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Orodispersible films based on blends of trehalose and pullulan for protein delivery

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ABSTRACT
During the last decades the number of therapeutic proteins increased tremendously on the pharmaceutical market. However, due to their often poor stability and limitations of the administration route, the application of therapeutic proteins is a major challenge. The oral mucosa has been suggested as a possible route for protein delivery. In this study, we prepared protein loaded orodispersible films (ODFs), based on blends of trehalose/pullulan by air- and freeze-drying. These two carbohydrates were selected based on the excellent protein stabilizing capacity of trehalose and film-forming ability of pullulan. ODFs were loaded with three model proteins. Ovalbumin was used to study the effect of protein incorporation on the mechanical properties, disintegration time, uniformity of weight and thickness of the ODFs. Lysozyme and β-galactosidase were used to evaluate protein stability. Ovalbumin loading did not significantly influence the mechanical properties of freeze-dried ODFs, while incorporation of ovalbumin in air-dried ODFs led to a substantial reduction in tensile strength. The trehalose/pullulan ratio had no impact on the stability of lysozyme, while the stability of β-galactosidase increased with increasing trehalose/pullulan ratios. Furthermore, freeze-drying appeared to be favorable over air-drying for process stability while the reverse was found for storage stability. In conclusion, trehalose/pullulan-based ODFs are from a technical point promising for possible protein delivery via the oral cavity.

1. Introduction
Since the first commercially available recombinant protein therapeutic, insulin, was approved by the US Food and Drug Administration (FDA) in 1982 [1], the number of therapeutic proteins, including vaccines, antibodies, enzymes, hormones and cytokines, has been grown tremendously. Over the last five years (2013–2017), even 111 therapeutic proteins were approved by the FDA Center for Drug Evaluation and Review (CDER) and the Center for Biologics Evaluation and Review (CBER) (“Purple Book” list of licensed biological products [2]). The reasons for the increasing interest in the application of therapeutic proteins are their highly specific biological activity combined with generally limited side effects.

However, there are two main barriers that hamper the application of therapeutic proteins. The first is their instability. Inherently to their production process, therapeutic proteins are obtained as aqueous solutions in which they are often susceptible to degradation, e.g. denaturation or aggregation. Consequently, most therapeutic proteins have to be stored and transported under refrigerated conditions, the so-called cold chain. But maintaining the cold chain is costly and even then the shelf life is often limited. Therefore, bringing the protein into a dry state in which it has low molecular mobility may be a practical strategy to solve this problem.

However, proteins are sensitive to stresses during the drying process. It is well accepted that proteins can be stabilized by drying them in the presence of sugars [3,4]. Among numerous types of sugars that have been used for stabilization of proteins, the disaccharide trehalose is often considered the gold standard [5,6]. The excellent stabilizing capacities can be ascribed to a number of favorable physicochemical characteristics. One these favorable physicochemical characteristics is the lack of reducing groups by which Maillard type reactions are prevented. Furthermore, due to its low molecular weight it can provide a tight molecular packing around a protein [3]. Hence, it can optimally vitrify the protein and optimally form hydrogen bonds with the protein.

The second key barrier to a widespread application of therapeutic proteins is the administration route. Currently, most therapeutic proteins are given parenterally, which is patient unfriendly, problematic for patients with needle phobia and requires trained health care
personnel. Oral administration is the more favorable as it does not require needles and syringes, it is preferred by patients and makes self-administration easy. However, proteins easily degrade in the harsh conditions of the gastrointestinal tract and are poorly absorbed from the gastro-intestinal tract, making oral delivery a challenge for protein drugs. Orodispersible films (ODFs) are a relatively new dosage form for several types of drug substances [7–9]. We hypothesized that ODFs might also be applicable for the administration of therapeutic proteins as it has been proposed that proteins can be absorbed directly via oral mucosa and that protein based antigens induce mucosal immunization via the oral cavity [10–12].

