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Original
Rational design of freeze-drying formulations: a molecular dynamics approach / Arsiccio, A., Pisano, R.. - STAMPA. - (2019). (7th European Conference “EuroDrying’2019” Torino July 10-12, 2019).

Availability:
This version is available at: 11583/2939674 since: 2021-11-23T14:57:48Z

Publisher:
Politecnico di Torino

Published
DOI:

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12 October 2026

Proceedings of Eurodrying'2019
Torino, Italy, July 10-12, 2019

Paper 130

RATIONAL DESIGN OF FREEZE-DRYING FORMULATIONS: A MOLECULAR DYNAMICS APPROACH

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Abstract
The freeze-drying process is often applied to biopharmaceuticals, but it may result in protein unfolding or aggregation. Suitable excipients must therefore be added to avoid loss of activity, but the choice of the formulation is currently empirical, due to a lack of knowledge about the phenomena involved. Here, molecular dynamics is used to understand the molecular mechanisms at the basis of protein stabilization, and guide the choice of suitable excipients.

Keywords*: freeze-drying, formulation, molecular dynamics, biopharmaceuticals*

1. Introduction

The most common technique for the storage of protein-based drugs in the solid state is freeze-drying, or lyophilization, which allows removal of water at low temperature. However, several stresses could arise during the process, that may result in loss of therapeutic activity.

For instance, the interaction of nonpolar residues with water is less unfavorable at the low temperature used during freezing, and this may lead to cold denaturation of the protein (Privalov 1990, Matysiak *et al.* 2012). The formation of ice may also be harmful to protein stability, because proteins may adsorb to the interface, and undergo surface-induced denaturation (Strambini and Gabellieri 1996). Also, the freeze drying process removes part of the hydration shell of the protein, which may disrupt the native state (Rupley and Careri 1991). This is related to both a decreased charge density on the protein surface in a water-poor environment, which promotes aggregation, and the potential removal of water molecules that are integral part of active sites.

Some excipients should therefore be added to stabilize the protein and prevent its denaturation (Wang 2000). The most common protectants include sugars, polyols, polymers, amino acids, and surfactants. When designing a freeze dried formulation, it should also be considered that the mechanisms of protein stabilization by excipients are not the same during the two stages of lyophilization, i.e., freezing and drying. The thermodynamic mechanisms prevail during freezing, as a not negligible amount of water is still present in the system, while the ability of the excipients to form a stiff, compact cake that kinetically inhibits the protein motions responsible for unfolding and aggregation becomes dominant in the dried
state (Ohtake *et al.* 2011).

One of the most widely accepted mechanisms of protein stabilization during freezing is preferential exclusion. According to this theory, a protective osmolyte should be excluded from the protein surface, and preferential hydration should therefore ensue. The presence of these co-solutes creates a thermodynamically unfavorable situation, which is augmented by an increase in the surface area exposed by the protein (Timasheff 1993). As a consequence, the native fold is stabilized, because denaturation would result in a greater contact surface area between the protein and the solvent.

During the drying steps of freeze drying, the preferential exclusion mechanism is no longer applicable because at this point there is essentially no water (Carpenter *et al.* 1993). Therefore, different theories have been developed to explain protein stability in the dried state. For instance, according to the water replacement mechanism (Crowe *et al.* 1984, Carpenter *et al.* 1990) protective

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osmolytes should hydrogen bond with the protein at the end of the drying process to satisfy the hydrogen bonding requirement of the polar residues.

One other major mechanism of protein stabilization by lyoprotectants is the formation of a viscous glassy matrix during lyophilization (Franks 1994), that increases protein stability by slowing down protein denaturation and unfolding (Hagen *et al.* 1995).

Here, a possible approach to the design of the formulation for biopharmaceuticals is discussed. More specifically, we propose Molecular Dynamics (MD) as an efficient tool for the pre-screening of a wide number of possible excipients. MD simulations can uncover the molecular mechanisms of protein stabilization by the surrounding osmolytes, thus supporting the scientists involved in formulation development. Our results suggest that not all the excipients are equally effective during freezing and drying, and allow the identification of some molecular properties that correlate with the stabilizing action of the osmolytes (Arsiccio and Pisano 2018).

2. Simulation approach

Molecular dynamics is here used to study the interaction between some model proteins and typical pharmaceutical excipients. In a common MD simulation, structure files describing the protein (in many cases a PDB file) and the excipient molecule, and topology files listing the forces acting on each atom, are needed to set up a simulation. A simulation box is then built, where the protein is surrounded by the desired number of excipient molecules and solvated in water, and counter ions are also added to guarantee the neutrality of the system (Fig. 1a). After an energy minimization step, the system is equilibrated at the desired values of temperature and pressure. Finally, the production run can start, where Newton's law of motion is integrated over time for all the atoms in the simulation box, and the coordinates are written to an output file at regular intervals.

