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The effect of γ-irradiation on PLGA/PEG microspheres containing ovalbumin

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Abstract

Poly(ethylene glycol) (PEG) and sodium chloride (NaCl) are excipients used in PLGA microsphere preparation to stabilize proteins and reduce their burst release. No information is till now available in the literature on the effect due to the use of such excipients on the biopharmaceutical performance of γ-irradiated microparticulate systems. On this purpose, different batches of microspheres containing ovalbumin (OVA) were prepared by using a PLGA 50:50 (average Mr: 13000), different amounts of PEG (Mr: 400 or 4000) and/or sodium chloride. The non-irradiated and irradiated microspheres were characterized in terms of morphology (SEM, particle size distribution), OVA and PEG content and in vitro OVA release. Radiolysis mechanisms of OVA and OVA loaded microspheres were investigated by EPR analysis.

Gamma irradiation affects either microsphere morphology or the release of OVA as a function of the amount of PEG, and the use of NaCl. Irradiation significantly reduces release rate of protein from the microspheres containing 15% and 30% of PEG and from controls (microspheres without additives), while no significative effect on protein release rate is highlighted on microspheres containing lower amounts of PEG. EPR investigation shows that increasing amounts of PEG up to 30% have a perturbation effect on OVA radiolysis path.

Keywords: Microspheres; Poly(lactide-co-glycolide); Poly(ethylene glycol); Ovalbumin; Biodegradable polymers; γ-irradiation

1. Introduction

Owing to the development of recombinant DNA technology, a large variety of protein drugs such as hormones, growth factors and vaccines have become commercially available to therapeutic purposes [1]. Proteins are typically administered by injection or infusion because of their poor oral bioavailability. Generally, they have short plasma half-lives and are incapable of diffusing through biological membranes. Many proteins currently being developed are aimed at

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curing chronic conditions where therapy may be required over months or years. Administration by frequent injections to keep the protein drug at effective concentrations is tedious, expensive and has poor patient compliance.

Encapsulation of proteins into microspheres made of biodegradable polymers, e.g. polylactide-co-glicolides (PLGA), offers an opportunity for protein sustained release injections [2]. Such microspheres are mainly produced by double (w/o/w) emulsion solvent evaporation/extraction methods. In these cases, the main problems related to the encapsulation of proteins in PLGA are the formation of aggregates, due to the denaturing conditions rising during microspheres double emulsion preparation processes, and the initial burst release. The literature reports several studies concerning the use of excipients to stabilize proteins and reduce their burst release. Among these excipients PEG and sodium chloride were proposed by several authors [3–9]. PEGs are usually added to the internal aqueous phase to limit the penetration of protein in the interfacial film of the primary w/o emulsion. Consequently, they stabilize the protein by reducing the contact with the organic phase [5]. Sodium chloride, added to the external aqueous phase to generate an osmotic pressure, cause a denser internal structure of the microspheres which reduces the burst release [10].

As the PLGA are thermosensitive materials, the final sterilization of microspheres is performed by means ionising radiations [11]. Ionisation events activate in the microspheres numerous chemical reactions which cause different effect on drug release, depending on the active ingredient used [12–18]. As an example, in the case of insulin-like growth factor-I (rhIGF-I), γ-irradiation causes rhIGF-I aggregation and an increased burst effect in the in vitro drug release experiments [17].

The effects of ionising radiation on PLGA and PLGA microspheres containing different active ingredients as well as the addition of PEGs to PLGA microspheres containing proteins are widely investigated [11–18,4,5,7–9]. However, no information is till now available in the literature on the effect due to the use of such excipients on the biopharmaceutical performance of γ-irradiated microparticulate systems. On this purpose, different batches of microspheres containing ovalbumin (OVA) were prepared by using PLGA 50:50, different amounts of PEGs and/or sodium chloride. The microspheres were γ-irradiated at a dose of 25 kGy [19]. Microsphere characterisation in terms of size, surface morphology, encapsulation efficiency, in vitro release of encapsulated ovalbumin has been performed before and after irradiation. Moreover, the intermediate radicals in the radiolytic process were investigated by matrix EPR spectroscopy after gamma irradiation at 77 K followed by gradual thermal annealing at room temperature. The interpretation of the spectra afforded information on the nature and reactions of the primary species and the interactions of the components in the irradiated microspheres.

2. Materials and methods

2.1. Materials

Poly(lactide-co-glycolide) (PLGA) Medi-sorb®, grade 50:50 DL-2A, inherent viscosity 0.16 dl/g, 13,000 Da Mw, T_g 39.9 °C, was from Alkermes Medisorb® Polymer (Wilmington Ohio, USA).

Sodium chloride (NaCl) was supplied by Carlo Erba (Milan, Italy), polyethylene glycols (PEG) (Mw 400 and 4000 Da), polyvinyl alcohol (PVA) (87–89% hydrolyzed, Mw of 85,000–146,000 Da) and ovalbumin (OVA) (Mw 44,287 Da, p*I* 4.63) were obtained from Sigma-Aldrich (Milan, Italy).

Unless specified, all other compounds were of analytical grade.

2.2. Microsphere preparation

All microspheres were prepared using a double emulsion solvent evaporation method, ovalbumin was chosen as the model protein for these studies because of its good water solubility, its high molecular weight and its thermal stability. Placebo batches were prepared for each composition of protein loaded microspheres (PL_1–PL_6).

An aqueous dispersion (phase W_1) of the protein (55 mg/ml) was dropped into 12 g of the organic solution containing the polymer (10% w/w) dissolved in methylene chloride (phase O).

Emulsification was performed at 9500 rpm with a homogenizer (Ultraturrax model T25 S25NI8G), the system was ice-cooled during the process. This first W_1/O emulsion was dispersed, under mechanical stir-

ring in a second aqueous phase (240 ml, W_2) containing 1% w/w PVA to form the double emulsion $W_1/O/W_2$. This emulsification step was performed by a Vibromixer apparatus (Vibromixer EI Chemapa-AG, Volketswil, CU) at 70 oscillations/s. Solvent evaporation of the double emulsion obtained, was performed at 37 °C under mechanical stirring. Solid microspheres were recovered through centrifugation, washed with distilled water, filtered through 0.8 μm Millipore membrane, and dried overnight under vacuum.

