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Irradiation as an alternative route for protein crosslinking: Cosolvent free BSA nanoparticles

Gustavo H.C. Varca *, Rodrigo G. Queiroz *, Ademar B. Lugão

Nuclear and Energy Research Institute (IPEN-CNEN/SP)-Av.Prof. Lineu Prestes, 2242, Cidade Universitaria, Zip Code 05508-000 São Paulo, SP, Brazil

HIGHLIGHTS

- Synthesis of BSA nanoparticles by irradiation.
- Tunable particle size by control of the absorbed dose.
- Overview of the effect of cosolvents over nanoparticle formation.
- Engineered BSA nanoparticles for biomedical applications.

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ABSTRACT

Recent studies reported the development of protein-based nanoparticles by the use of γ-irradiation for the production of advanced drug carriers and biomaterials at nanolevel. Basically, the technique combines protein aggregation by means of protein desolvation using a cosolvent, followed by crosslinking using irradiation. We hereby report the effect irradiation dose over the development of protein-based nanoparticles combined or not with cosolvents. BSA was used as a model protein and the samples were irradiated in phosphate buffer (pH=7.2) using a gammacell in absence or presence of ethanol or methanol at 30% and 40% (v/v) respectively. The irradiation dose effect was evaluated following the exposition of BSA to 2.5, 5, 7.5 and 10 kGy over particle size and protein crosslinking, as determined by photon correlation microscopy and fluorescence measurements. Optimized effects were achieved at 10 kGy, under the assayed dose range, with regard to higher particle size and protein crosslinking levels. The use of irradiation was suitable for the synthesis of BSA nanoparticles and tuning of particle size was achieved by controlling the absorbed dose. While the use of ethanol provided an additional control over BSA particle size if compared to the use of methanol at the concentrations assayed, the possibility to perform BSA crosslinking in absence of cosolvents unraveled a novel one-step procedure for the synthesis of protein nanoparticles with no toxicity generated by the use of cosolvents or monomers.

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1. Introduction

Albumins are globular proteins with wide range of applications and biological functions. Bovine serum albumin (BSA) and human serum albumin (HSA) are serum proteins that participate in several biological processes. The homology of structure and biochemical properties between both assured the applications of albumin for human clinical procedures, e.g. volume replacement, supplementation for nutritional purposes, support of colloid oncotic pressure, etc. (Soni and Margarson, 2004).

For instance, the uses of BSA and/or HSA as a drug carrier with the purpose of enhancing drug delivery properties like bioavailability and transportation or to provide controlled release properties are remarkable. In spite of the low cost BSA is also relatively stable and holds the ability to bind to many kinds of drugs (Hu et al., 2006; Kratz and Elsadek, 2012; Jahanshahi et al., 2008; Elzoghby et al., 2012).

In the seek for improvement of BSA properties nanoparticulate systems were constantly investigated to provide protection from degradation, enhancement of drug absorption and modification of pharmacokinetic and drug tissue distribution profiles among others (Jun et al., 2011; Elzoghby et al., 2012; Sripriyalakshmi et al., 2014). The biomedical and clinical relevance of albumin nanoparticles is subject of constant research and remain as a promising field, particularly for the administration of radiopharmaceutics and chemotherapeutics (Teng et al., 2013) as novel drugs combined with albumin enter the market.

* Corresponding authors.
E-mail addresses: varca@usp.br (G.H.C. Varca), rodgqueiroz@gmail.com (R.G. Queiroz).

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When it comes to protein nanoparticles conventional techniques are mainly based on desolvation methods achieved by the use of cosolvents, e.g. ethanol or acetone, followed by addition of a chemical crosslinker (Fernandez-Lafuente et al., 1995; Gerrard, 2002; Sabliov et al., 2015). While the use of cosolvent implies in changes in protein particle size, the crosslinker is responsible for covalently binding the formed structures, and as a consequence it also provides some additional size increase depending upon the crosslinker. In principle, the use of crosslinker is effective and capable of promoting the crosslinking with efficacy. However, if not properly removed, remaining traces of the crosslinker are known to induce undesirable reactions and toxicity to biological systems apart from the demand of extensive time-consuming dialysis stages.

