



Albumin-based nanoparticle trehalose lyophilisation stress-down to preserve structure/function and enhanced binding



Macarena Siri^a, Mariano Grasselli^b, Silvia del V. Alonso^{a,*}

^a Laboratorio de Biomembranas (LBM), and GBEyB, Grupo Vinculado IMBICE-CONICET; Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Saenz Peña 352, Bernal, B1876BXD Buenos Aires, Argentina

^b Laboratorio de Materiales Biotecnológicos (LaMaBio), and GBEyB, Grupo Vinculado IMBICE-CONICET; Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Saenz Peña 352, Bernal, B1876BXD Buenos Aires, Argentina

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ABSTRACT

The aim of this study was to preserve albumin nanoparticle structure/function during the lyophilisation process. Bovine serum albumin nanoparticles were obtained by γ -irradiation. Nanoparticles were lyophilised in buffer, miliQ water or in trehalose/miliQ solution. The size and charge of the nanoparticles were tested after lyophilisation by light scattering and Z potential. The most relevant results in size of BSA nanoparticle were those lyophilised in PBS between 20 and 350 nm, assembled in different aggregates, and negative Z potential obtained was 37 ± 8 mV in all, and those nanoparticles lyophilised with trehalose had a size range of 70 ± 2 nm and a negative Z potential of 20 ± 5 mV.

Structure determination of surface aminoacids SH groups in the BSA NP lyophilised in PBS showed an increase in the free SH groups. Different aggregates had different amount of SH groups exposure from 55 to 938 (from smaller to bigger aggregates), whereas BSA NP lyophilised with trehalose showed no significant difference if compared with BSA NP.

The binding properties of the BSA nanoparticle with a theragnostic probe (merocyanine 540) were studied after lyophilisation. Results showed more affinity between the BSA NP lyophilised with trehalose than that observed with non lyophilised BSA NP.

As a result, the lyophilisation condition in trehalose 100 μ M solution is the best one to preserve the BSA NP structure/function and the one with the enhance binding affinity of the BSA NP.

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1. Introduction

Bovine serum albumin (BSA) is widely used as drug delivery system because of its abundance, ease of purification, low cost, and ligand-binding properties [1]. Because of their physicochemical and biological properties, BSA nanoparticles (BSA NP) are considered a potential drug delivery system. BSA NPs obtained by Soto Espinoza et al. [2] by γ irradiation are 17–20 nm size. To date no lyophilisation process has been tested on the irradiated BSA NP. Other authors have reported lyophilisation of glutaraldehyde BSA NPs in presence of sugars like galactose and mannitol [3]. Others had obtained BSA or HSA nanoparticles lyophilised, but these were no clean nanoparticles [4]. Nevertheless, these authors use lyophilisation process as a mean of disposing off the solvent present, not as a way of long-term storage [5].

Lyophilisation, the process by which a frozen liquid is removed by sublimation, is used as a method to stabilise materials such as protein drugs and biotechnology products also used in long-term storage [6,7]. Proteins may suffer degradation or structure alteration during the process. To prevent this stabilisers are used, mainly, sugars [7,8]. These molecules are used to decrease stress during the lyophilisation process severe conditions [8]. Our purpose is to achieve a way of long-term storage, but imposing a condition that would maintain the functionality of the nanoparticle.

In this work, lyophilisation is considered successful when the NP preserved its structure/function after the process.

Different lyophilisation conditions are described to stress down the process conditions which is the objective of this study; (i) BSA NP in buffer solution without the use of any cryoprotector and (ii) BSA NP in miliQ water and (iii) BSA NP with increasing trehalose concentration solution. If condition (i) is an efficient way to lyophilise the BSA NP preserving its structure, it will be the best option to be considered being the simplest of them all.

* Corresponding author.

E-mail address: salonso@unq.edu.ar (S.d.V. Alonso).

Kumar et al. [8] proposed the use of trehalose in order to preserve the structure/function of albumin by lyophilisation. Considering that our structure is a complex one and considering the exposure of certain groups, in this study we propose the use of trehalose in different concentrations based on the relation of the interaction of trehalose OH group and NH surface groups in the NP that do not produce BSA NP aggregation or water crystals formation giving rise to a hygroscopic sample.

Trehalose is a disaccharide formed by an α,α -1,1-glucoside bond between two α -glucose units. Not only does this sugar have the advantage of protecting cells from disruption, but can also act as an antioxidant. It is used as a cryoprotector for nanoparticles, because of its ability to preserve their original size and structure after freezing [6].

The cryoprotection property is given by its several polymorphisms in both solid and liquid state. Trehalose has two polymorphisms: an amorphous and a dehydrate trehalose state. The latter can be dehydrated into two different anhydrous molecules: α or β . Each of the non-water states are capable of hydration going back to the dehydrated trehalose, characteristic of a sugar useful as a cryoprotector. Moreover, trehalose can rearrange the water molecules and decrease the freezing-temperature point, allowing more stability to the molecule in question. Mechanisms are Vitric, Exclusion and Water Replacement Theories;

Vitrification Theory: trehalose has a transition temperature to a more vitric phase where no crystal is formed during the process. It is here, inside the lattice formed, that the molecule is trapped gaining stability.

Preferential Exclusion Theory: there is no direct interaction between the NP and sugar. Instead, the trehalose molecules entrap bulks of water in order to stabilise the molecule.

Water Replacement Theory: the interaction is given between the molecule and trehalose. Here the latter is able to replace the water molecules forming hydrogen bonds with the stabilized molecule.

The next step after confirming the NP structure preservation will be to study its functionality. A theragnostic probe like Merocyanine 540 (MC540) will be useful to answer questions on functionality [8]. This experiment also aims to study the binding properties of the NPs through their interaction with MC540.

MC540 is an anionic cyanine dye which absorbs light in visible region (main peak in 532 nm in water solutions). Moreover, it has a dual role of photosensibiliser as a bactericidal agent and an immune reaction regulator [8]. Also, owing to its structure and function, MC540 is used as an optical and fluorescent probe in biomembranes and proteins. The interaction between protein and probe is mainly given by its fluorescence intensity. In our study, BSA NPs were freeze dried in the presence of phosphate buffer or miliQ water (with and without trehalose), and after reconstitution were evaluated regarding their physicochemical characteristics. The working hypothesis is that BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) and/or BSA NP lyophilised with trehalose has well preserved structure/function.