When PEG 400 was used as excipient, 500 μl of this polymer were added to the internal aqueous phase; when PEG 4000 was used, it was mixed to PLGA (PEG/PLGA ratio 1:40) and dissolved into the organic phase.

When NaCl was used, it was added to the external aqueous phase of the double emulsion at a 10% (w/v) concentration, this salt concentration represent the concentration commonly used to avoid leaking of protein in the external aqueous phase during microsphere preparation [20]. Table 1 reports the excipients used in the preparation of microsphere batches.

2.3. γ-irradiation

Placebo and OVA loaded microspheres were irradiated in air, at room temperature; a 25 kGy dose at 1 kGy/h dose rate was applied. ^{60}Co was used as irradiation source (Applied Nuclear Energy Laboratory (L.E.N.A.), University of Pavia). It was checked by thermometric control that sample temperature during the irradiation did not appreciably increase above room temperature. To evaluate the behaviour of PEG to γ-irradiation, samples of the polymer were irradiated at 25 kGy dose in the same conditions used for microsphere irradiation.

25 kGy is the minimum absorbed dose considered adequate for purpose of sterilizing pharmaceutical products without providing any biological validation [19]. This reason led us to consider these irradiation conditions that are the commonly used by the pharmaceutical industry.

Table 1
Additives used in the formulation of PLGA microspheres

Ms-batch	Excipient	Salt concentration in W_2 (w/v %)[a]
OV_1 (PL_1)	–	–
OV_2 (PL_2)	–	10
OV_3 (PL_3)	PEG 400[b]	–
OV_4 (PL_4)	PEG 400[b]	10
OV_5 (PL_5)	PEG 4000[c]	–
OV_6 (PL_6)	PEG 4000[c]	10

[a] Salt added to the PVA aqueous solution.
[b] Excipient added to the internal aqueous phase (W_1).
[c] Excipient co-dissolved with PLGA in CH_2Cl_2.

2.4. Nuclear magnetic resonance (NMR)

PEG determination was performed by NMR using a method from the literature [21]. Samples of at least 10 mg of microparticles were placed into glass NMR sample tubes with 1 ml of deuterated chloroformic solution (Sigma-Aldrich, Milano, Italy) containing 1% v/v tetramethylsilane. Samples of at least 10 mg of PEG non-irradiated and irradiated at 25 kGy, were prepared and analysed by NMR in the same conditions as done for microspheres.

The proton NMR spectra were acquired on a NMR spectrometer (Bruker AVANCE 400 spectrometer operating at 400 MHz, the temperature was regulated at 25 °C). ^1H NMR experimental parameters were as follows: an 8012.82 Hz spectra window, a 2.0444 s acquisition time, a 6 μs (90°) pulse width, 16 transients and 30 s pulse intervals. The hydrogen of the methine group of lactic acid unit of PLGA copolymer resonated at 5.2 ppm while those of methylene group of glycolic acid unit appeared at 4.8 ppm and the hydrogens of methylene group of PEG homopolymer appeared at 3.6 ppm. The areas under the peaks were integrated to determine the blend composition. All spectra were performed in triplicate to estimate relative standard deviation (r.s.d.) of peak area. All data were processed using XWIN-NMR 3.1 software.

2.5. Morphological characterization of microspheres

Microparticle size and morphology were evaluated before and after irradiation. The microsphere shape and surface were analysed by Scanning Electron Microscopy (SEM) (Jeol, Cx, Temcan, Jed, Tokyo, Japan). The samples were sputtered with an Au/Pd coating in argon atmosphere. The coating procedure was repeated three times.

To reveal the internal morphology, samples of microparticles were frozen (liquid nitrogen — 196 °C),

fractured and the fractured surface was imaged by SEM.

Granulometric analyses were performed by a conductive method using a Coulter Counter Apparatus (Coulter® Multisizer II, Coulter Electronics, Luton Beds, England). The orifice of apparatus tube used was 140 μm. Microspheres were suspended in a saline solution, the analyses were performed in a size range between 2 and 90 μm. Six analyses were performed for each sample. Results are expressed as $d_{50\%}$ and $d_{90\%}$, corresponding to the undersize cumulative diameters of 50% and 90% of microspheres, respectively.

2.6. Total protein content determination and "in vitro" dissolution test

Protein content was determined after microspheres digestion through alkaline sodium dodecil sulphate (SDS) solution.

The protein was determined with Bicinchoninic Acid Assay (BCA protein assay reagent kit), by colorimetric analysis at 562 nm with a Beckman DU 7500 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). Six replicates of all samples and standards were assayed.

Encapsulation efficiency was expressed as the percentage of protein entrapped compared to theoretical drug content.

Theoretical drug content was defined as the ratio between the amount of protein added to the formulation over the total amount of polymer plus protein, while actual loading was ratio between the amount of entrapped protein as determined by BCA assay and the microsphere batch mass.

"In vitro" protein release from microspheres, before and after irradiation, was evaluated by an "in vitro" dissolution test.

Amounts of 20 mg of microspheres were dispersed in 2 ml PBS (pH 7.4) containing 0.15% SDS and 0.01% NaN_3, the suspensions were kept at 37 °C and stirred moderately. At predetermined time intervals, 100 μl of medium from each sample were removed and replaced with fresh PBS buffer. The amount of protein released was determined spectrophotometrically at 562 nm by Bicinchoninic Acid Assay (BCA). Analyses were carried out on six samples for each batch of microspheres.

2.7. EPR analysis

EPR analyses were performed on OVA, placebo microspheres and OVA loaded microspheres.

Irradiation was performed at the Applied Nuclear energy Laboratory (L.E.N.A., Pavia University, Italy) by using a ^{60}Co gamma source calibrated against alanine and Fricke dosimeters. About 50 mg of sample were sealed under high vacuum in EPR quartz tubes and irradiated at 77 K (liquid nitrogen) at dose rate of 1 kGy/h, total dose 25 kGy.

Before irradiation the sample tubes were flamed by using the sliding technique in order to eliminate the radiation induced quartz paramagnetic centres. During this operation, the sample temperature was not allowed to rise above 77 K.

