Introduction

Therapeutic protein delivery may occur under unfavorable stress conditions, leading to aggregation or denaturation with unpredictable side effects, such as toxicity or immunogenicity. To mitigate these problems, proteins are often encapsulated into nanoparticles (NP). These carriers are submicron sized colloidal systems prepared from natural or synthetic polymers, suitable to deliver both small and macro-molecules such as proteins on a targeted or localized manner. They are able to further protect proteins from a harsh environment as observed for instance in the gastrointestinal tract due to pH and enzymes effects, and deliver it on a sustained manner avoiding repeated dose administration. Poly(lactic-co-glycolic acid) (PLGA) is one of the most used synthetic polymers on nanoparticles production mainly because of its good sustained release properties, biodegradability, biocompatibility, variable mechanical properties and nontoxic properties. A minimal systemic toxicity is observed on the use of this polymer for drug delivery and biomaterial applications.

As delivery systems, the most important characteristics of nanoparticles are the size, association efficiency (AE) and release profile. Their shape, surface charge and consistency are also important features to control. Since nanoparticles are produced to be administered to the human body and interact with cells, it is imperative to produce nanoparticles with a proper size, shape and surface charge, otherwise severe toxicity problems may occur. From an industrial and economic perspective, the AE is crucial especially in the case of proteins which are expensive products. To control all the discussed features of nanoparticles, different techniques of production may be employed. For hydrophilic molecules such as proteins, the double emulsion solvent evaporation technique is one of the most used methods. This technique of

Effect of cryoprotectants on the porosity and stability of insulin-loaded PLGA nanoparticles after freeze-drying

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Abbreviations: AE, association efficiency; HPLC, high-performance liquid chromatography; PBS, phosphate buffered saline; Pdi, polydispersity index; PEO, polyethylene oxide; PLA, polylactic acid; PLGA, poly(lactic-co-glycolic acid); PLGA-NP, PLGA nanoparticles; PVA, polyvinyl alcohol; SEM, scanning electron microscopy; TEM, transmission electron microscopy; UV, ultraviolet

PLGA nanoparticles are useful to protect and deliver proteins in a localized or targeted manner, with a long-term systemic delivery pattern intended to last for a period of time, depending on polymer bioerosion and biodegradability. However, the principal concern regarding these carriers is the hydrolytic instability of polymer in aqueous suspension. Freeze-drying is a commonly used method to stabilize nanoparticles, and cryoprotectants may be also used, to even increase its physical stability. The aim of the present work was to analyze the influence of cryoprotectants on nanoparticle stability and porosity after freeze-drying, which may influence protein release and stability. It was verified that freeze-drying significantly increased the number of pores on PLGA-NP surface, being more evident when cryoprotectants are added. The presence of pores is important in a lyophilizate to facilitate its reconstitution in water, although this may have consequences to protein release and stability. The release profile of insulin encapsulated into PLGA-NP showed an initial burst in the first 2 h and a sustained release up to 48 h. After nanoparticles freeze-drying the insulin release increased about 18% in the first 2 h due to the formation of pores, maintaining a sustained release during time. After freeze-drying with cryoprotectants, the amount of insulin released was higher for trehalose and lower for sucrose, glucose, fructose and sorbitol comparatively to freeze-dried PLGA-NP with no cryoprotectant added. Besides the porosity, the ability of cryoprotectants to be adsorbed on the nanoparticles surface may also play an important role on insulin release and stability.

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production may be responsible for the formation of pores on the nanoparticles surface due to the evaporation of solvent. These pores may also play a role on protein release rate and on its stability since pores are open pathways for protein denaturation by external factors. The use of nanoparticles formulation has some limitations mainly due to problems related to the integrity of the liquid suspension. Thus, to avoid some stability problems, limitations mainly due to problems related to the integrity of pores may also play a role on protein release rate and on its stability of nanoparticles surface due to the evaporation of solvent. These limitations may be responsible for the formation of pores on the nanoparticles and therefore protein release and stability.

The main objective of this work was to develop a formulation of insulin-loaded PLGA-NP with good physical properties and assess how freeze-drying the formulation with different cryoprotectants may influence nanoparticles stability and porosity which is an important feature on PLGA-NP release properties, constituting also a pathway for protein instability.

