Radiation Physics and Chemistry 105 (2014) 48–52

Contents lists available at ScienceDirect

Radiation Physics and Chemistry

journal homepage: www.elsevier.com/locate/radphyschem

Radiation synthesized protein-based nanoparticles: A technique overview

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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.

ARTICLE INFO

Article history:
Received 31 January 2014
Accepted 11 May 2014
Available online 23 May 2014

Keywords:
Protein-based nanoparticle
Papain
Ionizing radiation
Protease
Protein-crosslinking

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 pharmaceutics 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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http://dx.doi.org/10.1016/j.radphyschem.2014.05.020
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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 Nα-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 ^{60}Co as radioactive source in a gamma cell 220 irradiator. The samples were properly filtered using 0.22 μm filters and stored at ±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 λ=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 λ_{Ex}=325 nm λ_{Em}=340–500 nm, scan speed of 240 nm/min, $_{Ex}$slit of 5 nm and $_{Em}$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 acessibility 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

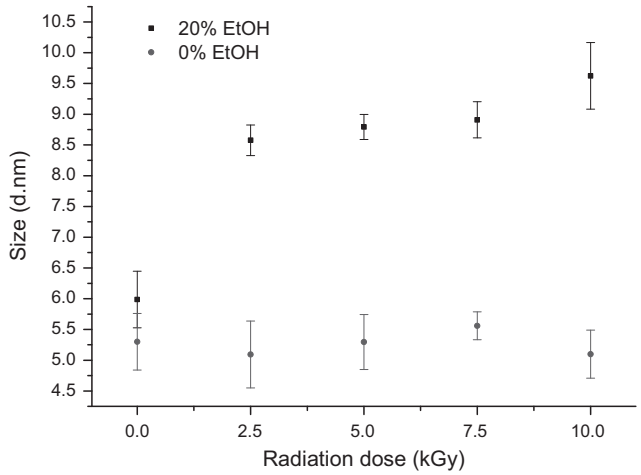

Fig. 1. Papain particle size increment as a function of irradiation dose in presence (20% v/v) and absence of ethanol.

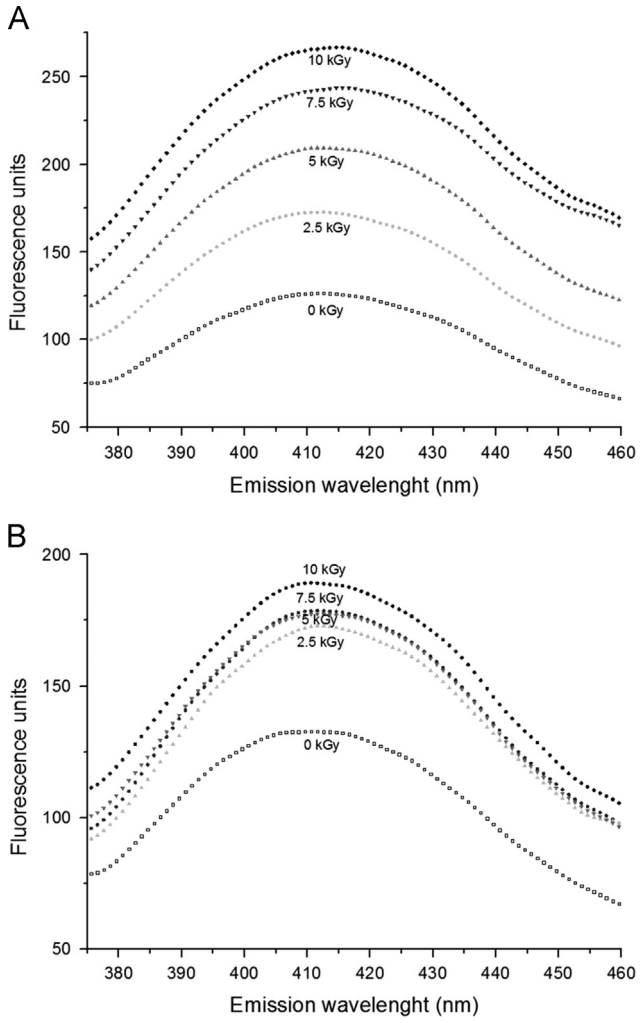

Fig. 2. Bityrosine monitoring in papain (12.5 mg mL^{-1}) as a function of irradiation dose in absence (a) and presence (b) of ethanol.

proposed mechanism, attributed to the protein crosslinking by means of bityrosine formation.

3.1.2. *Concentration effect*

As an attempt to understand to what extent the applications of the protein-based nanoparticles could be applied to, scale up experiments involving distinct papain concentrations – 12.5, 20 and 50 mg mL^{-1} – irradiated at the optimized dose of 10 kGy were performed as presented in Fig. 3.

Under the assayed conditions the particles ranged from 5 to 13 nm with minimum deviation for all concentrations assayed. These results revealed that the technique was effective at broad concentration range, from 12.5 to 50 mg mL^{-1}, considering that similar particle size and size distribution profiles, as a function of ethanol concentration, were obtained for the samples (Fig. 3). This evidenced a minor influence of the total protein concentration over particle size increment caused by the process.