EPR analysis was performed by using Varian X-band E_4, E_8 and a Brucker EMS 104 spectrometers equipped with a Stelar data acquisition and temperature control apparatus.

The EPR spectra were analysed by computer simulation using the Hamiltonian including the Zeeman electronic and nuclear terms and the hyperfine terms with isotropic and anisotropic components for the g and hyperfine tensors:

$$H = -\beta \cdot \vec{S} \cdot g \cdot \vec{H} + \sum_i \vec{S} \cdot A_i \cdot \vec{I}_i - \sum_i \vec{I}_i \cdot \vec{H}$$

The EPR spectra were recorded at first at 77 K, in order to identify the primary species, and subsequently after stepwise increases of temperature above 77 K up to room temperature.

The annealing procedure was intended to obtain suitable conditions for the reactions of primary species and for the identification of the secondary species.

3. Results and discussion

3.1. Nuclear magnetic resonance

NMR studies permitted to determine the amounts of PEG loaded in the microspheres. Results are shown in Table 2 where it is highlighted that the amount (mg) of PEG incorporated into the final formulations depended on PEG molecular weight. Microparticle formulations

Table 2
Amounts of PEG added and loaded in the microspheres

Batch	PEG added (mg)	Before irradiation		After irradiation	
		PEG loaded (mg)	PEG loaded* (%)	PEG loaded (mg)	PEG loaded* (%)
OV_3	563.4	4.363 ± 0.32	0.363 ± 0.05	n.d	n.d.
OV_4	563.4	0.483 ± 0.07	0.048 ± 0.01	n.d.	n.d.
OV_5	30	9.218 ± 0.73	30.726 ± 0.12	8.77 ± 0.43	29.23 ± 0.23
OV_6	30	4.317 ± 1.05	14.372 ± 0.15	3.59 ± 0.16	11.98 ± 0.18

* Weight (mg) of PEG loaded into microspheres divided by the initial weight (mg) of PEG added during microsphere preparation.

OV_5 and OV_6, prepared by adding PEG with molecular weight of 4000 Da in the organic phase, contained 9.218 and 4.317 mg of PEG. It is interesting to notice how the addition of PEG 400 (OV_3, OV_4) leads to lower amounts of polymer resident in the formulations. Thus, the higher the molecular weight, the higher the percentage of PEG incorporated in the microspheres: this behaviour is consistent also with the higher hydrophilicity of PEG 400 with respect to PEG 4000, that favours its mobility towards the aqueous phase. NMR data reported in Table 2, did not show significative changes of the amount of PEG loaded in the microspheres after irradiation. Fig. 1 reports, as an example, the NMR spectra of PEG 4000, these data permit some considerations about the polymer structure before and after irradiation. NMR spectrum of PEG before irradiation shows typical signals of PEG at 3.6 ppm (–CH_2–CH_2–). After irradiation (Fig. 1b) the peak at 3.6 ppm, related to –CH_2–CH_2– is modified in shape and intensity, the peak between 3.72–3.78 ppm increases, and new peaks at about 4.2–4.4 ppm appear. Moreover, the area of the peak at 2.2 ppm, related to alcoholic –OH, increases, and small signals related to oxidative products, at about 8 ppm, are highlighted. All these phenomena are ascribed to polymer fragmentation. From the ratio between the integrated peak areas it can be calculated that fragmentation due to irradiation is about 2%. This value suggests that PEG do not significantly change after irradiation.

3.2. Morphologic characterization

Microsphere characterization, both morphologic and functional is an important feature of microsphere technology. These two aspects are strictly connected: in fact the morphology of microspheres can vary depending on their composition and/or preparation method, and it can affect drug release behaviour from microspheres. Moreover, when microspheres are intended for parenteral use, their size must be in a well stated range, according to the administration route e.g. intramuscular or intraarterial (chemioembolization).

SEM analyses revealed that microspheres have spherical shape. The addition of excipients can lead to modifications of microsphere morphology. The

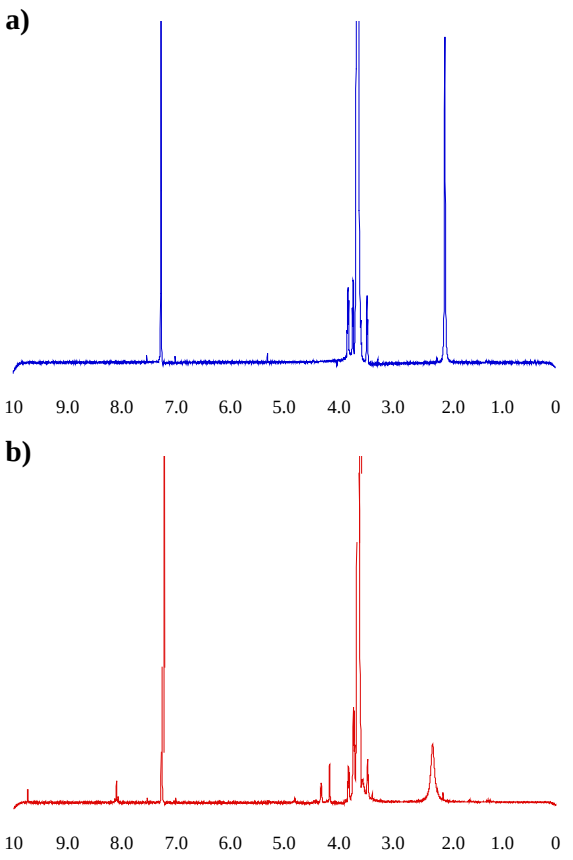


Fig. 1. NMR spectra of PEG 4000: (a) before and (b) after irradiation.

presence of PEG (OV_3 and OV_5) in the polymer matrix, independently of its amount and molecular weight, results in microspheres with very porous structure, pores are evident on microspheres surface and inside the polymeric matrix (Fig. 2a, b, c).

Simultaneous addition of PEG and NaCl in the preparation process permitted to obtain microspheres with a smooth, non-porous surface and compact internal structure (Fig. 2d): the same effect was obtained when only NaCl was used during the preparation process (batch OV_2, Fig. 2e). What observed by SEM highlights that the presence of NaCl in the external aqueous phase generates microsphere with a dense structure (Fig. 2f).