**Results**

**Optimization of PLGA nanoparticles formulation.** It was used two different ratios of PLGA (50:50 and 75:25), to produce the insulin-loaded PLGA-NP. Dichloromethane was used to dissolve PLGA in each formulation, as it is completely removable after nanoparticles preparation, thus avoiding its toxicity. It was also used three different surfactants (PVA, Pluronic and Tween) at two different concentrations (1% and 2%) in order to increase nanoparticles stability. Thus, preliminary studies using these different types of PLGA and surfactants, aimed to produce nanoparticles with the lower obtainable mean particle size and higher AE. Furthermore, the surface charge of nanoparticles must be negatively charged due to its polymeric matrix. The obtained results are shown in Table 1. This optimization was performed to obtain the formulation with the best physical-chemical properties to be used on further experiments.

**PLGA nanoparticles characterization.** Regarding the criteria used to select the optimized formulation, the insulin-loaded nanoparticles produced with PLGA 50:50/PVA 2% formulation was selected for further experiments. Such formulation after the optimization tests was produced with a higher concentration of insulin to be used on further experiments, and its physical-chemical properties are shown on Table 2. The physical-chemical properties of unloaded PLGA-NP are also shown. The loading of nanoparticles with insulin, increased the mean particle size up.

| Polymer | Surfactant | Particle size (nm) | PdI | Zeta potential (mV) | Insulin AE (%) |
|---------|------------|--------------------|-----|---------------------|----------------|
| PLGA 50:50 | PVA 1% | 437 ± 4a | 0.37 ± 0.03 | 10.1 ± 2.4e | 61.1 ± 11.4a |
| | PVA 2% | 256 ± 17a | 0.20 ± 0.07 | -13.2 ± 1.8e | 78.9 ± 4.1a |
| | Pluronic 1% | 1289 ± 232e | 0.57 ± 0.06 | 10.8 ± 2.9e | 21.7 ± 15.9a |
| | Pluronic 2% | 426 ± 13a | 0.22 ± 0.04 | -9.6 ± 3.4e | 31.4 ± 7.8p |
| | Tween 1% | 455 ± 23a | 0.21 ± 0.05 | -8.3 ± 0.5e | 0 |
| | Tween 2% | 565 ± 34a | 0.35 ± 0.01 | -8.3 ± 1.8 | 0 |
| | Pluronic 1% | 419 ± 28e | 0.31 ± 0.05 | -12.3 ± 1.5e | 12.9 ± 7.1 |
| | Pluronic 2% | 357 ± 22a | 0.21 ± 0.03 | 13.4 ± 2.3p | 8.7 ± 5.4 |
| | Tween 1% | 374 ± 61a | 0.25 ± 0.15 | 14.7 ± 1.6e | 6.6 ± 2.3 |
| | Tween 2% | 121 ± 12a | 0.49 ± 0.04 | -9.4 ± 2.3p | 7.2 ± 1.1 |

If the formulations were significantly different between them (a ≠ b; c ≠ d; e ≠ f; p < 0.05), differences between formulations were compared using a Tukey’s post hoc test.
to 446 ± 30 and the negative surface charge increased to -24.2 ± 3.4 mV, thus increasing the stability of the colloidal suspension.

As discussed above, the major problem limiting the use of nanoparticles in a colloidal suspension is its physical instability by aggregation and particle fusion or/and the chemical instability due to drug leakage, hydrolysis of the polymer and the chemical reactivity during extended periods of storage. Hence, freeze-drying comes to the front-line as an optimal method to stabilize nanoparticles. However, during freeze-drying in the freezing step, the concentration of the nanoparticles system increases during time, which may induce aggregation and sometimes irreversible fusion of nanoparticles. In addition, the ice crystallization induces a mechanical stress which leads to nanoparticles destabilization, so cryoprotectants must be added to nanoparticles formulation prior freezing to protect and further stabilize nanoparticles.

The produced insulin-loaded PLGA-NP were freeze-dried with and without cryoprotectants added, and its physical-chemical properties was assessed after freeze-drying (Table 3). The presence of cryoprotectants in formulation is important also to avoid aggregation after redispersion of the lyophilizate. In addition, the ice crystallization showed to facilitate the resuspension of polymeric nanoparticles (PEO) nanoparticles after freeze-drying.

