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Osmotic pressure and aggregate shape in BSA/poly (ethylene glycol)-lipid/Dextran solutions

Valeria Castelletto *, Ian W. Hamley, Luke A. Clifton, Rebecca J. Green

School of Chemistry, Food Biosciences and Pharmacy, The University of Reading, P.O. Box 226, Whiteknights, Reading, UK

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Abstract

In this work we study the colloidal osmotic pressure (COP) and aggregate shape in phosphate saline buffer solutions (pH 7.4) containing bovine serum albumin (BSA), poly(ethylene glycol) lipid (PEG_{2000}-PE) and Dextran (Dx). Dx was added to the BSA/PEG_{2000}-PE system in order to increase the COP of the solution to levels comparable to the COP of healthy adults, with the aim of using the solution as a blood COP regulator. Dynamic light scattering and small angle X-ray scattering results shown the formation of BSA/PEG_{2000}-PE/Dx aggregates in the solution. Osmometry results shown that the addition of Dx to the BSA/PEG_{2000}-PE system could successfully increase the COP, through the formation of BSA/PEG_{2000}-PE/Dx aggregates. The BSA/PEG_{2000}-PE/Dx solutions attained COP = 15 mm Hg, representing 60% of COP measured for healthy adults.

Keywords: Polyethylene(glycol)-lipid conjugate; Dextran; Bovine serum albumin; Light scattering; Small angle X-ray scattering; Osmometry

1. Introduction

The association of high molecular weight polyethylene (glycol) (PEG) or polysaccharides such as Dextran (Dx) to the model protein serum albumin (SA) has been studied in detail during the past few years [1–6]. Since SA is a major component of blood, while PEG and Dx have medical uses, the complexation of SA with PEG and Dx have a wide range of biomedical applications. Within these applications, the control of the colloidal osmotic pressure (COP) in blood has a particular relevance, since SA is a protein which maintains the 'osmotic pressure' that causes fluid to remain within the blood stream instead of leaking out into tissues.

The results presented in this work involve the binding of Dx and a PEG-lipid conjugate to SA. As detailed below, this study is framed in our previous publications on the subject [7,8], and represents a step forward in the control of the COP in solutions containing SA.

In a previous work [8] we reported the formation of complexes by self assembly of bovine serum albumin (BSA) with the poly(ethylene glycol) lipid conjugate 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000], namely PEG_{2000}-PE, in phosphate saline buffer solution (pH 7.4). [7,8] BSA was chosen due to the extensive information available on BSA properties in solution [9–11] and because it can be produced on an industrial scale. It was shown that the PEG-lipid conjugate binds to the protein such that each aggregate comprises a BSA macromolecule surrounded by a PEG_{2000}-PE shell [7]. Each aggregate exists isolated in solution and does not form BSA/PEG_{2000}-PE networks throughout the system. Since this configuration was proved to be stable at physiological conditions (pH 7.4, 37 °C) [7], it was consequently proposed that BSA/PEG_{2000}-PE aggregates can potentially be used as blood osmotic pressure regulators. Depletion forces were discounted as being the driving force for aggregate formation [8], while it was suggested that BSA/PEG_{2000}-PE complexation might take place due to the hydrophobicity of the PE block.

Despite all the experimental studies leading to these conclusions, no work has been done to determine the COP of the

* Corresponding author. Tel.: +44 113 343 7595; fax: +44 113 343 6551.
E-mail address: V.Castelletto@reading.ac.uk (V. Castelletto).

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BSA/PEG_{2000}-PE aggregates. In keeping with our previous idea of proposing these complexes as blood osmotic pressure regulators [7], it is desirable that, even under the addition of additives such as Dextran (Dx), individual complexes can be identified in the system and that the COP attained by the solution attains at least a few tens of the COP for healthy adults [12].

This work starts with the study of the COP in the BSA/PEG_{2000}-PE system. Then, Dx will be used as an additive to increase the COP of the BSA/PEG-lipid solution. Dynamic light scattering (DLS) and small angle X-ray scattering (SAXS) will be used to determine the size and shape of the aggregates in BSA/PEG_{2000}-PE/Dx solutions, while osmometry experiments will provide a measure of the COP attained by the ternary system.