In this work, human growth hormon (hGH) and lactate dehydrogenase (LDH) will be used as model proteins (see Fig. 1b), and the effect of sucrose, trehalose, cellobiose, lactose, glucose, sorbitol, glycine, mannitol, histidine and β-cyclodextrin as stabilizers will be investigated.

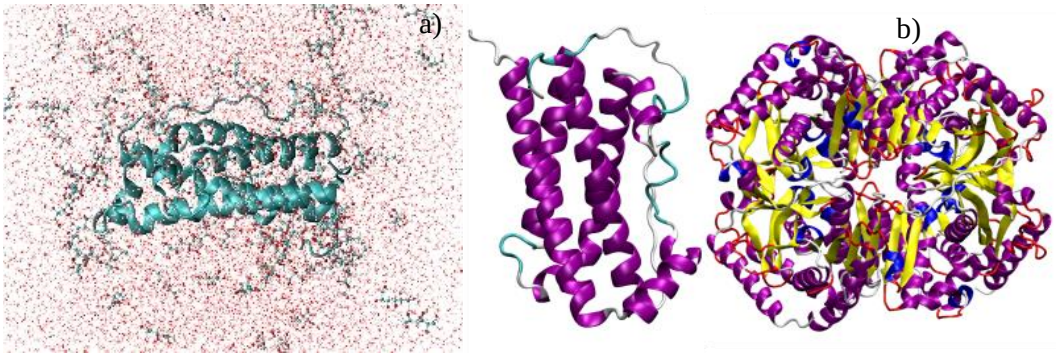


Fig. 1. a) Example of an MD simulation box, where native hGH is surrounded by sucrose molecules and solvated in water. b) Cartoon structures of hGH (left) and LDH (right).

The MD trajectory will therefore be analyzed to identify, and quantify, the mechanisms of protein stabilization by different osmolytes. As regards preferential exclusion, the cumulative radial distribution function, and thus the average number of excipient, $n_{exc}(r)$, and water, $n_w(r)$, molecules within a distance r from the protein surface can be computed. Hence, the degree of preferential exclusion can be calculated from the function,

$$\beta = \frac{n_{exc}(r)/n_w(r)}{n_{exc}(\infty)/n_w(\infty)} \tag{1}$$

The excipient is preferentially excluded from the protein if β is smaller than 1 for small values of r.

The vitrification theory can be tested by computing the viscosity μ of the protein-free solution; alternatively, the mean square displacement $\langle u^2 \rangle$ of the protein hydrogen atoms could also be

computed. The value of $\langle u^2 \rangle$ provides information on protein dynamics, since the hydrogen atoms reflect the motions of the side chains and backbone atoms to which they are bound.

Finally, the water replacement mechanism can be assessed by evaluating the parameter χ,

$$\chi = \frac{\text{number of protein} - \text{excipient hydrogen bonds}}{\text{total number of protein} - \text{excipient and protein} - \text{water hydrogen bonds}} \quad (2)$$

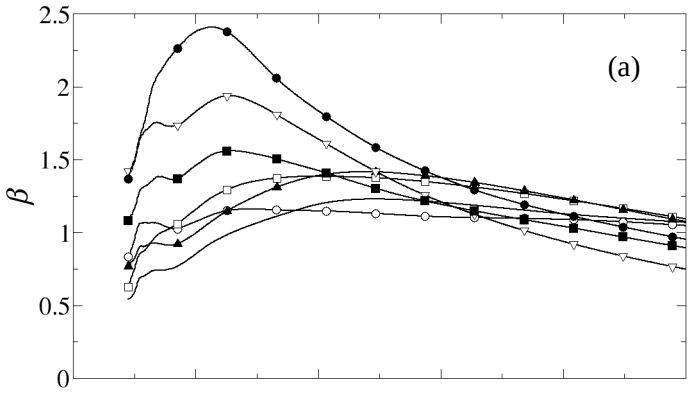
which represents the relative contribution of hydrogen bonding between the protein and the excipient with respect to the total number of intermolecular hydrogen bonds formed by the protein.

3. Results and discussion

The preferential exclusion of different excipients from hGH or LDH was computed using MD simulations, and the results are illustrated in Fig. 1a for hGH, and 1b for LDH (Arsiccio and Pisano 2017, Arsiccio *et al.* 2019).