Irradiation always led to microsphere aggregation. This phenomenon is evident for batch OV_6 (Fig. 3a) where incipient fusion is detected. Moreover, when microspheres have porous structure, they broke up very easily upon irradiation (Fig. 3b). Same behaviour was highlighted for placebo microspheres (images not shown).

Particle size distribution of microsphere was in the 15–50 μm range (Table 3). PEG addition in the formulations (OV_3–OV_6) led to obtain larger micro-

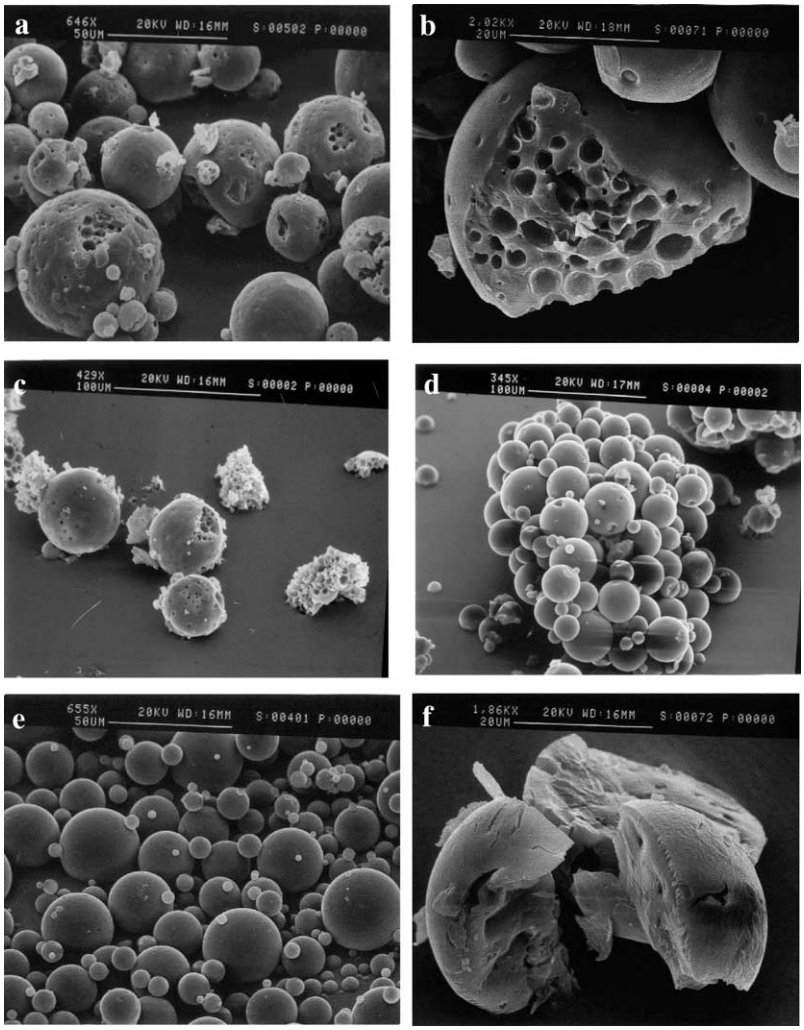


Fig. 2. Scanning electron microscopy of OVA loaded microparticles before irradiation: (a) batch OV_3, (b) batch OV_5 internal structure, (c) batch OV_5, (d) batch OV_6, (e) batch OV_2, (f) batch OV_2 internal structure.

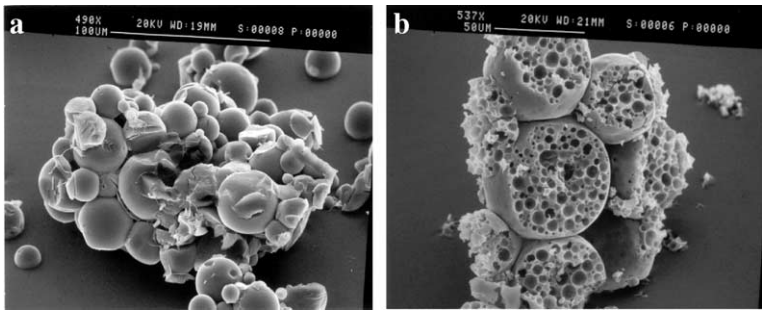


Fig. 3. Scanning electron microscopy of OVA loaded microparticles after irradiation at 25 kGy: (a) batch OV_6 and (b) batch OV_5.

spheres, this could be explained by an effect of water retention caused by PEG, that involves an increase in hydration of the polymer matrix. A change in particles size distribution was evidenced after irradiation at 25 kGy; this can be ascribed to modifications of microsphere structure as shown by SEM.

3.3. Protein content determination and "*in vitro*" release studies

Protein encapsulation efficiencies resulted to be among $88.27 \pm 0.44\%$ and $79.03 \pm 0.24\%$. These values did not vary significantly after irradiation, while a change in encapsulation efficiency was noticed depending on the presence of additives.

The highest value of encapsulation efficiency has been found in batch OV_2. This phenomenon find explanation the preparation conditions that involved addition of salt into aqueous phase W_2 with subsequent increase of the osmotic pressure in the external phase and emulsion stabilization through reduction of the osmotic pressure difference across the organic phase [20]. Addition of PEG during emulsification always reduced the encapsulation efficiency of OVA, this effect might be ascribed either to the increased microsphere porosity shown in SEM images for batches OV_3 and OV_5 or to the hydrophilicity of PEG that increases the time necessary for microsphere precipitation during the preparation process. In this way the prolonged contact of protein with the water phase enhances the chance for protein to leak out.

