RADIATION SYNTHESIS OF FUNCTIONAL NANOPARTICLES FOR IMAGING, SENSING AND DRUG DELIVERY APPLICATIONS

M. Grasselli, S. Soto Espinoza, V. Risso, E. Pawlak, E. E. Smolko; ARGENTINA

1. INTRODUCTION

Ionizing radiation has been industrially used for many years in polymer processing. The two main streams of applications belong to crosslinking and degradation reactions. More sophisticated commercial products are achieved under development such as hydrogel wound dressing, membranes and sensors [1].

Radiation technology can be used to generate polymer microparticles by precipitation polymerization with a very narrow particle size distribution. Additive-free initiation and easy process control are the main advantage of this technology. In this way, methacrylate-based microspheres can be prepared by radiation-induced polymerization of diethyleneglycol dimethacrylate (DEGDMA) and others cross linkers in organic solvent. Particle diameter can be achieved, from the range 0.8 to 8 μm, by selection of the appropriate organic solvent [2,3]. Tailor-made microspheres were further developed by copolymerization of DEGDMA with reactive monomers, such as glycidyl methacrylate, or particular monomers to rich functional microspheres [4,5], however lower-diameter particles still have not been achieved by this technique.

Small polymeric particles can be also reached by ionizing radiation technology, but via another strategy. Soluble synthetic polymers can be intramolecularly cross linked by quantum-ray, creating nanogels, which was first reported by Ulanski *et al.* [6,7]. Authors managed irradiation conditions to generate intramolecular crosslinking onto soluble polymer molecules in random coil conformation.

Many proteins are also soluble macromolecules; however, most of them have a very compact and defined three dimensional structure, which are known as globular proteins. The effect of gamma rays onto proteins was study for many years, mainly in features related to degradation. Proteins, as raw material, have been recently used for preparation of nanogels from gelatin, using ionizing techniques [8]. Gelatin comes from the hydrolysis of collagen, a fibrous and insoluble protein. Authors have been studying the changes in the intrinsic volume of these protein nanoparticles. However the first report of nanoparticle synthesis from proteins using radiation technology was done by Furusawa [9]. In both cases, row material was gelatin with average molecular weight of about 100 kDa, a mixture of polypeptides without a defined three-dimensional structure. As a consequence of lack of a defined conformation in solution, this protein is closer to synthetic polymers to enzymes and/or functional globular proteins.

In the present report we describe nanoparticle synthesis by ionizing radiation from globular proteins and methacrylate monomers. Dynamic light scattering and other spectroscopic methods were performed to characterize this new material.

2. MATERIALS AND METHODS

Bovine serum albumin, Fraction V (BSA), diethyleneglycol dimethacrylate (DEGDMA), and glutaraldehyde solution were obtained from Sigma (BA, Argentina).

BSA was dissolved in buffer phosphate 30 mM (BP) pH 7. Different amount of modifiers were added dropwise onto the protein solution at 5 ºC under constant stirring. BSA solutions were irradiated with gamma-ray from a ^{60}Co source (PISI CNEA Ezeiza) at a dose rate lower than 1 kGy/h at 5-10 ºC. After the irradiation, clear or slickly opaque solutions were observed in the samples. Protein solutions were diluted to a suitable concentration with BP pH 7 for analysis. Fluorescein isothiocyanate (FITC) was used to label BSA with fluorescein according to the method developed by Heuck [10]. Chemical cross-linking was performed by addition of glutaraldehyde solution (final concentration 2.6 %) to achieve particle. All other reagents were of analytical grade and used as received.

Particle size was determined by dynamic light scattering (DLS) at 25 °C by measuring the autocorrelation function at a 90-degree scattering angle in a 90Plus/Bi-MAS particle size analyzer. Circular Dicroism (CD) measurements were carried out at 20 °C on a Jasco 810 spectropolarimeter. BSA was subjected to thermal unfolding between 0 and 90 °C with a heating rate of 2 °C/min. Secondary structure break down was followed by measuring ellipticity at 220 nm, using the above-described spectropolarimeter and a 1.0-cm cell. UV-absorption spectra (240–340 nm, 0.1 nm sampling interval, 20 nm/s) were obtained with a Jasco V-550 spectrophotometer (Jasco Corporation, Japan). Fourth-derivative of the spectra was calculated applying two successive cycles of second order derivation. Fluorescence measurements were recorded in NanoDrop 3300 Fluorospectrometer. SEM pictures were done in electronic microscope JEOL JSM 5600 LV. For TEM analysis negative stain micrographs was performed with Uranyl Acetate stain with a Zeiss EM 109 Turbo transmission electron microscope.

