

Polyethylene Glycol-Based Low Generation Dendrimers Functionalized with β -Cyclodextrin as Cryo- and Dehydro-Protectant of Catalase Formulations

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Polyethylene glycol (PEG)-based low generation dendrimers are analyzed as single excipient or combined with trehalose in relation to their structure and efficiency as enzyme stabilizers during freeze-thawing, freeze-drying, and thermal treatment. A novel functional dendrimer (DG_o-CD) based on the known PEG's ability as cryo-protector and β -CD as supramolecular stabilizing agent is presented. During freeze-thawing, PEG and β -CD failed to prevent catalase denaturation, while dendrimers, and especially DG_o-CD, offered the better protection to the enzyme. During freeze-drying, trehalose was the best protective additive but DG_o-CD provided also an adequate catalase stability showing a synergistic behavior in comparison to the activities recovered employing PEG or β -CD as unique additives. Although all the studied dendrimers improved the enzyme remaining activity during thermal treatment of freeze-dried formulations, the presence of amorphous trehalose was critical to enhance enzyme stability. The crystallinity of the protective matrix, either of PEG derivatives or of trehalose, negatively affected catalase stability in the freeze-dried systems. When humidified at 52% of relative humidity, the dendrimers delayed trehalose crystallization in the combined matrices, allowing extending the protection at those conditions in which normally trehalose fails. The results show how a relatively simple covalent combination of a polymer such as PEG with β -CD could significantly affect the properties of the individual components. Also, the results provide further insights about the role played by polymer–enzyme supramolecular interactions (host–guest crosslink, hydrogen bonding, and hydrophobic interactions) on enzyme stability in dehydrated models, being the effect on the stabilization also influenced by the physical state of the matrix. © 2013 American Institute of Chemical Engineers Biotechnol. Prog., 000:000–000, 2013

Keywords: dendrimer, enzyme stability, β -cyclodextrin, dehydration, freezing and thawing, PEG, trehalose, catalase

Introduction

The low resistance of enzymes to processing conditions, i.e. freezing, drying, and thermal treatment have often limited the use of these proteins at the industrial level.¹

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Important efforts based on modifications of the protein surface (such as immobilization on solid supports,² chemical,^{3,4} enzymatic,⁵ and chemoenzymatic,⁶ among others) have been reported to improve enzyme thermo-stability. However, from the industrial point of view, the use of hydro-soluble additives appears as one of the most promising approaches, considering the ease and low cost of this procedure.¹ A common approach for increasing protein stability is to dehydrate or freeze proteins in the presence of polyols, where trehalose is one of the most used excipients.^{1,7-9} The stability of catalase in sugar freeze-dried matrices was previously studied, and trehalose proved to be one of the most effective protectors against both drying and prolonged heating.⁸ The action of trehalose can be ascribed to both kinetic level and specific interactions. At the kinetic level, it promotes the formation of amorphous systems. At the specific-interaction level, trehalose interacts by hydrogen bonding with biological structures thereby stabilizing them during drying.^{1,7-9} The higher proportion of unfrozen water of this disaccharide (3.98 mol H₂O/mol monosaccharide) in comparison with other saccharides can also have a beneficial effect during freezing and dehydration.¹⁰

A less frequent approach for increasing protein stability in dehydrated formulations is the use of cyclodextrins. β -Cyclodextrin (β -CD) is a cyclic non-reducing oligosaccharide composed of seven glucopyranose units bonded together via α (1-4) glycoside linkages, with a hydrophobic central cavity and a hydrophilic outer surface.¹¹ CDs are capable of including a variety of hydrophobic guest compounds such as aromatic amino acids located at the protein's surface.^{3,4} CD were previously used to modified polymers to act as protein stabilizers in freeze-dried formulations.^{1,9} A synthesized β -CD-branched alginate combined the physical stability provided by alginate with the protective effect on hydrophobic regions of the enzyme given by cyclodextrin.¹ A polycyclodextrin polymer was adequate to protect invertase during freeze-drying, and also provided protection of the enzyme under super-cooled conditions where trehalose crystallizes.⁹

Dendrimers are spheroid or globular nanostructures that are precisely engineered to encapsulate molecules in their interior void spaces or carry them attached to the surface.¹² Dendrimers are produced in an iterative sequence of reaction steps, in which each additional iteration leads to a higher generation material. Size, shape, and reactivity are determined by generation (shells) and chemical composition of the core, interior branching, and surface functionalities.¹² As a result, dendrimers are perfectly monodisperse macromolecules with a regular and highly branched three-dimensional architecture with surface functionalities.

In the past few years, research has focused in the so-called functional dendrimers, which are developed with particular core and surface functionalities according to the application (e.g., drug delivery, DNA or RNA transport, host-guest chemistry, and catalysis). Dendrimers have been used as steroids receptors,¹³ to mimic globular protein (for their spherical shape),¹³ and can act as selective pigment extractors.¹⁴ Some dendrimers were functionalized with α -, β -, or γ -cyclodextrin¹⁵ and were used for gene transfection.^{15,16} Evidence on the applications of dendrimers as enzyme stabilizers on freeze-dried formulations for potential food and pharmaceutical applications are scarce.

Catalase from bovine liver, an intracellular tetrameric hemoprotein, can be easily destabilized by freezing and drying where functionality is lost as a consequence of even

slight conformational changes.^{8,17} Catalase thus serves as a good model for analyzing the effectiveness of cryo- and dehydro-protectants. Moreover, the potential application of catalase as an antioxidant in the therapy of several diseases mediated by reactive oxygen species¹⁸ provides an impetus to search for stabilizing media.

Polyethylene glycol (PEG) is usually used as an adequate cryo-protective excipient of enzymes,¹⁹ but it is not efficient as a dehydro-protectant. Santagapita et al.^{1,9} demonstrated that β -CD was not adequate alone as a dehydro-protectant of enzymes during freeze-drying, but its efficiency can be enhanced by polymerization (poly-CD) or by covalent modification with polymers (like alginate- β -CD). Therefore, the modification of a PEG-based dendrimer with β -CD moieties could be an attractive alternative to protect enzymes during both freezing and drying (especially, for freeze-drying).

In the present work, PEG-based low generation dendrimers and one functionalized with β -CD were analyzed as single excipient or combined with trehalose in relation to their efficiency as catalase stabilizers for frozen and dried formulations.