The morphology of the obtained nanoparticles may be evaluated through the visualization of its microscopic appearance by TEM and SEM. On one hand, TEM may show us information essentially about the shape of PLGA-NP and SEM may show information about the surface of nanoparticles. However, using SEM microscopy to visualize the surface of nanoparticles with a good definition is very difficult, and focusing the electron beam on such a small area may also damage the nanoparticles. To avoid these drawbacks, during the visualization by SEM particles with the higher particle size that often occur in such formulations were chosen. Therefore, it is possible to visualize the larger particles and infer its morphology and surface features to the produced nanoparticles.

TEM allows the observation of the freeze-dried nanoparticles after their dilution, however the visualization of nanoparticles by SEM is very difficult when the cryoprotectant concentration is more than 5%, since a continuous matrix covering all the nanoparticles may be observed. Therefore, the purification of the performed freeze-dried nanoparticles, in order to remove the cryoprotectant is crucial to be possible to properly visualize the particles by SEM. Insulin-loaded PLGA-NP was visualized after formulation and after freeze-drying with no cryoprotectants added, by TEM (Fig. 1) and by SEM (Fig. 2). They were also visualized after freeze-drying with the cryoprotectants used by TEM and SEM and the results are shown in Figure 3 and 4, respectively.

**Discussion**

Optimization of PLGA nanoparticles formulation. Insulin-loaded PLGA-NPs were produced essentially to take advantage of the better controlled release properties and protection of the encapsulated protein. They were successfully produced by a modified solvent emulsification-evaporation method based on a w/o/w double emulsion technique. A clear advantage of this technique, is the avoidance of thermal or pressure stresses that can damage the structure of the encapsulated protein. Besides the production of polymeric nanoparticles, this technique allows also to produce other kinds of nanoparticles such as lipid-based nanoparticles. The preliminary study to assess the better formulation to proceed to further experiments (Table 1), showed that both PLGA ratios used were able to produce nanoparticles with the exception when PLGA 75:25 was combined with PVA. Thus, it may be concluded that such combination is not able to produce consistent nanoparticles with the methodology used. In addition, PLGA 50:50/Pluronic 1% formulation produced microparticles.
The mean particle size results showed that generally the higher concentrations of the surfactant resulted in a reduction of the particle size, however with just a significant difference (p < 0.05) for PLGA 50:50/Pluronic formulations. This is mainly because the surfactant is able to reduce the surface tension and promote the particle division during homogenization. Then, the decrease in particle size highly increases the surface area, stabilizing the nanoparticles, however, when higher concentrations of surfactants are used, the risk of toxic side effects increases.20 This problem was mitigated by removing the surfactant after nanoparticles production followed by freeze-drying. Possible agglomeration of some nanoparticles formulations can occur, however they showed to be easily resuspended. It was also verified that overall, when the surfactant concentration increased the polydispersity index (PdI) decreased, except for formulations where Tween was used. This may be once more explained by the higher stabilization of higher concentrations of surfactants during nanoparticles production.

The surface charge is an important feature, since nanoparticles are intended to interact with cells, and the charge may play an important role on such interaction. It is well known that PLGA-NP has negative surface charge due to its polymeric matrix.21 This negative surface charge was verified for the majority of the produced formulations, thus being an indicator of a good nanoparticles production. In other formulations the verified surface charge was positive, which may be explained by the maintenance of the surfactant presence on the nanoparticles surface even after nanoparticles purification. It is also shown in Table 1, that for the PLGA 50:50/PVA, PLGA 50:50/Pluronic, and PLGA 75:25/Tween formulations, at a surfactant concentration of 1% the surface charges of nanoparticles were positive and at 2% the surface charges were negative. This is because for those formulations, the higher concentration of surfactant better stabilized the nanoparticles, thus achieving the expected negative values characteristic of the polymeric matrix. Therefore, at a higher concentration of surfactants, the emulsification may have occurred more effectively and faster, forming a good emulsion and nanoparticles formulation. On the other hand, when a lower concentration of surfactant was used, the emulsification process was not so effective and the surfactant remained more attached to the polymer, becoming the surface charge of nanoparticles charged positively.