Being an excellent protein stabilizer, trehalose would be the ideal excipient of choice to prepare ODFs. However, trehalose is a very poor film former. Pullulan exhibits excellent film-forming properties and has been used as main material for ODFs before [13,14]. However, pullulan being a high molecular weight polysaccharide (45–2000 kDa) is too bulky to form a tight molecular packing around the protein. Therefore, we hypothesized that the use a combination of trehalose and pullulan for the preparation of protein loaded ODFs could benefit from both the protein stabilizing capacities of trehalose and the film forming capacities of pullulan. In addition, we recently showed that blends of trehalose/pullulan are excellent protein stabilizers in particular at high humidity conditions due the high Tg of pullulan [4].

Consequently, the aim of this study was to develop ODFs based on a combination of trehalose and pullulan for the delivery of therapeutic proteins via the oral cavity. The ODFs were prepared by air- and freeze-drying. A short-term storage stability study was carried out in order to elucidate the effects of trehalose/pullulan ratio and drying method on protein stability. Blank ODFs were prepared to investigate the residual water content, Tg and morphology. Ovalbumin (OVA) was incorporated at a high load to evaluate the effect of the presence of a protein on the uniformity of weight and thickness, disintegration time and on mechanical properties. Furthermore, lysozyme and β-galactosidase were incorporated to assess protein stability during ODFs preparation and subsequent storage up to four weeks. ODFs based on different trehalose/pullulan ratios (i.e. 0/100, 20/80, 30/70, 40/60; w/w) were prepared in order to determine the optimal combination with respect to the above mentioned quality parameters.

2. Materials and methods

2.1. Materials

Pullulan (average molecular weight 200–300 kDa) and trehalose were kind gifts from Hayashibara (Okayama, Japan). β-galactosidase (Mw = 540 kDa) was purchased from Sorachim (Lausanne, Switzerland). OVA (Mw = 44 kDa), lysozyme (from chicken egg white, Mw = 14 kDa) and Micrococcus lysodeikticus were obtained from Sigma-Aldrich (St. Louis, USA). Glycerol 85% and Tween 80 were obtained from Bufa (IJsstelstein, The Netherlands). All other excipients and chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of the casting solution and ODFs

In preliminary studies, it was found that ODFs prepared at a trehalose/pullulan ratio higher than 40/60 (w/w) were too brittle to handle. Incorporation of glycerol and Tween 80 in the casting solution was found to be essential in ODFs formation as these excipients improve spreadability of the casting solution as well as flexibility and appearance of the films. In addition, Tween 80, being a surfactant, facilitates disintegration of ODFs [15]. As shown in Table 1, the amount of glycerol was 17.5 wt-% with respect to the total amount of trehalose and pullulan. The concentration of Tween 80 in different trehalose/pullulan ratios was kept the same in the casting solution (0.58% (w/v)). Various casting solutions were prepared as listed in Table 1. All excipients were added to water under constant stirring. After the excipients were dissolved, the solution was stirred at low speed (100 rpm) overnight to remove air bubbles. These solutions were used to prepare blank ODFs. In case of the protein-loaded ODFs, OVA, lysozyme and β-galactosidase was added and stirred at low speed for an additional 4 h. The final concentration of OVA in the casting solution was 1% (w/v) and the amount of lysozyme or β-galactosidase was 0.4 wt-% with respect to the total amount of trehalose and pullulan.

ODFs were prepared using the solvent casting method as described previously [16]. Briefly, the solution was cast onto a release-liner (Prlimeliner 410/36, Loparex, Apeldoorn, The Netherlands), which was fixed to a film applicator (Erichsen, Hemer, Germany) by vacuum suction. The casting speed and height were 10 mm/s and 100 μm, respectively. Subsequently, the film layers were either air-dried for 48 h at 30 °C and a relative humidity (RH) of 60–80% or freeze-dried for 48 h using a Christ Epsilon 2–4 freeze-dryer (Salm & Kipp, Breukelen, The Netherlands). Freeze-drying was performed at a shelf temperature of −35 °C and a pressure of 0.220 mbar for 24 h after which the pressure was decreased to 0.050 mbar and the shelf temperature was gradually increased to 25 °C during 24 h. Blank ODFs were stored in a desiccator at 0% RH and protein-loaded ODFs were sealed in plastic bags and stored at −20 °C within 48 h before further analysis.