3. RESULTS AND DISCUSSION

In order to synthesize nano/micro particles we analyzed structured biopolymers usefulness, such as globular proteins as *´nano seed´* to produce particles. In this way, aqueous solutions of protein and DEGDMA were irradiated. In addition to water, ethanol was added as cosolvent in order to improve monomer solubilization. In Table 1 and 2 are described the composition of different mixtures studied. In the first experiment were irradiated different quantities of DEGDMA maintaining constant a BSA amount, as it is detailed in Table 1, dissolved in PB with ethanol added (40 % v/v). Mixtures were irradiated in a ^{60}Co gamma irradiation source at 10 kGy and temperature was kept at 0-5 °C during irradiation.

TABLE 1. BSA AND DEGDMA AMOUNTS DISSOLVED IN BUFFER WITH ETHANOL (40 % V/V) AND IRRADIATED FOR MICROSPHERES SYNTHESIS (TOTAL VOL: 1,5 ML)

Sample	**BSA** (μmol)	**DEGDMA** (μmol)
A1	0.7	45
A2	0.7	90
A3	0.7	135
A4	0.7	180

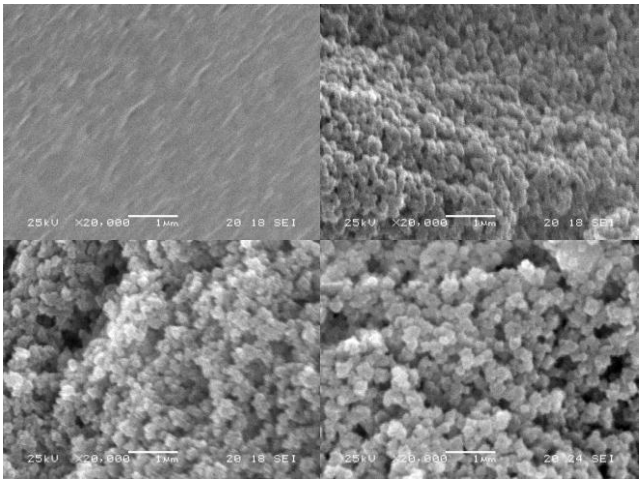

FIG. 1. SEM pictures of BSA/DEGDMA microspheres synthesized according Table 1, (Top left: sample A1; Top right: sample A2; Bottom left: sample A3 and Bottom right: sample A4).

With the exception of sample A1, particulate suspensions are yielded after irradiation. Scanning microscopy showed increments in the particle size, up to approximately 200 nm, upon increasing the addition quantities of DEGDMA (Fig. 1), as it can be expected. In the case of varying protein amount and keeping constant the DEGDMA quantity, as it is described in Table 2, all samples reached white dispersions after irradiation. After recovered by centrifugation and washed several times with water and ethanol and dried by lyophilization, SEM pictures were performed.

TABLE 2. BSA AND DEGDMA AMOUNTS DISSOLVED IN BUFFER WITH ETHANOL (40 % V/V) AND IRRADIATED FOR MICROSPHERES SYNTHESIS (TOTAL VOL: 1,5 ML)

Sample	**BSA** (μmol)	**DEGDMA** (μmol)
B1	0.23	225
B2	0.46	225
B3	0.58	225
B4	0.7	225

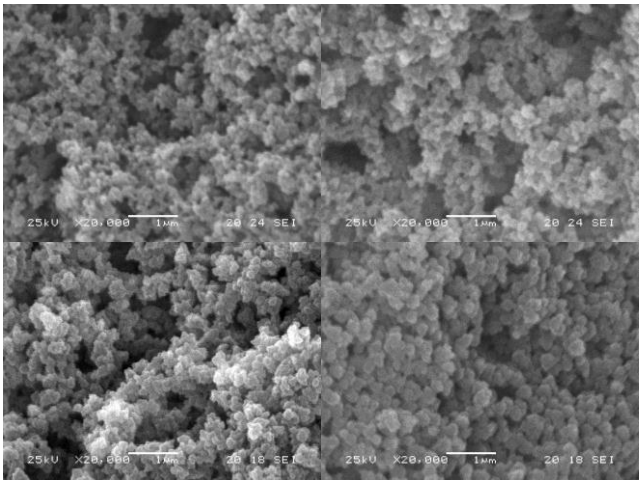

FIG. 2. SEM pictures of BSA/DEGDMA microspheres synthesized according Table 2 (Top left: sample B1; Top right: sample B2; Bottom left: sample B3 and Bottom right: sample B4).

