Rheological characterization and physical-stability of gamma irradiated anthocyanin-loaded albumin nanoparticles

Sofia L. Candido¹,²; Macarena Siri¹,²,†; Estefanía Achilli²,³; Juan C. Moreno³,⁴,⁵; Cristian Lillo¹; Patricia H. Risso⁶; Jeffrey Bodycomb⁷, Luis Martínez¹,²; Jorge Montanari¹,²; Fernando C. Alvira¹,²; Silvia del V. Alonso¹,²,*

¹Universidad Nacional de Quilmes, Departamento de Ciencia y Tecnología, Laboratorio de Bio-Nanotecnología, Bernal, Buenos Aires, Argentina.

²IMBICE, CONICET-CCT La Plata-UNLP-CICPBA, GBEyB, Grupo de Biología Estructural y Biotecnología, Instituto Multidisciplinario de Biología Celular, La Plata Argentina.

³Universidad Nacional de Quilmes, Departamento de Ciencia y Tecnología, Bernal, Buenos Aires, Argentina.

⁴CONICET, Consejo Nacional de Investigaciones Científicas y Tecnológicas, Argentina.

⁵Universidad Nacional de Avellaneda, Departamento de Tecnología y Administración, Ingeniería en Informática.

⁶Universidad Nacional de Rosario, UNR Departamento de Química Física, Rosario, Argentina.

⁷Horiba Instruments, Inc, Irvine, California, USA

† Departamento de Química Biológica, Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, CONICET), Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria, X5000HUA, Córdoba, Argentina.
KEYWORDS Anthocyanins, BSA-NPs, antioxidant capacity, nanoparticle-molecules number, rheological behavior.

ABSTRACT: The active blueberry compounds called anthocyanins have poor oxidation stability, but, if encapsulated by protein nanoparticles, they can be protected due to the slowing down of the oxidation process. This work describes the advantages of using a γ-irradiated bovine serum albumin nanoparticle bound to anthocyanins. The interaction was characterized biophysically, mainly by rheology. By computational calculation and simulation based on model nanoparticles, we estimated the number of molecules forming to the albumin nanoparticles, which allowed us to infer a ratio of anthocyanin/nanoparticles. Measurements by UV-VIS spectroscopy, FT-IR spectroscopy, fluorescence spectroscopy, dynamic light scattering (DLS), ζ potential, electron transmission microscopy and rheology at room (25 °C), and physiological (37 °C) temperatures were performed. The spectroscopy measurements allowed identifying additional hydrophobic sites created during the irradiation process of the nanoparticle. Based on the rheological studies, it was observed that for all the temperatures selected, the BSA-NP trend is a Newtonian flow behavior type, and there is a direct correlation between dynamic viscosity and temperature values. Furthermore, when anthocyanins are added, the system increases its resistance to the flow reflected in the morphological changes observed by TEM, confirming the relationship between viscosity values and aggregate formation.
1. Introduction

Anthocyanins are a natural product from different plants and specially from blueberries.\(^1\) They have high antioxidant capacity and other biological functional benefits \textit{in vivo} like scavenging of free radicals, regulation of blood-lipids, and anti-inflammatory and anticancer properties.\(^2\) Blueberry anthocyanins have a very poor shelf-life stability, degrading under prolonged illumination and storage.\(^3\) Moreover, when entering the digestive tract, they are easily metabolized or auto-aggregate, preventing cell membrane transposing.

Bovine serum albumin (BSA) has been reported to protect the DPPH free radical scavenging potential of dietary polyphenols and gallic acid.\(^4\) This work covers studies on gamma-irradiated albumin nanoparticles that explain the advantages of using BSA-NPs as drug delivery systems for this kind of compound.\(^5\)-\(^9\) Previous studies position the gamma irradiated nanoparticle as a potential drug delivery system due to its novel design, reproducible manufacture, non-toxicity and stability, among other advantages.\(^5\)-\(^9\)

The use of nanoparticles relies mainly on their design and, particularly, their size, shape, and density.\(^10\) Controlling nanoparticle size and shape is important, as these characteristics may influence the probability of margination when entering the organism. Alongside the parameters mentioned above, in this work we used rheology shear rate and dynamic viscosity to explore nanoparticle stiffness in depth.\(^11\),\(^12\)

Some authors, like Amin, et al., consider that formulations of stable protein nanoparticles with controlled rheological response is an area of high interest for the high growth of the biotherapeutic industry.\(^13\) Long circulating nanoparticles and with long lasting shelf-life that exhibit rheological properties that enhance/optimize the retention and delivery of therapeutic proteins will be the future panacea. Thorough characterization of the design nanoparticles is critical to successfully developing a powerful-built drug delivery system. The rheological analysis of the different systems studied (BSA, BSA-NP and anthocyanins) allows understanding the transport capacity of the active ingredients in the nanoparticles with an adequate controlled release. Considering this criterion, in this work we have focused on studying the effect of a shear rate for the different dispersions at different study temperatures (environment and human body) to determine the correlation between dynamic viscosity and temperature.
The BSA-NP reported here was prepared by $\gamma$-irradiation, which allows exposing new hydrophobic sites where drug interaction takes place, lengthening drug circulation time and possible degradation and protecting the organism from drug side effects.\textsuperscript{14} More importantly, the binding crosslinking between albumin molecules in the nanoparticle does not affect the protein’s main function as a carrier.\textsuperscript{8} Data obtained contain specific information on how protein molecules might be organized in the nanoparticle, as well as about the possible number of external molecules, their stability and how they can be modified by interacting with anthocyanins.

Up to date albumin-based nanomedicines have low stability of blood circulating times, resulting in early nanoparticle degradation and a loss of drug load before reaching the tumor site.\textsuperscript{15, 16} The way to improve the efficacy of albumin-based nanomedicines is to enhance their particle stability. To achieve this objective, we propose using a positive binding compound to enhance intermolecular interaction with the drug, increasing complex stability and, ultimately, improving shear stress flow profiles to enhance body temperature stability. Such drug-binding albumin-based nanomedicines have not been achieved by chemical modification because of the low conjugation yield, harsh reaction conditions, poor biocompatibility and limited improvement in stability. Thus, a critical issue is how to study and characterize the drug-albumin binding while the albumin retains its binding characteristics.\textsuperscript{17, 18} In this work, we complete the study and discussion of the macroscopic properties based on rheological studies of the nanoparticles. This study is critical because it exposes the behavior of the nanoparticles in contact with the external environment as a possible anticancer drug delivery.

2. Materials and methods

Bovine serum albumin (BSA) was obtained from Sigma Aldrich 99\% (MW 66.6 KDa). Blueberry anthocyanin was obtained from Mendoza, Argentina. It was extracted and purified according to Montanari et al., 2013.\textsuperscript{19} The total anthocyanin content was up to 950 mg every 100 g of extract whereas monomeric anthocyanins content was up to 80\%. All other reagents used were HPLC-grade and used as purchased.\textsuperscript{20}

2.1. BSA-NP Preparation
BSA was dissolved in milliQ (mQ) water, adding 0.6 ml of ethanol absolute drop wise to the protein solution up to a final concentration of 30%, keeping it at 0ºC under constant swirling. The BSA solution was \( \gamma \)-irradiated using 60Co source (PISI CNEA-Ezeiza, Argentina) with dosage below 5 kGy/h and keeping the temperature between 5-10 ºC.\(^5\),\(^6\)

The NP obtained was passed through a SEPHADEX G25 column to exchange the ethanol solution with mQ water, followed by G200 Sepharose molecular exclusion column purification.

2.2. **Blueberry Extract Preparation**

Blueberry extract was obtained from 25 g of the fruit by solid liquid extraction with ethanol 96% and HCl 0.1%. Fruits were then sonicated at 28ºC for 1 h at 40 kHz and the extract was filtered. Finally, the ethanol was evaporated in a roto-evaporator until constant weight was reached. The extract was then resuspended in mQ water. The concentration of monomeric anthocyanins was determined employing the method described in Nicoue et al., 2007,\(^2\) The concentration was calculated as indicated in Montanari et al., 2013,\(^2\) according to equation 1:

\[
\text{Anthocyanins (moles/l)} = \frac{\text{Abs} \times \text{DF}}{(\varepsilon \times 1)}.
\]

Equation 1

Where:

the Absorbance (Abs) is calculated according to the following equation, and DF is the dilution factor:

\[
\text{Abs} = (A_{520nm} - A_{700nm}) \text{ pH 1.0} - (A_{520nm} - A_{700nm}) \text{ pH 6.5}.
\]

Equation 2

Considering \( \varepsilon \): 26.900 (AU/cm mol l)

2.3. **BSA-NP/Anthocyanin interaction**

For these experiments we used different concentrations for BSA-NP (2 nM), free BSA (6.84 \( \mu \)M), and anthocyanin (7.43 \( \mu \)M). The following molar ratios were studied: 1:1, 1:10, 1:70, 1:100 and 1:200.

