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journal homepage: www.elsevier.com/locate/radphyschemRadiation synthesized protein-based nanoparticles:
A technique overviewGustavo H.C. Varca^{a,*}, Gabriela G. Perossi^a, Mariano Grasselli^b, Ademar B. Lugão^a^a Instituto de Pesquisas Energéticas e Nucleares, Centro de Química e Meio Ambiente (IPEN-CNEN/SP), Av. Prof. Lineu Prestes, no. 2242, Cidade Universitária, Zip Code, 05508-000 São Paulo, SP, Brazil^b Universidad Nacional de Quilmes– IMBICE (CONICET), Roque Sáenz Peña 352, Bernal, B1876BXD Buenos Aires, Argentina

HIGHLIGHTS

- Synthesis of protein-based nanoparticles by γ -irradiation.
- Optimization of the technique.
- Overview of mechanism involved in the nanoparticle formation.
- Engineered papain nanoparticles for biomedical applications.

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ABSTRACT

Seeking for alternative routes for protein engineering a novel technique – radiation induced synthesis of protein nanoparticles – to achieve size controlled particles with preserved bioactivity has been recently reported. This work aimed to evaluate different process conditions to optimize and provide an overview of the technique using γ -irradiation. Papain was used as model protease and the samples were irradiated in a gamma cell irradiator in phosphate buffer (pH=7.0) containing ethanol (0–35%). The dose effect was evaluated by exposure to distinct γ -irradiation doses (2.5, 5, 7.5 and 10 kGy) and scale up experiments involving distinct protein concentrations (12.5–50 mg mL⁻¹) were also performed. Characterization involved size monitoring using dynamic light scattering. Bityrosine detection was performed using fluorescence measurements in order to provide experimental evidence of the mechanism involved. Best dose effects were achieved at 10 kGy with regard to size and no relevant changes were observed as a function of papain concentration, highlighting very broad operational concentration range. Bityrosine changes were identified for the samples as a function of the process confirming that such linkages play an important role in the nanoparticle formation.

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1. Introduction

Proteins as a whole represent an important group of therapeutic agents available nowadays for the treatment of a wide range of disorders. Despite direct applications, these biomolecules may also be used to functionalize, confer biopharmaceutical advantages and constitute novel drug delivery systems for available drugs among other aspects (Banta et al., 2010; Sezaki and Hashida, 1985).

Perhaps the most relevant aspect to be taken into account with regard to globular proteins in pharmaceuticals and industrial processes is attributed to instability in unusual environments and

intrinsic limitations of such biomolecules. Thus, many approaches have been directed towards overcoming such limitations (Polizzi et al., 2007) including nanotechnological tools (Crommelin et al., 2003), chemical modifications (Fernandez-Lafuente et al., 1995), immobilization (Sheldon, 2007), use of additives such as sugars (Arakawa and Timasheff, 1982; Varca et al., 2010) among others, considering that overcoming such problems and intrinsic limitations would allow a great expansion in the use of such compounds (Arnold, 1993).

Particularly the use of high energy radiation is known to directly or indirectly damage or impair biological function of macromolecules and proteins (Saha et al., 1995; Davies, 1987; Furuta, 2002) and as a result its use is therefore limited. However, over the last decade some researchers have attempted to use radiation (Akiyama et al., 2007; Furusawa et al., 2004) to achieve nanometer-sized particles and nanogels based on proteins and

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peptides. A few years later, Soto Espinoza et al., 2012 evaluated the use of solvents combined with radiation to synthesize bovine serum albumin nanoparticles.

Specifically the use of ionizing radiation to achieve particle size control of enzyme nanoparticles with preserved bioactivity for biomedical applications was recently demonstrated by our group, using papain (Varca et al., 2014). This enzyme is a cysteine protease, widely applied as model enzyme for several studies due to its renowned biotechnological relevance (Hwang and Ivy, 1951) as well as its defined structure and biological properties (Kamphuis et al., 1984).

The approach given in this research reports an overview of the process in order to optimize the technique, including scale up and irradiation dose effects, and provides an experimental evidence to clarify the mechanism involved in the nanoparticle formation.

2. Experimental

2.1. Materials

Papain 30,000 USP-U/mg (EC 3.4.22.2) and ethylenediaminetetraacetic acid were purchased from Merck (Germany); L-cysteine hydrochloride monohydrate, dimethylsulfoxide, sodium chloride, ethanol, sodium hydroxide, chloridric acid, acetic acid and heptahydrate disodium phosphate from Synth (Brazil), and N α -benzoyl-DL-arginine p-nitroanilide were purchased from Sigma-Aldrich[®] (USA). All reagents were of analytical grade.

