceutical industry and the gradual shift towards continuous manufacturing, it is
992 imperative to pursue innovative strategies of product stabilisation.
993 In consideration of the literature, we have reviewed and proposed PTMs and controlled
994 structural mutations as one such strategy, enveloping their positive impact on thermal
995 stability, chemical stability, aggregation and interfacial stresses. Although excipients have
996 been successful in the past in the stabilisation of proteins and peptides for drying, they pose
997 some significant issues during storage and inhalable/parenteral formulation. Furthermore,
998 assuring stability prior to downstream processing of biomolecules has positive implications
999 on other unit operations imposing heat or interfacial stresses, such as filtration and
1000 chromatography, ultimately increasing protein yields, as well as a positive impact on
1001 subsequent solid-state storage. The costs and labour required for the upstream modification of
1002 biotherapeutics must be evaluated against the current cost of downstream stabilisation for
1003 each individual biopharmaceutical formulation. Considering the extensive research which
1004 needs to be conducted before utilising structural modifications as a stabilisation method, the
1005 approach may be considered viable only for the production and research of dried high-value
1006 biopharmaceuticals.

1007 ## CRediT authorship contribution statement

1008 Wiktoria Brytan: Conceptualisation, Investigation, Writing - original draft, Writing - review
1009 & editing, Visualisation. Luis Padrela: Conceptualization, Writing - review & editing,
1010 Supervision, Project administration, Funding acquisition.

1011 ## Declaration of Competing Interest

1012 The authors declare that they have no known competing financial interests or personal
1013 relationships that could have appeared to influence the work reported in this paper.
1014