Table 3
Particle size distribution of ovalbumin loaded microspheres before and after irradiation at 25 kGy

Batches	Size (μm) before irradiation		Size (μm) after irradiation	
	$d_{50\%}$	$d_{90\%}$	$d_{50\%}$	$d_{90\%}$
OV_1	13.90 ± 0.10	32.34 ± 0.08	15.45 ± 0.12	45.22 ± 0.05
OV_2	18.68 ± 0.15	29.70 ± 0.12	21.23 ± 0.10	30.90 ± 0.10
OV_3	25.12 ± 0.13	42.74 ± 0.06	26.53 ± 0.08	46.92 ± 0.13
OV_4	21.32 ± 0.10	32.69 ± 0.13	22.39 ± 0.12	33.22 ± 0.14
OV_5	38.48 ± 0.12	69.26 ± 0.12	30.29 ± 0.10	58.85 ± 0.12
OV_6	29.03 ± 0.10	56.02 ± 0.12	29.46 ± 0.12	48.03 ± 0.10

In vitro release studies were performed over 160 h. Protein release behaviour can be differentiated in two phases, a burst release defined as the percentage of protein released within the first 6 h, and a prolonged release upon 160 h. Fig. 4 shows the dissolution profiles of OVA from the microspheres, before and after γ-irradiation. OVA release from the control microspheres was almost completed, about 94% of protein released, after 6 h of incubation. The use of NaCl in the microencapsulation process significantly modified protein release profile leading to 10% of OVA released in the first 6 h and 50% after 7 days (OV_2).

Batches OV_3 and OV_4 show an effective control of OVA released either in the first burst effect or over 160 h. This effect is more evident in batch OV_4 obtained through concomitant addition of PEG 400 and NaCl: 6% of the protein was released in the first 24 h and 50% after 4 weeks. This can be related to the compact structure of the polymeric matrix obtained by addition of PEG 400 and NaCl that reduces OVA diffusion rate. PEG 4000, as shown by dissolution profiles of batches OV_5 and OV_6, was ineffective in controlling protein release rate. The different release patterns achieved in the presence of PEGs can be explained by the higher amounts of PEG 4000 contained in the microspheres that

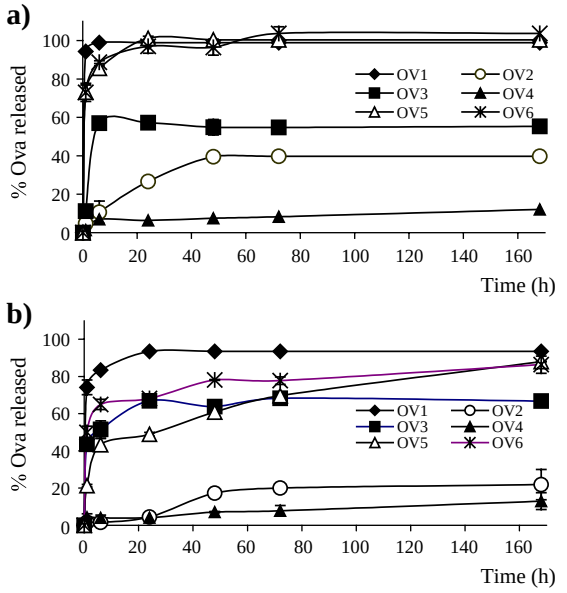


Fig. 4. In vitro release profiles of OVA loaded microspheres: (a) before irradiation, (b) after irradiation.

make the polymeric matrix more hydrophilic, promoting protein release. γ-irradiation induces a decrease in OVA release rate from the microspheres with the exception of batch OV_3 containing PEG 400.

The effects of γ-irradiation evidenced on the release rate of ovalbumin from the microspheres should be discussed taking into accounts parameters related both to the physical and chemical structure of microspheres. From the physical stand point γ-irradiation affects microsphere morphology, as shown by SEM. This reflects in unpredictable changes in protein release rate from the drug delivery system that can depends on the microsphere aggregation or rupture observed.

The effect of γ-irradiation on PLGA structure has been previously investigated and reported in the literature [22–24]. Upon irradiation at 25 kGy the polymer undergoes a decrease in its molecular weight [22]. The chain random scission of PLGA can generate an increase of the number of carboxylic polymer's end groups that interact, by ionic interaction, with the positively charged amine groups of the protein [25]. This factor contributes to the reduction of protein release rate that has been observed frequently in the microspheres after irradiation.

3.4. EPR investigation

3.4.1. EPR analysis of the raw materials

The vacuum low temperature gamma irradiation of neat ovalbumin followed by annealing at room temperature afforded an EPR spectrum consisting of two component: a nearly isotropic doublet of 17.8 G and g 2.003 and a sulphur radical pattern with a rhombic g tensor and a single proton splitting of 7.0 G (Fig. 5)

$$g_{zz} = 2.0580 \quad g_{yy} = 2.0269 \quad g_{gxx} \cong 2.00 \quad ;$$

$$A(\mathrm{H})_{\mathrm{av}} = 7.0\,\mathrm{G}$$

The doublet is commonly observed at room temperature in irradiated proteins and it is generally accepted that it should be assigned to the glycine radical [26]:

–CH–C(═O)–NH–

The sulphur pattern, which is also obtained from solid state irradiated low molecular weight thiols, sul-

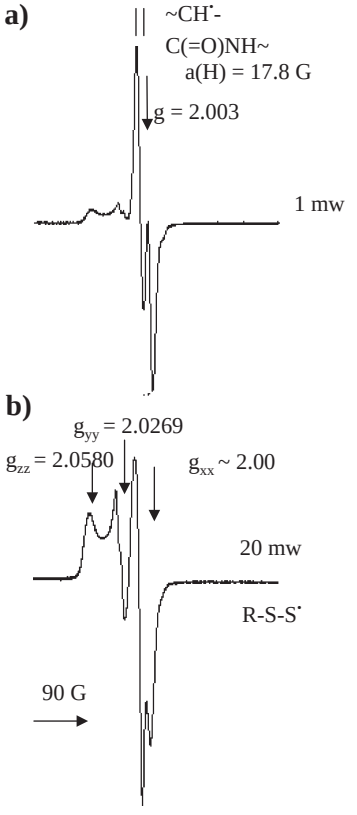


Fig. 5. EPR spectra of solid ovalbumin: (a) recorded at 113 K after vacuum γ-irradiation at 77 K and prolonged annealing at room temperature, (b) recorded at higher microwave power.

phides and disulphides, is assigned to perthiyl radicals $R–CH_2S–S^{\bullet}$, whose identification was recently obtained through EPR investigation of the molecular dynamics of the sulphur pattern within the channels of a thiourea clathrate [27]. As shown in Fig. 6b, the spectrum recorded at higher microwave power affords better evidence of the signal due to perthiyl radical.