From SEM pictures we can assert that for solutions prepared with increasing quantities of BSA, also yield particle with higher sizes, however, the effect is less intense than in the previous case. Thus, protein had a more complex behavior than can initially be speculated.

Having in mind that globular proteins are macromolecules very sensitive to microenvironment, the effect of ethanol addition during the irradiation of protein solution was studied. For the following work, looking for small particles, samples were analyzed by Dynamic Light Scattering to determine the population of different particle size in solution. Protein and protein/DEGDMA mixture were irradiated into PB solution with different amount of ethanol aliquots. For this experiment much less DEGDMA amounts were used (4 μmols) in order to avoid a detrimental solvent effect of the monomer onto the protein. Fig. 3 shown there is no measurable differences in the particle diameter of irradiated BSA protein at low ethanol concentration (less than 30%). However, when more than 30% of ethanol is used in the solution, sample reached nanoparticles.

Furthermore, irradiated protein in buffer/ethanol mixture yielded nanoparticles irrespective the presence of DEGDMA in the initial solution.

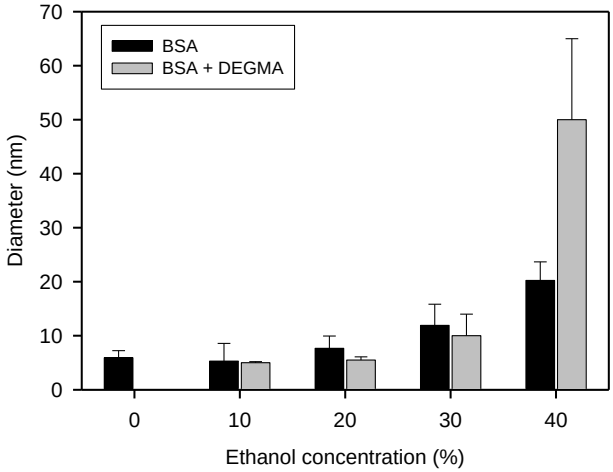

FIG. 3. DLS measurements of irradiated BSA, 0.7 μmol (black bars) and BSA/DEGDMA, 0.7 μmol / 4 μmol (grey bars) dissolved in buffer with different amount of ethanol added (Total vol: 1.5 mL).

The presence of DEGDMA reaches nanoparticles of higher sizes only when protein nanoparticle can be made. Taking into account sub-nanoscale reactive species created by gamma-rays and the nanoscale size of proteins we decide to study, in further detail, the influence of gamma-rays onto protein solutions without monomer addition.

Two initial conditions were studied; BSA dissolved in PB and a solution of PB with ethanol 40% v/v. Ethanol is a well-known protein precipitant, however more than 60 % ethanol is required to induce BSA precipitation. In this experiment, ethanol was used at sub-precipitating concentrations.

TABLE 3. AVERAGE PARTICLE DIAMETER OF BSA SOLUTIONS MEASURED BY DLS

Modifier	**Condition**	**Diameter**	**SD**
		(nm)	(nm)
BSA	No Irrad	4,8	1,3
BSA 40 % Ethanol	No Irrad	4,8	1,7
BSA	Irrad	3,8	1,7
BSA 40 % Ethanol	Irrad	20,5	3,5

In Table 3 are shown the DLS measurements of irradiated and non-irradiated protein samples. BSA irradiated in buffer shows a small diameter reduction, which can be related to degradation effect of

gamma rays [11]. However, the same protein in an aqueous/ethanol environment reached nanoparticles with dimensions higher than 20 nm after irradiation.

In a following experiment the two most common protein precipitants were studied under sub-precipitant concentration, an organic solvent and an inorganic salt. In both cases the precipitants, ethanol and ammonium sulphate, have a dewatering effect onto the solvated protein. In Fig. 4 are described the DLS measurments of irradiated protein solution at 10 kGy with addition of each modifier. The addition of increasing quantities of ammonium sulphate to the protein solution did not influence the average size of the particles after irradiation. However using ethanol, it can be found increment in the size of nanoparticles.