2.4. **UV-Vis absorption Spectroscopy**

Samples were measured with a Thermo Scientific Nanodrop 1000. Detection spectral range was 200-700 nm with a resolution of 2 nm. With UV-Vis spectroscopy, the concentration of BSA-NP at the elution of the molecular exclusion chromatography was obtained. Also, the concentration of anthocyanin in blueberry was measured by UV-Vis spectroscopy.
2.5. **FT-IR Spectroscopy**

The infrared profile of the BSA, BSA-NP, Anthocyanin and BSA-NP:A were measured in an FT-IR Affinity1-Shimadzu ATR. Measurements ran wavelength from 400 to 4000 cm\(^{-1}\), with 45 scans per sample and a resolution of 4 cm\(^{-1}\). Samples were blown-dried until a film was formed to obtain the spectra free of IR-water peaks. The concentration of the sample was 2 nM. The blank was subtracted by software.

For a better visualization of the overlapping components arising from the distinct structural elements, spectra were processed using Kinetic software developed at the Structure and Function of Membrane Biology Laboratory, Université Libre de Bruxelles, Brussels, Belgium. After subtraction of water vapor and side chain contributions, the spectra were baseline corrected and area normalized between 1700 and 1600 cm\(^{-1}\). The spectra were then deconvoluted using Lorentzian deconvolution factor with a full width at the half maximum (FWHM) of 20 cm\(^{-1}\) and a Gaussian apodization factor with a FWHH of 13.33 cm\(^{-1}\) to achieve a line narrowing factor K=1.5. Second derivative was performed on the Fourier original (K = 1) spectra. Bands were identified using both spectra (K=1.5 and second derivative) and used as initial parameter for a least squares iterative curve fitting of the K = 1 IR band in the amide I’ region, using mixed Gaussian/Lorentzian bands. Peak positions of each identified component were constrained within ±2 cm\(^{-1}\) of the initial value. FWHH initial values were 9 (β-sheet high wavenumber component), 17 (β-sheet low-wavenumber component), 43 (helix) and 20 (turn) cm\(^{-1}\), varying within ranges expected for each type of secondary structure, 8-11, 14-19, 30-56, and 5-30, respectively.

2.6. **Fluorescence Emission Spectroscopy**

A spectrofluorometric SCINCO model S2 was employed. The instrument employees a tungsten lamp as an excitation source, and PMT as detection. The excitation wavelength is selected with a diffraction grating of 1200 lines/mm and the detection. The excitation and detection slits were set at 2.5 nm. In the experiments performed to determine the binding constant at equilibrium, the excitation wavelength was set at 280 (Tyr and Trp) and 295 nm (Trp). The emission range was set between 290-500 nm and 305–500 nm, respectively. Results were analyzed with Origin software.
Samples were prepared for each set of experiments as follows: a constant concentration of 2 nM BSA-NP was maintained, and extract from 0 - 0.4 µM was added to separate samples of increasing anthocyanin (A) concentration. BSA-NP: Ratios from 1 to 200 (BSA-NP: A) were obtained using this method. All samples were diluted in mQ water.

Prior to any analysis, the fluorescence spectra obtained from BSA-NP: A were subtracted from those of A at the same concentration for each sample, because A exhibits fluorescent emission when excited at these wavelengths. All the spectra presented here were automatically corrected by subtracting the spectra of the respective polyphenol. This method for correction has already been validated by Papadopoulou et al., 2005.22

To determine the dissociation constant, the data treatment method described in Sevilla et al, 2007 and Siri et al., 2016 was followed.7-23. The dissociation constants Kd and Bmax, corresponding to the maximum of A bound to the protein for each binding site in the BSA-NP, were determined by following the changes in fluorescence signal. These changes are induced by the binding of the drug to the protein. The concentration of A attached to the vehicle (B) was the variable in the test. When BSA-NP was in excess, [A] would tend to 0. Therefore, the value of the area under the curve of each spectrum was plotted from the point of maximum emission (F), versus the [A] / [BSA-NP] ratio.

\[ F = F_0 + m_i v \]  
Equation 3

From equation (3), "mi" is determined as the slope of the curve and \( v = B / [\text{BSA-NP}] \). With the experimental data, then B can be calculated according to

\[ B = v * [\text{vehicle}] \]  
Equation 4

The amount of free ligand, L, is obtained from the difference between the amount of total ligand and the amount of bound ligand, calculated by equation (4). With this data, a Scatchard diagram can be obtained.

\[ \frac{v}{L} = n K_d - v K_d \]  
Equation 5

where "n" is the number of binding sites and "Kd" is the dissociation constant for complex formation. To obtain this constant, the data was analyzed with Origin software.

2.7. Antioxidant activity determination
Free radical scavenging activity of both the blueberry extract and the BSA-NP was assessed by the DPPH method. This is a stable free radical, which color changes after the reaction with the antioxidant compounds in the extract. Briefly, aliquots between 0 and 4E-6 M of anthocyanin were added by triplicates to a 3.1E-3 (w/v) solution of DPPH in methanol. Triplicates of samples were incubated in darkness for 30 min at room temperature and then the absorbance was read at 515 nm. This value is inversely proportional to the free radical scavenging of the anthocyanin. BHT was used as positive control of free radical scavenging. IC50 was calculated for each formulation as the concentration that inhibits 50% of the free radical. This value was obtained from a linear regression of the inhibition percentages (I%) produced by each sample concentration, which were calculated using Equation 6, in which A values correspond to the respective absorbances.

\[ I\% = \left[ \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100 \tag{Equation 6} \]

Thus, the I% of the working concentration of anthocyanins in BSA-NPs used in this work, (1:70 mol ratio) was also determined for the free extract and the BSA-NP.

2.8. Dynamic Light Scattering and \( \zeta \) potential

A Malvern Zetasizer Nano was used for all nanoparticle size determinations. The DLS samples dissolved in mQ water were always measured thrice, at 25 °C and 37 °C. Long term stability was measured with a Nanoparticle Tracking Analyzer (NTA), the HORIBA ViewSizer 3000. Zeta potential was determined with a HORIBA SZ-100.

Zeta (\( \zeta \)) potential is the potential in the interfacial double layer (DL) at the location of the shear plane compared to a point in the bulk fluid away from the interface. In other words, zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The zeta potential of a protein can be used as an indicator of stability. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles (proteins) in a dispersion. According to general colloid chemistry principles, a dispersed system typically loses stability when the magnitude of the zeta potential decreases to less than approximately 30 mV (regardless of whether the charge is positive or
negative). As a result, there will be some regions surrounding the condition of zero zeta potential (the isoelectric point, or IEP) for which the system is not particularly stable. Within this unstable region, the protein may agglomerate into larger size particles.

2.9. Microscopy

Transmission Electron Microscopy (TEM) was performed with a Philips EM 301 with an acceleration voltage of 60 kV, using carbon-coated 400 square mesh copper grids. The samples were diluted and then stained with uranyl acetate 2%. TEM images were taken before and after the rheological analysis.

For atomic force microscopy (AFM.), the BSA-NP sample was purified by exclusion chromatography in mQ water. A total of 20 μl of the sample was deposited on freshly cleaved mica. It was then dried for 10 min under flowing nitrogen. Images were obtained using a Dimension Icon in Peak Force QNM (PFQNM) (Bruker®). Measurements were using a rectangular silicon tip with a nominal spring constant of 42 N/m and a tip radius of 12 nm. This microscopy process was performed at the Institute of Biophysics, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

2.10. Rheological Analysis

Rheological characterization was performed with a Stress Controlled Rheometer AR-G2 (TA Instruments) with temperature control bath (Julabo AC100) and a 40 mm geometry cone-plate (55 μm truncation, 2° cone). The shear rate range was from 1 to 160 s⁻¹. In each assay, 0.7 ml of sample was placed on the plate at 25 ºC or 37 ºC. The sample on the plate came to temperature in 2 minutes. Then, before measurement, samples were stabilized with a pre-shear (0.3 s⁻¹ in 30 s). Next, the sample was left to rest for 30 s and only then the assay was analyzed.