The free radical products of the solid state radiolysis of PEG (data not shown) resembled closely those identified in the irradiated high molecular weight analogues [28]. The low temperature pattern was reckoned with C–H and C–C bond scissions with formation of the radicals $–OCH_2^{\bullet}$ and $–CH^{\bullet}–O–$; the post irradiation annealing at room temperature caused the gradual decay of the initial species and their

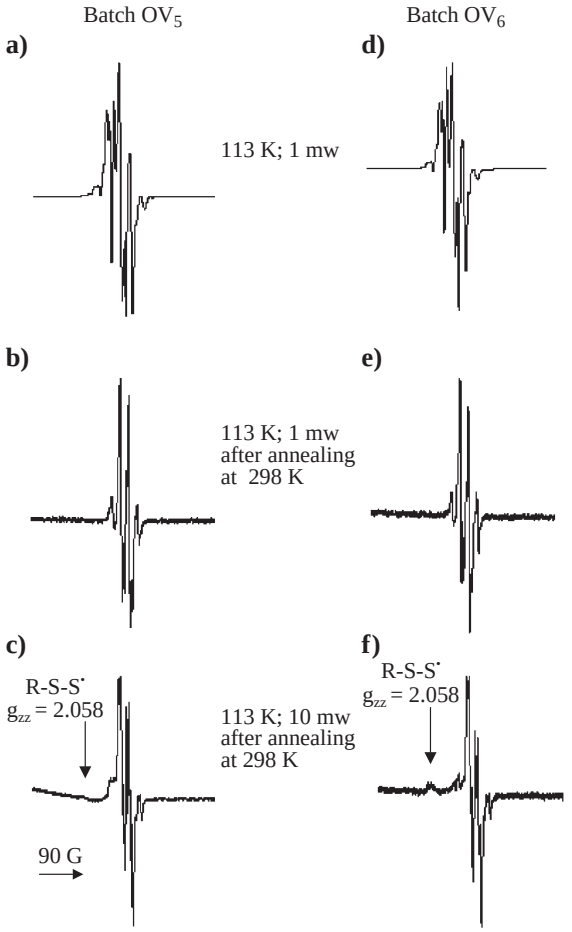


Fig. 6. EPR spectra obtained from ovalbumin loaded PLGA/PEG microspheres at two different PEG contents (batches OV_5, OV_6).

replacement with a doublet of 15 G centred at $g=2.005$; this latter signal is characteristic of the aldehydic radical [28]:

$$–CH^{\bullet} – CHO$$

The low field shift of the g value in the aldehydic radical is a distinctive feature which is a consequence of the delocalisation of the spin on the carbonyl oxygen.The formation of this species is reckoned with the cage reaction between neighbouring aldehyde-radical couples according to the mechanism:

$$\begin{aligned} &–CH_2CH^{\bullet} – O – CH_2CH_2O – \\ &\quad \rightarrow – CH_2CHO \cdots\cdots {}^{\bullet}CH_2CH_2O– \\ &\quad \rightarrow CH^{\bullet} – CH{=}{=}O + CH_3CH_2O– \end{aligned}$$

The aldehydic radicals are stable for months at room temperature under vacuum.

As previously assessed [15,22,29] the low temperature species from PLGA were diagnostic of C–H splittings both at the tertiary and secondary C–H bonds as well as C–C splitting leading to the species:

$$–CH^{\bullet}(CH_3), –CH_2^{\bullet}, –CH^{\bullet}–, –C^{\bullet}(CH_3)–$$

The post irradiation annealing under vacuum at room temperature caused the decay of the chain scission radicals and their replacement with the H abstraction radicals according to the scheme shown below, the tertiary radical being the most abundant species.

$$–\dot{C}H_2, –\dot{C}H(CH_3) \xrightarrow[298\ K]{PLGA} –\dot{C}H–, –\dot{C}(CH_3)–$$

The EPR spectra recorded after prolonged annealing at room temperature showed the presence of two quartets 1:3:3:1 characterized by different splitting which were proposed to arise from two different type of tertiary radicals $–C^{\bullet}(CH_3)–$ [22].