To some extent, similar profiles were observed for the samples in presence of ethanol without irradiation. However, as reported in our previous work (Varca et al., 2014) dilution tends to invert the process. Such fact did not take place when the samples were irradiated, supporting the occurrence of novel linkages and protein crosslinking as a consequence.

It is relevant to mention that some deviation ($\pm$ 1.5–2 nm) has been observed from our previous results (Varca et al., 2014).

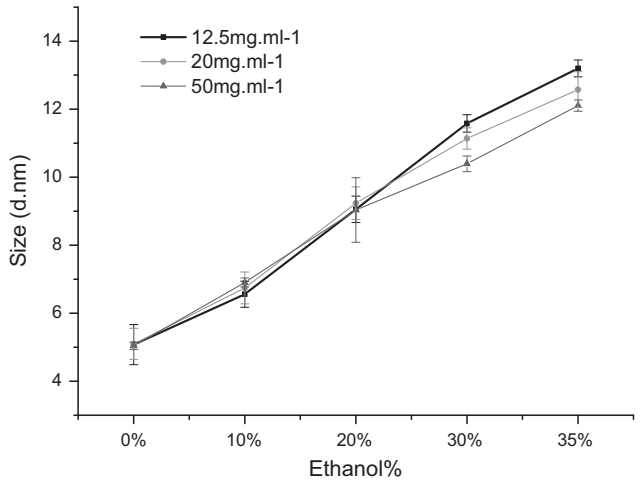

Fig. 3. Scale up experiments involving different papain (12.5, 20 and 50 mg mL^{-1}) and ethanol (0–35% v/v) concentrations irradiated at 10 kGy.

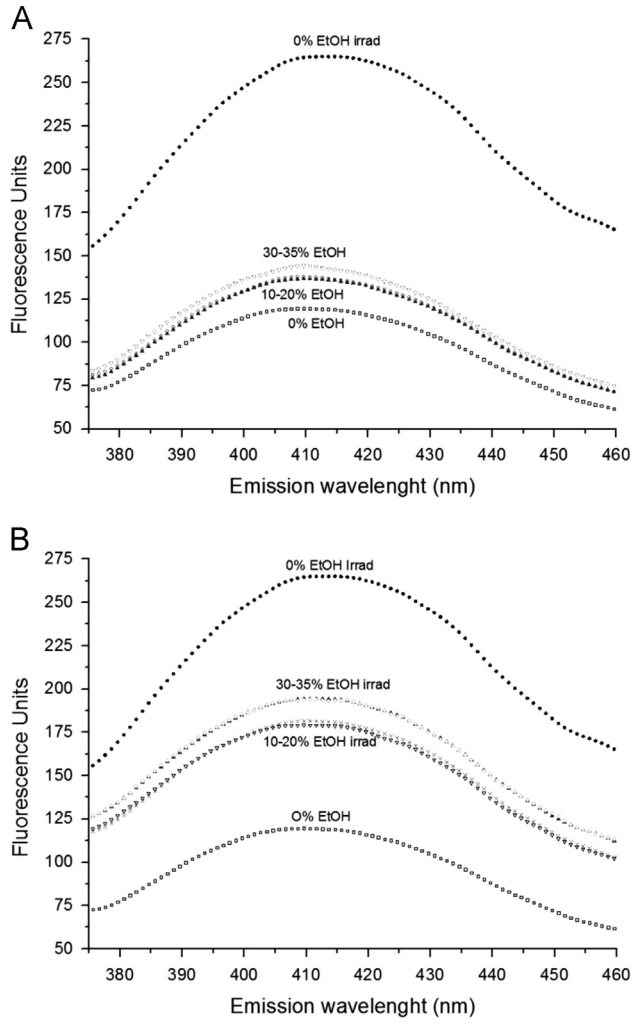

Fig. 4. Bityrosine monitoring in papain (12.5 mg mL^{-1}) non-irradiated (a) and irradiated (b) at 10 kGy under different ethanol concentrations (0–35%).

We support such changes considering that at this time the flaks were completely filled in order to minimize the effects of oxygen (Davies, 1987; Saha et al., 1995). In this case, a little shift was observed in terms of particle size and also a better size distribution was achieved up to 35% ethanol concentration, with a decrease in