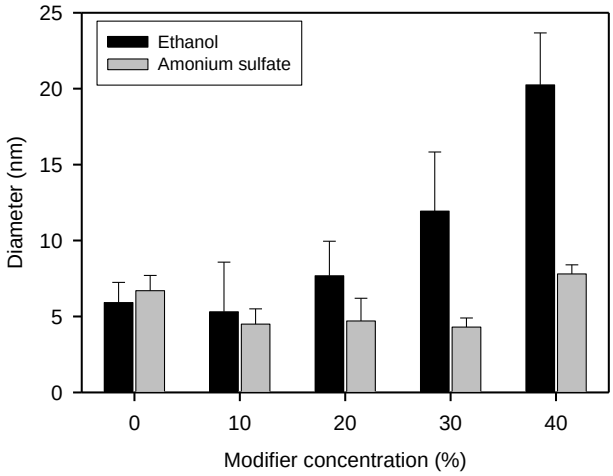

FIG. 4. Particle size for BSA irradiated samples with the addition of different additives: ethanol and ammonium sulfate. Standard deviation corresponds to 3 independent samples.

Other solvents were used to analyze the effect in nanoparticle formation, such as acetonitrile and isopropanol. They were used in an equivalent molar concentration to ethanol 40% v/v. As it is shown in Table 4 solvents compatible with globular proteins have similar effect than ethanol.

TABLE 4. AVERAGE PARTICLE DIAMETER OF BSA SOLUTIONS IRRADIATED AT 10 KGY WITH THE ADDITION OF THE MODIFIER. SAMPLES WERE DILUTED IN PB PREVIOUSLY TO BE MEASURED IN DLS.

Modifier	Condition	Diameter (nm)	SD (nm)
None		5,9	1,3
Ethanol	40 %	20,3	3,4
Acetonitrile	36 %	13,9	0,9
Isopropanol	52 %	34,9	3,5

Under the experimental condition of 40% v/v ethanol, samples containing different protein concentration were irradiated. As it is shown in Fig. 8 particle diameter becomes lower with the increases of initial protein concentration. Protein concentration of 30 mg/mL was choosen for the following experimental work because shows the minimun error dispersion.

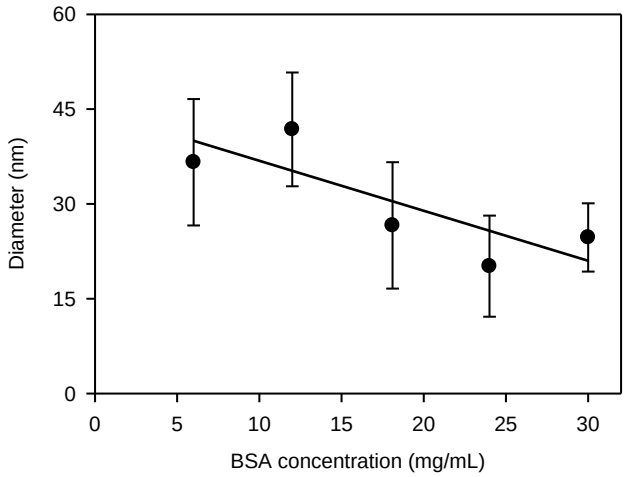

FIG. 5. Particle size for irradiated BSA samples at different initial BSA concentrations.

In the following experiment, the effect of irradiation dose was studied. BSA solution in buffer and buffer/ethanol 40% v/v was tested. Low dose rate (lower than 1 kGy/h) and different irradiation time was used for each condition. Light scattering measurments show than a minimun of 10 kGy was required to find out nanoparticles (Fig. 6). Protein size irradiated in buffer keep approximately their original size when it is irradiated in air. These results are in agreement with data reported of molecular weigth of BSA irradiated in oxygen atmosphere [12].