2. Experimental section

2.1. Materials

BSA (M_{BSA}=66,000 g mol^{-1}), Phosphate Buffer Saline (PBS, pH 7.4, ionic strength I=0.169 M) and Dx (M_{Dx}= 35,000–45,000 g mol^{-1}) were purchased from Sigma (U.S.A.), while PEG_{2000}-PE ($C_{133}H_{267}N_2O_{55}P$, $M_{PEG2000\text{-}PE}$=2806 g mol^{-1}) was obtained from Avanti Polar Lipids (U.S.A.).

The sample preparation procedure is detailed below. Two different sets of samples were prepared, one containing Dx and another free of Dx. In the following, we will refer as δ and Δ to the fraction $c_{PEG2000\text{-}PE}/c_{BSA}$ for samples with c_{Dx}=0 or c_{Dx}=1 wt.% respectively ($c_{PEG2000\text{-}PE}$, c_{BSA}, c_{Dx}: concentration measured in wt.%). All the samples were mixed using PBS as a solvent, while the experimental results presented in this work have been obtained at 20 °C.

Samples for osmometry, DLS and SAXS experiments were made by extensively mixing controlled amounts of PEG_{2000}-PE and BSA for several fractions Δ. The experiments were performed for a fixed c_{BSA}=1 wt.% and variable $c_{PEG2000\text{-}PEG}$. Then, enough Dx was added to the solution in order to obtain c_{Dx}=1 wt.%. Only one sample without Dx (δ=2, $c_{PEG2000\text{-}PE}$= 2 wt.%) was studied by osmometry.

2.2. DLS

Experiments were performed using an ALV CGS-3 system with 5003 multi-digital correlator. The light source was a 20 mW He–Ne laser, linearly polarized, with λ=633 nm. Scattering angles in the range $40° \le \theta \le 140°$ were used for all the experiments. Samples were filtered through 0.20 μm Anotop filters from Whatman into standard 0.5 cm diameter cylindrical glass cells.

2.3. SAXS

Experiments were carried out at the BM26 beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The samples were mounted in sealed 1 mm thick cells, with an inner spacer ring to hold liquids, contained between mica windows. The data was collected in the range q=(0.03–0.38) $Å^{-1}$ (q: scattering vector=4π sinθ/λ, λ: wavelength= 1.0 Å) using a two-dimensional Multiwire gas detector. SAXS data were corrected for sample transmission, background scattering and detector response. The data from the two-dimensional area detector were finally converted into one-dimensional intensity profiles by integration in a circular sector. The resulting corrected intensity curves are denoted $I(q)$.

2.4. Osmometry

Osmotic pressure was measured using a Gonotec Osmomat 090 membrane osmometer. Cellulose triacetate two layer membranes for aqueous solutions, with a cut-off corresponding to 20,000 Da, were used for the experiments. The membranes were placed in distilled water for 2–4 h before being mounted in the osmometer. Solutions were degassed before use. The machine was calibrated against a known hydrostatic pressure using water. The equilibration time for each experiment was about 15 min.

3. Theory

3.1. DLS

DLS experiments measured the intensity correlation function of the scattered light $g^{(2)}(q, t)$ [13]:

$$g^{(2)}(q,t) = 1 + A\left[g^{(1)}(q,t)\right]^2 \quad (1)$$

where A accounts for a correction factor depending on the alignment of the instrument, q=[4πn sin(θ/2)]/λ is the scattering vector (n=refractive index of the medium), t is the delay time and $g^{(1)}(q, t)$ is the electric field correlation function.

The relaxation rate distribution of the system, can be calculated by inverting the field correlation function $G(\Gamma)$ [14]:

$$g^{(1)}(t) = \int_0^\infty G(\Gamma)\exp(-\Gamma t)d\Gamma \quad (2)$$

The inverse Laplace transform of Eq. (2) provides a tool for calculating $G(\Gamma)$ and therefore the diffusion coefficient of the system. In this work, CONTIN program [14] was used to calculate $G(\Gamma)$ for $40° \le \theta \le 140°$. The relaxation rate distributions for different scattering vectors can be used to construct a plot of $\bar{\Gamma}$ vs q^2 (where, for a unimodal distribution, $\bar{\Gamma}$ is taken as the decay rate corresponding to the maximum in $G(\Gamma)$). The mutual diffusion coefficient is calculated as the slope $D=\bar{\Gamma}/q^2$ and it enables the apparent hydrodynamic radius R_H to be calculated according to the Stokes–Einstein equation:

$$R_H = \frac{k_B T}{6\pi\eta D} \quad (3)$$

where $k_B = 1.38\times10^{-23}$ J K^{-1} is the Boltzmann constant and η is the viscosity of water, taken to be $\eta = 1.003\times10^{-3}$ Pa s at 20 °C.