Data was captured with Advantage Software (TA Instruments). Duplicates were run and the data was adjusted to a rheological model (Newton Law) as seen in equation 4.

\[ \tau = \mu \gamma \]  

Equation 7

Where

\( \tau \): Shear Stress [Pa].
\[ \gamma \]: Shear Rate [s\(^{-1}\)].

\[ \mu \]: Dynamic Viscosity [Pa.s].
3. Results

3.1. BSA-NP characterization

3.1.1. Purification of the BSA-NP by molecular exclusion chromatography

After the BSA-NP synthesis process, the NP was passed through a Sephadex G25 column to exchange ethanol solution with water. Afterwards, the BSA-NP was separated from the remaining molecular BSA (c.a. 20 %) using G200 Sepharose molecular exclusion column purification. The final BSA-NP concentration was 2 nM. The NP molecular weight estimation was carried out following the method of Siri et al., 2020. Briefly, the molecular weight and quantity of albumin molecules in the BSA-NP are calculated from aqueous density measurements of the NP δ at 25 °C. Considering density δ:

\[
\delta_{BSA-NP} = \frac{m_{BSA-NP}}{V}
\]

Equation 8

where,

\[
\delta_{BSA-NP} = \delta_{el} - \delta_{H_2O} = 0.9722 g / ml
\]

Equation 9

Where \(\delta_{H2O}\) is the density of water at 25 °C and where \((V)\) is the volume in ml. It follows that the apparent experimental density of BSA-NP in dispersion is 0.9722 g / ml in an aqueous dispersion of 1 ml at 25 °C.

3.1.2. Atomic Force Microscopy (AFM)

Based on the data obtained by AFM microscopy, the BSA-NP observed has a value \(h = 64\) nm and an area obtained by the ratio pixel/nm equal to 21412.96 nm\(^2\) (Fig. 1), and with a value equal to 82.55, a sphere volume equal to 822476.17 nm\(^3\) and a size of 100-200 nm is calculated. Despite the heterogenicity in the volume of the NPs presented in Figure 1, the spherical shape is always conserved. Previous studies describe the colloidal suspension of BSA-NP as a monomodal population with very low percentage of higher aggregates based on TEM and DLS studies; Figure 1 shows a nonhomogeneous field of nanoparticles from 30-100 nm where the shape is always a sphere.
3.1.3. Experimental determination of the molecular weight of BSA-NP

This experimental determination was based on the DLS hydrodynamic radius and gravimetical studies carried out by Siri. et al., 2020. The theoretical model used to determine the molecular weight of BSA-NP assumed spherical particles. The shape of the particles was inspected by TEM and AFM. Conducting DLS experiments at 25 °C, the hydrodynamic radii of the nanoparticles were 15 nm ± 2. By calculating the volume of a sphere, and the definition of mass density, the mass of one nanoparticle can be determined. This calculation gives a result of 8.5 MDa, assuming a density of 0.97722 g/ml (value extracted from Singh, M. et al., 2005). The values obtained are described in Table 1.
Table 1 Experimental determination of the molecular weight of BSA-NP in water at pH = 6.5.

| Volume$_{NP}$ [nm³] | Area$_{NP}$ [nm²] | Radius$_{NP}$ [nm] | MW$_{NP}$ [MDa] | Total Molecules of BSA-NP |
|---------------------|-------------------|-------------------|------------------|-------------------------|
| 1.4E⁵               | 2827              | 15± 2             | 8.5              | 128                     |

3.1.4. BSA-NP Modeling Dimensions

Based on experiments and on an earlier model, a revised critical proposal of the BSA molecular structure drives consideration that it could be enclosed in a geometric figure with a triangular base and a given thickness. Under this premise, the ABC sides of this triangular base have an average length of 6.96 nm (Table 2) and a thickness of 2.71 nm. The most relevant outcome of this approach is the fact that internal angles have an average value of 1.047± 0.095 rad. These dimensions lead to a first approximation; namely, that the BSA could be estimated as a volume with two triangular-like equilateral faces (6.96 nm by side) and thickness 2.71 nm; acquiring a surface Area$_{BSA}$=20.83 nm² and volume Vol$_{BSA}$= 56.4 nm³.

Table 2 Approximations of the BSA dimensions

| Side | Length [nm] | Opposite Angle [rad] |
|------|-------------|----------------------|
| A    | 7.18        | 1.099                |
| B    | 7.20        | 1.105                |
| C    | 6.49        | 0.937                |
| Average | 6.96 | 1.047                 |

3.1.5. BSA-NP Model Dimensions.

The BSA-NP has been modeled as a spheroid composed of concentric internal shells with non-negligible thickness. Each of these spherical shells has an empty in-between layer and the rest of BSA on the surface (which is why shell thickness is 2.7 nm).
The peculiarity of modeling the BSA as an equilateral triangle whose ligature points are at the vertices is that it allows the configuration of new equilateral triangles formed by 4 BSA, 9 BSA, etc. The separation between the BSAs is of the order of 0.15-2.50 nm, so the triangular/hexagonal configuration approach remains valid. This separation is considered as it allows "curving" protein configuration.

This fact allows treating the spheroid as a regular polyhedron with equilateral triangular faces. Considering this, an icosahedron (solid with 20 equilateral triangular faces) was chosen. Similar results were obtained experimentally by TEM and AFM.

The next step is estimating concentric shell separation. Estimates are presented with separations of 0.3 nm, 0.8 nm, 1.3 nm and 1.8 nm. Obviously by increasing the value of the separation, the number and size DLS of inner shells is reduced. This model is applied to estimate the amount of BSA in a BSA-NP with a radius of approximately 15 nm, obtaining the results shown in Table 3 for the different thicknesses of separation between shells:

| Shell Separation [nm] | Approx. Nº of BSA Molecules | Nº of shells | Vol. occupied by BSA-NP (nm³) | Free Vol. of BSA-NP (nm³) | Free Vol. of BSA-NP (%) |
|-----------------------|-----------------------------|--------------|------------------------------|--------------------------|------------------------|
| 0.3                   | 237                         | 4            | 12820                        | 1320                     | 9.3                    |
| 0.8                   | 215                         | 3            | 11570                        | 2570                     | 18.1                   |
| 1.3                   | 198                         | 3            | 10580                        | 3560                     | 25.1                   |
| 1.8                   | 183                         | 3            | 10359                        | 3777                     | 26.7                   |

Comparing both simulation and DLS results, we conclude that the amount of BSA per NP is approximately 183 albumins. According to the calculations made previously, the BSA-NPBSA-NP can be approximated as a spheroid composed of 3 concentric, also spheroidal layers. The separation between layers would be 1.8 nm, and that involves a volume occupied by BSA of approximately 10300 nm³ and a free volume of 3800
nm$^3$ (27%). With this spacing between shells, the model leads us to conclude that the BSA-NP has a less compact spherical shape.

3.2. Interaction of BSA-NP with anthocyanins.

To further characterize BSA-NP interaction with the anthocyanins, UV-Vis, FT-IR and fluorescence spectroscopy assays were performed. Fluorescence spectroscopy was performed with the objective of determining the degree of interaction between BSA-NPs and their binding site.