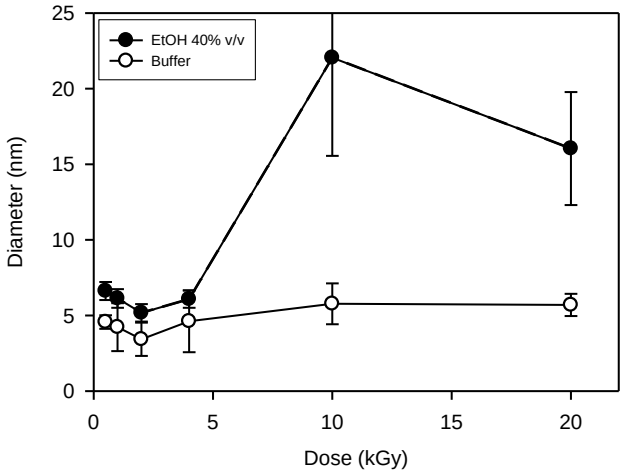

FIG. 6. Particle size for irradiated BSA samples in buffer and buffer/ethanol mixture at different doses.

Considering the study of differencial properties of irradiated BSA solutions the response to dye adsorption and behaviour to centrifugation and filtration was studied. Irradiated and non-irradiated BSA solutions were diluted in buffer and determined the amount of protein by Bradford assay. This assay is especific for proteins but it is based on an inespecific protein hydrophobic-dye interactions. As it is shown in Fig. 7, irradiated and non-irradiated samples showed the same amount of soluble protein. After the assay, samples was centrifugate at 14,000 rpm in a Epperdorf-type centrifuge for 30 min. Spectrophotometric determination of the dye-protein complex in supernatant shows protein depletion from the solution only in the sample corresponding to 40% ethanol, confirming the existance of nanoparticles.

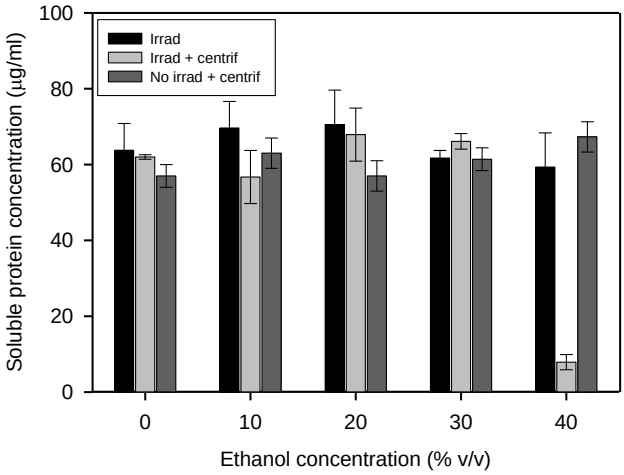

FIG. 7. Soluble protein concentration under different conditions.

In the following experiment fluorescein-labeled BSA was used to trace the BSA during the irradiation process. Fluorescence emission at 518 nm was measured after 10 kGy irradiation of

BSA with 5% of labeled BSA for the buffer/ethanol serie from 0 to 40% v/v. As it is shown in Fig. 8 the relative fluorescence of irradiated BSA against non-irradiated ones decrease proportional to the amount of ethanol in the solution. Fluorescence of BSA sample irradiated in ethanol 40% v/v has only 25% of the initial emission, which can be explained by a quenching process of aggregated BSA molecules in the nanoparticle. Irradiated samples without ethanol do not show any appreciable reduction in fluorescence emission.

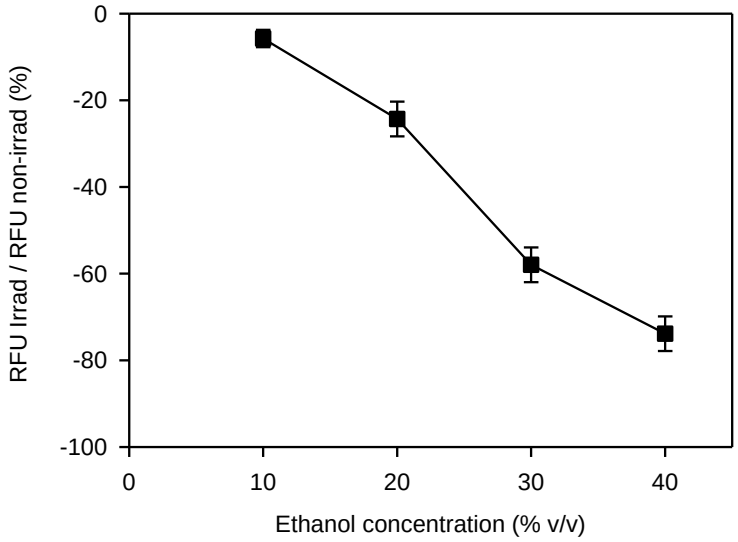

FIG. 8. Fluorescence ratio at 518 nm of irradiated and non-irradiated BSA samples in buffer/ethanol mixtures. RFU means Relative Fluorescence Units.