2.2. Methods

2.2.1. Particle synthesis

Phosphate buffer (50 mM), papain solution and ethanol 0–35% (v/v) were added dropwise to glass vials on ice bath and allowed to stabilize overnight prior to the beginning of experiments at refrigerated conditions (8 °C). Samples were exposed to γ -irradiation on ice bath and dose rate of 1.2 kGy h⁻¹ using ⁶⁰Co as radioactive source in a gamma cell 220 irradiator. The samples were properly filtered using 0.22 μ m filters and stored at \pm 8 °C prior to analysis. Controls were prepared under the same conditions.

2.2.2. Process overview

γ -irradiation dose: The effect of γ -irradiation dose over particle size changes was evaluated as a function of exposure to 2.5, 5, 7.5 and 10 kGy in presence and absence of 20% (v/v) ethanol as additive.

Scale up: The effect of papain concentration over the nanoparticle formation was evaluated in the range of 12.5–50 mg mL⁻¹ at 10 kGy.

2.2.3. Particle characterization

Particle size: Particle size measurements were performed by Dynamic Light Scattering analysis (DLS) on a Zetasizer Nano SZ90 device at 20 °C and 90° scattering angle in triplicates of 3 runs of 12 s each.

Bityrosine evaluation: The samples were properly diluted in buffer to reach equivalent absorbance at $\lambda=280$ nm using a Cary 1E UV-vis Varian[®] spectrophotometer. The samples were then checked for bityrosine emission on a F4500 Hitachi[®] Fluorescence spectrophotometer using $\lambda_{Ex}=325$ nm $\lambda_{Em}=340$ –500 nm, scan speed of 240 nm/min, E_{Ex} slit of 5 nm and E_{Em} slit of 10 nm.

3. Results and discussion

3.1. Technique overview

As previously established (Varca et al., 2014) gamma irradiation in presence of ethanol was capable of modifying papain particle size with smaller effects over bioactivity if compared to sodium chloride at 10 kGy. Best results were achieved at 20% ethanol concentrations and the computational evaluation of papain revealed higher accessibility values for the tyrosine residues, if compared to the other residues preferentially targeted by radiation (Saha et al., 1995). However questions regarding optimization of the process, by means of irradiation dose and scale up properties, involving higher papain concentration, as well as the experimental evidence of the bityrosine formation remained unclear.

3.1.1. Irradiation dose

In order to track down the effect of γ -irradiation over papain particle size increment and allow a proper selection of optimum irradiation dose for the synthesis of the nanoparticles the samples were submitted to distinct irradiation doses (Fig. 1). As reported in literature, the effects of irradiation exposure in proteins preferably led to chain scission and degradation effects (Furuta, 2002; Saha et al., 1995) and thus, the dose range evaluated did not exceed 10 kGy to preserve bioactivity and minimize protein degradation.

In absence of ethanol minor changes were observed as a function of dose ranging from 0 to 10 kGy by means of particle size as shown in Fig. 1. This profile was distinct in presence of ethanol (20% v/v), where papain did undergo changes at 2.5 kGy irradiation and similar effects were also observed up to 10 kGy, whereas minimum changes were registered among the doses ranging from 2.5 to 7.5 kGy. Based on these results optimized conditions for the nanoparticles synthesis were achieved at irradiation dose of 10 kGy. Similar results were observed in the case of BSA (Soto Espinoza et al., 2012), where albumin nanoparticles presented the highest particle size increase at 10 kGy.

The bityrosine evaluation of the samples is described in Fig. 2. The irradiation led to an increase in bityrosine formation in both cases. Particularly in absence of ethanol (Fig. 2A), the bityrosine linkages increased as the irradiation dose increased. The same profile was observed in presence of ethanol, but in this case (Fig. 2B) the increase was smaller and corroborated the DLS measurements (Fig. 1), where minimum changes took place between 2.5 and 7.5 kGy and more pronounced changes at 10 kGy. These data highlighted the optimized dose established to be 10 kGy, and experimentally supported the

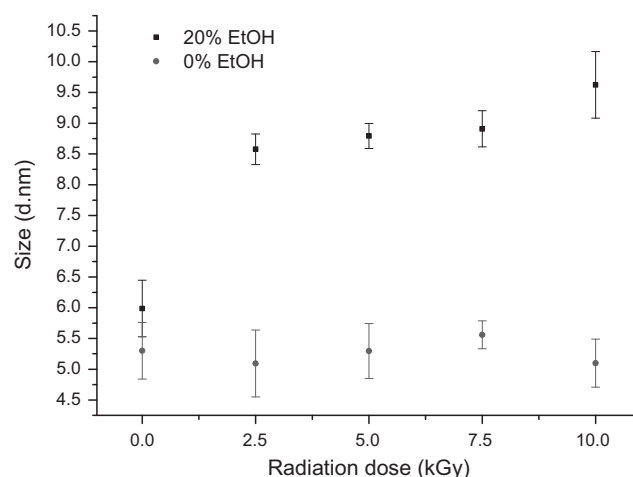


Fig. 1. Papain particle size increment as a function of irradiation dose in presence (20% v/v) and absence of ethanol.

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