Table 4 Concentration determination with the theoretically and experimental calculated molecular weight of BSA-NP.

| MW$_{\text{BSA}}$ [KDa] | Protein Concentration [M] | MW$_{\text{BSA-NP}}$ [KDa] | BSA-NP concentration [nM] |
|--------------------------|---------------------------|-----------------------------|---------------------------|
| 66                       | 2.4E\text{-}7             | 8500                        | 2                         |

To explore the BSA-NP:A interaction, FT-IR spectroscopy was carried out (Figure 2). The BSA protein was also measured in this experiment to study the structural differences between our NP and monomeric BSA. For both, albumin and NP, the characteristic maxima identified was 1645 and 1656 cm$^{-1}$, respectively. These peaks are associated to the $\alpha$-helix content of the albumin protein (Figure 2 a-b). For anthocyanin the characteristic maxima observed were 1722 and 1652 cm$^{-1}$ belonging to the vibration of C=O in C=C-COOR and benzene skeleton, respectively$^{30}$ (Figure 2 c). Shifts of these peaks in the BSA-NP:A spectrum denote interaction between both components (Figure 2d). The maxima at 1652 cm$^{-1}$ in the A spectrum and 1656 cm$^{-1}$ in the NP one, presented a shift towards lower frequencies in the BSA-NP:A spectrum c.a. 1640 cm$^{-1}$, while the maxima at 1722 cm$^{-1}$ in the A spectrum, presented a shift towards higher frequencies in the BSA-NP:A spectrum: c.a. 1727 cm$^{-1}$. These changes, together with the changes in intensity of the spectra suggest an alteration of the secondary structure of the protein NP when interacting with anthocyanins, probably via hydrogen bonding and hydrophobic attractions$^{31,3,32}$.
Figure 2 FTIR spectra from 1800 – 1200 cm⁻¹ of BSA (a), BSA-NP (b), anthocyanin (c) and BSA-NP:A (d). Remanent A was taken from the BSA-NP:A by centrifugation in AMICON centricons with a cut-off membrane of 10K.

The amide I’ region (1700 and 1600 cm⁻¹) correspond predominantly to the amide C=O stretching, being especially sensitive to the protein interaction with its environment³³,³⁴,³⁵. By using enhancement methods we were able to see the overlapping components arising from the distinct structural elements of the BSA, BSA-NP and BSA-NP:A spectra. At this point we would like to clarify that the accuracy of such procedure is prone to some error due to curve-fittings routines, the information provided is powerful about the relative changes and comparative values between individual components and BSA-NP:A formed.

A first visual examination comparing the BSA-NP to BSA spectra show a very different shape of the amide I’ region (Figure 3a-c) suggesting structural differences of the molecules. Both molecules depict main contributions of maxima around the 1650 cm⁻¹ peak. For BSA-NP this maximum is set at 1660 cm⁻¹ and 1646 cm⁻¹, and for BSA at 1646 cm⁻¹. While this band is generally assigned to the α-helix structure of BSA, we were unable to discern between α-helix and random structures for the NP. These bands have some disordered contribution which we were not able to unambiguously distinguish within the amide envelope. Nevertheless, this analysis allows to denote structural differences between BSA and BSA-NP. BSA has an
α-helix content 68.17%, whereas BSA-NP has a α-helix/random content of 81.12%. Further differences are observed when analyzing the band assigned to the β-sheet content of both molecules (c.a. 1620 cm⁻¹). BSA-NP has a β-sheet of 8.22 %, while BSA has a total of 15.47 %. The β-turn content also diminishes when the NP is formed, being 16.38 % for BSA and 10.65 % for the NP. This suggests that in the NP formation process most likely β-sheets are involved in the protein-protein interaction.

When the anthocyanin is in association with the NP further changes occur to the NP structure (Figure 3 d). The band assigned to α-helix/random is set at 1640 cm⁻¹ denoting a more restricted environment for the C=O to stretch in, the content of this structure also decreased from 91.12% in BSA-NP to 53.95 % in BSA-NP:A. Moreover, β-sheet content in the BSA-NP when anthocyanins is present increased to 43.3%, while the β-turn content decreased to 2.75 %. These changes not only confirm the interaction between BSA-NP and anthocyanins, but also shows how anthocyanins affects the NP structure. Similar results were observed by Wei et al., 2018. As hydrogen bonding and coupling between the transition dipoles are among the most important factors governing the conformational sensitivity of amide bands, the changes observed suggest an interaction of the benzene in the anthocyanins and the C=O, C–N groups of the BSA-NP through a combination of the hydrogen bond and hydrophobic interaction.
Both, anthocyanin and BSA-NP UV-vis spectra show an absorbance peak at 280 nm (Figure 4). This is due to the aromatic groups in both. Because of this we focus the analyses in the peak at 520 nm corresponding to anthocyanins. As the concentration of the cyanine increases in the BSA-NP solution, so does the absorbance of the 520 nm peak. This approach is further evidence of a conformational change in the NP induced by anthocyanins binding. The assay was performed in duplicate, with no ethanol in the solution. Figure 4A shows the probable formation of the complex BSA-NP by an increase in absorbance at 280 nm (BSA-NP) and 520 nm (Anthocyanin). As the concentration of anthocyanins increases, so does the absorbance of aromatic groups at 280 nm corresponding to the BSA-NP. This effect gives rise to a possible conformational change in NP sites when anthocyanins bind and is visualized when plotting the Abs increment at 280 nm vs concentration, exposing a different behavior of the molar coefficient of the BSA-NP after A concentration $5 \times 10^{-7}$ (Fig. 4B). Moreover, the increase in width of the maximum at 280 nm, is also indicative of a conformational change in the BSA-NP. Usually, these modifications in the absorbance
profile of a protein nanoparticle suggest an increase in size.  Figure 4C shows a linear Lambert-Beer plot corresponding to a complex (BSA-NP: A) formed with maximum at 520 nm.

According to Stanley and von Hippel (1989), the extinction coefficient depends on the amino acid sequence, especially Trp. They report an $\varepsilon$ of 43623 M$^{-1}$ cm$^{-1}$ for BSA and 38553 M$^{-1}$ cm$^{-1}$ for HSA, two and one Trp respectively. We obtained two coefficients at 280 nm for the BSA-NP, $\varepsilon$: 152803 M$^{-1}$ cm$^{-1}$ and 122444 M$^{-1}$ cm$^{-1}$. From these values, we can infer that one of Trp is hindered. At 520 nm, we obtained an $\varepsilon$ of 155100 M$^{-1}$ cm$^{-1}$, which points to complex formation.
The BSA contains two tryptophan residues (Trp-134 and Trp-212) and twenty-one tyrosines (Tyr). It also contains two hydrophobic sites, one in Domain II, governed by Trp 212, called the Sudlow I site, and the other governed by Tyr and Trp, called the Sudlow II site.36
By exciting the samples at 295 nm wavelength, only the contribution of the BSA-NP Trp is obtained, and when excited at the 280 nm wavelength, the contributions of both Trp and Tyr are obtained. This fluorescent emission profile provides more detailed information on the structure observed by UV-Vis absorbance, as well as the interaction between the NP and anthocyanins.

The fluorescence quenching pattern of the aromatic groups in BSA-NP at increasing concentrations of anthocyanins is shown in Fig. 5. An overview of BSA-NP obtained emission spectra depicts a decrease in the intensity of the spectra showing that the anthocyanins are interacting with the Trp and Tyr residues in the BSA-NP. This decrease in intensity, in turn, suggests that the main interaction sites remain and are still available once the BSA-NP is formed. After adding anthocyanins in excess (100 and 200-fold respectively to BSA-NP), a redshift in the emission peak at $\lambda = 280$ nm and $\lambda = 295$ nm can be observed (Figure 5). This redshift implies an increase of polarity and a reduction of hydrophobicity of the tryptophan residue region.

![Figure 5 Corresponding fluorescence emission spectra of BSA-NP interacting with increasing concentrations of A, excited at 280 nm (A) and 295 nm (B). The spectra are a result of the difference between the mixture spectrum and A at the same concentration.](image)

When samples were excited at 295 nm, a decrease in emission at 342 nm wavelength was observed (Figure 5b). This is due to the quenching effect of the Trp fluorescence in Sudlow I and II, according to Cahyana & Gordon, 2013. At pH=6.5, electrostatic interactions and hydrogen bonding become relevant to hydrophobic sites interactions.

A Scatchard fitting adjustment was made for each curve to obtain the binding parameters (Fig. 5). It is observed that the $K_d$ constants do not differ despite having excited the BSA-NP:A system at different
excitation wavelengths. This points to equal participation of the different Sudlow pockets of the albumins in the NP. According to the values found in literature for drugs belonging to the cyanine group, the values reported in this work indicate a great affinity of the BSA-NP towards anthocyanins.\textsuperscript{7,37}

The first experimental points of the BSA-NP:A interaction study that conserved linearity were used to draw a linear regression that led to the estimation of the values of $m_i$ and $F$ from the $F$ vs Ratio BSA-NP:A graph (Table 5).