Experimental Section

Materials

The following materials were used: PEG 6000, $M_w = 6,000 \text{ g mol}^{-1}$, (Sigma-Aldrich, St. Louis, MO); β -CD (Amaizo, Hammond, IN); α - α -trehalose dihydrate (T), (Hayashibara Co, Ltd., Shimoishii, Okayama, Japan/Cargill Inc., Minneapolis, MN); (2-hydroxypropyl)- β -CD (HP- β -CD), $M_w = 1,380 \text{ g mol}^{-1}$, with a substitution degree of 0.6 (Sigma-Aldrich); catalase (H₂O₂:H₂O₂ oxidoreductase, E.C. 1.11.1.6) from bovine liver (Sigma-Aldrich), $M_w = 225 \text{ kDa}$; optimum pH: 7.4. One unit of activity was defined as the amount of enzyme required to hydrolyze 1.0 μmol of H₂O₂ per minute at pH 7.4, at 25°C, meanwhile the H₂O₂ concentration decays from 10.3 to 9.2 mM measured by the rate of decline of absorbance at 240 nm.

Synthesis of dendritic structures

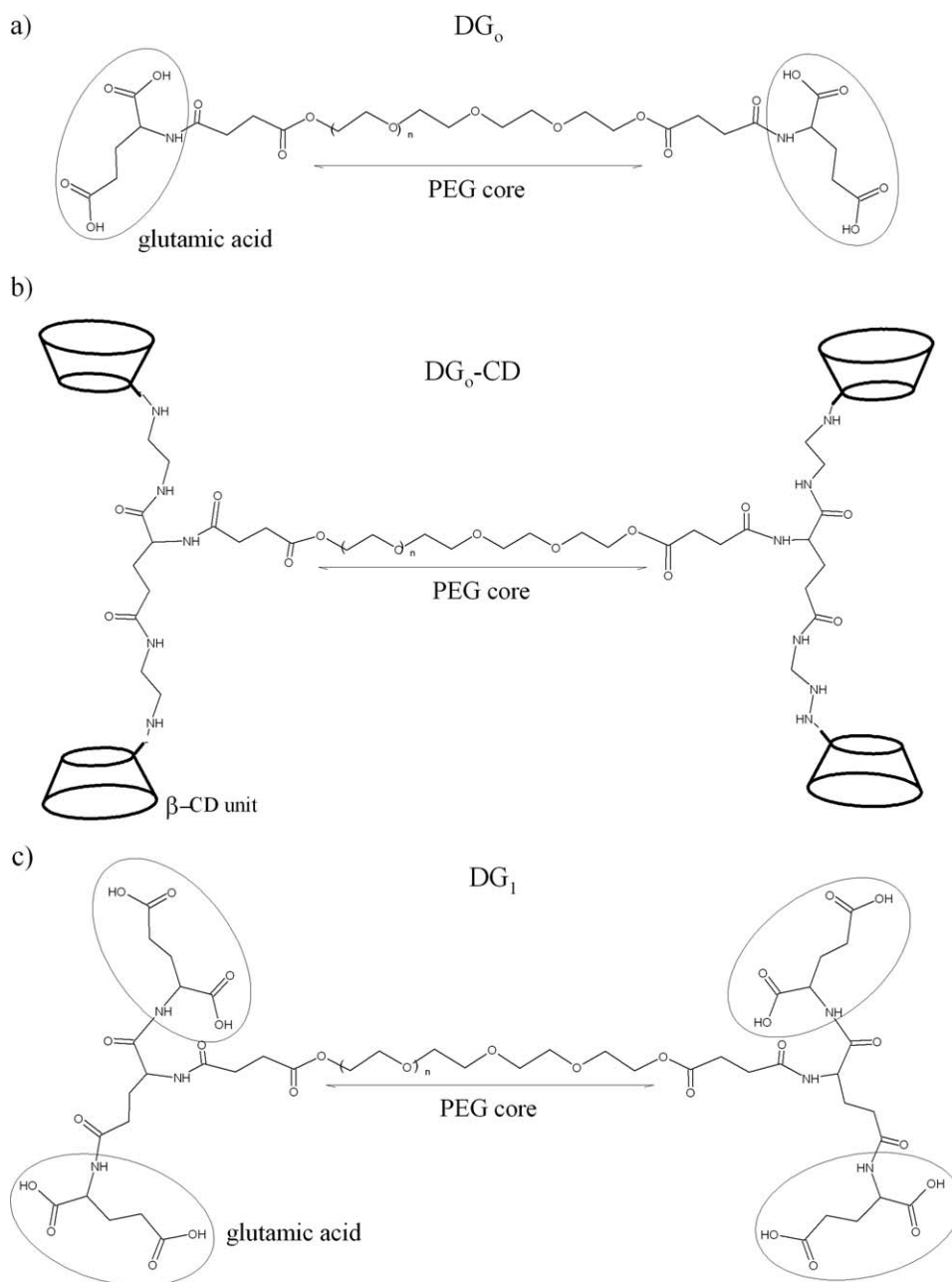
PEG 6000 was used as a core, and glutamic acid was used to divergently grow the dendrimer, which implies that the synthesis was performed from the core to the periphery. Three dendrimers were synthesized: PEG-based dendrimer of generation 0 and 1 (DG₀ and DG₁, respectively) and dendrimer DG₀ functionalized with (etano-1,2-diamine)- β -CD (DG₀-CD), whose structures are shown in Scheme 1.

Synthesis of precursor

Briefly, the synthesis of the precursor was performed by mixing 15 g of PEG and 0.63 g of succinic anhydride (SA) (1:2.5 molar ratio) in 10 mL of anhydrous dimethylformamide for 3 h at 100°C and PEG-SA was obtained. Once cold, the solution was precipitated in 40 mL of diethyl ether, washed with the same solvent and then was dried under vacuum. The yield was 93%.

Synthesis of DG₀ and DG₁

DG₁ was obtained by replacing OH groups of PEG-SA with *N*-hydroxy-succinimide/dicyclohexylcarbodiimide (NHS/DCC) in 30 mL of chloroform (1:2 PEG:NHS/DCC



Scheme 1. Dendrimers structures.