From an industrial point of view, the AE is a crucial feature since most of the therapeutic proteins encapsulated into nanoparticles are quite expensive. It is possible to conclude that nanoparticles produced with PLGA 75:25 showed that with this polymer ratio it was possible to obtain nanoparticles in a range of 100–450 nm but with low values of AE. Thus, the higher proportion of lactic acid of the polymer may hinder the ability of nanoparticles to encapsulate proteins using this methodology. Essentially, considering this feature the formulations containing PLGA 75:25 were not considered for further experiments. On one hand, formulations using PLGA 50:50/PVA, obtained significantly higher (p < 0.05) AE values for both surfactant concentrations, which was also higher for the higher surfactant concentration. On the other hand, formulations using PLGA 50:50/Tween obtained 0% of AE for both surfactant concentrations, because the surfactant did not promote the emulsification of the secondary emulsion properly, and therefore insulin was not encapsulated. This may be due to a possible interaction between the surfactant and the polymer itself leading to an extrusion of insulin from the inner core of the forming particle. This may be also the explanation why it was achieved such a low AE when Tween 1% and 2% was used to produce PLGA 75:25 nanoparticles.

Regarding all the obtained results, only one formulation of PLGA 50:50 nanoparticles was selected for further experiments because, as reported above, the AE obtained for PLGA 75:25 nanoparticles, even using different surfactants, was not satisfactory. Thus, the formulation produced using PLGA 50:50/PVA 2% was selected for further experiments regarding its lower mean particle size, higher AE and the negatively charged surface due to the polymeric matrix. The mean particle size of these nanoparticles is around 250 nm and the AE is about 80%, which is a good achievement. In fact, the AE of hydrophilic molecules is

![Figure 1. TEM microphotographs of insulin-loaded PLGA-NP after production (A) and after freeze-drying with no cryoprotectant added (B). (A) bar shows 200 nm and (B) bar shows 100 nm.](image-url)
After encapsulating a higher amount of insulin it was obtained an increase on particle size up to 446 ± 30 nm, and the surface charge became more negative which is once more explained by a higher adsorption of insulin on the particle surface. After production, the formulation was freeze-dried with the different used cryoprotectants and after resuspension in water the physical-chemical properties of nanoparticles were assessed (Table 3). A control sample was also performed. It was verified, that after freeze-drying with no cryoprotectant added, the particle size and the zeta potential values remained in the same order of values, a good indicator of an effective freeze-drying cycle. The negative surface charge even increased a little bit which proves the better stability of nanoparticles after freeze-drying. It was also verified, that further adding cryoprotectants prior freeze-drying, the negative surface charge increased more than 1.5-fold for all the cryoprotectants. The trehalose-added sample showed the higher value of -42.9 ± 1.7 mV, which may be due to the adsorption of cryoprotectants on nanoparticles surface. These results prove that the different cryoprotectants have a more stabilizing effect on nanoparticles during freeze-drying. Regarding the particle size values, it was verified that on one hand, adding trehalose and glucose decreased the particle size after freeze-drying respectively to 396 ± 16 nm and 365 ± 28 nm, and on the other hand sucrose, fructose and sorbitol increased the particle size to 559 ± 16, 712 ± 55 and 469 ± 23, respectively. These changes on particle size may be related with the behavior of each cryoprotectant during freeze drying, and the adsorption of cryoprotectants on nanoparticles surface.

As discussed above, the processing conditions employed in the nanoparticles preparation will influence the characteristics of the obtained nanoparticles, as the morphology, size, AE and insulin distribution and such parameters may influence the release of the improved by the double-emulsion technique allowing the encapsulation of therapeutic proteins. The PLGA-NP formulation containing PVA is indeed a good choice, since this surfactant is one of the most used surfactants to produce stable nanoparticles with a small size and a narrow PdI. It was also reported, that a fraction of PVA remains associated with the surface of nanoparticles even after nanoparticles washing. Thus, the presence of the PVA layer on nanoparticles surface may improve also their freezing resistance which stabilizes nanoparticles.