2. Materials and methods

2.1. Materials

The BSA 98% (agarose gel electrophoresis) powder, lyophilised ~7.1% in 0.15 M NaCl was acquired from Sigma-Aldrich, Co., St. Louis, USA. The Merocyanine 540 was from Molecular Probes, Inc., Eugene, Oregon, USA. The buffer used was phosphate buffer saline (PBS). Ethanol HPLC grade was acquired from Sintorgan®, SA, Buenos Aires, Argentina.

The lyophilisation of the BSA nanoparticle was performed in a Labconco Freeze Dry System/Freezone 4, 5 (La Química Quirúrgica, Buenos Aires, Argentina). The light scattering and Z potential measurements were obtained in a Zetasizer Nano ZS Malvern Instruments Ltd., Worcs, Malvern, UK. The UV-vis measurements were obtained in a UV-160 A UV-vis Recording Spectrometer, Shimadzu Corporation, Nakagyo-ku, Japan. Graphic analyses were carried out with GraphPad Prism v.5.0.

2.2. Methods

2.2.1. Nanoparticle preparation

BSA NPs were prepared according to Soto Espinoza et al. [2]. Briefly, BSA was dissolved in PBS (pH 7.0; 30 mM). Ethanol aliquots were added to the protein solution. The solution was then irradiated by γ -irradiation in a ^{60}Co source (PISICNEA-Ezeiza, Arg.) 1 kGy/h. The irradiation process lasted 10 h at an irradiation rate of 1 kGy/h, with a final dose of 10 kGy.

2.2.2. Lyophilisation procedure

The samples were lyophilised in two different ways:

- A millilitre of BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) solution 450 μM in buffer 35% ethanol solution was eluted with a size exclusion column (Sephadex 50 PD-10) in order to exchange the solvent to a 100% PBS (pH 7.0; 30 mM) solution. After that, the BSA NP solution was left in freezer at -80°C for 24 h. Then, they were lyophilised for another 24 h. The powder obtained was resuspended in buffer PBS (pH 7.0; 30 mM).
- The BSA NP obtained from γ -irradiation, was then passed through a size exclusion column (GE- Healthcare PD-10) in order to exchange the solvent from PBS/EtOH 35% v/v, to miliQ water.

Samples were prepared according to the molar ratio relationship $R = [\text{Trehalose}]/[\text{BSA NP}]$, keeping the BSA NP concentration at 0.30 μM . Trehalose concentration varied from 0.0 to 400.0 μM ($R =$ from 0.0 to 1000). After that, the BSANP solution was left in freezer at -80°C for 24 h. Then, they were lyophilised for another 24 h. The powder obtained was resuspended in buffer PBS (pH 7.0; 30 mM).

The lyophilisation process for both protocols lasted for 24 h, reaching a vacuum under 133 μbar .

2.2.3. Characterization of nanoparticles

To characterise the structure/function of NPs lyophilisation process, before and after, DLS, Z Potential and MC540 affinity experiments were carried out. The lyophilised nanomaterial was rehydrated in buffer PBS (pH 7.0; 30 mM) up to initial volume to maintain its concentration.

2.2.3.1. Particle size and Z potential. Size distribution and Z potential of NPs were measured by a commercial zeta-potential and particle size analyser size measurement, 90Plus/Bi-MAS particle size analyzer, 1 ml of the NPs in PBS was prepared. Subsequently 100 μl volume from the original concentration was taken up to 1 ml final volume with PBS. The particle size was measured at 25°C with a scatter angle of 90° . For the Z potential measurement, the NPs were dispersed in 1 ml of PBS, and measured at 25°C .

2.2.3.2. Microscopy.

2.2.3.2.1. Scanning electron microscopy (S.E.M). The lyophilised powder was analysed under a SEM Carl Zeiss NTS SUPRA 40 electronic microscope (Centro de Microscopía Avanzada, Pabellón I, Facultad de Ciencias Naturales y Exactas, Ciudad Universitaria, UBA, Buenos Aires, Argentina). Microscopies of randomly selected field of the sample were recorded in order to have a view of what was

happening with the sample. Microscopies were taken at several instrumental magnifications using tools in the microscope software.

2.2.3.2. Atomic force microscopy (A.F.M). The lyophilized powder was dissolved in buffer PBS (pH 7.0; 30 mM) and analysed under an AFM-DI-VEECO-MMAFM Nanoscope III (Centro de Microscopia Avanzada, Pabellon I, Facultad de Ciencias Naturales y Exactas, Ciudad Universitaria, UBA, Buenos Aires, Argentina).

For the BSA NP lyophilized with trehalose 30 μ l of a solution of nanoparticle were deposited in freshly cleaved mica. It was dried for 20 min with nitrogen gas. Images were obtained using a Dimension Icon in PeakForce QNM (PFQNM) (Bruker®), (Institute of Biophysics, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil). Rectangular, silicon tip with a nominal spring constant 2 N/m, with tip radius of 17 nm were used during the measurement.

Microscopies of randomly selected field of the sample were recorded in order to have an ample view of what was happening with the sample. Microscopies were taken at several instrumental magnifications using tools in the microscope software.

2.2.3.2.3. Transmission electron microscopy (T.E.M). A TEM PHILIPS EM 301 high resolution transmission electron microscopy was used for sample morphology observation (Centro de Microscopía Avanzada, Pabellon I, Facultad de Ciencias Naturales y Exactas, Ciudad Universitaria, UBA, Buenos Aires, Argentina). Samples were treated before the microscopy was taken by uranile acetate dye. Different microscopies were taken under different magnification as the instrument allowed it.

2.2.4. Fluorescence measurements

A triplicate of each sample was recorded in a FS2 SCINCO spectrofluorometer with an excitation wave set at 295 nm. The slits were 2.5 nm open. Concentration was 2.5 μ M; the buffer PBS pH 7.0; 30 mM. Trehalose concentration varied within the range 0.0–300.0 μ M.