2.2.2. Differential scanning calorimetry

The glass transition temperature (Tg) of anhydrous as well as moisturized ODFs was determined by modulated differential scanning calorimetry (MDSC) using a Q2000 DSC (TA Instruments, Ghent, Belgium). ODFs were accurately weighed (2–5 mg) in an aluminum pan and non-hermetically closed with an aluminum lid. To allow residual moisture to evaporate, samples were heated at 150 °C for 15 min. Subsequently, samples were equilibrated at 0 °C and then heated to 200 °C at 2 °C/min with a modulation amplitude of 0.318 °C every 60 s. After exposure to an atmosphere of 33% RH and 25 °C for 72 h using a desiccator containing a saturated aqueous solution of MgCl2·6H2O, the Tg of the moisturized ODFs was determined. ODFs were accurately weighed (2–5 mg) in a Tzero aluminum pan and directly hermetically closed using a Tzero lid. Samples were equilibrated at −50 °C and the temperature was subsequently raised to 150 °C at a rate of 20 °C/min. Then the samples were cooled to the initial temperature followed by heating to 150 °C at 2 °C/min with a modulation amplitude of 0.318 °C every 60 s. The inflection point in the reversing heat flow versus temperature curve was taken as the Tg.

2.2.3. Thermal gravimetric analysis

Thermogravimetric analysis (TGA) was performed in order to measure the water content of ODFs in a Discovery Thermogravimetric Analyzer (TA Instruments, Ghent, Belgium). Samples weighing around 3–5 mg were placed in a platinum pan. To allow residual moisture to evaporate, samples were heated at 80 °C for 60 min. In order to confirm that all residual moisture was evaporated, the samples were subsequently further heated to 300 °C. Upon subsequent heating to 300 °C, no further mass loss was measured indicating that all residual moisture had been evaporated.

Y. Tian et al.  European Journal of Pharmaceutics and Biopharmaceutics 133 (2018) 104–111
2.2.4. Scanning electron microscopy

The morphology of the surface and cross section of air- and freeze-dried ODFs was evaluated via scanning electron microscopy (SEM) in a JSM-6460F (Jeol, Japan) at an accelerated voltage of 10 kV. ODFs were fixed on metallic stubs with carbon tape and sputter-coated with gold at the thickness of 10 nm by using a JFC-1300 sputter coater (Jeol, Japan) with a FC-TM20 thickness control (Jeol, Japan).

2.2.5. Weight and thickness

The ODFs with a size of 1.8 × 1.8 cm were punched using an Artemio perforator (Artemio, Wavre, Belgium). Six randomly chosen ODFs were weighed individually on an analytical balance. The average weight and weight variation was calculated.

The thickness of the six different ODFs was measured at five different points: in the corners and in the middle of the ODF using a micro screw meter (Mitutoyo, Neuss, Germany). The thickness of each film was considered as the average of five points.

2.2.6. Disintegration time

The disintegration time was carried out in accordance to an adapted slide frame method as previously published [16]. The ODFs (n = 6) with a size of 1.8 × 1.8 cm were clamped in an arm, which moved up and down at a frequency of 30 ± 1 cycles per min, over a distance of 55 ± 2 mm in a water bath at 37 °C ± 1 °C. Disintegration time of ODFs was recorded when complete dissolution had occurred, which was judged by visual inspection.

2.2.7. Mechanical properties

Air- and freeze-dried ODFs were stored in an atmosphere of 33% RH and room temperature using a desiccator containing a saturated aqueous solution of MgCl₂·6H₂O for at least two days. The mechanical properties of the ODFs were evaluated using an Instron series 5500 load frames with a load cell of 100 N (Instron, Norwood, USA) [16,17]. Test specimens were cut according to ISO-527 standard (plastics – determination of tensile properties) (NEN-EN-ISO, 2012). The ODFs (n = 8) were fixed between two clamps positioned at a distance of 4 cm. The clamps were subsequently moved away from each other with a crosshead speed of 50 mm/min until tearing or breakage of the ODFs. Tensile strength (N/mm²), Young’s modulus (N/mm²) and elongation at break (%) were recorded and automatically calculated by using Instron Merlin (series IX).