The size of the β -CD ($C_{42}H_{70}O_{35}$) is not on scale respect to the DG_0 .

molar ratio) and then glutamic acid was added. Briefly, 6 g PEG-SA was mixed with 0.26 g of NHS in 15 mL of chloroform under constant agitation for 20 min. Then, a DCC solution (0.46 g of DCC was previously dissolved in 3 mL of chloroform) was dropped, with continuous stirring for 1 h. The final solution was filtered twice on a fritted funnel no. 4 in order to eliminate the DCC-urea precipitate, and the chloroform of the supernatant was evaporated in a rotavapor at 45°C. Then, the sample was resuspended in 5 mL of 0.1 M potassium phosphate buffer pH 7.8 and a glutamic acid solution (0.52 g of glutamic acid were previously dissolved in 16 mL of water and NaOH was added until complete dissolution was achieved) was added. The reaction was kept overnight at 4°C. The glutamic acid excess was eliminated by dialysis with a 1,000 Da membrane, and then water was eliminated in a rotavapor at 50°C. The final product (DG_0) was precipitated in diethyl ether, and dried under vacuum.

About 1 g of DG_0 was obtained (the yield was 16%). DG_1 (0.07 g) was obtained by repeating the previous procedure using DG_0 (0.8 g). The yield was 9%.

Synthesis of β -cyclodextrin-ethylenediamine- G_0 dendrimer ($DG_0\text{-CD}$)

Mono-6-(ethylenediamine)-6-deoxy- β -CD was previously obtained by treating mono-6-*O*-tosyl- β -CD with ethylenediamine in dimethylformamide.^{1,20,21} To introduce the β -CD derivative into the functional group of the dendrimer, 220 mg of β -CD derivative and 200 mg of DG_0 were dissolved in 5 mL of distilled water and then 46 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were added. It is important to keep in mind that the $COOH:NH_2$ ratio was 1:1 and the DG_0 have four $COOH$ groups. The solution was stirred for 16 h at 25°C and then dialyzed against distilled

H₂O for 1 day in a 1,000 Da membrane. Finally, the solution was frozen at -20°C for 24 h and freeze-dried in a Heto Holten A/S, cooling trap model CT 110 freeze-dryer (Heto Lab Equipment, Denmark) operating at a condenser plate temperature of -111°C and a chamber pressure of 4×10^{-4} mbar. The secondary drying was performed at 25°C . The final product β -CD-ethylenediamine-DG₀ (DG₀-CD) was obtained as a powder (0.2 g). The yield related to PEG backbone was 54%.

Preparation and thermal treatment of solid systems

Solid systems consisted of freeze-dried solutions containing 1% (wt/vol) of the dendrimer (DG₀, DG₁, or DG₀-CD) or of the unmodified excipients (PEG, β -CD, and HP- β -CD) or their blends with 5% (wt/vol) of trehalose; in all systems the enzyme catalase was added to a final concentration of 0.042 mg mL⁻¹. Aliquots of 0.1 mL of each model system were placed in micro-centrifuge tubes, frozen for 24 h in a conventional freezer (-20°C) and subjected to immersion in liquid nitrogen immediately before freeze-drying. After freeze-drying the samples were transferred to vacuum desiccators and some of them were exposed to relative humidity (RH) of 52% (saturated solution of Mg(NO₃)₂) at $(25 \pm 1)^{\circ}\text{C}$ for one week.²² Then, all the samples were hermetically sealed and some freeze-dried samples were stored at $(55 \pm 1)^{\circ}\text{C}$ in a forced air convection oven. At suitable interval, two samples were removed from the oven and the remaining activity of the enzyme was determined as describe below.

Freezing-thawing treatment in liquid systems

Liquid systems were prepared containing 1% (wt/vol) of the dendrimer (DG₀, DG₁, or DG₀-CD), the unmodified excipients (PEG, β -CD, and HP- β -CD), or their blends with 5% (wt/vol) of trehalose (T), with a final catalase concentration of 0.042 mg mL⁻¹. Aliquots of 0.1 mL of each model system were placed in micro-centrifuge tubes and were subjected to three successive cycles of freezing for 24 h at -20°C and thawing at 4°C .

Catalase activity

Catalase activity was measured spectrophotometrically by determining H₂O₂ decomposition as a function of time, which follows first-order kinetics.²³ An absorbance measurement at 240 nm was taken every 15 s for 4 min and the slope of the linear regression was calculated from the log (Abs) vs. time plot. The samples were resuspended in 0.1 mL of 50 mM sodium phosphate buffer pH 7.4 and were kept at 5°C until complete dissolution was achieved. The enzymatic activity of catalase was determined by adding 0.20 mL of H₂O₂ solution (190 mM in 50 mM sodium phosphate buffer pH 7.4) and 1.78 mL of the buffer to 0.02 mL of the dissolution sample. The samples were stirred by tube inversion prior to starting the measurement. For each system, the H₂O₂ decomposition rate after a given time of thermal treatment (*S*) was related to the corresponding rate before the thermal treatment (*S*₀) and the remaining activity (RA) was expressed in percentage as: $\text{RA} = 100 S/S_0$. Duplicate measurements were performed for each analysis.

In order to evaluate the effect of the different additives on enzyme stability after a certain treatment, an activity index was calculated as shown in Eq. (1):

$$\text{Activity Index} = \frac{(\text{RA})_i}{(\text{RA})_{w/a}} \quad (1)$$

where (RA)_i is the remaining enzyme activity in any of the studied systems with additives, and (RA)_{w/a} is the remaining activity without additives.

Degree of trehalose or PEG crystallization

Endothermal peak areas (related to the amount of crystalline sugar or PEG in the freeze-dried systems) were determined by differential scanning calorimetry (DSC) by means of a Mettler Toledo 822 DSC (Mettler Toledo AG, Switzerland) and STARe Thermal Analysis System version 3.1 software (Mettler Toledo AG). The melting of crystalline trehalose dihydrate or crystalline PEG present in a sample was observed as an endothermic peak at around 97°C ,⁹ or 55°C ,²⁴ respectively. The instrument was calibrated using standard compounds (cyclopentane and indium) of defined melting point and heat of melting. All measurements were made in duplicate with 5–10 mg sample mass, using hermetically sealed aluminum pans of 40 μL inner volume (Mettler), heated from 0°C to 130°C at $10^{\circ}\text{C min}^{-1}$; an empty pan was used as a reference. The confidence intervals estimated for temperature and enthalpy values were 2°C and 10 mJ, respectively.