**Table 5 Parameters from the linear regression from the BSA-NP interaction with A (Fig.5).**

| Parameters | 280 nm | 295 nm |
|------------|--------|--------|
| $M$        | -2235.3| -2369.3|
| $F_0$      | 58371  | 55877  |

**Table 6 Binding values between BSA-NP: A according to Fig.5**

| Parameters | 280 nm | 295 nm |
|------------|--------|--------|
| $B_{max}$  | $6.761E^{08} \pm 3.37E^{08}$ | $5.92E^{08} \pm 3.61E^{08}$ |
| $K_d$      | $1.023E^{07} \pm 1.42E^{07}$ | $1.02E^{07} \pm 1.72E^{07}$ |
| $r^2$      | 0.72   | 0.53   |
Our findings are consistent with the literature, where it is reported that additional hydroxyl groups strengthened the association of polyphenols with albumin at pH 7.4.\textsuperscript{41-43} The hydroxyl substituents of anthocyanins may form hydrogen bonds with the polar groups in the protein. According to Cahyana et al., the effect of pH on binding depends on both the structure of anthocyanins at different pH values and the
change of state of albumin.\textsuperscript{38} The hydrophobic effect plays a major role in the interaction of anthocyanins at pH 4, but is less important at pH 7.4. Our work considers a pH value closer to 7.4 (6.5) than to pH 4.

By fluorescence spectroscopy we were able to further characterize the interaction of the BSA-NP with anthocyanins. Although this interaction causes a conformational change in the NP, as observed in the FT-IR and UV-vis spectroscopy experiments, the natural binding sockets of the BSA (Sudlow site I and II) are still functional and used for the interaction with the drug as shown by fluorescence spectroscopy. As the emission spectra show a decrease in intensity at both $\lambda_{\text{ex}}$ (280 nm and 295 nm), it is estimated that both Sudlow sites participate in the interaction with anthocyanins; because of its lower K value, a higher affinity is inferred with Sudlow I (280 nm).

3.3. Percentage determination of BSA-NP capture (partition coefficient)

Considering the results obtained in UV-Vis and fluorescence assays, where the interaction of BSA-NP with anthocyanins and the molar ratio in which they begin to saturate with A is demonstrated, the capture rate was determined. The principle of solid-liquid ethanol extraction was considered to obtain the active principles of blueberries. Using the value of absorbance intensities, the calculated capture percentage was 77\% (c.a. 107.8e-8 nM drug bound).

\[
\text{BSA-NP:A (1:70)} \times \frac{100}{(\text{BSA NP – EtOH – BSA NP:EtOH})} = 77\% \quad \text{Equation 10}
\]

Compared to the results in the fluorescence experiments mentioned above, 40\% more of A bound to the BSA-NP is detected by this method. This suggests that the additional drug detected here is not bound to the
Sudlow sites of the BSA-NP, but to the new hydrophobic sites formed during the gamma irradiation process of the NP.8

![Graph](image)

**Figure 7 UV-Vis spectra for the determination of the quantity of anthocyanins captured.**

During this NP preparation process, the BSA-NP has a higher number of -SHs on its surface (c.a. 50 per NP) than BSA.8 Thus, this NP has a better binding capacity than molecular BSA to carry A, positioning the vehicle as a potential drug delivery system with some advantages over the molecular protein. Moreover, the conformational changes that the BSA-NP experiments because of their interaction with anthocyanins favor the abound. BSA-NP b-sheet content increase when the drug is bound, as observed in the FT-IR spectroscopy experiments. This secondary structure is hydrophobic, adding more possibilities of unspecific interaction between BSA-NP and A, together with the more specific one happening in the Sudlow sites and the SH surface groups.

### 3.5. BSA-NP antioxidant capacity

The value of free radical scavenged was calculated as mentioned in *Materials and methods* above. The IC50 of radical scavenging assessed by DPPH method was 1.75 µM, equivalent to 3’,4’-Dihydroxyflavone’,4’; OOOHOH with a MW of 254.2 and an IC50 of 4.8±0.1 µM.

These results are in agreement with the view that phenolic groups with an ortho-dihydroxyl unit have a good ability to scavenge the DPPH radical.44
The IC% for free anthocyanins and those bound to BSA-NP are shown in Fig. 8, where the quenching effect of DPPH of anthocyanins was maintained even in the case when they are bound.

![Figure 8 Absorbance value, normalized to DPPH.](image)

### 3.6. Determination of rheological parameters by flow analysis

To obtain a formulation that can be administered, rheological tests were performed. The rheological analysis provides important information on colloidal system structures. In this sense, some systems that at first glance seem homogeneous, are actually very complex dispersions that contain different phases and that, under relaxed conditions, show characteristics that change, such as viscosity, when they are subject to external deformations. An accurate detection and characterization of protein nanoparticles or aggregates – including physical characteristics like size, structure, morphology, interactions and rheology in therapeutic protein formulations – are critical to minimize important safety issues like immune response and to assure different physical and chemical stabilities to obtain an optimal cargo delivery.

If protein nanoparticles show a Newtonian flow, the force needed to get them through the syringe hollow tube at high velocity with low injection time might be significantly overestimated due to shear thinning, according to Rathore et al., 2012.\(^45\) This issue is not an easy task if mimicking other existing biological systems, unless the rheological properties of the nanoparticle designed under high shear conditions are properly and efficiently mimicked.
Sharma et al., 2011 conclude that relative viscosity is similar for all samples when applying the low shear rate range, regardless of concentration (expected behavior for colloidal dispersions). They also propose that this behavior at low deformation rates must be related to the formation of an interfacial layer of adsorbed protein. They report concentrations in the range between 10 and 250 mg/ml with viscosity values of the order of 10^-2 Pa.s for 25 °C and with highly shear thinning response behavior, while our protein nanoparticles with active ingredients (anthocyanins) but with concentrations of less than 1 µg/ml would be expected to show Newtonian fluid characteristics and values even lower than those reported by these authors.

The system, however, remains unchanged when we analyze the properties mentioned above, which can be altered when a high shear rate is used. This alteration is seen, for example, when the intravenous (IV) solution passes through the needle at the time of injection and when the solution crosses the circulatory system and enters the body as it travels through arteries and veins. Steffe et al., 1996 mention that blood flow corresponds to a shear rate of 100-103 s^-1. Therefore, a drug transported by a drug delivery system (DDS) with a controlled release profile at the time of injection, by the effect of the shear stress caused by the movement of the fluid (blood) would seriously affect the performance of the IV system that contains the drug.

Considering the above, the effect of shear stress under different shear deformation rates applied to the DDS system with antioxidants was studied. The rheological parameters were studied at different temperatures (25 °C and 37 °C) and using different shear rates.

Analyzing the results, it is observed that for both temperatures, all samples tend to show Newtonian flow. However, both BSA-NPs and BSAs with the incorporated extract increase their viscosity compared to when they are separated.
| Samples            | µ (Pa.s)       | R²   | µ (Pa.s)       | R²   |
|--------------------|----------------|------|----------------|------|
| Temp (ºC)          | 25             |      | 37             |      |
| BSA                | 9.12 x 10⁻⁴ ± 1.22 x 10⁻⁵ | 0.994 | 8.32 x 10⁻⁴ ± 9.06 x 10⁻⁶ | 0.996 |
| BSA-NP             | 2.01 x 10⁻³ ± 1.33 x 10⁻⁵ | 0.998 | 5.07 x 10⁻⁴ ± 5.71 x 10⁻⁶ | 0.996 |
| BSA-NP: A          | 2.65 x 10⁻³ ± 3.63 x 10⁻⁵ | 0.992 | 1.46 x 10⁻³ ± 3.47 x 10⁻⁵ | 0.984 |
| BSA: A             | 2.70 x 10⁻³ ± 7.06 x 10⁻⁵ | 0.995 | 1.48 x 10⁻³ ± 7.79 x 10⁻⁶ | 0.999 |
| A                  | 4.17 x 10⁻³ ± 3.73 x 10⁻⁵ | 0.997 | 2.78 x 10⁻³ ± 1.53 x 10⁻⁵ | 0.999 |

The analytical values (µ) obtained confirm the results shown in Fig 7 (A and B). The value of µ at 25 ºC for the BSA-NP sample is higher than that of BSA, while at 37 ºC they are both of the same order of magnitude. Both decrease as temperature increases. A similar behavior is observed when the samples are BSA: A and BSA-NP: A.
3.7. DLS size and size stability determination for BSA-NP: A

Particle size was determined by DLS or NTA with and without anthocyanins added to BSA-NP and/or BSA. These tests were carried out at the same two temperatures as before (25°C and 37°C). A six-month DLS stability study of BSA-NP was carried out (Table 8). Size increased from 30 nm to 200 from day 15 to day 180 (Fig. 8). Previously, we had published a stability study of the BSA-NP in buffer solution for 60 days, where size was duplicated from the 15th day. This is a clear sign of the solvent effect on the BSA-NP.