2.2.8. Protein stability

2.2.8.1. Lysozyme activity assay. The stability of lysozyme was determined using a turbidimetric assay. Lysozyme causes lysis of bacteria, which leads to a decrease in the turbidity of a bacterial suspension. Therefore, the activity of lysozyme can be measured from the rate of turbidity decrease of a Micrococcus lysodeikticus suspension [18]. Briefly, ODFs (1.8 × 0.9 cm) were dissolved with 1.0 mL of 0.1 M phosphate buffer (pH 7.3). Micrococcus lysodeikticus bacteria were suspended in 66 mM phosphate buffer pH 6.2 at a concentration of 2.5 mg/mL. Samples (n = 3) with a volume of 20 μL were pipetted into the wells of 96-well plates. After 200 μL of the bacterial suspension was pipetted into each sample, the absorbance was measured at 415 nm during 4 min at room temperature with an interval of 18 sec (Synergy HT Microplate Reader, BioTek Instruments, Winooski, VT). Solutions of lysozyme in phosphate buffer (pH 7.3) at concentrations ranging from 0 to 125 µg/mL were used to construct a calibration curve. The activity of lysozyme was calculated from slope of test samples and lysozyme activity was corrected for the weight of ODFs.

2.2.8.2. β-galactosidase enzymatic activity assay. The stability of β-galactosidase was determined using a kinetic enzymatic assay, based on the rate of conversion of a colorless substrate, o-nitrophenyl-galactoside, into the yellow colored product, o-nitrophenol, by β-galactosidase [3,4]. First, ODFs (1.8 × 0.9 cm) were dissolved in
1.0 mL of 0.1 M phosphate buffer (pH 7.3) and diluted 10 times with enzyme diluent (0.1% BSA and 1 mM MgCl₂ in 50 mM phosphate buffer). Subsequently, 20 μL samples (n = 3) were pipetted into each well of a 96-well microplate (Greiner Bio-One, F shape), followed by 200 μL of 1.4 mM MgCl₂ in 0.1 M phosphate buffer. The plate was incubated at 37 °C for 10 min. Thereafter, 20 μL of 50 mM o-nitrophenyl-galactoside was pipetted into the wells and the absorption was measured at 405 nm for 15 min at 37 °C with intervals of 30 s (Synergy HT Microplate Reader, BioTek Instruments, Winooski, VT). The activity of β-galactosidase in ODFs was calculated from the slope of this conversion and β-galactosidase activity was corrected for the weight of ODFs.

2.2.9. Statistical analysis

The results were statistically analyzed using two-way analysis of variance (ANOVA). A p-value < 0.05 was considered as significantly different. The graphs and curve fittings were performed using Graphpad Prism version 6.0 (GraphPad Prism Software, Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. SEM pictures of the top layer, bottom layer and cross section of trehalose/pullulan-based ODFs are presented in Fig. 1. Generally, SEM micrographs indicated that both air- and freeze-dried ODFs were homogeneous and no trace of crystallization was observed. Air-dried ODFs (Fig. 1A, B, G and H) showed smooth surfaces, with no major differences in morphology between top and bottom layer and between the ODFs composed of different trehalose/pullulan ratios. Furthermore, the cross-sections of these ODFs showed rather dense structures (Fig. 1C and I). In contrast, freeze-dried ODFs (Fig. 1D, E, J and K) showed porous surfaces with the bottom layer having more pores than the top layer. The micrographs of the cross-sections of freeze-dried ODFs (Fig. 1F and L) clearly showed highly porous structures, which is a morphology typically observed for freeze-dried materials when no collapse has occurred. They showed a mirror image of the ice crystals formed during freezing.

ODFs with trehalose/pullulan weight ratios of 20/80 and 30/70 had similar morphologies (data not shown).

3.2. Glass transition temperature and water content

The thermograms of ODFs at all trehalose/pullulan ratios showed a single Tg (data not shown), indicating the formation of a homogeneous blend of the two sugars and other excipients. The glass transition for pure trehalose and pullulan was observed at 121.5 and 242.3 °C, respectively. As the Tg of pullulan is much higher than that of trehalose, the Tg of anhydrous ODFs decreased with increasing trehalose/pullulan ratio with only minor differences between ODFs prepared by air- or by freeze-drying (Table 2). The Tgs of ODFs were considerably lower than those of pure trehalose/pullulan blends published previously [4]. These differences can be ascribed to the presence of other excipients, especially glycerol, which has a strong plasticizing effect. The same tendency was found for ODFs exposed to 33% RH having lower Tgs than the corresponding anhydrous ODFs. This difference can be ascribed to the plasticizing effect of water.