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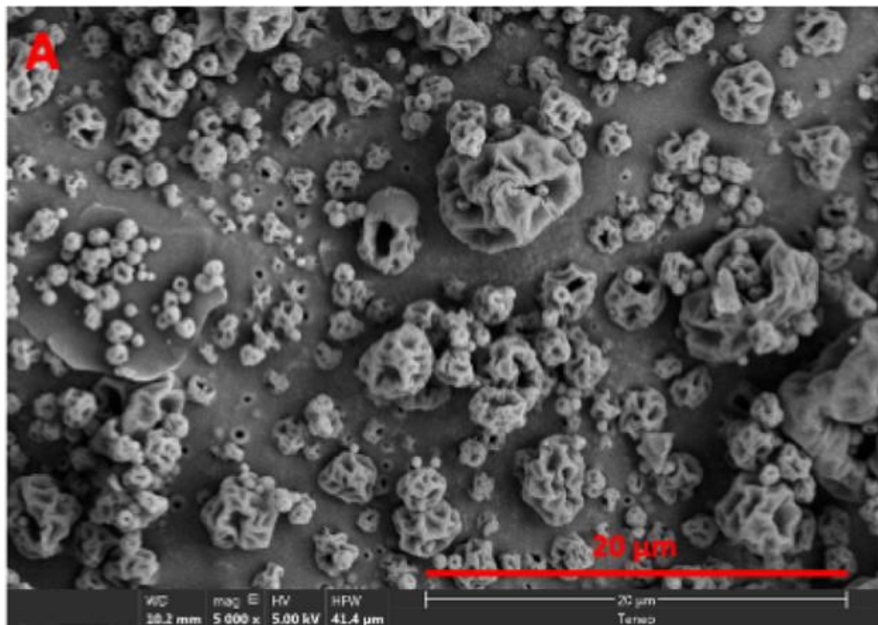
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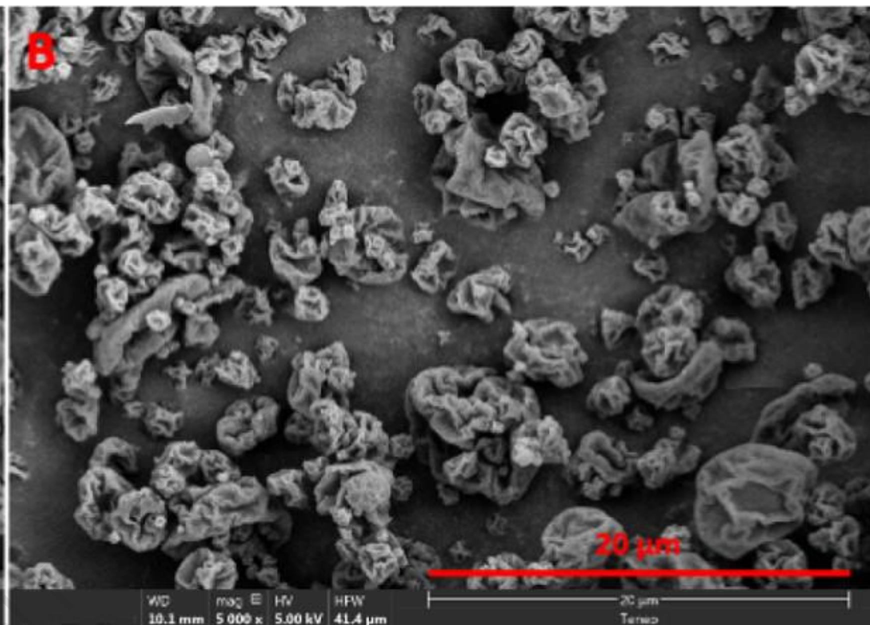