As regards hGH (Fig. 1a), the β parameter was sometimes found to be smaller than 1 at short distance from the protein surface, indicating that at least some of the excipients were preferentially excluded from the protein. More specifically, sucrose was slightly more excluded than trehalose, while cellobiose and lactose had an intermediate behavior. Glucose, sorbitol and glycine were characterized by a significantly lower extent of preferential exclusion if compared to the disaccharides. This means that, according to our analysis, they should not have a remarkable cryoprotective effect.

As regards LDH (Fig. 1b), it is clear that mannitol and histidine were not excluded from the protein surface, but, rather, they preferentially interacted with LDH, displacing the water molecules. In fact, their β parameter was greater than 1 for small values of the distance *r* from the protein surface. In contrast, the β-cyclodextrin molecules seemed to be the most excluded from the protein surface, showing values of β which were remarkably smaller than 1 close to the surface of LDH. According to this analysis, therefore, the cyclodextrins may be the best cryoprotectants, even better than the disaccharides. Also in the case of LDH, sucrose was slightly more excluded than trehalose from the protein surface, while cellobiose behaved similarly to sucrose.



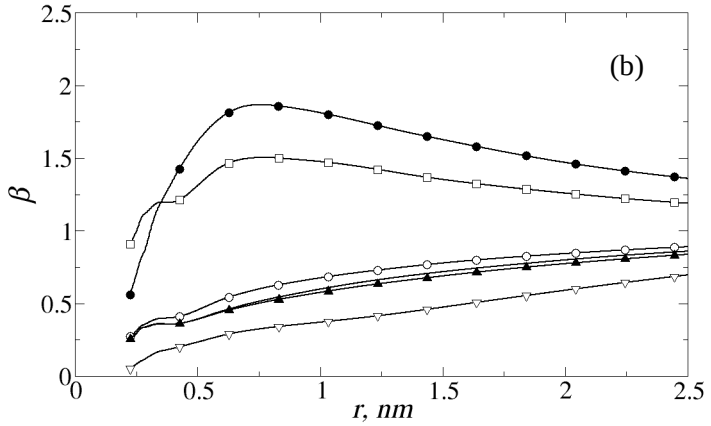


Fig. 1. (a) Plot of β parameter vs distance from hGH in 0.38 M sucrose (—), trehalose (-○-), lactose (-□-), cellobiose (-▲-), glucose, (-▽-), glycine (-●-), and sorbitol (-■-). (b) Plot of β parameter vs distance from LDH in 0.83 M sucrose (—), trehalose (-○-), mannitol (-□-), cellobiose (-▲-), histidine (-●-), and 0.25 M β-cyclodextrin (-▽-).

The results for hGH and LDH compare fairly well with one another. This suggests a generally applicable behavior of the osmolytes toward different proteins, which is not sequence-specific. Moreover, the predictions of MD simulations are confirmed by experimental data. For instance, Nema and Avis (Nema and Avis 1992) studied the residual activity of LDH after freeze-thawing cycles, in the presence of different excipients. They found that the addition of 0.9% (w/v) β-cyclodextrin protected the protein more than a 5 % (w/v) trehalose solution, with a remaining activity of 31 % and 26 %, respectively. As regards the disaccharides, the MD simulations suggest that sucrose is more excluded than trehalose from the protein surface. This, again, is in line with the experimental work by Nema and Avis, where a 5 % (w/w) sucrose formulation is reported to preserve the LDH activity much better (73 %) than a trehalose solution (26 %) at the same solid content.

Finally, the efficiency of mannitol and histidine as protectants, according to our simulations, should be quite poor. They are generally used in typical formulations not as protein stabilizers, but as buffer components, in the case of histidine, or bulking agents, in the case of mannitol. In line with our simulations, Nema and Avis observed that LDH showed only 6% recovery of enzymatic activity after freeze-thawing when mannitol was used as stabilizer, confirming its poor efficiency as a cryoprotectant.

Beside the thermodynamic mechanism of preferential exclusion, water replacement and vitrification could arise when the osmolyte concentration is sufficiently high. The viscosity μ for protein-free formulations at different concentrations was therefore computed, and the results are shown in Fig. 2a.

It is possible to notice that the viscosity increased with excipient concentration, as expected during the cryoconcentration and dryng processes. The simulations performed allow the identification of a clear trend, with the increase in viscosity being highly non-linear. The disaccharides showed higher values of viscosity than glucose and glycine, especially at the highest concentration tested. Furthermore, the viscosity of sorbitol was intermediate between those of disaccharides and glucose or glycine.