2.2.5. Ellman's reaction: free thiol groups detection

Samples of BSA (450 μ M) and NPs (555 nM) were diluted 1/10 to a concentration of 45 μ M and 55 nM, respectively. PBS buffer pH 6.0 and 8.0, both 0.1 M were made for this assay. For the thiol groups detection, a solution of DTNB 3.9 mg/ml PBS 0.1 M was required.

Procedure was as described in Ellman [9] with modifications. Samples were incubated at room temperature for an hour. Samples were then incubated with 5 μ l of the DTNB solution. UV–vis spectra were recorded of each sample after 15 min at an absorbance of 425 nm. Measurements at 280 nm were also made in order to calculate the protein concentration of the sample.

Using the equation below and the molecular coefficient extinction of $\epsilon_{\text{BSA}_{280\text{nm}}} = 44,200 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{\text{DTNB}_{425\text{nm}}} = 12,400 \text{ M}^{-1} \text{ cm}^{-1}$, the total amount of thiol groups was calculated:

$$n = \frac{\left(\frac{\text{Abs}_{\text{DTNB}}^{425\text{nm}} - \text{Abs}_{\text{Blank}}^{425\text{nm}}}{\epsilon_{\text{DTNB}}^{425\text{nm}}} \right)}{l} - 1 / C_{\text{BSANP}} \quad (1)$$

where n is the thiol mol per protein mol and C_{BSANP} is the NP concentration.

2.2.6. Binding affinity: fluorescent probe MC540

BSA NP concentration was kept constant at 6 nM. From the Merocyanine 540 (MC540) stock solution (450 μ M) dilutions were made in order to cover a range from 0.0–1.5 e04 ratio between BSA NP and drug [MC540]/[BSA NP]. In that way the maximum of drug concentration was 90 μ M. Then, 15 min incubation was needed to ensure the interaction between the BSA NP and MC540. The determinations of the binding constants were carried out as explained in Sevilla et al. [10]. Briefly, the amount of MC540 in every

Table 1

Z potential and size of lyophilised nanoparticles. Each value corresponds to duplicates of 10 scans per sample.

Sample	Z Potential (mV)
BSA NP	-25 ± 14
BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM)	-37 ± 8
BSA NP lyophilised in miliQ water	-28 ± 19
BSA NP lyophilized: trehalose 162:1	-24 ± 7
BSA NP lyophilized: trehalose 325:1	-20 ± 5

binding site of the nanoparticle was observed by quenching of the nanoparticle's fluorescence signal: Kd corresponds to the dissociation constant and Bmx to the maximum of MC540 bound to the nanoparticle. With the samples of BSA NP with increasing MC540 concentrations and plotting Trp and/or Tyr fluorescence intensity, vs, [MC540]/[BSA NP], we obtained a slope. From this slope we calculate the free MC540 in each sample. Then, applying the Scatchard equation after plotting the free MC540 vs the MC540 bound we obtained Bmx and Kd. This is carried out by fitting the points to a Hyperbola One site-binding Fitting. Experimentally, a S2 Scinco Fluorspectrophotometer was used, where samples were excited at a wavelength of 295 nm, with emission at 340 nm; slits were open at 1.5 nm. For graphs analysis GraphPad Prism V.5 was used.

3. Results

Once the BSA NP was obtained, according to a novel radiation-induced crosslinking method (Soto Espinoza et al. [2]), the lyophilisation process under different experimental conditions took place. In order to study the BSA NP integrity, after re-suspensions of the samples, characterisation by biophysical studies took place.

3.1. Nanoparticle size and Z potential

The magnitude of the measured Z potential is an indication of the repulsive forces present and can be used to predict the stability of the product. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and there will be no tendency for the particles to aggregate. However, if the particles have low zeta potential values, then there is no force to prevent the particles from getting together and flocculating [10]. The minimal charge for repulsion depends on the size of the nanoparticle. For nanoparticles to be stable in a colloidal suspension, a minimum charge of 20 mV is required to gain certain stability and avoid agglomeration.

Charge and size are the characteristics that best illustrate nanoparticles. All the Z potentials values obtained were negative. Data are comparable with that of non lyophilised BSA NP (-25 ± 14 mV), pH 7.0 [11], BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) without trehalose has a Z potential of -37 ± 8 mV and BSA NP lyophilised in miliQ water has a Z potential value of -28 ± 20 mV. This slight difference in the experimental conditions is probably due to the presence of ions from salts of the original buffer. For the BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) in presence of trehalose the value was of -20 ± 5 mV (Table 1).

It is possible to observe that while the BSA NP lyophilised in buffer offered a wide range of diameter sizes. Population mono- and bimodal are observed: from 30 nm to 340 nm as indicated with arrows in Fig. 1a. However, only a monodispersed population is obtained when stabilisers are used (Fig. 1b). D.L.S. measurements obtained for BSA NP lyophilised with trehalose were in the range of 70–100 nm. After five experimental repetitions of each condition, BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) certain physical differences were observed (Fig. 1c). Samples with a more powdery appearance were the ones with the smallest size while