Fluorescent labeled-BSA samples (irradiated and non-irradiated) was filtrated through 0.22 μm membrane, the usual esterilization filters. After filtration all samples show more than 95% of the initial fluorescence. This experiment verifies most of the nanogel solutions fulfill with the occurrence of particles of nanometric size.

In order to study protein structure in the nanoparticles we performed UV-vis and Circular Dicroism (CD) spectra. Near CD gives information about ternary protein structure and the simetry around aromatic aminoacids where only folded proteins have CD signal in this range. In Fig. 9 are shown the CD characterization of irradiated BSA solutions. CD spectra of the irradiated samples shows no changes in the general spectral shape, thus main conformational features of the ternary structure of the protein are preserved. However, from 20% to 40% ethanol concentration shows an increment in the CD signal. This effect could be assigned to a more rigid conformational structure of the protein.

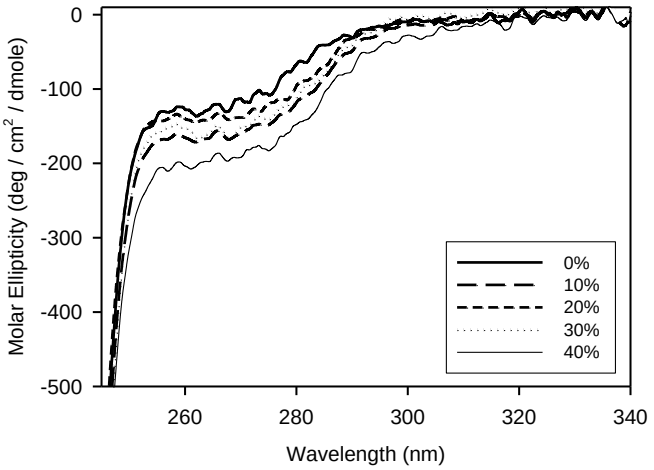

Fig. 9. Circular dicroism of the irradiated BSA samples with different ethanol proportions.

In order to confirm a covalent chemical link between proteins, irradiated samples were diluted in Guanidinium chloride (GdmCl) 6 M, a chaotropic salt used to unfold (denaturate) proteins. In the Table 5 are determined the average particle diameter in this denaturing solution. All recorded data reached higher diameters than values plotted in Fig. 4, as can be expected for unfolded and open protein structures. The higher dispersion in data can be assigned to the random coil unstructured particles in this solvent condition. Additionally, it can be shown an inverse correlation between the initial ethanol concentration during irradiation and particle diameter in unfolded condition. Thus, it seems than higher amount of ethanol during the irradiation induce to more protein molecules keeping closer and in that way higher probability to be cross linked.

TABLE 5. AVERAGE PARTICLE DIAMETER OF BSA SOLUTIONS IRRADIATED AT 10 KGY WITH THE ADDITION OF THE ETHANOL. SAMPLES WERE DILUTED IN GDMCL 6 M 20 H PREVIOUSLY TO BE MEASURED IN DLS.

	Particle size	
EtOH (%)	**Diam (nm)**	**SD (nm)**
0	630	500
10	650	200
20	440	400
30	190	140
40	100	80

Fourth derivative UV-vis spectra have been shown to be very sensitive to sense minute protein conformational changes. Alteration of the microenvironment (polarity, hydration, hidrophobic interactions, packing density) of tyrosine and tryptophan aminoacids can be follow by this

spectroscopic technique. The forth-derivative UV-vis spectra of the irradiated BSA samples were shown in Fig. 10. All curves have the same shape indicating that the microenvironment of aromatic aminoacids are keeping the same conformational features into the protein.