3.2. SAXS

The coherent part of the SAXS intensity from an isotropic solution of globular objects, $I(q)$, can be written as [15]:

$$I(q) = kP(q)S(q) \quad (4)$$

where k is a normalization constant proportional to the number density of scatterers, $P(q)$ is the form factor and $S(q)$ is the structure factor. The systems studied in this work correspond to dilute systems. Therefore $S(q)\sim 1$ in Eq. (4), so $I(q)$ is proportional to the form factor $P(q)$.

For dilute systems, $P(q)$ at low q obeys the Guinier law [16]:

$$\lim_{q\to 0} I(q) = I(0)\exp\left(-\frac{q^2 R_G^2}{3}\right) \quad (5)$$

where $I(0)$ is the scattering at $q=0$. The radius of gyration of the particle, R_G, in Eq. (5) can be evaluated from a $\ln[I(q)]$ vs q^2 Guinier plot in the regime $qR_G<1$.

4. Results and discussion

As already mentioned in the introduction, BSA/PEG_{2000}-PE complexes in solution have been previously characterized by us for concentrations $\delta=0.15$–2 ($c_{BSA}=1$ wt.%, $c_{Dx}=0$) [7,8]. It was shown that a BSA/PEG_{2000}-PE complex consists of a BSA core surrounded by a PEG_{2000}-PE shell [7]. Recent isothermal titration experiments on this system shown that enthalpy changes due to PEG-lipid/BSA interactions do not make an appreciable contribution to the complexation. Instead, the PEG-lipid/BSA binding is driven by changes in the entropy of the system, which favour the association of the PE block to the BSA, due to the high hydrophobicity of the lipid block.

PEG makes an important contribution to the COP in BSA/PEG_{2000}-lipid solutions [17]. Therefore, in order to determine the highest COP attained by the system, this quantity was measured for a sample with $\delta=2$. However, the measured COP=4.5 mmHg was relatively low, representing only 18% of the COP=25 mm Hg corresponding to healthy adults [12]. It was therefore worth finding an alternative method to increase the COP in the solution while preserving stable BSA/PEG_{2000}-PE aggregates in the system.

Dx is a polysaccharide which has been proved to bind to BSA forming stable BSA/Dx complexes in solution [1,2]. In addition, Dx solutions have medical applications as plasma substitutes or additives for blood transfusions. In this work, we expect that for a fixed δ, the addition of Dx to BSA/PEG_{2000}-PE aggregates will increase the COP, due to the formation of high molecular weight BSA/PEG_{2000}-PE/Dx complexes.

In order to confirm the hypothesis above, DLS and SAXS experiments were performed to determine the size of the aggregates in BSA/PEG_{2000}-PE/Dx solutions, for $\Delta=0$–1 and fixed $c_{Dx}=c_{BSA}=1$ wt.%. The COP was then determined for the same samples.

DLS experiments were performed for the samples mentioned above. All the resulting $G(\Gamma)$ functions were dominated by one peak centred in R_H of the order of tens of angstroms. A second peak, centred in R_H of the order of thousands of angstroms was also observed for some $G(\Gamma)$. However, the peak at higher R_H will be excluded from our analysis, since its intensity was ~0.09 lower than the peak at lower R_H and it did not systematically appeared in all $G(\Gamma)$.

The dependence of R_H on Δ, is displayed in Fig. 1, showing that the size of the scattering object increases upon increasing the polymer-lipid conjugate concentration.

Fig. 1 shows that $R_H=38.6$ Å for $\Delta=0$ ($c_{BSA}=c_{Dx}=1$ wt.%). This value is higher than $R_H=35.6$ Å previously reported by us for $c_{BSA}=1$ wt.% ($c_{Dx}=0$) [8], denoting Dx binding to BSA. Simultaneously, the increase in R_H upon increasing $c_{PEG2000\text{-}PE}$ for a fixed c_{Dx} (Fig. 1) shows that the PEG-lipid conjugate binds to BSA for $c_{Dx}=1$ wt.%.