DLS was also used to assess if there are changes in size in the BSA-NP: A formulation, which was then exposed to a shear stress to determine if it could maintain low degradability under blood flow.

Figure 9 Experimental Flow data of BSA-NP and BSA, Blueberry extract, anthocyanins (A) and the BSA-NP and BSA/extract at two temperatures – 25 °C and 37 °C (A and B) – and fitting to the proposed model (Newton Law).
**Table 8 BSA-NP stability determination, from day 1 to day 180 (6 months).**

| Days | Sizes (nm) |
|------|------------|
| 1    | 30         |
| 30   | 30         |
| 60   | 180-200    |
| 180  | 200        |

Having reached a stable size of 200 nm and conserved shape (Fig. 8), from day 60th to 180th, nanoparticles can then be exposed to a shear stress that exceeds that of blood flow (101 -102 S-1). Siri et al. (2019), described an increase in size of the BSA-NP in buffer from 70 nm to 140 nm by day 15, reaching a 10-fold increase of its initial size by day 60.9 Differences in these results may be due to different buffer media and pH used. Another possibility may be that anthocyanins help preserve the BSA-NP stability for a longer time. Charges in the NP may change while bound to the drug, causing less interaction with the solvent, preventing any changes in the structure.

Luebbert et al. (2017) conclude with further studies that the increased nanoparticle diameters were due to the bound fatty acids modulating electrostatic interactions between albumin nanoparticles during the desolvation process, and not to changes in protein structure, stability or generation of additional albumin oligomers.47

An analysis of the results obtained confirms that BSA-NPs elute with a size of 30 nm at 25 °C, while BSAs do so at a size of 7 nm at 25 °C, which represents the non-aggregation diameter. The interaction with anthocyanins causes in both cases an increase in hydrodynamic diameter from 7 nm (BSA) to 30 nm, and from 25 nm (BSA-NP) to 50 nm when anthocyanins bind. When anthocyanins bind, there is a change in binding forces based on the appearance of a second mode in size distribution obtained by DLS. This increment in size is due to the possible influence of anthocyanin tendency to form aggregates on both BSA and BSA-NPs.
Figure 10 A) DLS analysis of size distribution of BSA-NP at 25 °C and 37 °C from day 15th to 180th. B) NTA analysis of size distribution of BSA-NP at 25 °C.

It was also observed that samples, after being exposed to mechanical action under shear stress, form aggregates in absence of anthocyanins. It should be noted that hydrodynamic diameter remains the same in the BSA-NP: A at 37 °C. However, this represents only 20% of the population, the remaining percentage has sizes larger than microns, out of the equipment detection limit, like the BSA sample at this temperature and at 25 °C.
3.8. Electron Transmission Microscopy (TEM)

To complete the results obtained by DLS, zeta potential and rheology, and to elucidate morphology changes produced in the samples according to our hypothesis due to the interaction of the samples with the blood flow, electron transmission microscopy (TEM) was performed. Based on these findings, we infer that most probably these fatty acids become loose and that originates new electrostatic bindings generating microparticles.

The photographs shown below correspond only to eluate 1 samples, i.e. BSA-NP and BSA-NP: A before and after being exposed to the shear stress of the rheology test, since this is the formulation of interest to be used as a drug transporter for intravenous administration.

As the images taken by TEM that the BSA-NP show, after being exposed to rheological stress, aggregates were formed (Fig. 10 A1). Similarly, to BSA-NPs, BSA: A NPs formed aggregates after rheology (Fig. 10 B1). Additionally, most NPs interact with A (dark circles). The size of the BSA-NP is c.a. 28 nm (Fig. 10 A) while, after rheology, aggregates of around 150 nm were observed (Fig. 10 A1) which represents a 300% increase in size. When BSA-NPs interact with anthocyanins (Fig. 10 B), the average size is around 100 nm, with up to 3 particles added together. After rheology, the smaller aggregates are around 200 nm (Fig. 10 B1).

With samples from the tests at 25°C, BSA-NPs of around 40 nm can be observed (Fig. 10 A). After being subjected to the mechanical stress of the rheological test, they do not form visible aggregates (Fig. 10 A1), maintaining their size. In turn, when they are brought into contact with anthocyanins, interaction efficiency increases; since, as previously observed, BSA-NPs do not present sizes in the order of 90-135 nm, changes can only be due to interaction efficiency (Fig. 10 B).

In the samples used for tests at 37°C, BSA-NPs have sizes of around 30 nm (Fig. 11 A). After being subjected to the mechanical stress of the rheological test, they formed large aggregates of more than 500 nm (Fig. 11 A1) creating concentric structures. In turn, when they associate with anthocyanins, interaction efficiency is high and aggregates with sizes of the order of 100 nm and larger can also be observed (Fig. 11
B). Additionally, certain particles of bigger size, after rheological stress BSA-NPs: A, formed larger aggregates of the order of 250 nm (Fig. 11 B1).

3.9. Stability determination of BSA-NP: A by DLS in comparison with ζ potential

![Graphs showing particle size distribution](image)

**Figure 11** Particle size distribution of BSA-NP and BSA with and without anthocyanins (A) at 25°C and 37°C (A and B). Images showing the before (left) and after (right) of the shear stress rheology experiment.

Considering the results obtained by DLS, where changes in sample size are observed depending on the temperature set, such as the decrease in the size of the NP at 37°C compared to 25°C, a zeta-potential analysis was carried out to determine sample stability.

The samples analyzed were BSA-NP and free BSA. Tests of the BSA-NP: A formulation at different molar ratios was also carried out considering the previous results of UV-Vis and fluorescence to evaluate the modifications in potential and stability values.
Figure 12 TEM images obtained at 25°C with a magnification of 46000. A) BSA-NP before rheology assay, A1) BSA-NP after rheology, B) BSA-NP: A before rheology, B1) BSA-NP: A after rheology.

The literature shows that the zeta potential of BSA varies with pH. Taking as reference the values of -20, -30 corresponds to a range of pH = 6-7, since samples were prepared in water mQ, pH = 6.5. Data was obtained from the Internet.\textsuperscript{48}
Looking at the results obtained, it can be seen that BSAs have a greater zeta potential than BSA:NP, being more stable than the free BSAs. Similarly, an increase in the potential of BSA-NPs is observed as the concentration of anthocyanin increases. However, this increment is relevant only at a temperature of 37 °C. Zeta potential values of BSA-NP at 37°C match those obtained in previous studies on the BSA-NP in buffer medium.\textsuperscript{7}
Table 9 Values of \( \zeta \) potential at room temperature and at body temperature, at pH of 6.5 (mQ water dilution).

| Samples                  | \( \zeta \) at 25 ºC | \( \zeta \) at 37 ºC |
|--------------------------|-----------------------|-----------------------|
| BSA                      | -23.0                 | -30.0                 |
| BSA-NP                   | -36.0                 | -22.0                 |
| BSA-NP: A (1:70)         | 0.5                   | 15.0                  |
| BSA-NP: A (1:100)        | 10.0                  | 16.0                  |
| BSA-NP: A (1:200)        | 10.0                  | 12.0                  |
| BSA-NP: A (1:500)        | 16.0                  | 18.0                  |

When considering future applications, it should be considered that if anthocyanin concentrations are higher, as observed by DLS, aggregates of approximately 100 nm are generated, meaning that stability is relative to anthocyanin concentration. As observed by FT-IR measurements, BSA-NP and anthocyanins forge a stable interaction governed by hydrophobic bonding and protein structure rearrangement. Moreover, in the fluorescence studies, we observed that the BSA-NP tryptophan emission decreased as the concentration of the anthocyanins increased, inducing changes in the NP conformation. The fluorescence of the complex may be weakened, as suggested by Li et al. 2020 due to the non-exposure of aromatic amino acid residues because of changes in the molecular conformation. Several works in which FT-IR and fluorescence spectroscopy studied the interaction of anthocyanins with different proteins suggested that the structural changes occurred because this interaction enabled the protein stability.\(^3,31\)

A is linked to BSA-NPs with anthocyanins, with the overall negative zeta potential diminishing as the concentration of interacting anthocyanins increases. It was also observed by fluorescence that the NP: A molar ratio (1:70) is when the NP active sites are saturated. Thus, at higher molar ratios, the zeta potential values obtained correspond to the formation of NP aggregates. This aggregation is caused by the
anthocyanins that remain on the surface of the BSA-NPs, thus favoring intermolecular interactions. This aggregation effect was also noted by Zhou et al., 2014.