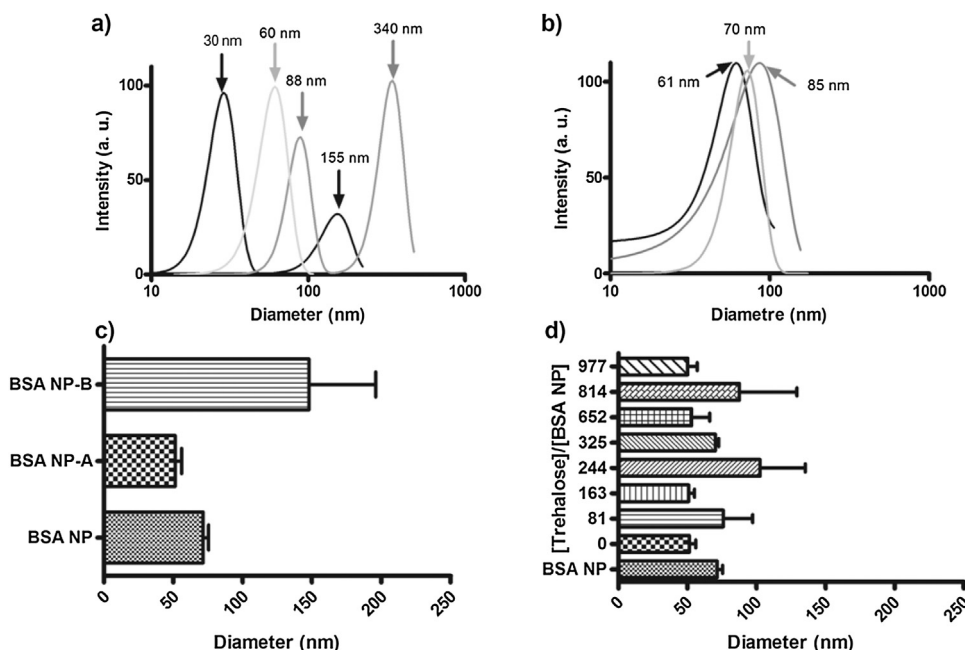


Fig. 1. This figure depicts the measurements obtained in D.L.S of BSA NP lyophilised with buffer PBS (pH 7.0; 30 mM) and BSA NP lyophilised with trehalose samples. Arrows pointing at the mean population from each batch tested using the same colour as the batch's profile. (a) Describes the diameter size (nm) of the BSA NP lyophilised while in PBS (5 independent samples). (b) Describes the diameter size (nm) obtained from the lyophilisation of the BSA NP in miliQ water and increasing trehalose concentrations (5 independent samples). (c) Represents the difference among BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM)-MQ and BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) samples compared to the BSA NP. (d) Represent the difference among BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) BSA NP lyophilised with trehalose with increasing sugar concentration compared to that of BSA NP. The populations described in (c) and (d) are those which had the highest diameter distribution population of NPs concentration. (a) and (b) show arrows pointing at the mean population from each batch tested using the same colour as the batch's profile.

those with cotton like appearance had a larger size at the same pH; lyophilisation depends on the surface exposure and the starting water content. The multiple data obtained are indicative of a non-reproducible lyophilisation process as regards preservation of the original NP structure; ions present disorganised water distribution around the BSA NPs and surface exposed.

Fig. 1d shows the main distribution group of the different populations present in each sample of BSA NP lyophilised in trehalose with different sugar concentrations. Coincidentally, those samples with the higher deviation are those with higher polydispersity (data not shown to ease the manuscript understanding). On the other hand, it was also observed that polydispersity varies according to the concentration of trehalose used; ergo the water content not replaced by trehalose. Given its capacity to absorb humidity, the sugar might start forming crystal instead of preventing their formation at certain concentrations between the BSA NPs according to Kumar et al. [12]. We must remember that by interacting with water, trehalose prevents the formation of water crystals when the samples are under the freezing process. Despite this, if the sugar is present in high concentration in the sample, this capability is lost.

As observed, each sample with a different trehalose concentration presents different NP diameters. Despite this, there is no marked tendency as how the sugar affects the size of the BSA NP lyophilised with trehalose. If looked closely it is evident that the ratio capable of achieving an almost equal size to the non-lyophilised NP ($72 \text{ nm} \pm 4$) in buffer solution (Fig. 1c and d) is that of a molar ratio of 325:1 ($[\text{Trehalose}]/[\text{BSA NP}]$) ($70 \text{ nm} \pm 2$). In this concentration there are in between 300 to 400 trehalose molecules for every BSA NP in solution. In Fig. 1b the three batches obtained are represented as diameter size. We can observe that the samples do not present as large polydispersity as BSA NP lyophilised in buffer PBS Z potential and size of BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) nanoparticles (Fig. 1a and c). None of the samples examined present sizes larger than 100 nm.

According to Kumar et al. [12], there are several risks to be considered when freezing the BSA protein sample without any cryoprotector and in buffer. As stated before, for samples dissolved in buffer PBS (pH 7.0; 30 mM), the lyophilisation process is not a reproducible one. When freezing the BSA sample or any aggregates of this protein the hydrophobic forces increase, and depending on the solvent used, pH value may vary by two units either way; these might cause protein degradation and denaturation, as well as aggregation [11].

When the BSA NP drying process takes place, the removal of water may affect the stability and function of the protein, thus when resuspended again, our BSA NP formed aggregates. This situation is present for samples studied in presence of water and in absence of a cryoprotectant like trehalose (Fig. 1a, and c). In contrast, when we used trehalose/miliQ water solution, we obtained a monodispersed Gaussian population with no aggregates (Fig. 1b). This proved that the trehalose in solution works as a cryoprotectant and lyoprotectant [12]. Alongside, we observed similar results for the samples lyophilised in miliQ water and in absence of trehalose (Fig. 1c). It may be because the absence of salts in the solvent prevents the drastic change in pH value avoiding NP destabilisation and/or aggregation.

When lyophilised in miliQ water alone, the D.L.S. main size measured was of $51.3 \text{ nm} \pm 4.7$ (Fig. 1c) and standard deviation was low for the different samples measured. BSA NP diameter before lyophilisation was 70.0 nm . Therefore, it is thought that even though measurements among batches were similar, the lyophilisation in absence of salts is not enough to preserve the original size. Thus, the BSA NP lyophilised with trehalose preserves the original diameter ($72.0 \text{ nm} \pm 4.0$) (Fig. 1d). Trehalose is then thought to be essential in preserving the NP size structure during the lyophilisation process.