In systems containing trehalose, the degree of trehalose dihydrate crystallization (ϕ) was calculated from the ratio of the area of the endothermic melting peak in the sample thermogram (ΔH_m) and the melting enthalpy of pure trehalose dihydrate (ΔH_{mT} , 139 J g^{-1}) measured by DSC in the same conditions, as shown in Eq. (2):

$$\phi = \frac{(\Delta H)_m}{(\Delta H)_{mT}} \quad (2)$$

In systems containing PEG, the crystallization degree was calculated by using Eq. (2), but taking into account the melting enthalpy of pure PEG ($\Delta H_{mPEG} = 188 \text{ J g}^{-1}$) as measured by DSC under the same conditions.

Dendrimer characterization

Nuclear Magnetic Resonance. The DG₀-CD, DG₀, and DG₁ dendrimers and PEG (core) in solution of 20 mg/mL in D₂O at 25°C , were characterized by ¹H-NMR (500 MHz Bruker equipment (Bruker Optics, Rheinstetten, Germany)). The experiments were conducted by using a Bruker's defined pulse program (zg30), the acquisition mode was DQD (digital quadrature detection), the number of scans and dummy scans were 16 and 2, respectively, the size of the free induction decay was 65,536, the spectral width was 15 ppm, and the acquisition time was around 4.4 s. The spectra were analyzed by TopSpin 2.0.b.5 software (Bruker Biospin, Germany) with exponential apodization (0.1 Hz line broadening was used). A standard Fourier transform was performed (32,768 points) and the phase and baseline were corrected as required in each case.

Particle Size Determination. Dynamic light scattering (DLS) measurements were carried out in a Zetasizer Nano-Zs (Malvern Instruments, Malvern, UK) provided with a He-Ne laser (633 nm) and a digital correlator (model ZEN3600, Malvern Instruments). Measurements were carried out in a range between 0.6 nm and 6 mm at a fixed scattering angle

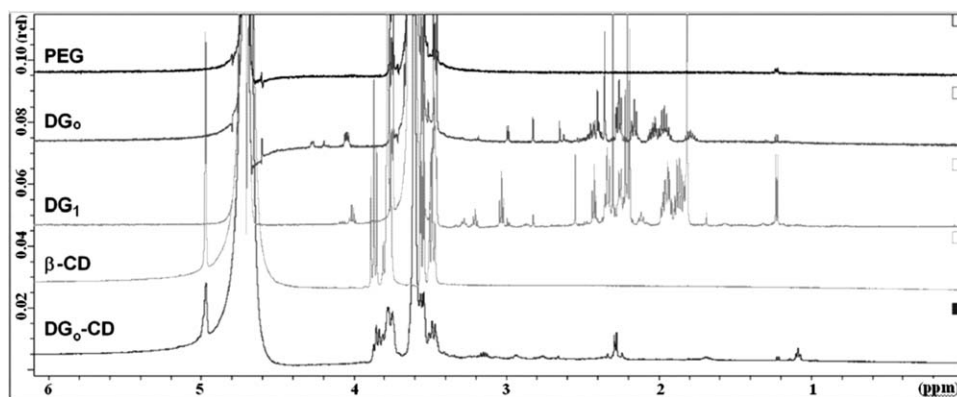


Figure 1. ^1H -NMR spectrum for dendrimers ($\text{DG}_0\text{-CD}$, DG_0 , DG_1) and PEG (dendrimer's core).

All spectra are at the same scale.

of 173° and at 25°C (holding by at least 10 min before measurement). Samples were contained in a disposable polystyrene cell.

DLS measures the time dependent fluctuations in scattered light from molecules in a solution to determine the translational diffusion coefficient and subsequently determine the hydrodynamic radius (R_H) from the Stokes–Einstein equation. The given R_H corresponds to the radius of a sphere diffusing with the same speed as the molecule in solution. The R_H depends thus on the shape of the molecule, with an increased R_H for elongated molecules compared to spherical molecules of the same molecular weight. The size distribution of the sample in each size peak was obtained from the number size distribution, converted using Mie theory from the intensity size distribution. The samples (0.1% wt/vol of DG_0 , DG_1 , and $\text{DG}_0\text{-CD}$ in water) were filtered through a $0.22\text{-}\mu\text{m}$ microfilter Whatman International Ltd. (Maidstone, England) before measurements. The Dispersion Technology Software (Malvern Instruments) have empirical mass vs. size calibration curves available for use in DLS applications, for each specific “family” or type of macromolecule ((a) Malvern Instruments Ltd web page. Can the M_w be measured with dynamic light scattering? Frequently asked questions KB000773, (b) Characterization of polymers using light scattering techniques. Application note MRK568-01, (c) Size Measurement and Molecular Weight Estimation of Globins. Application note MRK1293-01. All available at <http://www.malvern.com/labeng/support/support.htm> (accessed October, 2012)). For reference purposes, it is noted that all of the proteins in the protein family are globular; the linear polymers are pullulans or linear polysaccharides; the branched polymers are ficolls or densely branched polysaccharides; and the spherical polymers are starburst type dendrimers, described as spherical, with a density that increases with radial distance from the core ((a) Malvern Instruments Ltd web page. Can the M_w be measured with dynamic light scattering? Frequently asked questions KB000773, (b) Characterization of polymers using light scattering techniques. Application note MRK568-01, (c) Size Measurement and Molecular Weight Estimation of Globins. Application note MRK1293-01. All available at <http://www.malvern.com/labeng/support/support.htm> (accessed October, 2012)). Then, an “apparent molecular weight” could be estimated by the R_H of the samples (which is the half of the hydrodynamic diameter (D_H) given by the soft) by assuming a molecular conformation. The assay was performed by triplicate on three individual samples.

Statistical analysis

Significance of the effect of treatment (freezing-thawing (f/t), freeze-drying, thermal treatment, or humidification at RH 52%) and the effect of matrix composition on catalase activity were evaluated by one-way ANOVA (significance level $\alpha = 0.05$ %) with Tukey post-test using Prism 5 (GraphPad Software Inc., San Diego, CA).