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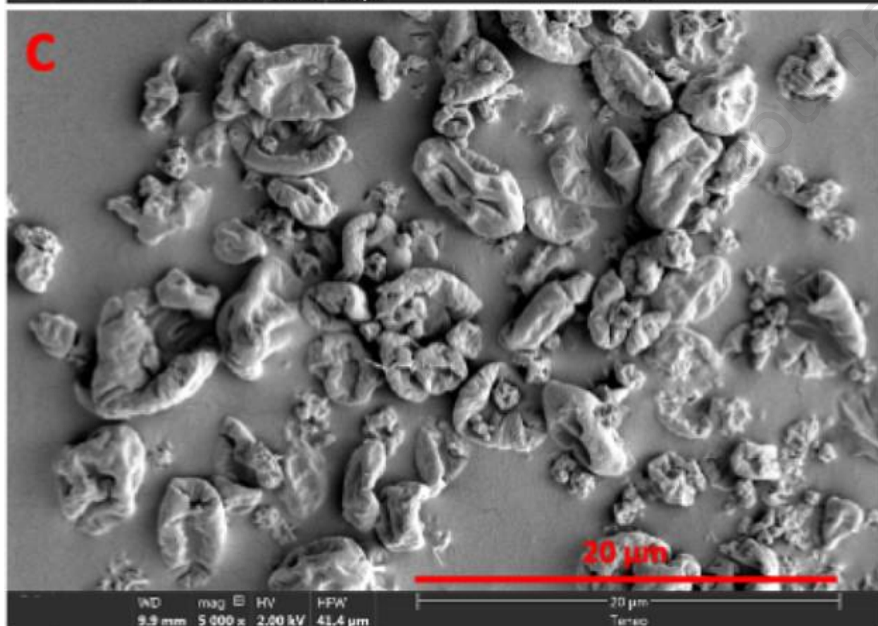
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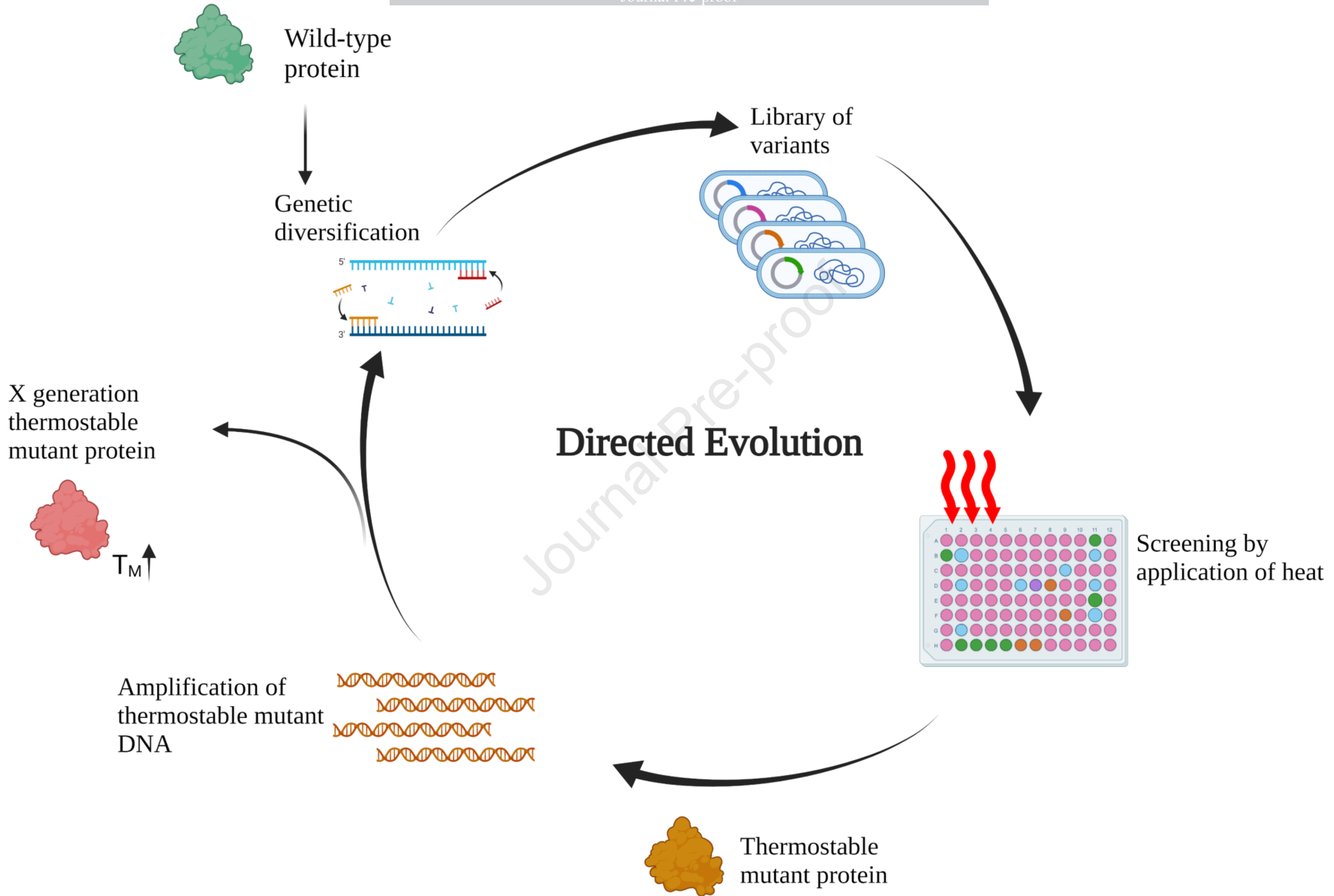