The use of irradiation on the other hand offers the possibility to produce crosslinked protein-based structures at a nanoscale (Varca et al., 2014a, 2014b; Soto Espinoza et al., 2012), combined or not with simultaneous sterilization as observed for other systems (Kadlubowski, 2014), inside the final package with no toxic products or residuals. Within this context, the main goal of the study was evaluate the effect of irradiation dose on the development of BSA-based nanostructures, combined or not with cosolvents in order to explore the potential of the technique for the development of BSA nanocarriers for biomedical applications. For such purpose, BSA irradiation was performed in presence and absence of cosolvents, ethanol or methanol, under dose range of 2.5–10 kGy and characterized according to particle size and the formation of protein crosslinks.

2. Experimental

All materials and reagents were of analytical grade and used as provided. Ethanol and methanol were kept in freezer at −20 °C for at least 6 h prior to use.

2.1. Nanoparticle synthesis

2.1.1. BSA solutions

BSA, heat shock fraction, fatty acid free (purity ≥ 98%, from Sigma-Aldrich®-USA) was solubilized in 50 mM phosphate buffer solution (PBS) at pH 7.2 to reach final concentration of 20 mg mL^{-1} and transferred to 5 mL glass vials on ice bath. After preparation, the samples were hermetically sealed and allowed to stabilize overnight at ±4 °C prior to irradiation.

2.1.2. Effect of cosolvents

Aliquots of 1 mL of the BSA stock solution (100 mg mL^{-1}) were transferred to glass vials followed by slow addition of freeze-cooled cosolvent to reach BSA concentration of 20 mg mL^{-1} and 30% Ethanol or 40% methanol (Synth®-Brazil) concentrations (v/v). The procedure was performed on ice bath and handled as described for the BSA solutions.

2.1.3. BSA irradiation

The vials were exposed to irradiation in a gammacell 220 Irradiator (Atomic Energy of Canada Limited, Canada) at dose rate of 1.03 kGy h^{-1}, established by alanine dosimetry (ISO/ASTM 51607:2013, 2013), using ^{60}Co as radioactive source. Irradiation was performed at low temperatures using a mini-cooler with synthetic ice packs. The samples were stored at ±4 °C before and after irradiation. The selected irradiation doses were 2.5, 5, 7.5 and 10 kGy. Non-irradiated BSA was used as a control.

2.2. Characterization

2.2.1. BSA particle size

BSA particle size was evaluated by photon correlation spectroscopy (PCS). Mean particle diameter was determined using 3 sets of 3 runs of 12 s each at 20 °C and 90° scattering angle on a Zetasizer Nano ZS90 (Malvern Instruments GmbH, Germany). The samples were filtered using 0.45 μm cellulose acetate syringe filters prior to analysis.

2.2.1.1. Particle size versus number of molecules. The approximate number of BSA molecules involved in each assayed condition was estimated by applying the mean particle diameter obtained by PCS into the volume of a sphere ($V=\frac{3}{4}\pi r^3$) where $r=\frac{\text{Mean particle diameter}}{2}$. Native papain mean particle diameter was taken as reference value of 1.

2.2.2. Protein crosslinking-bityrosine

The BSA solutions were diluted in PBS to reach equivalent absorbance of 0.25 at $\lambda=280$ nm (±0.75 mg mL^{-1}). Bityrosine emission was then evaluated by fluorescence spectroscopy according to parameters described in literature (Di Marco and Giuliv, 2006; Varca et al., 2014b) $\lambda_{ex}=325$ nm, $\lambda_{em}=350–500$ nm and scan speed of 240 nm/min. The measurements were performed on a Spectramax i3 (Molecular Devices, USA) device. Normalization was calculated based on the maximum emission values registered for native BSA.

3. Results and discussion

3.1. BSA crosslinking by γ-irradiation

The effects of irradiation over albumin have been previously studied by Gaber (2005) and other researchers in the past (Plonka et al., 1991). while the direct effects of irradiation require high irradiation doses to cause significant changes in protein structure, the majority of the effects occur as a result of the indirect effects of the exposure. in more specific way, the interaction of the water or solvent radiolysis products and other free radicals generated by irradiation, including mechanisms and pathways for the formation of bityrosines as well as other products formed during the process over proteins and peptides have been detailed and reviewed by Houée-Lévin et al. (2015).