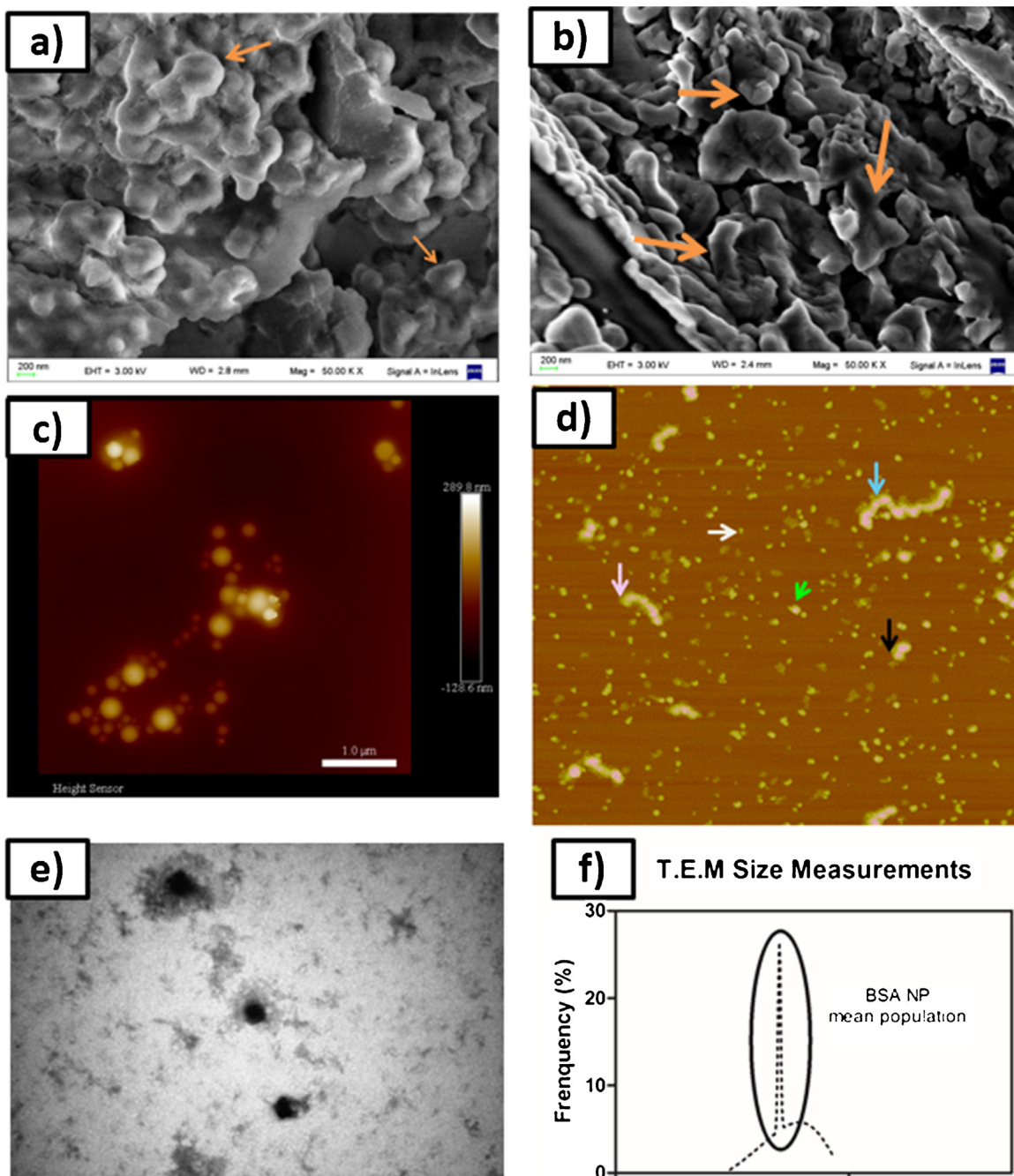


Fig. 2. (a) SEM microscopy of lyophilised BSA NP in buffer and (b) BSA NP lyophilised in miliQ water and trehalose. Both have a Mag. 50.00 K \times , scale depicts 200 nm, EHT 3.00 kV, WO 2.8 mm and signal A = In Lens. (c) AFM of BSA NP lyophilised in trehalose dissolved in buffer PBS pH 7.0; 30 mM. Aggregates of different sizes that are formed after the freeze-drying process are observed between 25 and 250 nm. (d) AFM of BSA lyophilised with trehalose and then dissolved in buffer PBS pH 7.0; 30 mM. Aggregates of different sizes that are formed after the freeze-drying process are observed; 20 nm (white arrow), 40 nm (green arrow), 60 nm (black arrow), 100.97 nm (pink arrow) and 200.19 nm (blue arrow). Dots in the background correspond to BSA molecules. Scale depicts 200 nm. (e) TEM microscopy of BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) BSA NP lyophilised with trehalose in buffer after lyophilisation. Scale depicts 200 nm. (f) Plot profile of (e) depicts that major population present in the sample belongs to that of 35 nm; 250 nm aggregates are in small concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Bearing these results in mind, and from the D.L.S. data, it is thought that the best results obtained were when trehalose 100 μ M was used to stabilise the NP.

There are researchers who worked on the lyophilisation process and albumin nanoparticles, but this nanoparticles were synthesised by different methodology mainly by chemical reaction (desolvation method) [3,4]. Each method renders nanoparticles of different sizes and with different stability properties. Kumar et al. [12] determines the best condition to lyophilise BSA molecules in presence of different sugars, Anhorn et al. [4] reports condition for HSA nanoparticles

obtained by desolvation method in presence of glutaraldehyde. The condition used is saline, buffer (pH 8.0) and miliQ water in presence of sucrose, mannitol and trehalose. The best cryoprotectant is either sucrose or trehalose and the percentage of the excipient present depends on the sample's nature (pegylated or not). They tested different concentrations of trehalose to achieve their goal; between 2 and 3% w/v were the chosen for them. In our work, we achieve the size preservation of the BSA NP with 3.4% w/v of the sugar. Wendorf et al., [3] also tested a nanoparticle core made of PEG with a shell of BSA and using buffer PBS (pH and mM) or trehalose buffered. In