Results and Discussion

Dendrimers characterization

Two low generation dendrimers, DG_0 and DG_1 , and the modification of DG_0 with $\beta\text{-CD}$ were tested as catalase protective excipients. The structure of the obtained dendrimers (DG_0 and DG_1), the $\text{DG}_0\text{-CD}$, and PEG was confirmed by ^1H -NMR spectroscopy as shown in Figure 1. The multiple signals between 3.44 and 3.78 ppm correspond to the protons of the repetitive units of PEG, as previously reported,²⁵ and were observed in all the spectra. An increasing complexity was observed from DG_0 to DG_1 (multiple signals between 1 and 3 ppm), since more glutamic acid residues are added. In the spectrum corresponding to $\text{DG}_0\text{-CD}$, the appearance of multiple signals in the region between 3.5 and 4.0 ppm correspond to hydrogen nucleus in the $\beta\text{-CD}$ rings,²⁶ and confirms the presence of the $\beta\text{-CD}$ units. Also, the signal of anomeric proton of $\beta\text{-CD}$ at 5.0 ppm was clearly observed. In addition, the resonances associated to the methylene groups of the ethylenediamine bridges are evident at higher fields (1.0–1.4 ppm).¹ The peak corresponding to water was observed at 4.7 ppm in all the spectra.

The average $\beta\text{-CD}$ content in the polymeric derivative was estimated from the ^1H -NMR spectra by the integration ratio between the signals at 1.0 and 1.4 ppm (corresponding to the CH_2 groups of the alkyl spacer), the anomeric protons of the $\beta\text{-CD}$ moieties (5.0 ppm) and PEG signal (3.4–3.8 ppm), considering the number of protons involved in each groups of signals. An average of 3.95 mol of $\beta\text{-CD}$ was estimated to be attached to each mol of dendrimer, which means that more than 98% of the dendrimer moieties are substituted with 4 units of $\beta\text{-CD}$, while the rest are substituted with 3 units of $\beta\text{-CD}$.

The theoretical dendrimers molar mass (M_w) evolved from 6,000 Da (average M_w of PEG) to 6,458 Da, 6,974 Da, and 11,164 Da for DG_0 , DG_1 , and $\text{DG}_0\text{-CD}$, respectively. The size distribution of PEG and the synthesized dendrimers has been determined using DLS. DLS allows the determination

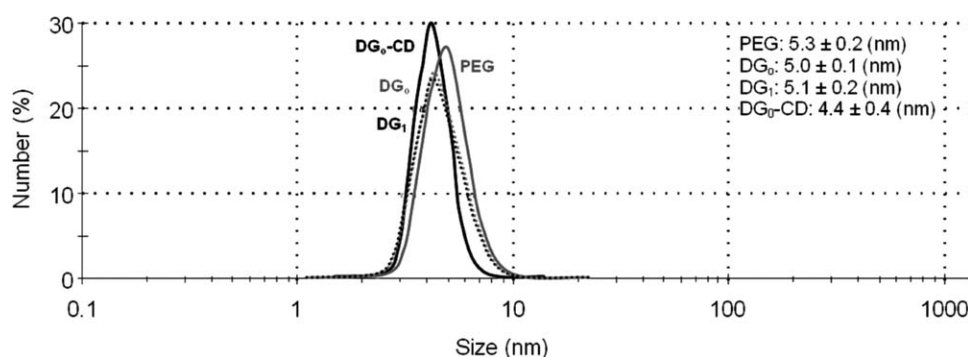


Figure 2. Hydrodynamic diameter distribution of PEG and dendrimers solutions at 0.1% (wt/vol) determined by dynamic light scattering.

Table 1. Hydrodynamic Diameter (D_H) and Estimated Apparent Average M_w Determined by DLS (Using Software Calibration Tool) and Theoretical Molar Mass of PEG and Dendrimers

Sample	D_H (nm)	Estimated "Apparent" M_w (Da)		Theoretical Molar Mass (Da)
		Linear	Branched	
PEG	5.3 ± 0.2	12,300	17,500	6,000*
DG ₀	5.0 ± 0.1	10,700	14,700	6,458
DG ₁	5.1 ± 0.1	11,100	15,400	6,974
DG ₀ -CD	4.4 ± 0.4	8,500	11,000	11,164

*From manufacturer.

of the hydrodynamic diameter (D_H) of the samples, whose size distribution is shown in Figure 2. Also, it has been shown that DLS can be effectively used to estimate the M_w by establishing a calibration curve for similar compounds, and it was previously used with linear, branched, and spherical polymers and globular proteins ((a) Malvern Instruments Ltd web page. Can the M_w be measured with dynamic light scattering? Frequently asked questions KB000773, (b) Characterization of polymers using light scattering techniques. Application note MRK568-01, (c) Size Measurement and Molecular Weight Estimation of Globins. Application note MRK1293-01. All available at <http://www.malvern.com/labeng/support/support.htm> (accessed October, 2012)).

Table 1 shows the D_H , the theoretical molar mass, and the estimated M_w determined by empirical mass vs. size calibration curves available in DLS software. As the glutamic or β -CD groups attached to the PEG backbone affect the molecule's diffusion, the calibration curve should only be used to estimate the corresponding weight of polymer/dendrimer that diffuses with the same speed, and hence an "apparent" molecular weight could only be estimated. The average hydrodynamic diameter of DG₀ and DG₁ were similar to PEG diameter, assuming a more branched structure of dendrimers, which is consistent with the M_w estimation. It is known that for the same molecular weight the hydrodynamic size is higher as the polymer structure becomes more linear.²⁷ From the estimated apparent M_w (Table 1) it can be concluded that both DG₀ and DG₁ dendrimers were dispersed as dimers (assuming a branched structure) and PEG molecules are aggregated either as dimers or trimers (assuming a linear or branched structure, respectively). In the case of DG₀-CD, the inclusion of four β -CDs caused a major change in both the molar mass and in the shape of the dendrimer. DG₀-CD presented a smaller hydrodynamic diameter (4.4 ± 0.4 nm), which is consistent with the estimated molar mass of a branched polymer for the proposed theoretical size, with no

molecular association. Probably, the presence of β -CD avoids the tendency of PEG chains to interact with each other. A decrease in the hydrodynamic diameter due to increased branching has been previously reported for other dendrimers.²⁸

Dendrimers as excipients for catalase stabilization after freezing and thawing

The effect of the additives on catalase activity was first studied in liquid systems prior to any denaturation event. None of the additives reduced the enzyme activity with respect to catalase free of additives. Some of them (PEG; DG₁; and HP- β -CD) even increased the activity up to 20%, while others (DG₀ and β -CD) up to 10% and in the case of T and DG₀-CD no changes in activity were observed in comparison with the samples free of additives.