In a previous work we have determined $R_H=86.4$ Å for PEG_{2000}-PE micelles in PBS [8], while in this work we measured $R_H=46.8$ Å for 1 wt.% Dx in PBS. Data in Fig. 1 does not support the existence of free PEG-lipid micelles or unbounded Dx in the system. It can be concluded that the polymer-lipid conjugate and the polysaccharide are preferentially bounded to the protein within the range of concentrations plotted in Fig. 1. This information, together with the paragraph above show the formation of BSA/PEG_{2000}-PE/Dx aggregates in the solution.

In our previous study of the BSA/PEG_{2000}-PE system, R_H increased from 35 to 59 Å upon increasing δ ($c_{BSA}=1$ wt.%, $c_{Dx}=0$) from 0 to 2 [8]. In this work, R_H increases from 38 to 70 Å in the range $\Delta=0$–2 ($c_{BSA}=c_{Dx}=1$ wt.%). Since R_H values measured in this work can only be higher due to Dx binding, results in Fig. 1 confirm that in a BSA/PEG_{2000}-PE/Dx solution, Dx binds to BSA without avoiding the simultaneous complexation of PEG_{2000}-PE with the protein.

Data in Fig. 1 shows that a depletion interaction between the aggregates induced by the Dx can be discarded, since that effect should be mirrored by a reduction of R_H with increasing Δ, as already discussed in literature in relation to PEG/BSA [5] and BSA/PEG_{2000}-PE [8] complexation in water.

SAXS experiments were carried out for the same concentrations studied by DLS. Fig. 2 shows some representative Guinier plots for $\Delta=0$–2 ($c_{BSA}=c_{Dx}=1$ wt.%). The SAXS curves in Fig. 2 present only one Guinier region, showing that there is only

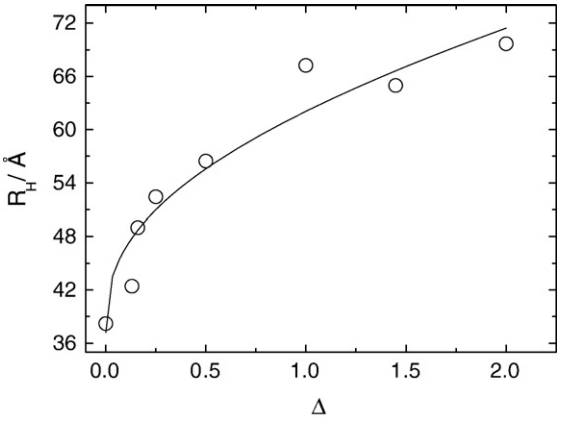

Fig. 1. Hydrodynamic radius obtained for the BSA/PEG_{2000}-PE/Dx system as a function of Δ ($c_{BSA}=c_{Dx}=1$ wt.%). The full line is a guide for the eyes.

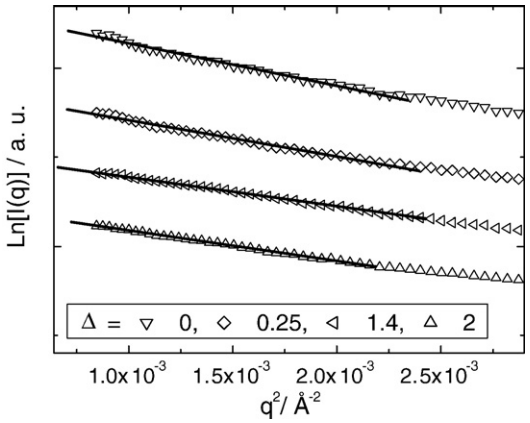

Fig. 2. Guinier plots for the BSA/PEG_{2000}-PE/Dx system as a function of Δ ($c_{BSA}=c_{Dx}=1$ wt.%).

one distribution of particle sizes in the system in the Guinier range (measurable overall particle sizes $\sim 2\pi/q=(209–134)$ Å for $q=(0.03–0.457)$ $Å^{-1}$), in good agreement with DLS results.

The radius of gyration of the scattering particle was calculated from the fitting of Eq. (5) to the SAXS data in Fig. 2. Fig. 3 shows the dependence of R_G on Δ. R_G increases with $c_{PEG2000\text{-}PE}$, from 37.7 Å ($\Delta=0$) to 45.3 Å ($\Delta=2$). The radius of gyration obtained here for $\Delta=0$ ($c_{Dx}=c_{BSA}=1$ wt.%), is higher than that obtained for the BSA in water $R_G=28$ Å measured by SAXS [8] and $R_G=30.5$ Å measured by SANS [10], suggesting an initial binding of Dx to the BSA for the protein in the polysaccharide solution.