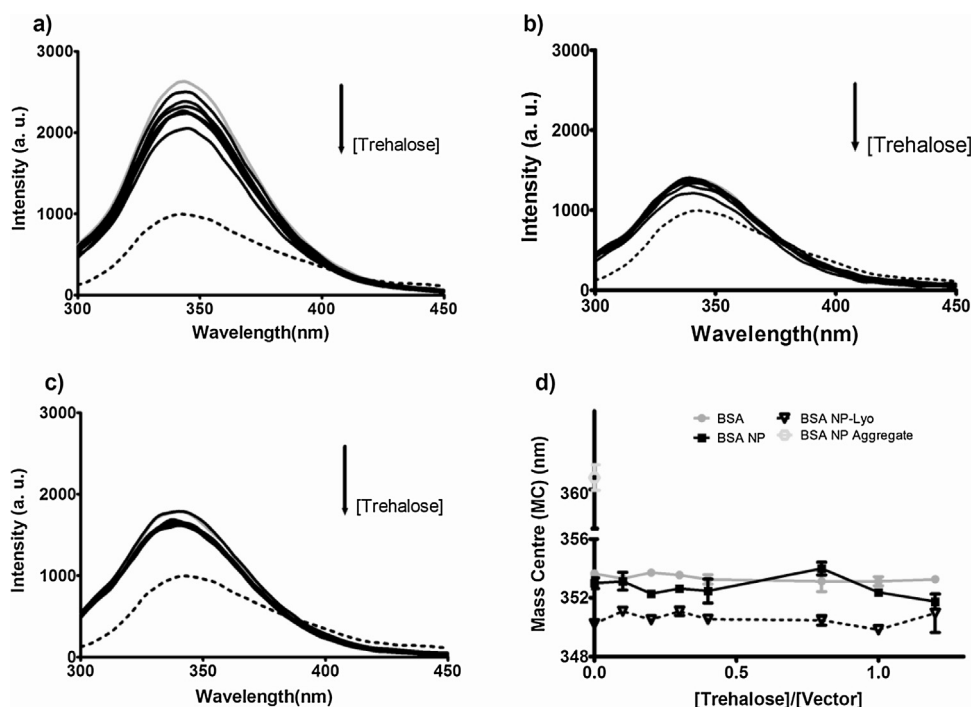


Fig. 3. This figure shows the fluorescence spectra obtained from BSA (a) and BSA NP (b) and BSA NP lyophilised with trehalose (c) and the aggregation curve with mass centres (d). The measurements are triplicates. The dotted line in all figures represents the BSA NP in an aggregation state. All samples were excited at 295 nm; slits were opened at 1 nm.

both conditions aggregates are observed, sizing up to 50 nm bigger when trehalose is present from 113 nm to 170 nm (the trehalose concentration used to achieve this was 5% w/v).

Our data is the first to obtain BSA NPs conserving size after lyophilisation with a concentration molar ratio of 325 (trehalose/BSA NP) without aggregation. More even so our contribution is also finding the condition in which the shape is preserved.

Lins et al. [13], proved that trehalose competes against water for hydrogen bonds surrounding the NP. According to Lins et al. [13] and to Chang et al. [7], trehalose may be acting as the Water-Replacement and Preferential Exclusion Theories indicate. They also state that despite being stable, the protein may still be able to fluctuate in solution slightly changing its native conformation. When lyophilised with trehalose, the protein or the proteins in the NP tend to preserve their native conformation [7,13].

3.2. Physicochemical characteristics of BSA NP

The structures of NPs were examined by different microscopies according to sample characteristics and experimental requirements; SEM (Fig. 2a and b); TEM (Fig. 2c) and AFM (Fig. 2d) microscopy.

All four pictures (Fig. 2a–e) have arrows pointing to different round shape NPs. Fig. 2a and b describes a SEM microscopy of the BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) and BSA NP lyophilised with trehalose (Trehalose/BSA NP, molar ratio 325:1). It is possible to detect bigger aggregates when there is salt present and higher monomodal definition of the NP in absence of salts, but presence of trehalose. When lyophilised the NP in PBS (pH 7.0; 30 mM) does not stay in a monomeric form but aggregate into larger NPs. The larger NPs shown have a diameter around 200–400 nm (Fig. 2a). This is probably favoured by the lyophilisation process that gives the closeness of void water spaces, allowing stronger Van der Waals forces and non-water hydrogen bonds between adjacent proteins.

Fig. 2b shows the S.E.M microscopy of the BSA NP lyophilised with trehalose. The definitions among the NP as individually differentiated can be observed. Contrary to the ones observed when lyophilising BSA NP in buffer and without trehalose, the shape observed in the 2D AFM image is worm-like (Fig. 2d).

TEM microscopy (Fig. 2e) shows NP lyophilised in presence of trehalose. This is a crucial microscopy showing that NPs do not aggregate when redissolved after lyophilisation and preserve their shape. Fig. 2d is an AFM microscopy of BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) showing that aggregation is present in all fields.

Assuming that the NP is one entity formed by a number of BSA molecules, it is of concern then based on DLS measurements to assume a shape and molecular weight taking a hydrodynamic ratio of the dimer as 70 nm in buffer PBS (pH 7.0; 30 mM); being the nanoparticle c.a. 35 nm from TEM measurements and previous findings [2,15]. Besides, based on microscopies (SEM and TEM) and on Erickson's paper [14], an approximate calculation of the molecular weight of the NP can be calculated using equations assuming that the NP has a spherical shape the volume is: $V = 4/3\pi R^3$.

Assuming that the partial specific volume is equivalent to the protein (0.73 cc/g), the volume obtained was 12,628 nm³ considering the volume formulae and replacing the volume in $V = 1.212 \times 10^{-3} \text{ nm}^3/\text{Da MW}$, the MW was obtained. Considering the hydrodynamic diameter as 35 nm (Fig. 1b) for the BSA NP and the molecular weight of the BSA NP lyophilised with trehalose is 4.5 10^7 Da gives a total of 620 BSA molecules per NP. Calculi were made to estimate the molecular weight of the different BSA NP studied (Table 2).

Note that figures in Table 2 NPs have a molecular weight a thousand-fold and ten thousand-fold than a BSA molecule. These molecular weights were used as reference for the calculation of molar base data and molar ratios.

Table 2
Molecular Weight values of the BSA NP obtained in this study.

SAMPLE	Molecular Weight (MDa)
BSA NP	45
BSA NP lyophilised with trehalose	45
BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM)	8–144
BSA	66E-03

3.3. Fluorescence study

By means of a fluorescence study we were able to observe if the trehalose *per se* added had any effect on the sample increasing or reducing the aggregation process.

A titration with increasing concentration of trehalose was carried out after and before lyophilisation. This was pursued in order to prove that the sugar did not favour aggregation of BSA, BSA NP, nor of BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM). For better illustration, a spectra corresponding to a BSA NP aggregate solution was included (dotted line). Thus, if there were aggregation in presence of trehalose, the spectra should be similar to that of the aggregated BSA NP.