3.1.1. Effect of γ-irradiation dose

γ-irradiation over BSA was capable of inducing particle size changes as detailed in Table 1. While native BSA particle size was estimated around 6–7 nm in buffer, at 2.5 kGy irradiation no considerable changes in particle size were observed, whereas at 5 kGy particle size increased up to 8.8 nm. As irradiation dose

Table 1
Mean native BSA particle size changes as a function of irradiation dose and the presence of cosolvents.

Irradiation dose (kGy)[a]	Mean particle size (d nm ± SD)		
	Native BSA + PB 50 mM	30% Ethanol (v/v)	40% Methanol (v/v)
0	6.7 ± 0.5	14.3 ± 1.3	10.6 ± 2.3
2.5	7.7 ± 1.5	14.9 ± 1.7	11.7 ± 1.1
5.0	8.8 ± 1.8	13.9 ± 2.4	10.2 ± 1.7
7.5	12.4 ± 3.7	16.2 ± 2.2	11.3 ± 2.1
10	16.6 ± 2.3	25.1 ± 2.9	15.7 ± 2.4

[a] Irradiation was performed at a dose rate of 1.03 kGy h^{-1}.

increased to 7.5 and 10 kGy, particle size reached 12.4 and 16.6 nm respectively.

These results evidenced a irradiation dose dependant effect over particle size increase. Literature described that regarding irradiation of papain in buffer, the effect irradiation dose over particle size was negligible, as desolvation by ethanol was essential for size change (Varca et al., 2014a, 2014b) when the enzyme was irradiated at the same dose-range applied in this work.

To some extent this result pointed out possible distinct mechanism of nanoparticle formation between these biomolecules and evidenced the possibility to provide BSA nanoparticle size control promoted by the use of irradiation exclusively, which was not achieved in the case of papain (Varca et al., 2014b). One possible explanation to support such difference is related to the presence of methionine and the higher amount of disulphide bonds presence in BSA, among other structural aspects, which in principle would allow more pathways for nanoparticle formation. Detailed information regarding the mechanism of BSA nanoparticle formation by gamma irradiation on a structural based approach has been recently studied and detailed by Queiroz et al. (2016).

3.1.1.1. Effect of γ-Irradiation dose versus protein crosslinking. The question to be clarified is whether caused changes were permanent or just a result of an agglomeration of the BSA particles induced by irradiation. In such terms an evaluation of the possible caused protein crosslinking is of high importance to the study. Bityrosines are products of protein irradiation, which have been extensively studied for distinct applications (Giulivi et al., 2003; DiMarco and Giulivi, 2006), and have been identified by Varca et al. (2014b) as one of the main mechanism of nanoparticle formation in the case of the radiation synthesis of papain nanoparticles.

Regarding BSA nanoparticles, although previous researchers identified possible crosslinking by irradiation in presence of distinct solvents, the nature of such crosslinks remained unclear (Soto-Espinoza et al., 2012). Recently, Queiroz et al. (2016) evaluated the role of cosolvent concentration over BSA nanoparticle formation and highlighted the formation of bityrosine crosslinks in the nanoparticle formation.

Our experiments revealed that BSA irradiation led to a higher magnitude of almost 8-fold in the bityrosine signal if compared to native BSA (Fig. 1). In a more specific way, a 3, 4.5 and 6-Fold increase was registered followed BSA irradiation at 2.5, 5 and 7.5 kGy respectively. To assure the reliability of the bityrosine results, BSA concentration was brought to equivalence prior to the experiments, considering the high sensitivity of the technique.

Corroborating the particle size measurements, bityrosine measurements also revealed a gradual, dose-dependant bityrosine formation as irradiation dose increased up to 10 kGy. The bityrosine emission registered for native BSA was negligible. Advancing the discussion in terms of mechanism this clearly provides another experimental evidence of the role of bityrosine over BSA nanoparticle formation by irradiation, in accordance to literature (Queiroz, et al., 2016).

3.1.2. Effect of cosolvent

Cosolvents and additives have been widely applied for production of protein particles in a micro- to nanoscale for a wide variety of different final applications (Sabliov et al., 2015; Gerrard 2002). The addition of solvent brings about changes in the protein-solvent microenvironment, which may dramatically alter protein structure depending upon the dose. By adding the proper amounts of such compounds may create the milieu suitable for intermolecular protein crosslinking.