Both R_H (Fig. 1) and R_G (Fig. 3) increase upon increasing Δ, showing an initial binding of Dx to BSA, such that the PEG_{2000}-PE also binds to the BSA when added to the system. This leads to a progressive growth in size of the BSA/PEG_{2000}-PE/Dx aggregates upon increasing $c_{PEG2000\text{-}PE}$ for a fixed c_{Dx}. However, the growing rates of R_H and R_G are different, suggesting that the aggregates do not have a spherical shape. Indeed, the average $R_H/R_G=1.4$ obtained using data in Figs. 1 and 3, is different from $R_H/R_G=1$ or $R_H/R_G=0.75$ expected for a sphere or a hard sphere respectively.

According to the paragraph above, it is worth confirming the shape of the BSA/PEG_{2000}-PE/Dx aggregates from the data

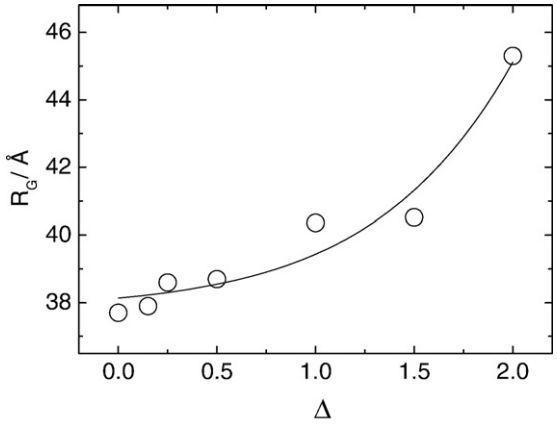

Fig. 3. Radii of gyration obtained for the BSA/PEG_{2000}-PE/Dx system as a function of Δ ($c_{BSA}=c_{Dx}=1$ wt.%). The full line is a guide for the eyes.

plotted in Figs. 1 and 3. In a previous work we showed that BSA/PEG_{2000}-PE aggregates present an oblate shape for $\delta=0.15–2$ [7]. Thus, it seems to be a natural choice to propose an ellipsoidal oblate shape for the BSA/PEG_{2000}-PE/Dx aggregates.

The radius of gyration of an oblate ellipsoid with half axis (a, $b=\nu a$, $b=\nu a$, $\nu<1$: anisometry) can be calculated according to:

$$R_G = \left[\frac{a^2+2b^2}{5}\right]^{1/2} \tag{6}$$

In order to evaluate if SAXS results are in good agreement with DLS results, it is possible to use Perrin's formula for an oblate ellipsoid [18]:

$$R_H = \frac{a\gamma}{\tan^{-1}\gamma} \tag{7}$$

where $\gamma=[(b/a)^2-1]^{1/2}$.

Eqs. (6) and (7) provide a tool to express R_H as a function of a, b and R_G in a single equation:

$$R_H = \frac{\gamma + a}{\tan^{-1}\left\{\left(5R_G^2-a^2\right)^{1/2}/2^{1/2}(a^2-1)\right\}} \tag{8}$$

Using R_G in Fig. 3, it is possible to solve Eq. (8) numerically and find which a and b provide R_H values similar to those plotted in Fig. 1 for a determined Δ. Results are listed in Table 1.

Parameters in Table 1 provide an oblate ellipsoidal shape for BSA/PEG_{2000}-PE/Dx aggregates, with a relatively high anisometry, nearly close to spherical ($\nu=1$) for $\Delta=1$, 1.5. The volume of the aggregates $V=\frac{4\pi ab^2}{3}$ (Table 1) increases with Δ and it is on average 1.6 times the volume of BSA/PEG_{2000}-PE aggregates already determined by us at 37 °C ($V=[1.5–4.5]\times 10^5$ $Å^3$ for $\delta=0.5–2$) [7].

Calculations following the same methodology used in Eqs. (6)–(8) were performed for a prolate ellipsoidal shape, but it was not possible to find any physical solution for that alternative shape.

DLS and SAXS results point to the existence of BSA/PEG_{2000}-PE/Dx aggregates in the system. The osmotic pressure of such aggregates was measured for $\Delta=0.15–2$, using a fixed $c_{Dx}=c_{BSA}=1$ wt.%. Results are displayed in Fig. 4, showing that the osmotic pressure increases upon increasing Δ. Simultaneously, the value obtained COP = 15 mm Hg for $\Delta=2$ ($c_{BSA}=c_{Dx}=1$ wt.%) is higher than COP = 4.5 mm Hg reported above for $\delta=2$ ($c_{BSA}=1$ wt.%, $c_{Dx}=0$).