BSA (Fig. 3a) and BSA NP (Fig. 3b) and BSA NP lyophilised with trehalose (Fig. 3c) samples show no significant alteration under the influence of trehalose (Fig. 3). Hence, the sugar does not affect the structure of either BSA or BSA NP, nor does it favour aggregation. There is no change in the fluorescence emission of the Trp-214 throughout the experiment. This means that the tryptophan is not affected and does not interact with the molecule.

We can learn about the protein (forming the NP) denaturation by studying the mass centre given by the spectrum of each sample (Fig. 3d). At higher wavelength displacement, higher is the protein denaturation. As observed in Fig. 3d BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) is the one with a more hydrophobic structure (displacement to lower wavelengths). BSA NP lyophilised with trehalose presents a slight displacement towards a more hydrophobic wavelength than the BSA NP. This supports recent theory describing the role of trehalose as an efficient cryoprotector.

If both NPs resulting from the different lyophilising protocols were compared; we are biased into thinking that the one with miliQ water and the aid of trehalose is the most efficient in preserving the structure of the original BSA NP. Nonetheless, with miliQ water even if the structure is preserved, the size is smaller than the original NP.

3.4. Free thiol groups detection

The number of free thiol groups in protein samples may change during the formation of the NPs by γ irradiation. For this to happen, the number of sulphide bonds has to be altered modifying the number of SH groups present in the forming BSA NP. Due to the results obtained so far, a free thiol group detection experiment was performed to elucidate if those groups are also involved in the aggregation of the NP. Ellman's Reagent method [9] enable us to detect free thiol groups in the molecule and to compare BSA and non lyophilised BSA NP samples.

BSA NP lyophilised with trehalose free thiol groups (Fig. 4) is similar to BSA NP, so in the mentioned samples the structure of the protein in the NPs is preserved if compared to the original one.

Table 3
Number of surface –SH groups of BSA NP, BSA NP lyophilised with trehalose in miliQ water and BSA NP lyophilized in buffer PBS (30 mM; pH 7.0).

	BSA NP	BSA NP lyophilised with trehalose	BSA NP lyophilised in buffer
N Thiols/BSA NP	37.1 ± 3.3	46.9 ± 2.8	54.7 ± 7.3
N Thiols/BSA NP (dim.)	295.7 ± 26.3	374.3 ± 22.3	938.3 ± 124.9

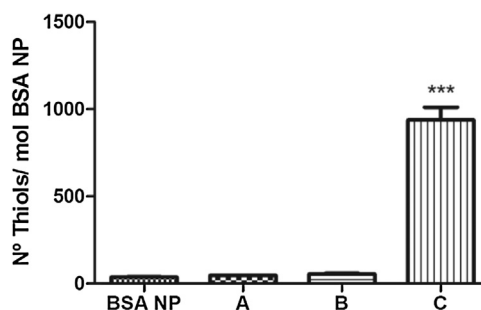


Fig. 4. This figure describes the values obtained in the free thiol group detection assay for all systems studied BSA NP and BSA NP lyophilised with trehalose (A) and BSA NP lyophilised in buffer PBS (pH 7.9; 30 mM) divided into smallest population (B) and largest population (C). Data represent triplicates of the measurement. Statistical analysis was by 1-way ANOVA, $P < 0.0001$.

To elucidate the changes of the BSA NP studied before and after lyophilisation is important to discuss what the amount of free thiols means (Fig. 4). So based on the calculations of the MW of the BSA NP in a former section (DLS) an estimation of the number of free thiol group per NP was performed.

Results showed that not all of the molecules are exposed to the surface. The concentration used for experimental purposes contained 1×10^{13} BSA NPs units, so for every BSA NP there are c.a. 40 free surface thiol groups for the BSA NP and 295 free surface thiol groups for the dimer BSA NP. As for BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) the amount of free surface thiol groups is divided in two groups, giving a total of 54 for the smaller population and 983 for the larger ones of the BSA NP lyophilised in buffer PBS. Finally, for BSA NP lyophilised with trehalose, the total amount of free surface thiol groups is 47 for a single BSA NP and 295 for the BSA NP dimer (Table 3). This comparison is made considering that the BSA NP is seen in dehydrated conditions (T.E.M., S.E.M. A.F.M sample preparation) and the dimer is found in solution (D.L.S, Fig. 2b).

When the NP was not lyophilised with trehalose, higher concentration of SH- were detected (Fig. 4). Even though according to Kumar et al. [12] while in powder-state, the humidity absorbed by the buffer salts increased the chances of gaining flexibility and inducing chemical reactions such as thiol-sulfate exchange and β -elimination, and a rearrangement of the protein structure [12] should be detected. Our data suggests fewer thiol groups for BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) than expected. This probably is a consequence of the interaction between NPs lyophilised in buffer. All of these would induce cross-linking and aggregation processes. Thus, the BSA NP lyophilised experienced loss of original structure.

The difference between BSA NP and BSA NP lyophilised with trehalose is not a marked one. Nevertheless more –SH groups were detected in the surface of BSA NP lyophilised with trehalose. This might be because even though the NP is protected by trehalose, some reactions might still occur during the process of lyophilisation.

The hypothesis pursued is that while the irradiation process takes place, some thiol groups are formed from S–S bonds inside the BSA. Others, although not broken, are rearranged in a position nearer to the surface of the molecule due to the energy spent in the process. Lyophilisation of the NP may produce, as stated before, a new arrangement of the –SH position modifying their exposure.