3.4.2. EPR analysis of microspheres

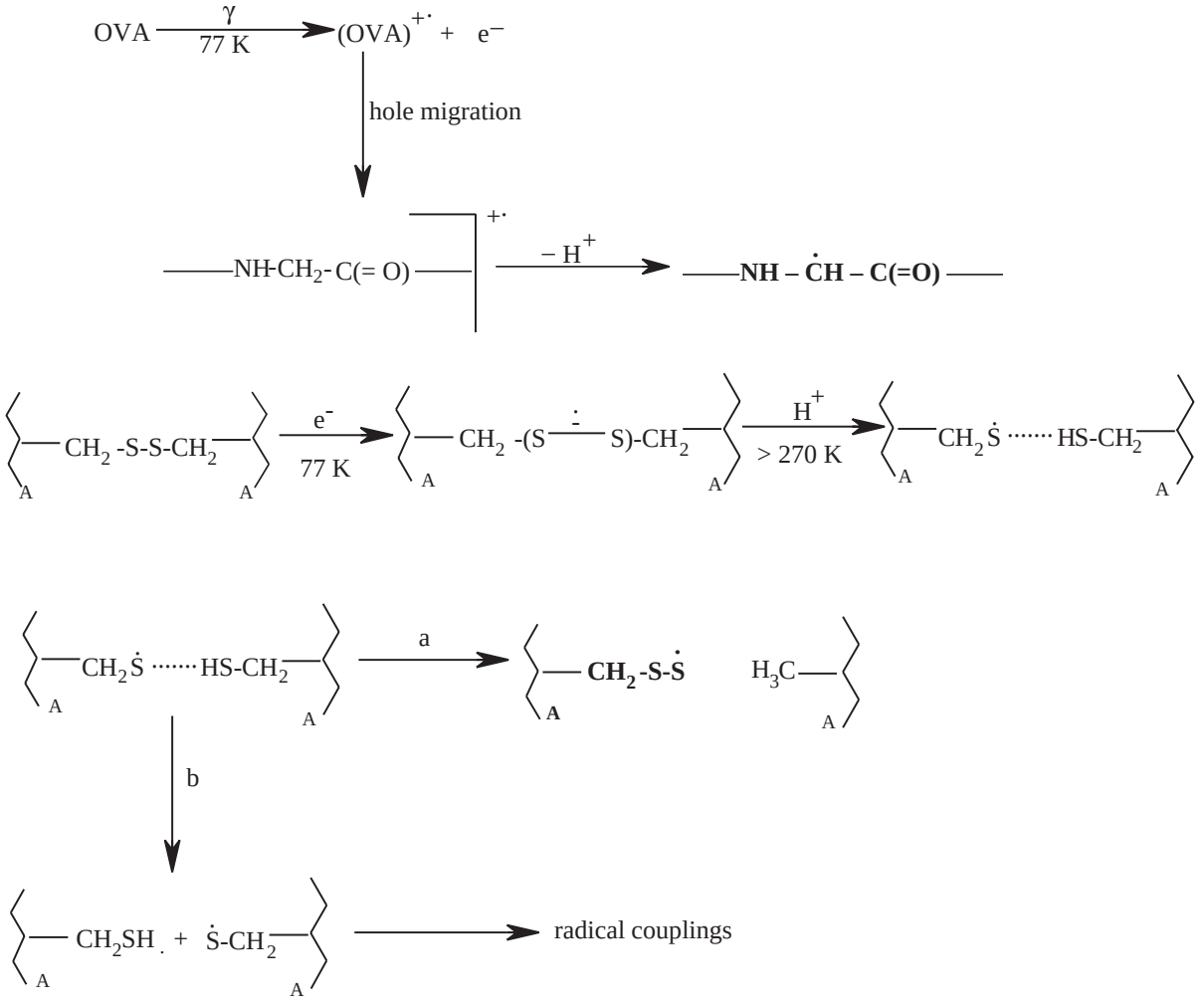
The irradiation of the PLGA/PEG microspheres under vacuum gave to radicals products arising essentially from the radiolysis of the PLGA component, the tertiary radical $–C^{\bullet}(CH_3)–$ being the most abundant, after annealing at room temperature. The lack of detection of PEG radicals can reasonably be explained in terms of smaller radiolytic yields as compared to PLGA and/or in terms of scavenging of PEG radicals by PLGA. The tertiary C–H bonds in PLGA are

expected to be especially reactive in the H abstraction reactions by $–CH_2^{\bullet}$ and $–CH^{\bullet}–O–$ PEG radicals.

The EPR spectra recorded after vacuum irradiation of ovalbumin loaded PLGA/PEG microspheres containing different concentrations of PEG (batches OV_6 and OV_5) are shown in Fig. 6. The major radical products either at low or room temperature were the ones usually detected in pure irradiated PLGA spectra; however when the spectra were recorded at higher power after annealing at room temperature, the g_{zz} and g_{yy} features of the ovalbumin RS–$S^{\bullet}$ perthiyl radical were clearly identified (Fig. 6 c, f). A significant difference between the two spectral series was represented by the lower intensity of the perthiyl radical signal detected from the sample with a higher PEG content (batch OV_5, Fig. 6c). This ability of PEG to affect the probability of the protein sulphur radicals formation has some interesting mechanistic implications which are discussed below.

3.4.3. The mechanism of formation of perthiyl radicals

What it is known from the literature about the radiolytic behaviour of organic disulphide suggests that disulphide bridges within the ovalbumin structure can act as trap of the electrons stemming from primary ionisations giving the anion radicals $–CH_2(–S–S–)^{\bullet-}CH_2–$ [30]. Such species are proposed to be the precursors of perthiyl radicals according to the following mechanism:



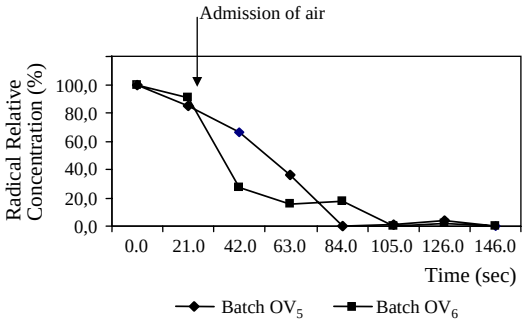


Fig. 7. Enhancement by oxygen of the radicals decay kinetics in irradiated PLGA/PEG microspheres samples with different content of PEG. The EPR signal intensities do not reflect differences of radiolytic yields.

The electron capture by the disulphide bridge gives the anion radical which by protonation generates a reacting couple $–CH_2S^{\bullet}\cdots HSCH_2–$. Path **a** must be considered negligible in solution since solution thiyl radical chemistry studies including pulse radiolysis, have not yet afforded experimental evidence of intermediate perthiyl radicals; instead, the products observed are always reckoned with the self-reactions of thiyl radicals or reactions with added substrates [30].

In the solid state within the protein structure however a different situation seemed to apply with the reacting centres $–CH_2S^{\bullet}$ and $–CH_2SH$ being generated and maintained in a geometrical position favouring the reaction path **a** with respect to the competition with other more thermodynamically favoured reactions (path **b**). The formation of perthiyl radicals may therefore be considered a peculiar consequence of the control on reactivity exercised by the protein structure in the solid state. This conclusion is supported by the results of specific EPR experiments performed on the reaction $RS^{\bullet}+RSH$ within the channels of a long chain alkanethiol thiourea clathrate [27].

As previously observed, the radiolytic yield of perthiyl radicals is consistently lower in the microsphere samples containing higher concentration of PEG (Fig. 7). This difference may be imputed to a chemical interference of PEG on the protein radiolysis (that is, scavenging of perthiyl radicals precursors) and/or to an effect of PEG on the topochemical factors controlling the formation of perthiyl radicals. The latter hypothesis implies that PEG favours the geometrical mismatch of the reacting centres $–CH_2S^{\bullet}\cdots HSCH_2–$ thus enhancing the path **b** at the expense of the path **a**. This effect may be a consequence of an enhancement by PEG on polymer chain mobility within the matrix or it may follow from the hydrogen bonding of reacting centres with neighbouring PEG units.