Since the protein is excluded from the ice crystals during freezing, protein molecules are subjected to the chemical and physical changes that occur in the non-ice phase.²⁹ Ice formation and the presence of interfaces also induce protein denaturation affecting enzyme stability.^{30,31} The synthesized dendrimers were tested as catalase protective excipients. The remaining activity of catalase in the dendrimers systems, and in their combined matrices with trehalose after three cycles of f/t is shown in Figures 3a,b, respectively. PEG, β -CD, and HP- β -CD were used as controls. It is known that f/t cycling over a short time-frame decreases protein stability to a higher extent than long-term frozen storage.²⁹ Without additives, 60% of catalase remaining activity was recovered after the first f/t cycle, and less than 10% after the 3rd cycle. The presence of PEG or any of the studied dendrimers protected the enzyme against the first f/t cycle (Figure 3a, white bars). In subsequent f/t cycles, PEG failed to prevent enzyme denaturation (remaining activity was less than 20%) while dendrimers, and especially DG₀-CD, offered a better protection of the enzyme (more than 70% of the activity was recovered in DG₀-CD after the 3rd cycle). Taking into account that the remaining activity after the third f/t cycle was around 30% for β -CD systems and less than 10% for PEG, a synergistic effect was obtained in the new functionalized dendrimer. Although β -CD could interact with hydrophobic groups of the enzyme through host-guest supramolecular associations, preventing denaturation-derived changes, it was not a good protectant (Figure 3a). This can be attributed to the low solubility in water of β -CD (1.8 g/100 mL), which is directly related to low hydration ability and high proportion of frozen water for this oligosaccharide in comparison to other

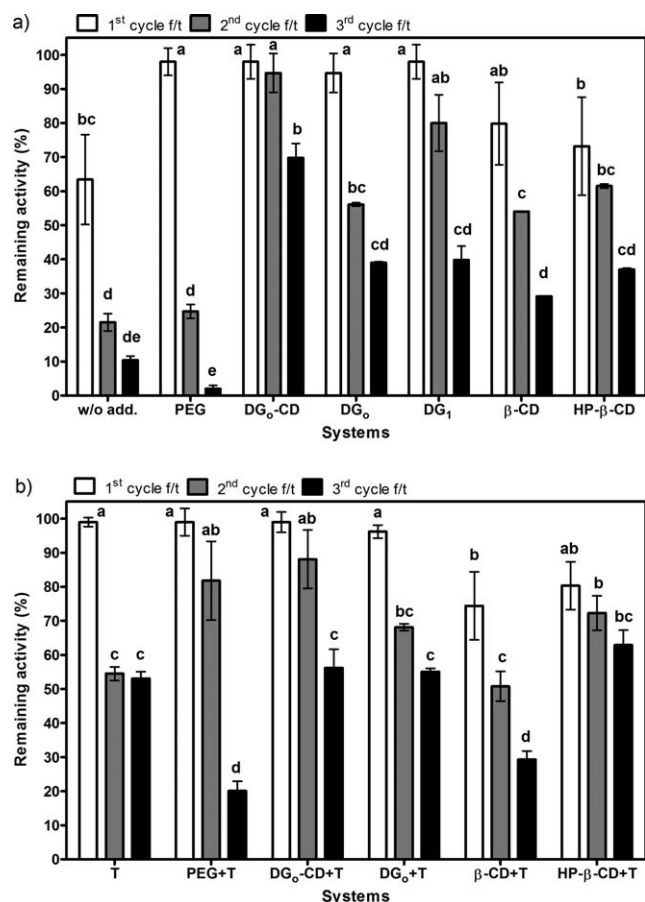


Figure 3. Remaining activity of catalase after three cycles of f/t in the dendrimers, PEG, β -CD, or HP- β -CD as one additive system (a) and in their combined matrices with trehalose (b).

Each cycle consists of freezing at -20°C for 24 h and thawing at 4°C . Different letters on the bars (a–e) indicate significant differences ($P < 0.05$) on remaining activity values. Standard deviation values are included.

saccharides (about 1.78 mol H_2O /mol monosaccharide remains unfrozen in the β -CD system).³² The driving force of the guest- β -CD complex formation is the substitution of the high-enthalpy water molecules inside the β -CD cavity by appropriate guest molecules, less polar than water, to occupy the relatively hydrophobic β -CD cavity.¹¹ The guest molecule and the β -CD are usually dispersed in water and stirred to equilibrium for several hours to favor inclusion complex formation.³³ However, for the encapsulation of labile compounds, this procedure could affect biomolecule stability, being inappropriate for our systems. HP- β -CD has a structure and molecular weight similar to β -CD but it is 100 times more soluble than β -CD,³⁴ and was included for comparison in Figure 3a. HP- β -CD provided better enzyme activity conservation than β -CD, after the 2nd and 3rd f/t cycles, confirming that β -CD must be soluble in order to interact and protect the enzyme. When included in the dendrimer, β -CD has higher solubility in water and could interact better with the protein than when it is alone.

In the combined systems with trehalose (T) (Figure 3b), the protective pattern was similar to the observed for the dendrimers as one additive, but with higher remaining activity values. Through the addition of trehalose a synergistic effect was observed during the 1st and 2nd f/t cycles. However after the third f/t cycle, enzyme activity in trehalose

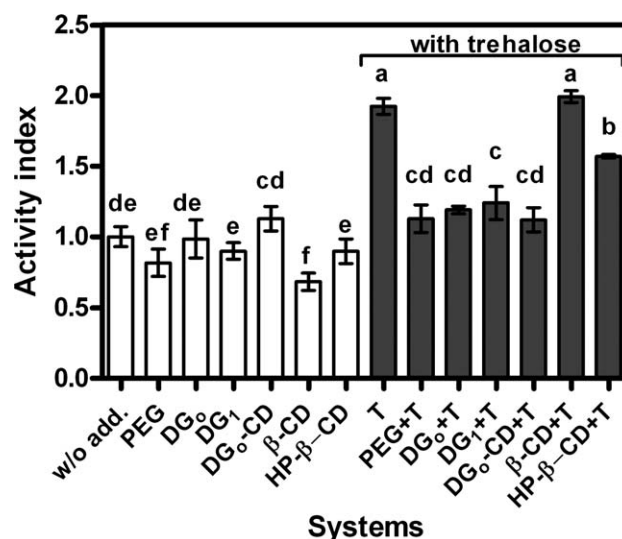


Figure 4. Activity index of catalase after freeze-drying in the synthesized dendrimers as one additive system or in their combined matrices with trehalose.