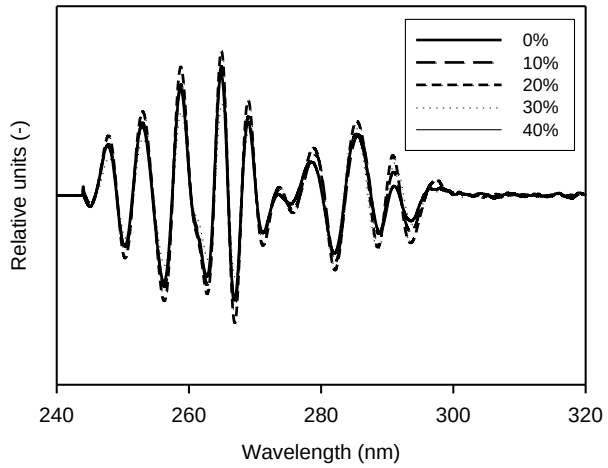

FIG. 10. FOURTH-DERIVATIVE UV-VIS SPECTRA OF THE IRRADIATED BSA SAMPLES WITH DIFFERENT ETHANOL ALIQUOTS.

From CD and UV-vis data the main amount of BSA protein in the samples are keeping its native structure. Thus nanoparticles should be composed by an agregation of protein molecules in their native-state.

Recently Akiyama *et al* [8] have reported the preparation of gelatin nanoparticles based on gamma irradiation of an aqueous protein solution. They also described an increase in the median particle size when a high protein concentration sample is irradiated in the range of 10 to 20 kGy. However, these results cannot be compared with ours because gelatin is not a native globular protein, thus it has not a compact and defined three-dimensional structure such as BSA.

In order to compare with a chemical crosslinking method, such as glutaraldehyde treatment, particles from BSA in BP ethanol 40% v/v have a mean diameter of 17.5 +/- 3 nm. This value is not statistically different from 20.5 +/- 3.5 nm of irradiated ones, thus seem that aggregation and crosslinking step are independent process.

When the aqueous solution involving a polymer was irradiated with gamma-rays, reactive oxygen species such as hydroxyl radicals were formed by radiolysis of water, and they eliminate the hydrogen atom from the carboxyl group or the hydroxyl group to form polymeric radicals. The three kinds of reactions such as cross-linking, main chain scission, and side chain scission occurred by these polymeric radicals.

In order to construct an hypothesis (to hypotetize an explanation) of the experimental results Radiation Target theory is used to analyze the possible source of crosslinking effect by direct hit. Considering the transfer energy that produce ionizations, the minimun energy in a primary ionization (PI) would be the ionization potential of orbital electrons. The most probable energy

transfer is ~20 eV and the average energy transfer can be considered ~60 eV. In diluted protein solutions most of the PI are in water molecules, ths radiation damage is occurring via the radiolytic products of water, this is called indirect effect. It was described up to 99.9% of degradation radiation effects are via this indirect mechanism [13].

Radiation sensitivity of proteins depend on the mass of the molecule and is independent of the molecular volume or shape [14]. From the Poisson equation, the fraction of non-damage molecules (*F*) after a radiation dose, *D,* can be calculated as:

$$F = N / N_0 = e^{-qmD} \quad (1)$$

Where *N* and N_0, is the number of non-damage and initial molecules, respectively. The *m* is the mass an *q* include the average energy deposition (65 eV) and conversion factors [13]. From the Eq.(1) and considering 10 kGy as the minimum dose to reach nanogels, and 66 kDa of BSA molecular weight, the *F* of BSA is equal to 0.90. Thus only 10% of the BSA molecules has the probability to have at least one direct PI. The energy deposited in that interaction ultimately results in breakage of chemical bonds randomly throughtout an entire polypeptide. Considering than a single molecule has a 2.4 nm radii and nanoparticle around 10 nm, a rough estimation gives around seventy close packed molecules to reach a nanoparticle. Taking into account the direct PI onto the macromolecule previously described, only around ten crosslinked bonds are created per nanoparticle. Thus direct hit should be not enoght to explain the nanoparticle stabilization and some indirect effect should be considered.

4. CONCLUSION

Radiation effect onto the proteins, such as fragmentation and aggregation process, has been studied by many authors, as well as modifications of primary, secondary and ternary structure of BSA [15,11]. However proteins have not been used as building blocks combined with radiation technology.

In this work, for the first time, a soluble and globular protein such as BSA was used as basic unit to build nanoparticles without the addition of any additional chemical crosslinker. Detectable protein nanoparticles were reached by irradiation of protein solution in the presence of polar organic solvents. By changing the environmental condition, such as the solvent composition, aggregation clusters could be dynamically created in solution, which are radiation crosslinked. From theoretical data direct hit onto proteins should be not enough to explain the nanoparticle formation. Spectroscopic data showed protein molecules keep its general three-dimensional structure into the created nanoparticles.

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