In conclusion, PEG affects the perthiyl radicals yield in ovalbumin loaded PLGA/PEG microspheres through a perturbation of the specific reaction path **a** in the radiolysis mechanism leading to perthiyl radicals.

3.4.4. Radical decay kinetics under vacuum and on admission of air

The vacuum radicals decay kinetics at room temperature placebo and ovalbumin loaded PLGA/PEG microspheres were found to be very slow so that intense EPR radical signals could be detected after months. In solid polymer matrices where true translation diffusion of the radicals is negligible, the rate constant k_t for radical decay has been modelled by assuming a mechanism of spin migration via H

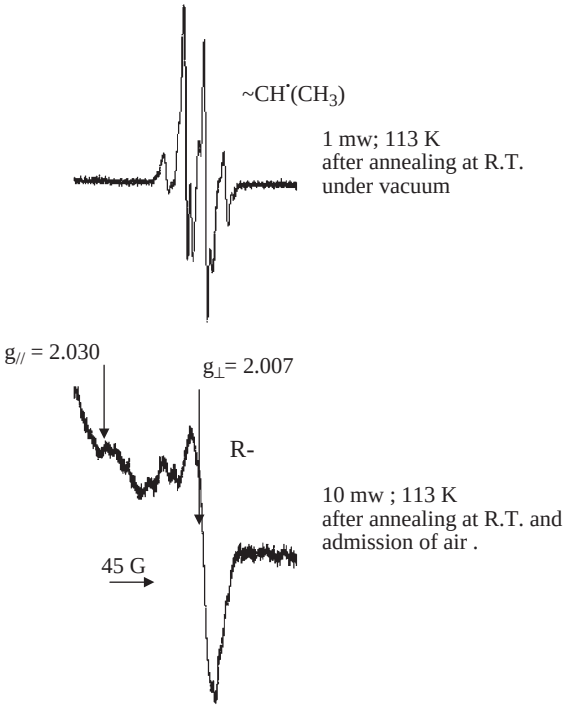


Fig. 8. EPR spectrum showing the conversion of the PLGA tertiary carbon radicals into peroxyl radicals in irradiated PLGA/PEG (15% of PEG) microsphere samples exposed to air at room temperature after vacuum gamma irradiation at 77 K.

abstraction steps coupled with the spatial oscillation of the free radical centres [31]

$$k_t = 4\pi \cdot d \cdot D \cdot N_{\underline{A}}/1000\left(\mathrm{LM}^{-1}\mathrm{s}^{-1}\right)$$

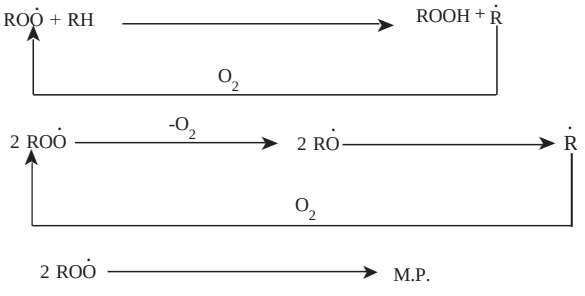
$$D = \lambda^2 \cdot k[\mathrm{RH}] \cdot [\mathrm{R}^{\bullet}]/6.$$

$$-\mathrm{d}[\mathrm{R}^{\bullet}]/\mathrm{d}t = 2k_t \cdot [\mathrm{R}^{\bullet}]^2$$

where d is the minimum reaction distance, D is the diffusion coefficient for the spin migration, λ is the jump length at each step and k•[RH]•[R$^{\bullet}$] is the rate of the H abstraction (corresponding to the jump frequency). The parameter λ is related to the amplitude of the segmental motion in the polymer chains. According to this model, the observed radical stability can be imputed to the rigidity of the matrix (small jump amplitude λ) coupled with the unfavourable thermodynamics for the spin diffusion via H abstraction at the C–H bonds in the PLGA and PEG chains which was expected to be endothermic or at most thermoneutral for the carbon and sulphur radicals trapped at room temperature.

On admission of air the free radical population in both placebo and ovalbumin loaded PLGA/PEG microspheres started decaying rapidly, the $t_{1/2}$ being about 40–50 s (Fig. 7). The decay was accompanied by the expected formation of the intermediate peroxy radicals whose characteristic EPR signal with a nearly axially symmetric g tensor with principal values $g_{//}$=2.030 and $g_{\perp}$=2.007 (Fig. 8) is detected at high power.

The dramatic decrease of the radical life-time under oxygen was diagnostic of the permeability of the polymer matrices which was enhanced by the short diffusion path within the microspheres. A concomitant factor was the enhancement of the spin diffusion rate through the relay H abstraction mechanism based on peroxy and alkoxy radicals:



Further contribute to the enhancement of the radical decay rate under oxygen can arise from peroxy and alkoxy radicals fragmentation reactions leading to species of smaller molecular size, as OH, CHO(OO$^{\bullet}$) and $CH_3OO^{\bullet}$, which may be expected to be capable of diffusing within the polymer matrix.

4. Conclusions

The experimental results obtained afford the following conclusions.

The presence of PEG in the microspheres modifies both their structure and the hydrophilicity of polymeric matrix. As a consequence protein release profile changes. The presence of small amounts of PEG in the microspheres achieves the control of protein release rate, this is probably due to improved protein incorporation inside the polymeric matrix. The dense and compact matrix, obtained by concomitant addition of small amounts of PEG and NaCl (or by addition only of NaCl during microsphere preparation process) is responsible for slow release rates with low burst release of protein. Vice versa higher amounts of PEG, even in the presence of NaCl (batch OV_6) lead to a more hydrophilic matrix giving higher protein release rate.

Γ-irradiation affects microsphere morphology leading to microparticle aggregation and, for highly porous systems, to their rupture. The global result, as shown by granulometric analysis, is particle size spreading towards larger size.

The changes in OVA release rate from the microspheres, induced by γ-irradiation, can be ascribed to concomitant phenomena: the modifications of microsphere physical structure, polymer degradation and its interaction with the protein.

EPR investigation shows that PEG has a perturbation effect on the perthiyl radicals yield in ovalbumin loaded PLGA/PEG microspheres leading to formation of molecular products by radical coupling. This mechanism could contribute to the different ovalbumin release highlighted after irradiation.

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