The water content of all moisturized ODFs was within the range of 5.5–7%, and freeze-dried ODFs always had a slightly higher water content than air-dried ODFs. This phenomenon is probably due to the porous structure of freeze-dried ODFs, leading to higher specific surface area and thus more adsorbed water.

3.3. Weight and thickness

As shown in Fig. 2, an increasing trehalose/pullulan ratio resulted in an increased weight of both air- and freeze-dried ODFs. This can be explained by the fact that ODFs with increased trehalose/pullulan ratio were prepared from solutions in which the pullulan concentration was kept the same while the trehalose concentration was increased. Freeze-
dried ODFs were much thicker than air-dried ODFs, which can be ascribed to their highly porous structures. The incorporation of OVA hardly affected the weight and thickness of the ODFs. Furthermore, all ODFs showed acceptable uniformity of weight and thickness with low standard deviation.

3.4. Disintegration time

Disintegration time is an important parameter for quality control of ODFs [19–21]. Disintegration time was recorded as the time in which ODFs were completely dissolved in the frame. For both air- and freeze-dried ODFs an increase of disintegration time was observed with increasing trehalose/pullulan ratios (Fig. 3). This increase can be ascribed to the increased weight of the ODFs with increasing trehalose/pullulan ratios. Furthermore, freeze-dried ODFs had shorter disintegration time than air-dried ODFs, which is explained by the porous structure of freeze-dried ODFs. The addition of OVA did not significantly influence the disintegration time. In pharmacopoeias no requirements for the disintegration time of ODFs are described. However, all ODFs disintegrated within the time limits mentioned in the Ph. Eur. monograph for orodispersible tablets [22,23].

3.5. Mechanical properties

The effect of trehalose/pullulan ratio and the incorporation of OVA on the mechanical properties of the ODFs were evaluated. OVA was incorporated at a high load (3.2–5.0 wt-%) compared to the lysozyme and β-galactosidase loaded ODFs (0.33 wt-%), to amplify possible effects of protein incorporation on the mechanical properties. Tensile strength, elongation at break and Young’s modulus of the ODFs are shown in Fig. 4. For air-dried ODFs without OVA, a trend towards the decreased tensile strength, elongation at break and Young’s modulus...
with increasing trehalose/pullulan ratios was observed, although differences were not significant in all cases. Incorporation of OVA in the air-dried ODFs resulted in a substantially decreased tensile strength, while elongation at break and Young’s modulus were less affected. Compared to air-dried ODFs, in particular the tensile strength and Young’s modulus for freeze-dried ODFs were much lower, which can be ascribed to their porous structure. The considerable decline of tensile strength and Young’s modulus in freeze-dried ODFs indicate the ODFs were more brittle and less rigid, respectively, than air-dried ODFs. Furthermore, incorporation of OVA in the freeze-dried ODFs hardly affected the mechanical properties.

3.6. Protein stability

3.6.1. Lysozyme

As can be seen from the Fig. 5, the enzymatic activity of lysozyme after both air- and freeze-drying was around 100%. After storage at 30 °C/0% RH for 4 weeks, there was no significant decrease in enzymatic activity of lysozyme incorporated in either air- or freeze-dried ODFs.

For the ODFs stored at 30 °C/33% RH, a slight decrease of the enzymatic activity of lysozyme was seen after storage for 4 weeks, with a remaining activity of around 95%. There was no significant difference.
in enzyme activity when the two drying methods or the trehalose/pullulan ratios were compared. As shown in Table 2, air- and freeze-dried ODFs with a trehalose/pullulan ratio of 0/100 and air-dried ODFs with a trehalose/pullulan ratio of 20/80 stored at 33% RH had Tgs higher than the storage temperature was, (30°C) while the others had lower Tgs. However, no significant differences for lysozyme’s enzymatic activity were found (Fig. 5C and D). It is well known that lysozyme is a relative stable protein. Jovanović et al. [24] incorporated lysozyme in a trehalose or sucrose formulation using superficritical fluid drying. The enzymatic activity of lysozyme immediately after drying was above 95%, independent on the presence of sugars. After one-month storage at 4°C, no significant decrease in enzyme activity was observed. With these results we showed that vitrification of lysozyme is apparently not required for maintenance of its enzymatic activity during storage.