Each BSA-NP is formed of 183 BSA molecules. Thus, the occupancy of anthocyanins should be of at least 183 anthocyanins (1:1 molar ratio). Nevertheless, the kinetics studies showed an occupancy between 1:70 and 1:100. This occupancy also depends on factors such as shape size and charge. Based on zeta potential data, it can be inferred that this also depends significantly on the binding compound. At the concentrations mentioned above, potential drops from -36 mV to a range between 0.5-10 mV, so aggregation and electrostatic interaction forces lay in between, and that is a direct influencer to make them less efficient in occupancy, dropping a 40% of the expected value.

There is a correlation between the viscosity values obtained and temperature. At higher temperatures, there is a decrease in viscosity for the samples tested. Also, viscosity values are linked to aggregate formation, which is a consequence of the stress generated by a shear force, regardless of temperature. In the future, a viscoelastic analysis of the samples will be carried out from the rheological point of view to determine if there are changes in the solid-elastic (G1) and liquid-viscous (G2) character considering temperature and scanning frequency.

Conclusions

In this work we studied the interaction of anthocyanins with BSA-NP and how they might affect the NP function. The result was a complex of strong hydrophobic forces which the changes in structure induced by the anthocyanins bonding, enhanced the stability of the BSA-NP. More importantly, at 37°C there are no morphological changes for BSA-NP: A and BSA: A, and after rheology, and their viscosity is similar. This result implies that the RES system will not differentiate the albumin from the nanoparticle plus anthocyanin, so it will not be phagocytized and will favor its permanence in circulation. Moreover, the antioxidant capacity property of anthocyanins was maintained at the same level even in the case when they bound. And last but not least one of the important findings is that the BSA-NP: A captured permitted also the knowledge that the additional drug detected is not only bound to the Sudlow sites of the BSA-NP, but to the new
hydrophobic sites formed by the conformational changes induced by its interaction with A. Thus, the studies characterizing the interaction of BSA-NP and anthocyanins in this work place the gamma-irradiated albumin NP as a promising nanovehicle for a wide usage from the pharmaceutical to the food industry.

*Corresponding Author*

Silvia del Valle Alonso, PhD
Laboratorio de Bio-Nanotecnología
Departamento de Ciencia y Tecnología
Universidad Nacional de Quilmes
GBEyB, IMBICE-CONICET
Roque Sáenz Peña St. No 352
BXD1876B Bernal, Buenos Aires, Argentina.
TE: +54.11.4365.7100 (ext.5625)
e-mail: silviadelvalle@gmail.com, salonso@unq.edu.ar.

Current Addresses

4. Author Contributions
All authors contributed to writing this manuscript and have approved its final version.

5. Funding Sources
This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (PIP-CONICET #11220170100806C), Universidad Nacional de Quilmes (PUNQ 1388/19), Ministerio de Ciencia y Tecnología (PICT-2014-0511), and Nuclear Atomic Energy Agency (IAEA, Project Code F22064).

6. ACKNOWLEDGMENTS
Macarena Siri; Juan C. Moreno; Patricia H. Risso; Jorge Montanari; Fernando C. Alvira and Silvia del V. Alonso are members of the Argentinean Scientific Council (CONICET). Sofia L. Candido is grateful for the PhD fellowship granted by Research Program from the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICPBA)

7. ABBREVIATIONS
A= anthocyanin; BSA= Bovine Serum Albumin; ε= Molar Extinction Coefficient; EtOH=ethanol; NP=Nanoparticle; NPs=Nanoparticles; BSA-NPBSA-NP= Bovine Serum Albumin Nanoparticle; BSA-NPBSA-NPs= Bovine Serum Albumin Nanoparticles; HAS= Human Serum Albumin; UV-VIS= Ultraviolet-Visible; DPPH= diphenylhexatriene; BHT=butylated hydroxytoluene; mQ=milliQ; DLS=Dynamic Light Scattering; NTA=Nanoparticle Tracking Analysis; Trp=tryptophan; Tyr = tyrosine.

8. HIGHLIGHTS

- Anthocyanins bind to bovine serum albumin and rheological properties change.
- There is a correlation between the viscosity values obtained and temperature
- At 37°C, there are no morphological or viscosity changes for BSA-NPBSA-NP: A and BSA: A after rheology.
- Formation of aggregates linked to the stress of the shear force, regardless of temperature.

9. Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

10. Bibliography

1. Wang, Y.; Zhu, J.; Meng, X.; Liu, S.; Mu, J.; Ning, C., Comparison of polyphenol, anthocyanin and antioxidant capacity in four varieties of Lonicera caerulea berry extracts. *Food chemistry* 2016, 197, 522-529.
2. Bornsek, S. M.; Ziberna, L.; Polak, T.; Vanzo, A.; Ulrih, N. P.; Abram, V.; Tramer, F.; Passamonti, S., Bilberry and blueberry anthocyanins act as powerful intracellular antioxidants in mammalian cells. *Food chemistry* 2012, 134, (4), 1878-1884.
3. Lang, Y.; Li, E.; Meng, X.; Tian, J.; Ran, X.; Zhang, Y.; Zang, Z.; Wang, W.; Li, B., Protective effects of bovine serum albumin on blueberry anthocyanins under illumination conditions and their mechanism analysis. *Food Research International* 2019, 122, 487-495.
4. Cao, H.; Chen, X.; Yamamoto, K., Bovine serum albumin significantly improves the DPPH free radical scavenging potential of dietary polyphenols and gallic acids. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents) 2012*, 12, (8), 940-948.
5. Espinoza, S. L. S.; Sánchez, M. L.; Risso, V.; Smolko, E. E.; Grasselli, M., Radiation synthesis of seroalbumin nanoparticles. *Radiation Physics and Chemistry* 2012, 81, (9), 1417-1421.
6. Achilli, E.; Casajus, G.; Siri, M.; Flores, C.; Kadłubowski, S.; Alonso, S. d. V.; Grasselli, M., Preparation of protein nanoparticle by dynamic aggregation and ionizing-induced crosslinking. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 2015, 486, 161-171.
7. Siri, M.; Grasselli, M.; Alonso, S. d. V., Albumin-based nanoparticle trehalose lyophilisation stress-down to preserve structure/function and enhanced binding. *Journal of pharmaceutical and biomedical analysis* **2016**, 126, 66-74.
8. Siri, M.; Ruocco, M. J. F.; Achilli, E.; Pizzuto, M.; Delgado, J. F.; Ruysschaert, J.-M.; Grasselli, M.; Alonso, S. d. V., Effect of structure in ionised albumin based nanoparticle: Characterisation, Emodin interaction, and in vitro cytotoxicity. *Materials Science and Engineering: C* **2019**, 103, 109813.
9. Siri, M.; Grasselli, M.; Alonso, S. d. V., Media interaction and stability of a gamma irradiated albumin nanoparticle. *Journal of Biotechnology* **2019**, 306, 169-176.
10. Gentile, F.; Ferrari, M.; Decuzzi, P., The transport of nanoparticles in blood vessels: the effect of vessel permeability and blood rheology. *Annals of biomedical engineering* **2008**, 36, (2), 254-261.
11. Carboni, E.; Tschudi, K.; Nam, J.; Lu, X.; Ma, A. W., Particle margination and its implications on intravenous anticancer drug delivery. *Aaps Pharmscitech* **2014**, 15, (3), 762-771.
12. Carboni, E. J.; Bognet, B. H.; Cowles, D. B.; Ma, A. W., The Margination of Particles in Areas of Constricted Blood Flow. *Biophysical journal* **2018**, 114, (9), 2221-2230.
13. Amin, S.; Barnett, G. V.; Pathak, J. A.; Roberts, C. J.; Sarangapani, P. S., Protein aggregation, particle formation, characterization & rheology. *Current Opinion in Colloid & Interface Science* **2014**, 19, (5), 438-449.
14. Singh, M., Studies of Molecular Interactions of alpha-Amino Acids in Aqueous and Cationic Surfactant Systems Investigated from Their Densities and Apparent Molal Volumes at 283.15, 288.15 and 293.15 K. *Pakistan Journal of Scientific and Industrial Research* **2005**, 48, (5), 303.
15. Miele, E.; Spinelli, G. P.; Miele, E.; Tomao, F.; Tomao, S., Albumin-bound formulation of paclitaxel (Abraxane® ABI-007) in the treatment of breast cancer. *International journal of nanomedicine* **2009**, 4, 99.
16. Elzoghby, A. O.; Samy, W. M.; Elgindy, N. A., Albumin-based nanoparticles as potential controlled release drug delivery systems. *Journal of controlled release* **2012**, 157, (2), 168-182.
17. Liu, F.; Mu, J.; Xing, B., Recent advances on the development of pharmacotherapeutic agents on the basis of human serum albumin. *Current pharmaceutical design* **2015**, 21, (14), 1866-1888.
18. An, F.-F.; Zhang, X.-H., Strategies for preparing albumin-based nanoparticles for multifunctional bioimaging and drug delivery. *Theranostics* **2017**, 7, (15), 3667.
19. Montanari, J.; Vera, M.; Mensi, E.; Morilla, M.; Romero, E., Nanoberry for topical delivery of antioxidants. *Journal of cosmetic science* **2013**, 64, (6), 469-481.
20. Montanari, J.; Vera, M.; Mensi, E.; Morilla, M.; Romero, E., Nanoberry for topical delivery of antioxidants. *J Cosmet Sci* **2013**, 64, (6), 469-81.
21. Nicoue, E. E.; Savard, S.; Belkacemi, K., Anthocyanins in wild blueberries of Quebec: extraction and identification. *Journal of agricultural and food chemistry* **2007**, 55, (14), 5626-5635.
22. Papadopoulou, A.; Green, R. J.; Frazier, R. A., Interaction of flavonoids with bovine serum albumin: a fluorescence quenching study. *Journal of agricultural and food chemistry* **2005**, 53, (1), 158-163.
23. Sevilla, P.; Rivas, J. M.; García-Blanco, F.; García-Ramos, J. V.; Sánchez-Cortés, S., Identification of the antitumoral drug emodin binding sites in bovine serum albumin by
spectroscopic methods. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **2007**, 1774, (11), 1359-1369.