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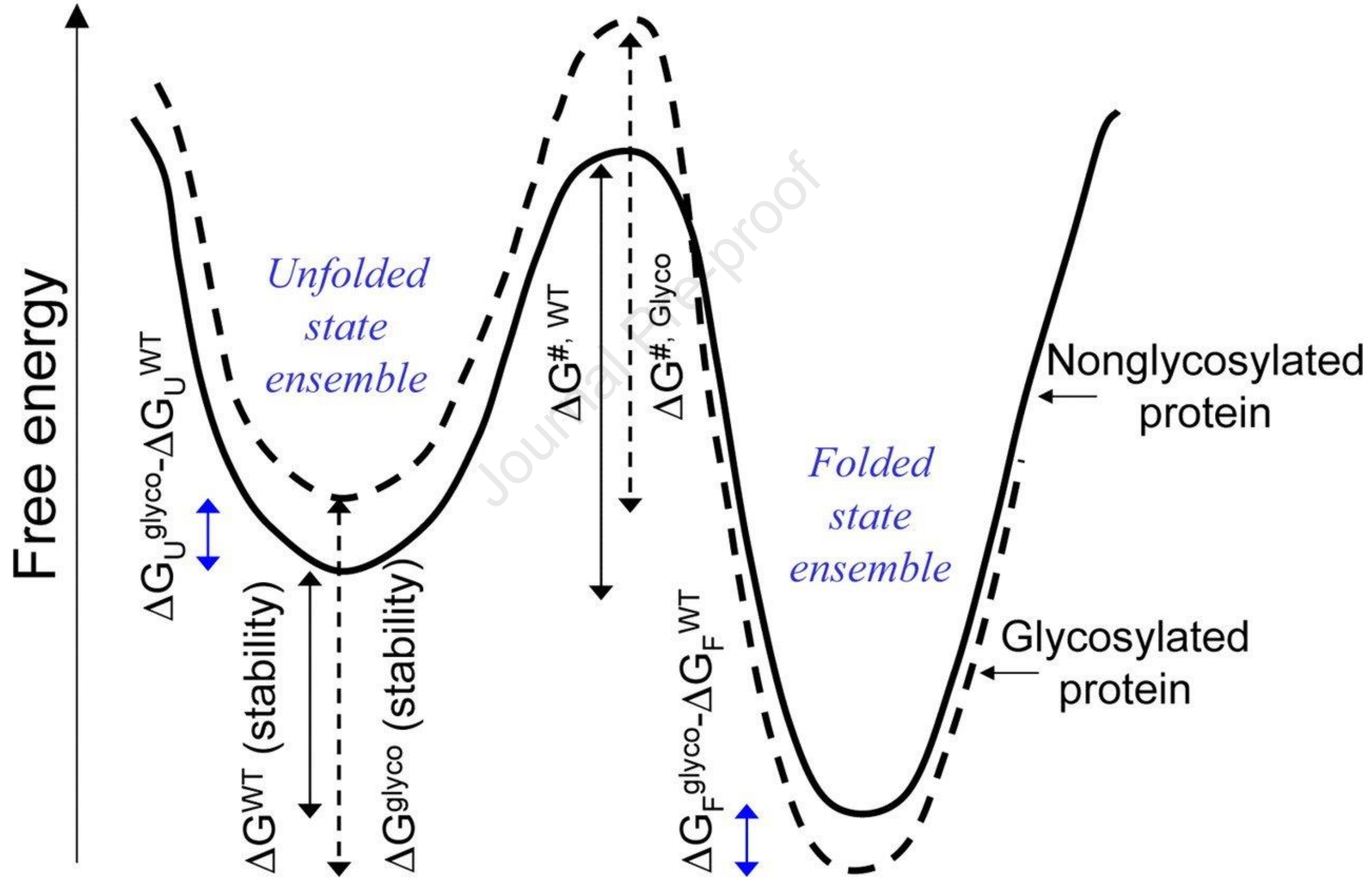