PEG, β -CD, or HP- β -CD was included for comparison. Different letters on the bars (a–f) indicate significant differences ($P < 0.05$) on activity index values. Standard deviation values are included.

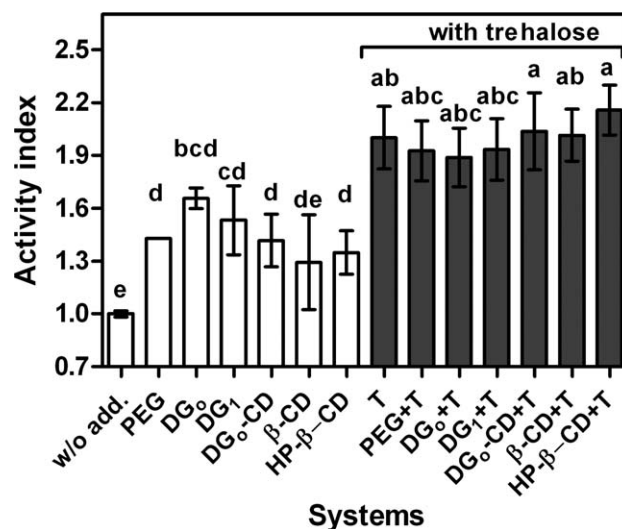


Figure 5. Activity index of catalase in freeze-dried systems thermally treated for 15 min at 55°C .

Dendrimers, PEG, β -CD, or HP- β -CD were used as one additive or in their combination with trehalose. Different letters on the bars (a–e) indicate significant differences ($P < 0.05$) on activity index values. Standard deviation values are included.

alone was higher than in PEG or β -CD containing matrices, and similar to DG_o-CD+T and DG_o+T. Even though the addition of trehalose represented a substantial improvement to PEG systems, the functionalization of a based-PEG dendrimer with β -CD (DG_o-CD) generated a more efficient system to protect catalase against f/t.

Effect of dendrimers on catalase stability of freeze-dried formulations

Figure 4 shows the effect of dendrimers and their mixture with trehalose on catalase stability during freeze-drying, as

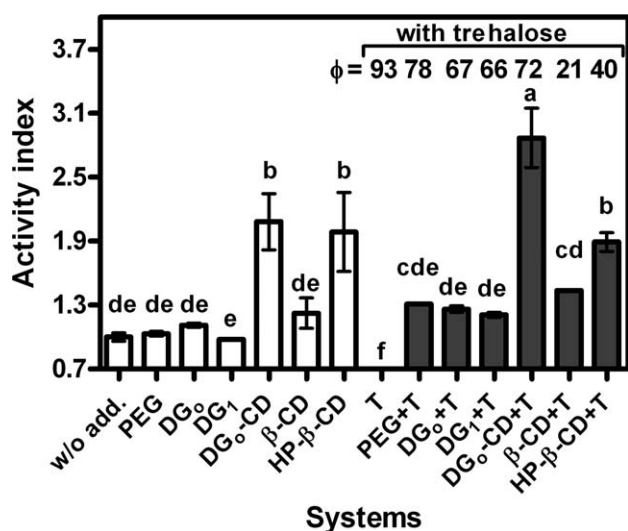


Figure 6. Activity index of catalase in the synthesized dendrimers as one additive or in their combined matrices with trehalose in freeze-dried systems humidified for 9 days at 52% RH and 25°C.

PEG, β -CD, or HP- β -CD were included for comparison. Different letters on the bars (a–f) indicate significant differences ($P < 0.05$) on activity index values. Standard deviation values are included. The degree of trehalose crystallization (ϕ) was indicated above each bar for the combined trehalose systems. The standard deviation of ϕ was $< 2\%$ for all the combined systems.

expressed by the activity index (Eq. (1)). During freeze-drying the enzyme was better protected in the trehalose matrix than in any of the other additives used as single component. The dendrimers or their mixtures with trehalose were not as good as the saccharide to protect the enzyme. Carpenter et al.¹⁹ studied the stability of two enzymes (phosphofructokinase and lactate dehydrogenase) with several additives and found that the T+PEG combination was the most efficient strategy to protect the enzymes during freeze-drying. However, in this work, the suggested T+PEG synergy was not evident since the system containing only trehalose offered the best protection. The stability of the enzyme in DG_o-CD was higher than in the single components PEG or β -CD. The addition of trehalose enhanced the stability of the enzyme in comparison to the single additives systems, especially with HP- β -CD or β -CD matrices. The combined matrix T + β -CD was also suitable for stabilizing the enzyme invertase in freeze-dried formulations.^{1,9} The presence of trehalose was likely to improve the solubility of β -CD thereby improving its ability to interact with the protein through supramolecular interactions.^{1,9} It is known that saccharides protect proteins by direct interaction, and the concentration of saccharides necessary to form a monomolecular layer on the protein surface is the minimum to achieve the maximum stabilization.³⁵ Therefore, the stabilization of catalase was found to depend not on the bulk concentration of the sugar but on the weight ratio of sugar to catalase, where the minimum required ratio is around 0.4.³⁵ In our case, the weight ratio excipient:catalase was much higher than 0.4 (between 238 and 1429, for 1% and 6% wt/wt excipient concentration, respectively). Thus, the different observed behavior in the several matrices cannot be attributed to an excipient-concentration factor, since all the excipients in the systems are far beyond the 0.4 limit to form the required monomolecular layer.