3.1.2.1. The case of ethanol. As detailed in Table 1 the effect of cosolvent leads to an additional abrupt increase in particle size. Before irradiation, the addition of ethanol at 30% (v/v) was capable changing particle size from 6–7 to 14 nm, which occurs as a result of desolvation caused (Yoshikawa et al., 2012). As 2.5 and 5 kGy were applied, no significant changes were registered. At 7.5 and 10 kGy BSA particle size increased up to 16 and 25 nm respectively. The relationship between irradiation dose and particle size was not altered in presence of ethanol as relevant particle size changes were registered starting from 7.5 kGy and the highest achieved at 10 kGy.

Bityrosine spectra in presence of ethanol increased up to about 2.5-fold and also a dose-dependant relationship was observed (Fig. 2). If compared to BSA irradiation in buffer, the reduction in bityrosine intensity observed in presence of ethanol was attributed whether to the scavenging properties held by the cosolvent (Adams et al., 1965) or the combined aggregation implied, which possibly led to higher formation of intermolecular bityrosines over intramolecular ones, which supports the higher particle size increase with reduced intensity observed in the bityrosine spectrum.

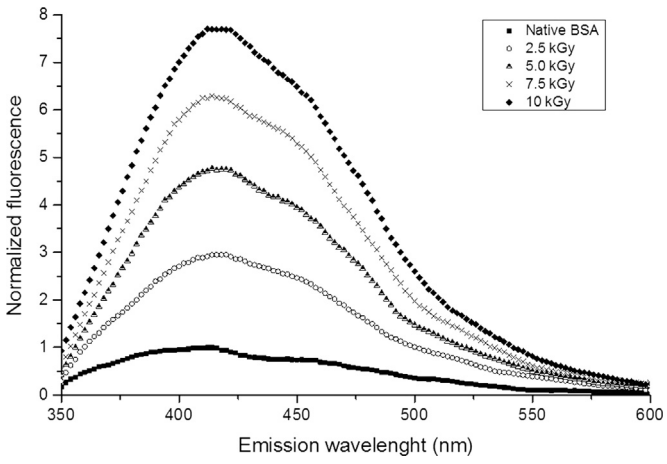

Fig. 1. Evaluation of bityrosine formation of non-irradiated (native) and irradiated BSA samples at different doses (2.5, 5.0, 7.5 and 10 kGy). The fluorescence spectra excitation used (λ_{Ex}) was at 325 nm and emission (λ_{Em}) was collected from 350 to 600 nm. Irradiation conditions: dose rate of 1.03 kGy h^{-1}, absorbed doses of 2.5, 5, 7.5 and10 kGy, and total protein concentration of 20 mg mL^{-1}.

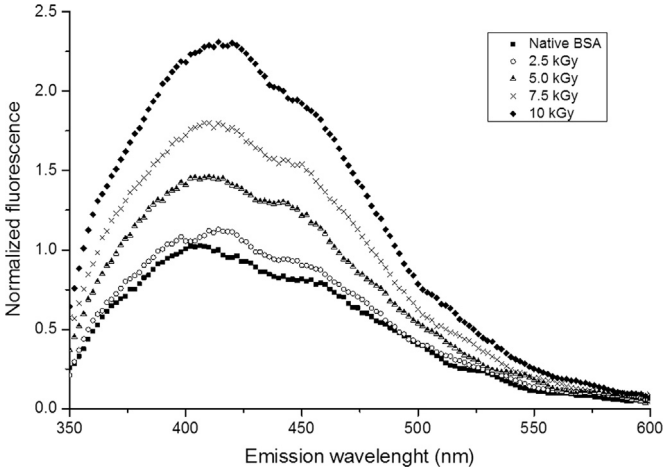

Fig. 2. Evaluation of bityrosine formation of non-irradiated (native) and irradiated BSA samples at different doses (2.5, 5.0, 7.5 and 10 kGy) in presence of 30% ethanol (v/v). The fluorescence spectra excitation used (λ_{Ex}) was at 325 nm and emission (λ_{Em}) was collected from 350 to 600 nm. Irradiation conditions: dose rate of 1.03 kGy h^{-1}, absorbed doses of 2.5, 5, 7.5 and10 kGy, and total protein concentration of 20 mg mL^{-1}.