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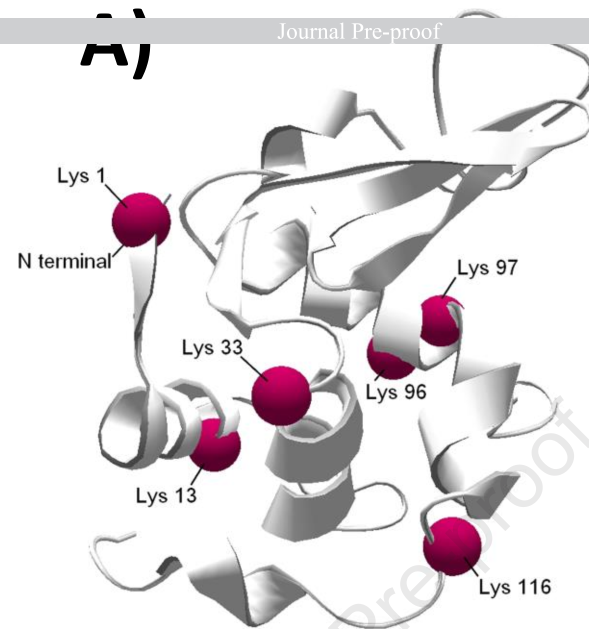

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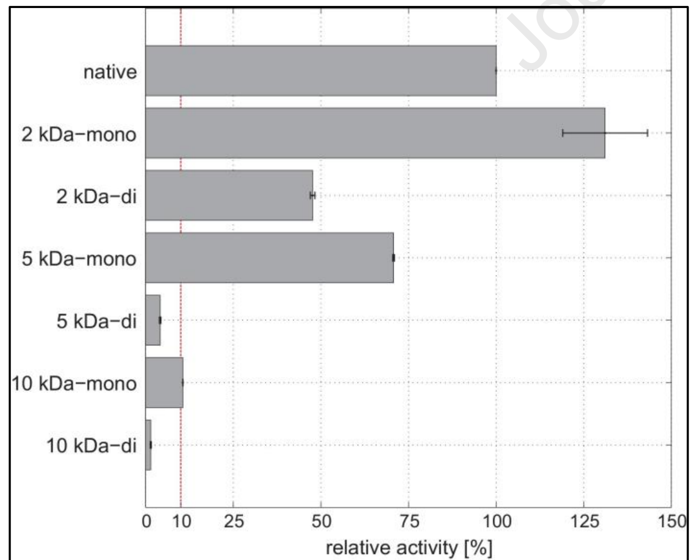

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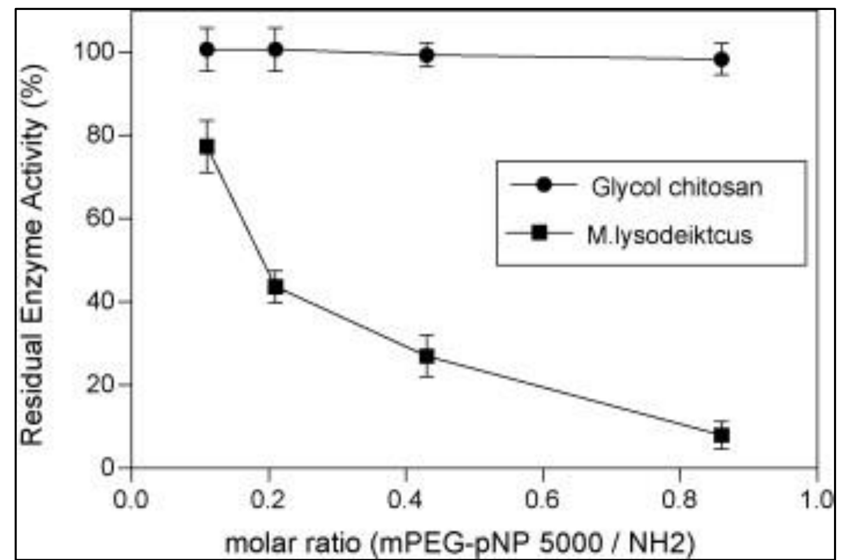