24. Shimada, K.; Fujikawa, K.; Yahara, K.; Nakamura, T., Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of agricultural and food chemistry* **1992**, 40, (6), 945-948.

25. Burits, M.; Bucar, F., Antioxidant activity of *Nigella sativa* essential oil. *Phytotherapy research* **2000**, 14, (5), 323-328.

26. Adiguzel, A.; Ozer, H.; Sokmen, M.; Gulluce, M.; Sokmen, A.; Kilic, H.; Sahin, F.; Baris, O., Antimicrobial and antioxidant activity of the essential oil and methanol extract of *Nepeta cataria*. *Polish Journal of Microbiology* **2009**, 58, (1), 69-76.

27. Siri, M.; Grasselli, M.; del V Alonso, S., Correlation between assembly structure of a gamma irradiated albumin nanoparticle and its function as a drug delivery system. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2020**, 125176.

28. Rohiwal, S. S.; Satvekar, R. K.; Tiwari, A. P.; Raut, A. V.; Kumbhar, S. G.; Pawar, S. H., Investigating the influence of effective parameters on molecular characteristics of bovine serum albumin nanoparticles. *Applied Surface Science* **2015**, 334, 157-164.

29. Gill, S. C.; Von Hippel, P. H., Calculation of protein extinction coefficients from amino acid sequence data. *Analytical biochemistry* **1989**, 182, (2), 319-326.

30. Fei, P.; Zeng, F.; Zheng, S.; Chen, Q.; Hu, Y.; Cai, J., Acylation of blueberry anthocyanins with maleic acid: Improvement of the stability and its application potential in intelligent color indicator packing materials. *Dyes and Pigments* **2021**, 184, 108852.

31. Li, J.; Wang, B.; He, Y.; Wen, L.; Nan, H.; Zheng, F.; Liu, H.; Lu, S.; Wu, M.; Zhang, H., A review of the interaction between anthocyanins and proteins. *Food Science and Technology International* **2021**, 27, (5), 470-482.

32. Wei, J.; Xu, D.; Zhang, X.; Yang, J.; Wang, Q., Evaluation of anthocyanins in *Aronia melanocarpa/BSA* binding by spectroscopic studies. *Amb Express* **2018**, 8, (1), 1-9.

33. Chirgadze, Y. N.; Nevskaya, N., Infrared spectra and resonance interaction of amide-I vibration of the antiparallel-chain pleated sheet. *Biopolymers: Original Research on Biomolecules* **1976**, 15, (4), 607-625.

34. Goormaghtigh, E.; Ruysschaert, J.-M.; Raussens, V., Evaluation of the information content in infrared spectra for protein secondary structure determination. *Biophysical journal* **2006**, 90, (8), 2946-2957.

35. Byler, D. M.; Susi, H., Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers: Original Research on Biomolecules* **1986**, 25, (3), 469-487.

36. Peters, T., *All about albumin: biochemistry, genetics, and medical applications*. Academic Press: San Diego, 1996; p 432 p.

37. Banerjee, M.; Pal, U.; Subudhhi, A.; Chakrabarti, A.; Basu, S., Interaction of Merocyanine 540 with serum albumins: photophysical and binding studies. *Journal of Photochemistry and Photobiology B: Biology* **2012**, 108, 23-33.

38. Cahyana, Y.; Gordon, M. H., Interaction of anthocyanins with human serum albumin: Influence of pH and chemical structure on binding. *Food chemistry* **2013**, 141, (3), 2278-2285.

39. Zang, Z.; Chou, S.; Tian, J.; Lang, Y.; Shen, Y.; Ran, X.; Gao, N.; Li, B., Effect of whey protein isolate on the stability and antioxidant capacity of blueberry anthocyanins: A mechanistic and in vitro simulation study. *Food Chemistry* **2021**, 336, 127700.
40. Gallo, M.; Vinci, G.; Graziani, G.; De Simone, C.; Ferranti, P., The interaction of cocoa polyphenols with milk proteins studied by proteomic techniques. *Food research international* **2013**, 54, (1), 406-415.
41. Kang, J.; Liu, Y.; Xie, M.-X.; Li, S.; Jiang, M.; Wang, Y.-D., Interactions of human serum albumin with chlorogenic acid and ferulic acid. *Biochimica et biophysica acta (BBA)-general subjects* **2004**, 1674, (2), 205-214.
42. Min, J.; Meng-Xia, X.; Dong, Z.; Yuan, L.; Xiao-Yu, L.; Xing, C., Spectroscopic studies on the interaction of cinnamic acid and its hydroxyl derivatives with human serum albumin. *Journal of Molecular Structure* **2004**, 692, (1-3), 71-80.
43. Xiao, J.; Suzuki, M.; Jiang, X.; Chen, X.; Yamamoto, K.; Ren, F.; Xu, M., Influence of B-ring hydroxylation on interactions of flavonols with bovine serum albumin. *Journal of agricultural and food chemistry* **2008**, 56, (7), 2350-2356.
44. Zheng, C.-D.; Li, G.; Li, H.-Q.; Xu, X.-J.; Gao, J.-M.; Zhang, A.-L., DPPH-scavenging activities and structure-activity relationships of phenolic compounds. *Natural product communications* **2010**, 5, (11), 1759-1765.
45. Rathee, J. S.; Hassarajani, S. A.; Chattopadhyay, S., Antioxidant activity of Nyctanthes arbor-tristis leaf extract. *Food chemistry* **2007**, 103, (4), 1350-1357.
46. Steffe, J. F., *Rheological methods in food process engineering*. Freeman press: 1996.
47. Luebbert, C. C.; Clarke, T. M.; Pointet, R.; Frahm, G. E.; Tam, S.; Lorbetskie, B.; Sauvé, S.; Johnston, M. J., Nanoparticle size and production efficiency are affected by the presence of fatty acids during albumin nanoparticle fabrication. *PloS one* **2017**, 12, (12).
48. Entegris Zeta Potential BSA. [http://pssnicomp.com/zeta-potential-bsa/](http://pssnicomp.com/zeta-potential-bsa/) (4/1/2020)