This difference in fluorescence intensity observed between BSA irradiation in absence and presence of cosolvents also highlighted a possible distinct bityrosine formation, possibly due to a different crosslinking nature or a controlled formation of such linkages. This theory whatsoever remains a subject of further study considering that more techniques are required to properly distinguish between the inter or intramolecular nature of the bityrosine formation.

3.1.2.2. The case of methanol. The use of methanol as a cosolvent is particularly useful for drug loading considering that it allows the solubilization the poor water-soluble drugs at later stages of production that could not be solubilized in ethanol/water systems. However, from a toxicity point of view, unlike ethanol, such cosolvent implies in further extensive solvent removal or washing stages as a consequence, as methanol toxicity to biological systems is well known.

In the case of BSA, at 40% methanol, BSA particle size shifted to 10.6 nm. As irradiation was applied minor changes occurred up to 7.5 kGy, where at 10 kGy particle size shifted to 15.7. This value was similar to the values obtained for BSA irradiation in buffer and thus this information highlights that methanol, at the given concentration, does not provide any additional effects in terms of particle size if compared to irradiation in absence of cosolvents.

The levels of bityrosine were also smaller if compared to irradiation exclusively but, if compared to ethanol, the values were higher. At 2.5 kGy 1.25 normalized fluorescence was registered, 2 and 2.5 for 5 and 7.5 respectively, and almost 4-fold at 10 kGy (Fig. 3). They presented a reduced signal in all doses when compared to the samples without cosolvent but, their signals were higher than the samples at 30% (v/v) ethanol. It is interesting to point out that the highest particle size was achieved with reduced the bityrosine intensity if compared to the irradiated protein in buffer, and this was also clear in the case of papain (Varca et al., 2014b).

3.1.2.3. Particle size versus number of molecules. In order to distinguish and elucidate the effect of each cosolvent and irradiation dose over the number of BSA molecules involved in the process, an approximation by means of the volume of a sphere was performed (Fig. 4). Although the calculations to estimate the number of BSA molecules involved in each experimental condition were limited to the extent of approximate values, a good correlation may be established as BSA is a globular protein by nature, which makes the

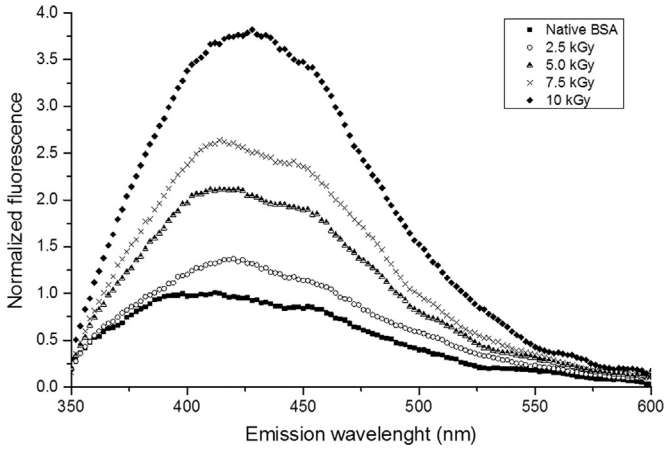

Fig. 3. Evaluation of bityrosine formation of non-irradiated (native) and irradiated BSA samples at different doses (2.5, 5.0, 7.5 and 10 kGy) in presence of 40% methanol (v/v). The fluorescence spectra excitation used (λ_{Ex}) was at 325 nm and emission (λ_{Em}) was collected from 350 to 600 nm. Irradiation conditions: dose rate of 1.03 kGy h^{-1}, absorbed doses of 2.5, 5, 7.5 and10 kGy, and total protein concentration of 20 mg mL^{-1}.