**PLGA nanoparticles characterization.** After the discussed optimization, the selected formulation using PLGA 50:50/PVA 2% was formulated encapsulating a higher amount of insulin (0.2 mL of 150 mg/mL insulin solution) to be used in further experiments. Thus, it was produced loaded and unloaded PLGA-NP and their properties are shown on Table 2. After insulin loading into PLGA-NP, its particle size increased about 1.8-fold in comparison with the unloaded nanoparticles. This may be explained by a higher adsorption of insulin on the particle surface increasing the particle size, which may also explain the increase of the negative charge on the loaded nanoparticles surface up to -24.2 ± 3.4 mV, since at pH 7.4 the charge of insulin is negative. This increase allows the increment of nanoparticles stability in the formulation. Concerning to the AE obtained value, it was observed an AE of 87.4 ± 0.2%, which is a very good achievement. Therefore, even encapsulating a higher amount of insulin, the AE was even higher. This is explained not by a higher amount of insulin, that is encapsulated into nanoparticles because the volume of the aqueous inner core was the same of the preliminary study, but by a higher amount of insulin that is associated with the nanoparticles surface and more electrostatically linked, thus increasing insulin AE. Furthermore, regarding the results shown on Table 1 for the PLGA:PVA 2% formulation, after encapsulating a higher amount of insulin it was obtained an increase on particle size up to 446 ± 30 nm, and the surface charge became more negative which is once more explained by a higher adsorption of insulin on the particle surface. After production, the formulation was freeze-dried with the different used cryoprotectants and after resuspension in water the physical-chemical properties of nanoparticles were assessed (Table 3). A control sample was also performed. It was verified, that after freeze-drying with no cryoprotectant added, the particle size and the zeta potential values remained in the same order of values, a good indicator of an effective freeze-drying cycle. The negative surface charge even increased a little bit which proves the better stability of nanoparticles after freeze-drying. It was also verified, that further adding cryoprotectants prior freeze-drying, the negative surface charge increased more than 1.5-fold for all the cryoprotectants. The trehalose-added sample showed the higher value of -42.9 ± 1.7 mV, which may be due to the adsorption of cryoprotectants on nanoparticles surface. These results prove that the different cryoprotectants have a more stabilizing effect on nanoparticles during freeze-drying. Regarding the particle size values, it was verified that on one hand, adding trehalose and glucose decreased the particle size after freeze drying respectively to 396 ± 16 nm and 365 ± 28 nm, and on the other hand sucrose, fructose and sorbitol increased the particle size to 559 ± 16, 712 ± 55 and 469 ± 23, respectively. These changes on particle size may be related with the behavior of each cryoprotectant during freeze drying, and the adsorption of cryoprotectants on nanoparticles surface.

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lead to protein denaturation. This loss of protein structure may occur in different extents depending on the different nanoparticles used, since they may have different porosity. In addition, the influence of the different cryoprotectants used on nanoparticles porosity after freeze-drying which affects protein release rate and ultimately its stability is not well established.

The Figure 1 shows the microscopic appearance by TEM of PLGA-NP, produced using PLGA 50:50/PVA 2% after protein. The nanoparticle porosity, for instance, plays an important role on insulin release since a large amount of pores may increase the rate of protein release. The porosity of nanoparticles may be determined by its hardening during the evaporation of dichloromethane in the preparation process and by the rate of its evaporation, or even by the temperature of nanoparticles preparation. Furthermore, porosity may also influence insulin structure, since pores may open a pathway to external factors that can lead to protein denaturation. This loss of protein structure may occur in different extents depending on the different nanoparticles used, since they may have different porosity. In addition, the influence of the different cryoprotectants used on nanoparticles porosity after freeze-drying which affects protein release rate and ultimately its stability is not well established.

Figure 1. TEM microphotographs of insulin-loaded PLGA-NP after freeze-drying with: 10% (w/w) trehalose (A); 10% (w/w) sucrose (B); 10% (w/w) glucose (C); 10% (w/w) fructose (D) and 10% (w/w) sorbitol (E). [A and B] bar shows 100 nm, [C] bar shows 50 nm and [D and E] bar shows 100 nm.

Figure 3. TEM microphotographs of insulin-loaded PLGA-NP after freeze-drying with: 10% (w/w) trehalose (A); 10% (w/w) sucrose (B); 10% (w/w) glucose (C); 10% (w/w) fructose (D) and 10% (w/w) sorbitol (E). [A and B] bar shows 100 nm, [C] bar shows 50 nm and [D and E] bar shows 100 nm.
Figure 4. SEM microphotographs of insulin-loaded PLGA-NP after freeze-drying with: 10% (w/w) trehalose (A); 10% (w/w) sucrose (B); 10% (w/w) glucose (C); 10% (w/w) fructose (D) and 10% (w/w) sorbitol (E) (bar shows 30 μm).
The presence of such depressions and pores may be also confirmed by SEM in Figure 2A. By the analysis of Figure 3, it is noticed that nanoparticles freeze-dried with trehalose (Fig. 3A) and glucose (Fig. 3C) added, better maintained the nanoparticles morphology regarding the shape and presence of such depressions where pores may be found. It is also shown in Figure 4, that freeze-drying the nanoparticles significantly increases the number of pores on nanoparticles surface. This is because, after solidification of all solutes and water, the ice-vapor is evacuated and the shelf temperature increases supplying energy for sublimation, and thus beginning the primary drying. The elimination of the ice crystals by sublimation creates an open network of pores, which are pathways for water removal from nanoparticles.  