3.6.2. β-galactosidase

As lysozyme was too stable to discriminate between the stabilizing capacities of the different formulations and the two preparation methods, a highly unstable protein was selected, i.e. β-galactosidase [3,4]. Indeed, as shown in Fig. 6, the stability of β-galactosidase both during preparation of the ODFs and during subsequent storage indeed appeared to be considerably less than that of lysozyme. Immediately after preparation, the enzymatic activity of β-galactosidase incorporated in air-dried ODFs with a trehalose/pullulan weight ratio of 0/100 was around 45% and gradually increased with increasing trehalose content to around 75% for ODFs with a trehalose/pullulan weight ratio of 40/60, indicating the beneficial effects of trehalose for protein stability. During storage at 30°C/0% RH, the activity of β-galactosidase decreased in more or less the same rate for all air-dried ODFs. Upon 4 weeks storage, ODFs with a trehalose/pullulan weight ratio of 0/100 had almost completely lost their activity, while in ODFs with trehalose incorporated the remaining activities were all above 30%.

Compared to air-drying, the process stability of β-galactosidase during freeze-drying was much better. This difference may be caused by the fact that during the freeze-drying process the solution is immediately solidified after casting by placing it on a shelf of the freeze dryer which was pre-cooled to −35°C, while during the air-drying process, the casted solution remained for a long time in the liquid or rubbery state. In the liquid or rubbery state, the molecular mobility is high and because the protein concentration gradually increases, degradation reactions, such as aggregation, may easily occur. In addition, also for the freeze-dried ODFs, it was found that the activity of β-galactosidase increased with increasing trehalose content. The storage stability of β-galactosidase in the freeze-dried films, however, was much less than for air-dried ODFs. In all cases the enzymatic activity of β-galactosidase was reduced to below 25% after 2 weeks and negligible after 4 weeks of storage. However, similar to the air-dried films, the storage stability of β-galactosidase clearly increased with increasing amounts of trehalose incorporated in the freeze-dried ODFs.

As compared to the air-dried ODFs, freeze-dried ODFs had a larger specific surface area due to their porous structure. Therefore, assuming a homogeneous distribution of the protein molecules over the matrix, more protein molecules will be situated near or at the surface, which might lead to less protection by the sugars. Hence, β-galactosidase incorporated in freeze-dried ODFs will degrade faster than when it is incorporated in air-dried ODFs. Based on the results, we conclude that air-drying is more suitable to prepare ODFs in order to retain the storage stability of a highly unstable protein such as β-galactosidase.

4. Conclusion

Protein containing trehalose/pullulan based ODFs with fast disintegration properties could successfully be prepared by air-drying and freeze-drying. Based on the small standard deviations found for weight and thickness of the ODFs and enzymatic activity of the protein, we conclude that the ODFs showed a good uniformity of content. Air-dried ODFs are preferred as they exhibited better mechanical properties than freeze-dried ODFs. The mechanical properties of the air-dried ODFs were generally negatively influenced by the incorporation of a protein and by an increase in the trehalose/pullulan ratio but they were still superior to those of the freeze-dried ODFs. Lysozyme exhibited an excellent stability during incorporation in the ODFs and during subsequent storage independent of the drying method and the trehalose/pullulan ratio. For β-galactosidase loaded ODFs, however, both process stability and subsequent storage stability of β-galactosidase were less than that of lysozyme. In addition, both air- and freeze-dried ODFs showed a clear tendency towards increased β-galactosidase activity when the trehalose/pullulan ratio was increased. Furthermore, β-galactosidase incorporated freeze-dried ODFs showed better process stability, while air-dried ODFs showed better storage stability. To the best of our knowledge, this is the first study in which protein stability in ODFs is investigated. Therefore, we conclude that with this study, a step towards the development of ODFs for oral protein delivery has been made.

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