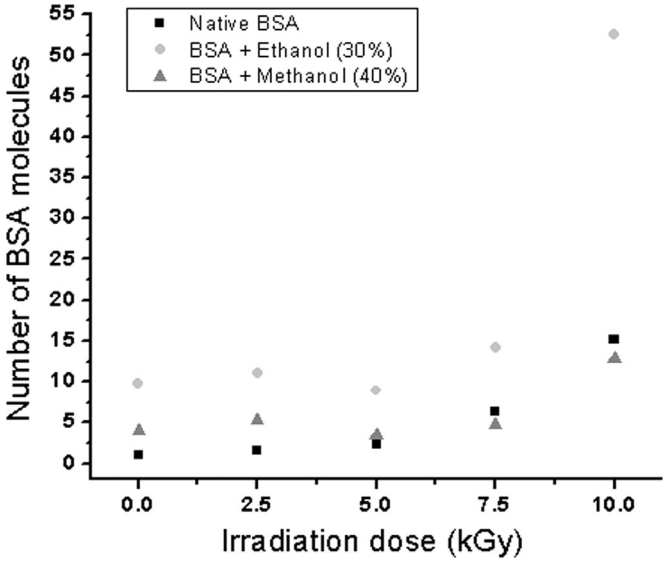

Fig. 4. Effect of absorbed dose and the presence of cosolvents-ethanol (30% v/v) or methanol (40%v/v) over the number of BSA molecules involved in the process. Irradiation conditions: dose rate of 1.03 kGy h^{-1}, absorbed doses of 2.5, 5, 7.5 and10 kGy, and total protein concentration of 20 mg mL^{-1}.

volume of a sphere an adequate correlation to be used.

In terms of number of BSA molecules, ethanol combined with irradiation involved about 55 units while irradiation in presence of methanol and in absence of cosolvents achieved a value of about 13-15 molecules. In absence of irradiation a similar profile was observed considering about 10 BSA molecules for ethanol, about 4 for metanol. The selection of cosolvent concentrations, 30% ethanol and 40% methanol, aimed to bring such cosolvents near equivalence in mass and thus allow a proper comparison between the effects promoted by each compound.

The results revealed that the effect of irradiation over BSA in buffer were very similar to those obtained in presence of methanol, whereas ethanol was capable of dramatically altering particle size in addition to the effects promoted by irradiation, and thus indicating a very different behaviour between the effect of the two cosolvents. With regard to irradiation dose, under the given experimental conditions and parameters, optimized conditions in terms of highest particle size and bityrosine formation were established as 10 kGy in presence and absence of cosolvents. This evidences the positive effect of ethanol and the nearly negligible effect promoted by methanol over particle size at the assayed conditions and concentrations.

In terms of aggregation the considerable differences observed between the effects of each cosolvent over BSA occur a result of the protein desolvation induced by each solvent, whereas its polarity plays a fundamental role (Sabliov et al., 2015). A similar behaviour was reported by previous researchers where the agglomeration effects promoted by both solvents at distinct concentrations over BSA were different from one another (Queiroz et al., 2016).

Another point to be considered is related to the scavenging effects promoted by each cosolvent. From a mechanism point of view, in presence of such compounds, the radicals responsible for the BSA crosslinking are more likely to be ethanol or methanol derived radicals rather than direct producs of water radiolysis, as these last ones are scavenged by the cosolvent (Queiroz et al., 2016). At a first glance, the difference in cosolvent concentrations applied in this research, 30% for ethanol and 40% for methanol respectively, could influence the results as an increase in scavenging activity would be expected as cosolvent concentration increases. However, the such concentrations were high enough to

assure that the scavenging differences promoted by each solvent were of negligible or minimum relevance (Adams et al., 1965).

4. Conclusions

The use of γ-irradiation was suitable for the synthesis of BSA nanoparticles in absence of chemical agents, bifunctional crosslinkers, or cosolvents of any nature. Protein crosslinking was evaluated by means of bityrosine formation and revealed that a control of irradiation dose assured controllable BSA particle size and crosslinking as a consequence, and some correlation between crosslinking and particle size was established. Advances of the technique are related to the lack of cosolvents and possible residual monomer or toxicity from their use, as well as the possibility to produce the nanoparticles in a one-step procedure, which depending upon the irradiation conditions, may allow simultaneous crosslinking and sterilization.

The effect of cosolvents was different at the assayed concentration versus dose range. Ethanol provided an additional control over particle size increment and crosslinking as expected. Specifically highest particle sizes were achieved at 10 kGy in presence and absence of cosolvents. Whatsoever, the effect of methanol was not effective, considering that similar particle sizes were achieved for native BSA and in presence of methanol, despite the differences observed in the bityrosine crosslinks, more likely to be composed in majority by crosslinks of intramolecular nature.

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