Figure 5. Cumulative release profile of insulin from PLGA-NP after formulation (solid line) and after freeze-drying with no cryoprotectant added (dotted line). (n = 3, bars represent SD).

Figure 6. Cumulative release profile of insulin from PLGA-NP after freeze-drying with: no cryoprotectant (dotted line), 10% (w/w) trehalose (circle); 10% (w/w) sucrose (square); 10% (w/w) glucose (triangle); 10% (w/w) fructose (diamond) and 10% (w/w) sorbitol (dash) added. (n = 3, bars represent SD).

production (Fig. 1A) and after freeze-drying (Fig. 1B). It is noticed that nanoparticles exhibited a spherical shape and a smooth surface, most probably due to the polymeric matrix. These characteristics are further maintained after freeze-drying with no cryoprotectant added (Fig. 1B) and with cryoprotectants added (Fig. 3). Once more this maintenance of nanoparticles morphology after freeze-drying proves that the freeze-drying step using or not cryoprotectants was effective on nanoparticles stabilization.

The presence of cryoprotectants on nanoparticles even after purification, may be noticed in Figure 3 that gives an aspect that nanoparticles are embedded in the sugar solution. In Figure 1A it is possible to see white circles on nanoparticles, which may be some depressions on nanoparticles surface where some pores may occur. The presence of such depressions and pores, may be also confirmed by SEM in Figure 2A. By the analysis of Figure 3, it is noticed that nanoparticles freeze-dried with trehalose (Fig. 3A) and glucose (Fig. 3C) added, better maintained the nanoparticles morphology regarding the shape and presence of such depressions where pores may be found. It is also shown in Figure 4, that freeze-drying the nanoparticles significantly increases the number of pores on nanoparticles surface. This is because, after solidification of all solutes and water, the ice-vapor is evacuated and the shelf temperature increases supplying energy for sublimation, and thus beginning the primary drying. The elimination of the ice crystals by sublimation creates an open network of pores, which are pathways for water removal from nanoparticles.  

This increase in nanoparticle porosity after freeze-drying
with cryoprotectants added, may open a pathway for a faster insulin release from nanoparticles and for its degradation by external factors. The freezing step may also influence the porosity of nanoparticles, since this step may affect the morphological characteristics of the freeze-dried cake.27 Therefore, the freezing step influences the size of ice crystals and subsequently the drying steps and thus, the surface of nanoparticles and porosity of the final cake strongly depends on it. The presence of such pores are really necessary on a lyophilicate, since the absence of a porous structure such as may happen in a collapsed cake, becomes the reconstitution of the lyophilizate very hard to accomplish.28,29

Both the higher release rate and insulin degradation may be mitigated by the adsorption of the cryoprotectant on nanoparticles surface obstructing some of the formed pores. This is particular evident in Figure 4A–C.

Evaluation of insulin in vitro release from nanoparticles.

In Figure 5, it is shown the cumulative release profile of insulin from nanoparticles in 48 h, after formulation and after freeze-drying with no cryoprotectant added. The release pattern for both samples is very similar with an initial burst release within the first 2 h and a sustained release pattern until the 48 h, which is characteristic of the PLGA-based nanoparticles.16 The initial burst release may be explained by the release of insulin which is adsorbed on nanoparticles surface, and then the encapsulated insulin is released during time, achieving a sustained release pattern.

Thus, the PLGA-NP after formulation released about 57% of the insulin in the first 2 h, and maintained a sustained release achieving 65% of insulin released after 48 h. On the other hand, PLGA-NP after freeze-drying with no cryoprotectant added released about 75% of insulin in the first 2 h, which is an increment of 18% of insulin released comparatively to nanoparticles after formulation. Such increment of insulin release may be related to the increase of the porosity of the cake after freeze-drying, or even by the possible increase of pores size of nanoparticles as may be visualized in Figure 3B. Thereby, a method to precisely assess the diameter of nanoparticles pores is required to support this possibility. These freeze-dried nanoparticles further released about 86% of insulin after 48 h.