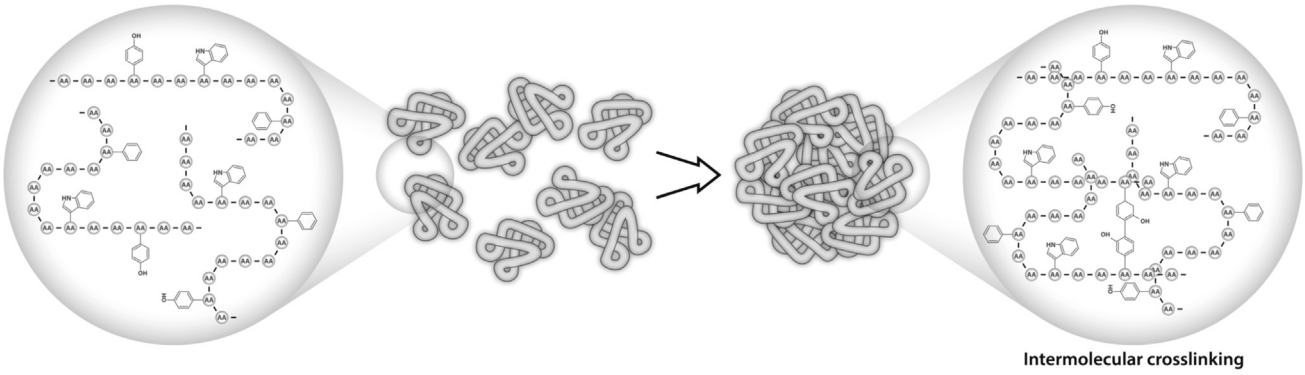

Fig. 5. Representative scheme of the proposed mechanism involved in the papain nanoparticle formation induced by irradiation.

the formation of aggregates. However, more experiments shall be conducted in order to establish the role of the atmospheric conditions and oxygen itself over the irradiation process.

In absence of irradiation, the presence of ethanol led to a minimum increase in bityrosine fluorescence units (Fig. 4A). These changes were attributed to microenvironmental changes in the solvent which holds some effects over protein and might lead to distinct values.

At defined irradiation dose of 10 kGy Fig. 4B, the samples containing ethanol presented considerably higher bityrosine intensity if compared to non-irradiated papain indicating an increase in the formation of such linkages if compared to the native form of the enzyme. At 30–35% ethanol, the values were a little bit higher if compared to lower ethanol concentrations (10–20%). The same profiles were observed for samples prepared at higher papain concentrations 20 and 50 mg mL^{-1} (data not shown).

The interaction of ethanol over proteins is well established. This solvent is widely applied as protein precipitant agent, which is known to induce changes on protein structure and denaturation as well, depending upon ethanol concentration, the protein itself and experimental conditions (Yoshikawa et al., 2012).

Regarding the radiation induced nanoparticle, the role of ethanol in the process was evidenced by comparing irradiated papain in absence and presence of ethanol. This compound created a specific environment suitable for the particle size increase, which induced the formation of bityrosine in a controlled manner, possibly related crosslinks of intermolecular nature, which was not achieved otherwise. To experimentally support such information (Figs. 1A and 4A), irradiation in absence of ethanol led to higher levels of bityrosine crosslinks with no relevant changes in particle size whatsoever, and thus indicated that the formation of such linkages, in this case, was at intramolecular level.

In addition, the bityrosine experiments provide clear evidence that the mechanism involved is a result of building of novel tyrosine linkages, possibly of intermolecular nature, and they are sufficient to demonstrate a direct relation between the linkages and the particle formation itself. Whatsoever, more experiments shall be performed in order to clarify the nature of such linkages.

3.1.3. Mechanism approach

The results supported the proposed mechanism involved in the nanoparticle formation induced by irradiation (Fig. 5) and revealed that it occurred in a similar way of the so called protein crosslinking (Baylei, 1991; Matheis and Whitaker, 1987) and the changes caused by UV exposition (Garcia-Castinaras et al., 1978), where the main mechanism involves changes – linkages at intermolecular level – in tyrosine residues.

In the case of gamma irradiation, the radiation induced formation of bityrosine occurs as a result of the interaction between the tyrosine residues, if properly accessible, and the species produced as a result of the water radiolysis. Even though the dimerization of tyrosine residues by irradiation is not yet established (Saha et al., 1995), there is evidence to support the crosslinking formation by C–O–C bonds induced by interaction with OH-radical (Karam et al., 1984, Saha et al., 1995) and in parts by the generated tyrosine phenoxyl radicals (Casas-Finet et al., 1984).

4. Conclusions

The radiation-induced synthesis of papain nanoparticles under optimized conditions led to the formation of protein based nanoparticles with size ranging from 5 to 13 nm depending upon ethanol concentration and irradiation dose. The optimized irradiation dose was confirmed at 10 kGy and scale up experiments revealed that protein concentration 12.5 -50 mg mL^{-1}) did not hold a major influence in the particle formation. Therefore, these results indicated that the radiation induced technique may be carried out in a wide protein concentration range with minimum size variation.

Bityrosine changes as a function of the process were observed and provided experimental evidence that such linkages were directly related to the particle formation process. This effect was attributed to irradiation in specific conditions which led to a controlled formation of bityrosine, possibly of intermolecular nature, which is not achieved otherwise. Ethanol was capable of promoting this microenvironment suitable for the nanoparticles synthesis. Possible applications of the technique concern the development of protein-based nanostructured bioactive nanoparticles and/or drug carriers for distinct biomedical applications.

Acknowledgments

The authors would like to thank Laboratório de Biofísica (IF – Universidade de São Paulo.), Fundação de Amparo a Pesquisa do Estado de São Paulo – FAPESP (Process number 2010/10935-9) for a scholarship and the Nuclear Atomic Energy Agency – IAEA for financial contribution. M.G. is member of CONICET.

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