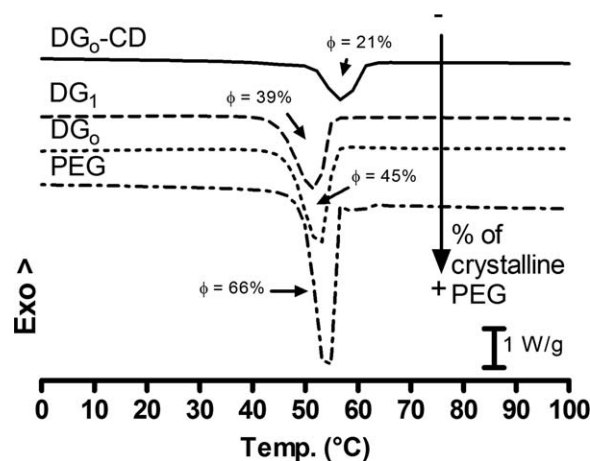


Figure 7. DSC thermograms for dendrimers (DG_o, DG₁, DG_o-CD) and PEG freeze-dried samples.

The degree of PEG crystallization (ϕ) was indicated. The standard deviation values of ϕ were $< 1\%$ for all the systems.

The effect of dendrimers or their combination with trehalose to protect the enzyme during a thermal treatment is shown in Figure 5. The freeze-dried systems were thermally treated at 55°C and the corresponding activity indexes were calculated. The synthesized dendrimers (DG_o-CD, DG_o and DG₁) improved catalase stability (between 35 and 60% in comparison with the control system without additives) and even respect to the other matrices studied. It can be observed that the systems containing trehalose (either alone or in combination with other additives) were the most suitable matrices for stabilizing the enzyme during thermal treatment.

Effect of additives crystallization on catalase stability

Crystallization of the solid phase considerably affects the mechanisms by which amorphous sugars manifest protective effects on biomolecules and, consequently, the shelf life of a product.^{1,9,36} Therefore catalase activity during storage was evaluated in relation to both trehalose and dendrimer crystalline degree in the formulations.

Figure 6 shows the stability of catalase in the freeze-dried studied systems and in their combined matrices with trehalose after humidification at 52% RH at 25°C. Although trehalose was an effective protectant of the enzyme during both freeze-drying and thermal treatment (Figures 4 and 5, respectively), this effect was lost at 52% RH (Figure 6). This behavior was previously related to trehalose crystallization.^{1,9,37,38} At RH $\geq 43\%$ and 25°C trehalose is above the glass transition temperature and both the molecular mobility and the amount of water are enough to allow trehalose dihydrate crystallization.³⁶

Therefore, the degree of trehalose crystallization was evaluated by DSC and is indicated in Figure 6. Trehalose was almost completely crystallized as single additive ($\phi = 93\%$) and its crystallization was delayed in all the combined systems. It can be concluded that when the matrix is completely crystallized (as in the case of T), enzyme activity was rapidly lost. However, in the combined matrices containing at least 20% of amorphous sugar the stability depended not only on the amount of crystalline sugar, but also on other factors such as the nature and physical state of the second excipient. Figure 6 also shows that DG_o and β -CD were not effective to protect catalase. However, their combination

with the synthesized DG₀-CD resulted in a suitable matrix to protect the enzyme even in the absence of trehalose, being the activity index similar to the obtained in HP- β -CD. These results demonstrate once again that the β -CD must be soluble (which occurs in the dendrimer) in order to interact through supramolecular interactions and protect the enzyme. In addition, DG₀-CD could preserve the active multimeric structure of catalase, probably by the formation of host-guest supramolecular crosslinks between the hydrophobic residues at the protein chains and the CD-branched dendrimer molecules.

Catalase stability was also analyzed regarding the dendrimer crystalline degree. Figure 7 shows the DSC thermograms for the freeze-dried dendrimers (DG₀, DG₁, DG₀-CD) and for PEG. The endothermic peak around 55°C corresponds to the melting of PEG and was used to calculate ϕ , which is indicated for each dendrimer in the figure. The order of degree of PEG crystallinity is PEG > DG₀ > DG₁ > DG₀-CD, indicating a decreasing crystallinity with increasing generation/complexity. Hawker et al.²⁸ reported that certain generations of other dendrimers are completely amorphous, while their corresponding linear structures are crystalline.

The increased stability observed in the dendrimer DG₀-CD, as compared to DG₀, DG₁, and PEG, after freeze-drying (Figure 4) and after humidification (Figure 6) was associated not only with the presence of β -CD, but also with the lower crystallinity of the dendritic core (Figure 7). In heat-treated systems at 55°C (Figure 5), the presence of amorphous trehalose was critical to improve the thermal stability of the enzyme. At 55°C (the melting temperature of PEG) the presence of PEG crystals was avoided and stability differences of catalase in the dendrimers (DG₀, DG₁, and DG₀-CD) and PEG were not observed.

Conclusions

A novel functional dendrimer was successfully synthesized through a new chemical synthetic route, and based on the known PEG's ability as cryo-protector and β -CD as supramolecular stabilizing agent. Of the studied systems, DG₀-CD was the best catalase stabilizing additive during the critical conditions of freeze-thawing cycling over a short time-frame. Thus, good performance is expected in long-term frozen storage. During freeze-drying, trehalose was the best protectant agent, while DG₀-CD exhibited an adequate protection of catalase and showed a synergistic behavior in comparison to the activities recovered employing PEG or β -CD as individual stabilizers. During thermal treatment, although all the studied dendrimers improved the remaining activity of the enzyme, the presence of amorphous trehalose was critical to enhance the stability of the enzyme.

The crystallinity of the protective matrix, either of PEG derivatives or of trehalose, negatively affected the stability of catalase in the freeze-dried systems. When humidified at 52% RH, the dendrimers delayed trehalose crystallization in the combined matrices thereby extending the trehalose protective effect at those conditions where trehalose normally fails. DG₀-CD could interact with the enzyme probably by CD supramolecular interactions (host-guest supramolecular crosslinks between the hydrophobic residues of the protein chains and the CD-branched dendrimer molecules), enhancing protein stability. At the studied conditions, β -CD as single additive could not develop adequate interactions to protect the enzyme.

Although the experiments were performed with catalase as a model of a labile protein, the obtained results have a more general application since they show how a relatively simple covalent combination of polymers such as PEG with CD can significantly affect their protein-protective properties. Further insights about the role played by specific interactions polymer-enzyme on enzyme stability in dehydrated models were also provided. Supramolecular and specific interactions are involved in the protective effect of the excipients where their effects on the stabilization are also influenced by the physical state of the matrix.

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