Regarding the release patterns obtained for cryoprotectant added samples shown in Figure 6, it is possible to conclude that the release pattern of insulin remained quite the same due to the polymeric matrix, with an initial burst release in the first 2 h and a sustained release pattern until 48 h. Comparatively to the freeze-dried sample with no cryoprotectant added, the sample with trehalose added achieved a higher released amount of insulin while samples with the other used cryoprotectants added led to a lower amount of insulin released. In fact, trehalose added nanoparticles released almost 91% of the insulin amount in the first 2 h, releasing up to 96% of insulin after 48 h. This may be due to the increase of the porosity of nanoparticles, as stated in Figure 4A and to trehalose properties as cryoprotectant. In fact, it was reported that trehalose seems to be the best cryoprotectant for biomolecules, due to its many advantages comparatively with the other sugars. Such advantages are a higher glass transition temperature Tg, less hygroscopicity and the absence of internal hydrogen bounds which during freeze-drying, allows a more flexible formation of hydrogen bonds with nanoparticles.30 Due to these more flexible bonds, trehalose is removed more easily from nanoparticles surface and insulin is released in a higher amount.

Even increasing the porosity after freeze-drying, the others cryoprotectant added samples showed a lower insulin released amount, mainly due to the presence of the sugars on the nanoparticles surface, which may obstruct the pores leading to a lower release. Indeed, cryoprotectants may stabilize nanoparticles during the drying steps due to the formation of hydrogen bonds between the cryoprotectant and the polar groups, at the nanoparticles surface at the end of the drying step.31 Therefore, it is harder to remove these cryoprotectants from the nanoparticles surface and the insulin release rate is lower. The glucose-added nanoparticles sample released the lowest amount of insulin with just almost 30% released in the first 2 h, which is less 45% comparatively to the non-added cryoprotectant sample, and releasing just 44% of the total amount of insulin after 48 h.

This result shows that the porosity of the nanoparticles and the presence of cryoprotectants which are sugars on nanoparticles surface may affect the release of insulin from nanoparticles. This is particularly important because the increase of the porosity of nanoparticles after freeze drying may open a pathway for insulin release and simultaneously for insulin degradation by external factors. On the other hand, the cryoprotectants bonding to nanoparticles surface may obstruct nanoparticles pores leading to a lower insulin release rate.

Materials and Methods

Materials. For the production of nanoparticles, PLGA 50:50 (Evonik Industries AG, Resomer® RG 503 H), PLGA 75:25 (Purac Biomaterials, Purasorb® PD LG 7502), PVA (Sigma-Aldrich, P1763), Pluronic® F-127 (Sigma-Aldrich, P2443), Tween® 80 (Merck, 822187), dichloromethane (Sigma-Aldrich, 91077C) were used. The cryoprotectants used were trehalose (Fluka Analytical, 90210), sucrose (Fluka Analytical, 84100), fructose (Fluka Analytical, 47740), glucose (Fluka Analytical, 49152) and sorbitol (Fluka Analytical, 97336). Acetonitrile HPLC Gradient Grade (Fischer Scientific, A/0627/17) and trifluoroacetic acid (Acros Organics, 139721000) were used in the HPLC measurements and phosphate buffered saline (PBS) (Sigma-Aldrich, P4417) was used in the in vitro release study. MilliQ-water was produced in-house.

Preparation of PLGA nanoparticles. Different formulations of nanoparticles were prepared with PLGA 50:50 and PLGA 75:25. For each polymer used to produce the nanoparticles, different types of surfactants at two different concentrations were used, namely Polysine Alcohol (PVA, 1% and 2%), Pluronic (1% and 2%) and Tween (1% and 2%). The chosen method for the nanoparticle preparation was a modified solvent evaporation method based on a w/o/w double emulsion technique.32,33 Briefly, 200 mg of polymer was dissolved in 2 ml of dichloromethane. Then, 0.2 mL of a 20 mg/mL insulin solution in HCl 0.1 M was added to the polymeric solution, and
homogenized using a Bioblock vibracell 75186 sonicator (Fischer Bioblock Scientific), during 30 sec with 70% of amplitude. This primary emulsion was poured into 25 mL of each PVA, Pluronic or Tween solution at the two different concentrations and then homogenized for 30 sec using the same equipment. The organic solvent was then removed by evaporation during 3 h under magnetic stirring. The produced nanoparticles were purified three times by centrifugation using a Heraeus Megafuge 1.0 R centrifuge (Thermo Scientific) at 4300 rpm for 50 min, and redispersed in water before storage at 4°C for further analysis.