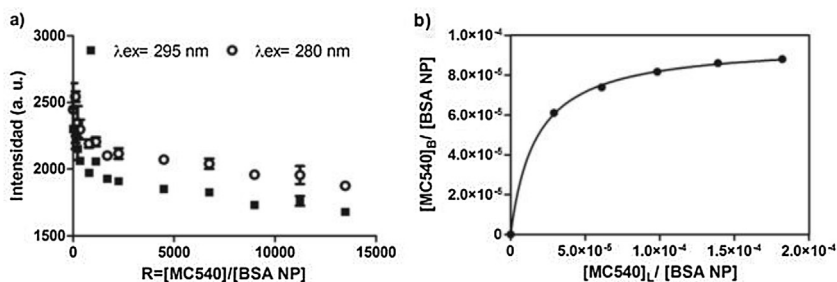


Fig. 5. The figure the quenching process that happens due to the interaction of the probe with the BSA NP lyophilised with trehalose (a) and the Scatchard plot (b) where we extract the binding constants: dissociation constant (K_d) and minimum drug loaded (B_{mx}). Data responds to triplicate measures. Graphic analysis (b) was “One site binding (hyperbola)” from Non-linear regression fit curve.

The absence of trehalose may increase this kind of modification in the lyophilised BSA NP, leading then to the difference between BSA NP lyophilised in buffer PBS (pH 7.0; 30 mM) and BSA NP lyophilised with trehalose. Values obtained here say that not all of the BSA molecules are exposed to the NPs surfaces. All in all, this leads our thinking towards a more compact structure of a golf ball-like appearance of the BSA NP. In accordance to the results, the BSA NP lyophilised with trehalose preserves the structure of the original BSA NP.

3.5. Binding properties with a fluorescent probe: MC540

Despite the fact that the structure of the BSA NP is preserved when using trehalose/miliQ water, function is the other main characteristic we are concerned for. To tests its functionality, we carried out a binding fluorescence experiment with a fluorescence probe (MC540). Aromatic aminoacids can be preferentially excited according to the excitation wavelength. Trp-214 is excited at 295 nm but all Tyr in addition to Trp when a 280 nm excitation light is used. This experiment is carried out repeatedly with different samples of BSA NP lyophilised with trehalose keeping its concentration constant, but increasing MC540 concentration. If there was any interaction between the NP and MC540, a quenching effect would be observed in the fluorescence emission.

A fluorescence intensity curve with increasing MC540 molar ratio was obtained and a specific interaction between MC540 and BSA NP was observed resulting in a quenching effect. The quenching curves for both excitation wavelength 295 nm (Trp only) and 280 nm (Trp and Tyr), were similar (Fig. 5). The decaying curves had slopes with differences within the error [12]. If both curves are similar means that only Trp are involved in the quenching effect.

We think the binding pocket where Tyr is present may be not available to interact with MC540 in accordance with other authors [15]. If this were not to be true, then differences between data obtained when the excitation is at 295 nm and/or at 280 nm from the quenching curves should have been observed.

The next step is to calculate the binding parameters, dissociation constant (K_d) and maxima drug loaded (B_{mx}), with a Scatchard equation (see Section 2.2.3 (e) from Materials and Methods) with data derived from Fig. 5a. The curve reported as MC540 bound vs MC540 free corresponds to the 295 nm excitation wavelength and represented in Fig. 5b. The fitting performed on the curve (Fig. 5b) describes the binding process to be a one-step process similar to the one between MC540 and BSA NP non lyophilised [15]. Therefore, the binding constants K_d and B_{mx} (Table 4) from Scatchard plot (Fig. 5b) were obtained and the carrier function is preserved.

We observed BSA NP in a dryer environment and its dimer in buffer solution. Table 4 depicts the maxima drug loaded for the BSA NP lyophilised and its dimer. No difference were observed, both showed the same B_{mx} value ($5.31E-6 \pm 0.28E-6$

Table 4

This table depicts the values obtained from the Scatchard Plot in Fig. 5b.

Parameters	$\lambda_{ex}=295$	$\lambda_{ex}=295$ (dimer)
B_{mx}	$5.31E-6 \pm 2.79E-7$	$5.31E-6 \pm 3.51E-7$
K_d	$2.22E-6 \pm 8.30E-7$	$3.55E-6 \pm 1.21E-6$
R^2	0.93	0.91

and $5.31E-6 \pm 0.35E-6$, respectively). Then, both entities are equal.

The drug loaded by the lyophilised NP is significantly less than the one loaded by the BSA NP non lyophilised; $0.9E-4 \pm 0.3E-4$ [15]. This could be due to the trehalose present in solution, preventing drug loading.

When comparing BSA NP/MC540 interaction K_d value ($2.76E-4 \pm 1.34E-4$), from Achilli et al. [15] with that of the, BSA NP lyophilised with trehalose, the K_d value obtained in this study has a greater affinity (hundred-fold) for the probe. Reasons may be that after lyophilisation the NP acquires a new depth on hydrophobicity. But this higher affinity does not necessarily imply a change in the structure/function of the NP.

When comparing with others K_d reported by other authors; e.g. BSA-Genticic Acid with a $K_d = 2.76E-4 \pm 1.82E-4$ [16]; our results are within the same range of binding affinity with a different drug.

During lyophilisation, a water removal process takes place. When the sample is resuspended, despite not having any alteration in its structure, it becomes more compact due to the lack of water. Then the BSA NP lyophilised with trehalose would be more hydrophobic, allowing the probe to interact with higher affinity. Therefore, functionality was not compromised but enhanced after lyophilising the BSA NP in trehalose/miliQ solution.

4. Conclusion

We described that lyophilisation of a novel albumin nanoparticle, in presence of trehalose in a relationship of 300–400 to 1 molar ratio (Trehalose: BSA NP), being the maximum protection efficiency that allows the preservation of the structure and function of the BSA NP formed.

Lyophilisation in buffer rendered different aggregates in the range of 35 nm up to 350 nm, proving not to be a reproducible method to preserve the NP structure. In contrast, when trehalose was used and lyophilisation was in miliQ water, diameter sizes were preserved. We also observed no differences in number as regards the free thiol groups available in the NP.

We also obtained a higher affinity for the drug comparing BSA NP with BSA NP lyophilised with trehalose. MC540 as a probe biased our thinking into a preserved functionality of the BSA NP lyophilised with trehalose as a drug carrier. Nevertheless, the BSA NP lyophilised with trehalose renders in a higher efficiency of the affinity site when MC540 is used as a theragnostic probe.

All in all, we think that the lyophilisation in trehalose/miliQ at a ratio of 325:1 (trehalose:BSA NP) as a suitable protocol thinking for long-term storage of the NP where structure and function are both preserved.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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