As previously mentioned, the vitrification hypothesis generally associates a higher viscosity to slower chemical and physical degradation rates of proteins. Viscosity is closely related to the α relaxation processes, i.e., the slowest and strongly temperature dependent motions of a glass (Yoshioka and Aso 2007). However, some studies seem to suggest that no correlation exists between the α relaxation time and protein stability (Davidson and Sun 2001).

In contrast, it was reported (Cicerone and Douglas 2012) that the fast β relaxation processes have a strong relationship with the protein degradation rates. The correlation between β relaxation dynamics and protein stability in lyophilized products can be experimentally observed by measuring the mean square displacement $\langle u^2 \rangle$ of hydrogen atoms using incoherent inelastic neutron scattering (Cicerone and Soles, 2004). $\langle u^2 \rangle$ is directly proportional to the amplitude of the fast β processes and was

demonstrated to be strongly related to protein stability in sugar glasses (Cicerone and Douglas 2012). In those studies, $\langle u^2 \rangle$ measurements were made on a nanosecond timescale, and a similar procedure was reproduced in simulations of LDH as model protein. The results of this analysis are shown in Fig. 2b, where different formulations have been considered.

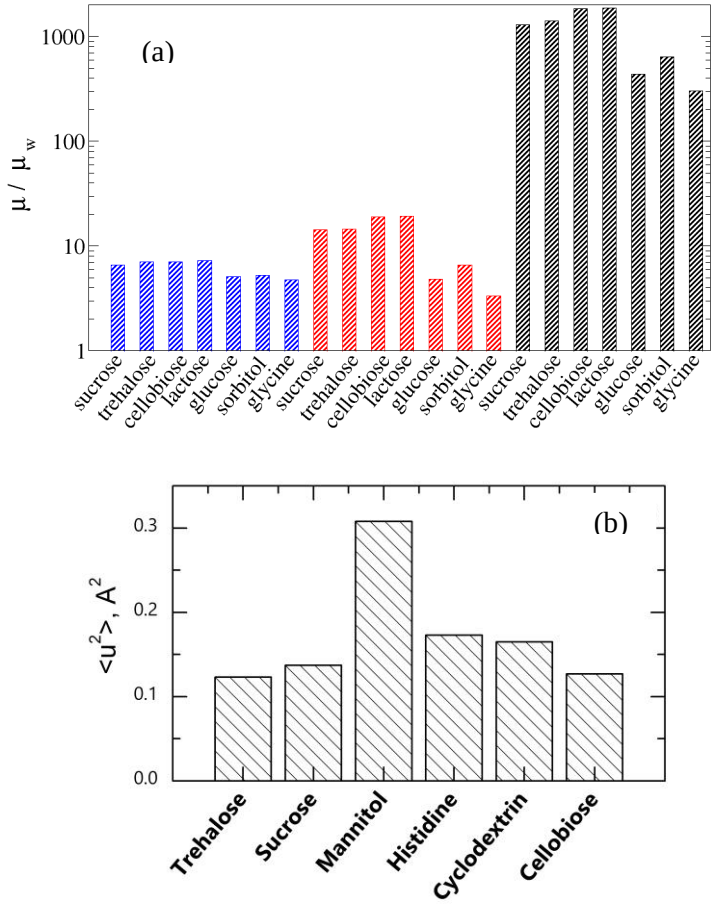


Fig. 2. (a) Viscosity of protein-free formulations at 0.38 M (blue bars), 1.15 M (red bars) and 2.5 M (black bars) concentration, relative to the viscosity μ_w of water at 300 K and 1 bar, as measured from MD simulations. (b) Amplitude of the mean square displacement of LDH hydrogen atoms on a nanosecond timescale in 2.5 M sucrose, trehalose, mannitol, cellobiose, histidine, and 0.75 M β-cyclodextrin.

It is apparent that the disaccharides should provide the best kinetic stabilization of the protein structure, also if we consider the β relaxation time as critical parameter. According to our results, mannitol should be the worst stabilizer, followed by histidine and the β-cyclodextrin. Among the disaccharides, trehalose and cellobiose should again be slightly better than sucrose.

The MD results obtained are therefore in accordance with the experimental work by Al-Hussein and Gieseler (Al-Hussein and Gieseler 2012), where sucrose was proven to be a significantly better lyoprotectant than histidine or mannitol. Also Izutsu *et al.* (Izutsu *et al.* 1995) found that a 200 mM sucrose solution protected LDH (30 % remaining activity) from freeze-drying stresses more than a 400 mM mannitol formulation (14 % residual activity only), in line with the MD simulations.