After formulation optimization, the optimal formulation was produced by the same methodology using PLGA 50:50 and PVA 2% as a surfactant and 0.2 mL of a 150 mg/mL insulin solution in HCl 0.1 M. The produced nanoparticles were then purified three times by centrifugation at 4300 rpm for 50 min at 4°C, and redispersed in water prior to freeze-drying and storage.

**Freeze-drying of nanoparticles.** The effects of five cryoprotectants on the stability of PLGA-NP were assessed. The cryoprotectants used were trehalose, sucrose, fructose, glucose and sorbitol at a concentration of 10% (w/w). A control group of nanoparticles freeze-dried with no cryoprotectant was also included in study, and all the different cryoprotectant conditions were performed in triplicate. Samples were poured into semi-stoppered glass vials with slotted rubber closures and frozen at -80°C for 2 h and then were transferred to a Modulyo 4K freeze-dryer (Edwards, Crawley) at 0.09 mbar for 72 h, being maintained at the condenser surface temperature of -60°C.

**Freeze-dried samples reconstitution.** After freeze-drying, the samples were reconstituted by injecting slowly distilled water in the inside wall of the vial, and then it was maintained during 10 min to ensure the proper cake wetting. After such period of time, samples were gently shaken in a Vortex Mixer ZX Classic (Velp Scientifica) for 3 min to completely homogenize the samples. After reconstitution, samples were physico-chemically characterized.

**Particle size and zeta potential analyses.** Samples were diluted with MilliQ-water to a suitable concentration for both particle size and zeta potential analyzes. Particle size was analyzed by dynamic light scattering using a 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation.). The zeta potential was determined by phase analysis light scattering using a ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments Corporation). All measurements were performed in triplicate.

**Insulin association efficiency.** The AE was determined indirectly. The amount of insulin entrapped into the PLGA-NP was calculated by the difference between the total amount used to prepare the systems, and the amount of insulin that remained in the aqueous phase, after nanoparticles isolation by ultracentrifugation in a Beckman Optima TL ultracentrifuge (Beckman Coulter) at 20,000 rpm for 15 min at 4°C. The following equation summarizes this concept:

\[
AE = \frac{\text{Total amount of insulin} - \text{Free insulin in supernatant}}{\text{Total amount of insulin}} \times 100
\]

The insulin concentration in the supernatant was then determined by a HPLC-UV method previously developed and validated by our group. Thus, the measurements were performed on a Merck-Hitachi LaChrom HPLC instrument (Merck) equipped with an XTerra RP 18 column, 5 μm particle size, 4.6 mm internal diameter × 250 mm length (Waters) and a LiChrospher 100 RP-18, 5 μm particle size guard column (Merck). The sample was run in triplicate.

**Transmission electron microscopy.** Nanoparticles were observed by transmission electron microscopy (TEM), in order to characterize its morphology. Samples were placed on a grid, treated with uranyl acetate and then observed in a JEOL JEM-1400 Electron Microscope (JEOL Ltd.).

**Scanning electron microscopy.** The surface morphology of nanoparticles was observed by scanning electron microscopy (SEM) on a FEI Quanta 400 FEG SEM (FEI). Nanoparticles were resuspended and purified three times with distilled water by ultracentrifugation in a Beckman Optima TL ultracentrifuge (Beckman Coulter) at 20,000 rpm for 15 min at 4°C, to remove the dissolved cryoprotectant. Then, samples were mounted onto metal stubs and vacuum-coated with a layer of gold/palladium before observation in the SEM microscope.

**Insulin in vitro release study.** Insulin-loaded PLGA-NP were dispersed in 20.0 mL of pH 7.4 phosphate buffered saline (PBS) solution and incubated at 37°C under magnetic stirring at 100 rpm. Samples were taken at predetermined time intervals of 30 min, 1, 2, 4, 8, 24 and 48 h and replaced with fresh medium maintained at the same temperature. The collected samples were ultracentrifuged, and the content of insulin in the supernatant was determined by the described HPLC methodology. All samples were run in triplicate.

**Statistical analysis.** All the performed statistical analysis was done using the GraphPad Prism Software vs. 5.0 (GraphPad Software Inc.), and differences between the formulations were compared within a Tukey post hoc test, and considered to be significant at a level of p < 0.05.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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