When particles experiencing repulsive interactions are forced to crowd together, the related osmotic pressure represents the resistance that the concentrated suspension opposes to a decrease in the available volume of particles [19].

Table 1
Parameters calculated using Eqs. (6)–(8) and data plotted in Figs. 1 and 3

Δ	a/Å	b/Å	ν	V/$Å^3$
0.25	29.7	57.3	0.52	4.1×10^5
0.5	37.3	55	0.68	4.7×10^5
1	51.3	53.6	0.96	6.2×10^5
1.5	49.5	53.6	0.92	5.9×10^5
2	50	62.3	0.80	8.1×10^5

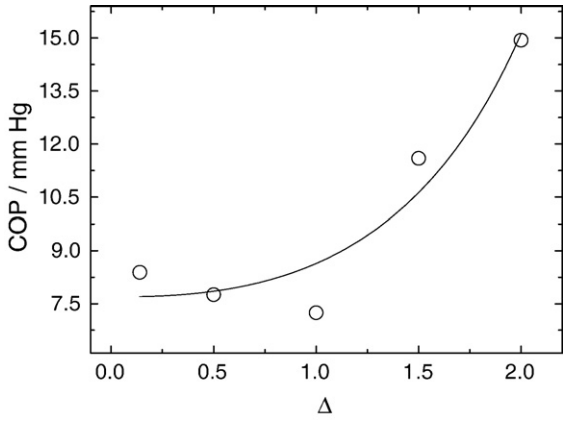

Fig. 4. Colloidal osmotic pressure measured as a function of Δ ($c_{BSA}=c_{Dx}=1$ wt.%) for the BSA/PEG_{2000}-PE/Dx system. The full line is a guide for the eyes.

According to the paragraph above, the increase of COP upon increasing Δ (Fig. 4) denotes a resistance of the BSA/PEG_{2000}-PE/Dx aggregates to crowd together under addition of PEG_{2000}-PE. It is well known that PEG and Dx are immiscible in solution. Indeed, thanks to their immiscibility, aqueous Dx/PEG solutions are traditionally used in protein separation processes [20]. But DLS and SAXS results in this work (Figs. 1–3) have shown that both Dx and PEG_{2000}-PE bind to BSA in solution. As mentioned at the beginning of this section, the PE block of the PEG-lipid binds to BSA due to its high hydrophobicity. In contrast, the PEG block probably remains exposed to the solution, reinforcing the resistance of the BSA/PEG_{2000}-PE/Dx aggregates to crowd together through repulsive interactions with the Dx bounded to neighbouring BSA/PEG_{2000}-PE/Dx aggregates.

5. Conclusions

We have studied the COP and aggregate shapes in solutions containing BSA, PEG_{2000}-PE and Dx dissolved in PBS (pH 7.4), using osmometry, DLS and SAXS.

The COP measured by osmometry for $\delta=2$ ($c_{Dx}=0$, $c_{BSA}=1$ wt.%) was relatively low compared to the COP for healthy adults. Thus, Dx was added to the BSA/PEG_{2000}-PE system in order to increase the COP for a fixed Δ ($c_{Dx}=c_{BSA}=1$ wt.%). Osmometry results shown that the addition of Dx to the BSA/PEG_{2000}-PE system could successfully increase the COP, through the formation of BSA/PEG_{2000}-PE/Dx aggregates. In particular, COP = 15 mm Hg obtained for $\Delta=2$ ($c_{Dx}=c_{BSA}=1$ wt.%), corresponds to 60% of COP = 25 mm Hg measured for healthy adults [12], providing enough COP to play an important role in equilibrating blood COP and maintaining the overall blood volume.

DLS and SAXS results confirmed the formation of individual BSA/PEG_{2000}-PE/Dx aggregates in the solution. The size of the aggregates increased upon increasing Δ ($c_{Dx}=c_{BSA}=1$ wt.%) showing that the binding of Dx to BSA does not prevent the simultaneous complexation of PEG_{2000}-PE with BSA. Geometrical shape calculations using the hydrodynamic radii and radii of gyration of the complexes, measured by DLS and SAXS respectively, favoured an oblate ellipsoidal shape for the BSA/PEG_{2000}-PE/Dx aggregates in solution.

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