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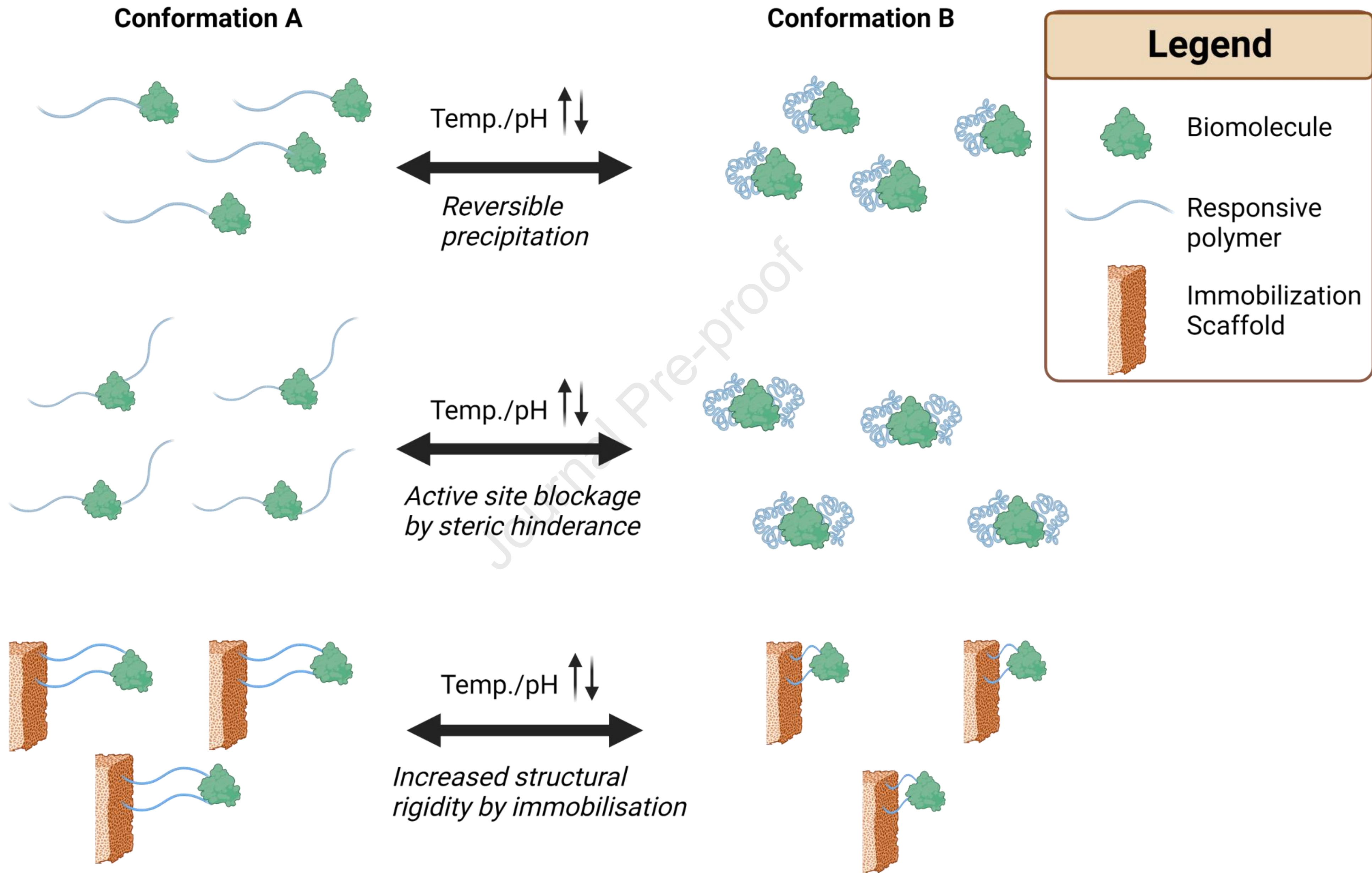

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Highlights

- Both established and novel biomolecule drying techniques are discussed in light of their effects on protein and peptide stability
- Current stabilisation methods for maintaining biomolecule structural integrity during drying are reviewed and their effect on drying applications are recognized.
- Protein engineering, artificial conjugation and post-translational modifications are evaluated in their ability to protect protein integrity during thermal, interfacial and chemical stresses, amongst others.
- Methods to induce beneficial protein modifications for drying applications are discussed

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CRediT authorship contribution statement

Wiktoria Brytan: Conceptualisation, Investigation, Writing - original draft, Writing - review & editing, Visualisation. Luis Padrela: Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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