The hydrogen bonding network within each simulation box was also analyzed. As evident from Fig. 3, the relative contribution of hydrogen bonding between hGH or LDH and the osmolytes increased with increasing excipient concentration.

In particular, it was small when the number of water molecules was sufficiently high, while it became predominant when the number of water molecules was no more sufficient for a complete protein hydration. Hence, these data suggest that water replacement becomes dominant in the solid

state, when most of the water has been removed, while preferential exclusion should be the prevailing mechanism in the liquid state.

Remarkably, the disaccharides, which formed the greatest number of hydrogen bonds with the protein, also increased the solution viscosity the most (Fig. 2a), or resulted in the slowest β relaxation processes (Fig. 2b). Among the disaccharides, trehalose and cellobiose could form a larger number of hydrogen bonds with hGH than sucrose or lactose (see Fig. 3a), and a similar conclusion can be drawn from Fig. 3b for LDH if we compare cellobiose, trehalose and sucrose.

On the contrary, the excipients which were the least effective in hydrogen bonding to the protein were glucose, sorbitol and glycine in the case of hGH, or histidine and mannitol for LDH as model protein.

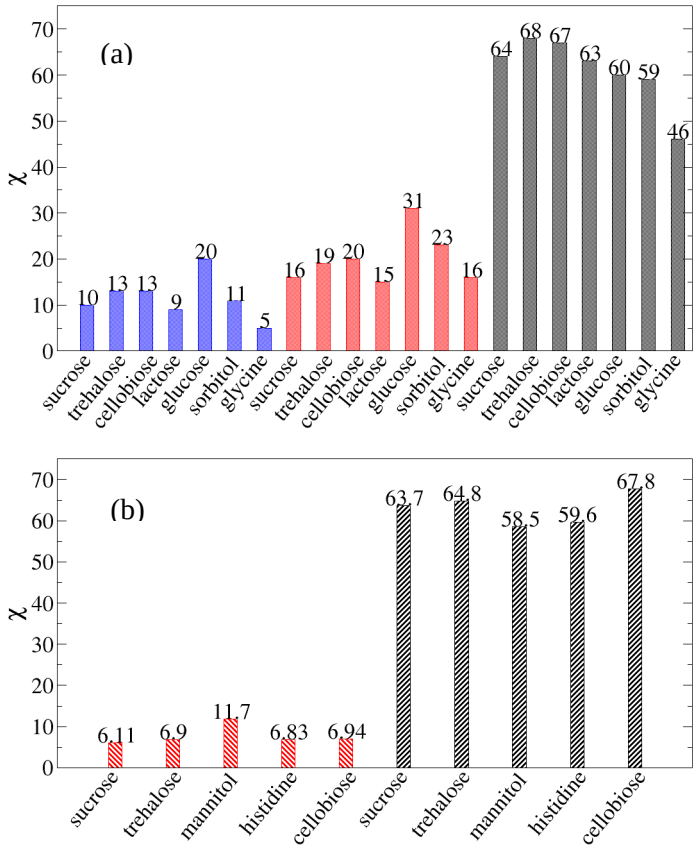


Fig. 3. (a) χ parameter for hGH as model protein, during simulations in excipients formulations at 0.38 M (blue bars), 1.15 M (red bars) and 2.5 M (black bars) concentration. (b) χ parameter for LDH as model protein, during simulations in excipients formulations at 0.83 M (red bars), and 2.5 M (black bars) concentration.

4. Conclusions

In this work, an MD simulation approach has been proposed to support the selection of a formulations for biopharmaceuticals to be freeze dried. From the molecular dynamics simulations here performed, the following conclusions could therefore be drawn:

- The mechanisms of protein stabilization change significantly during the freeze drying process, mostly as a result of the increase in excipient concentration. Preferential exclusion prevails during freezing, while vitrification and water replacement become dominant in the dried state;
- Not all the excipients are equally effective. The disaccharides should be better than polyols, monosaccharides and amino acids both during freezing and in the dried state;

- Also among the disaccharides, small differences may exist, with sucrose and lactose being extremely good cryoprotectants, and trehalose and cellobiose being slightly better for lyoprotection.

Moreover, the results obtained for hGH and LDH seem to be in line with one another, suggesting that the analysis performed should not be protein-specific, but should rather be generally applicable. The proposed approach may therefore prove extremely valuable for scientists involved in formulations development, and could be combined with experimental techniques to improve the stability of freeze-dried biopharmaceuticals.

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