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Research paper

# γ-Irradiation effects on biopharmaceutical properties of PLGA microspheres loaded with SPf66 synthetic vaccine

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**Abstract**

γ-Irradiation is currently the method of choice for terminal sterilization of drug delivery systems made from biodegradable polymers. However, the consequences of γ-sterilization on the immune response induced by microencapsulated antigens have not yet been reported in the literature. The aim of the present work was to evaluate the effect of γ-irradiation on the biopharmaceutical properties of PLGA microspheres containing SPf66 malarial antigen. Microspheres were prepared by a (w/o/w) double emulsion/solvent extraction method. Once prepared, part of the formulation was irradiated at a dose of 25 kGy using $^{60}$Co γ as radiation source. The *in vitro* results obtained showed that the γ-irradiation exposure had no apparent effect on SPf66 integrity and formulation properties such us morphology, size and peptide loading. Only the release rate of SPf66 was slightly faster after γ-irradiation. Subcutaneous administration of irradiated and non-irradiated microspheres into mice induced a similar immune response (IgG, IgG1, IgG2a levels) and was comparable to that obtained with SPf66 emulsified with Freund's complete adjuvant. These observations illustrate the applicability of γ-irradiation as a method of terminal sterilization of microparticulate delivery systems based on chemically synthesized antigens encapsulated into biodegradable PLGA microspheres.


*Keywords:* SPf66 malarial antigen; Microspheres; PLGA; γ-Irradiation; Sterilization

## 1. Introduction

Microparticle systems based on PLGA polymers have been intensively investigated in the last decades for vaccine development by administering them via parenteral or mucosal routes [1–7]. When administered parenterally, these polymeric microparticles have to meet the pharmacopoeial requirements of sterility. However, microparticle manufacturing often involves multiple stages, which made difficult aseptic processing in a clean room environment under Good Manufacturing Practice (GMP) conditions. Therefore, terminal sterilization is preferential to aseptic processing since it is easier from a technological point of view and economically more convenient [8].

The common terminal sterilization methods are steam, dry heat, ethylene oxide gas, and ionizing radiations [9]. Among them, dry heat and steam sterilizations are carried out at high temperature and can cause severe degradation and hydrolysis of polymeric microparticles, and ethylene oxide is not applicable due to its toxic residues. Thus, γ-irradiation currently remains the more frequently used method for terminal sterilization of these formulations. A minimum radiation dose of 25 kGy is considered as adequate for the purpose of sterilizing pharmaceutical

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products without providing any biological validation [10]. Nevertheless, it is well known that γ-sterilization may cause radiolytic degradation of the polymer and incorporated drug [11]. Previous studies have reported that γ-irradiation of biodegradable polyesters such as PLGA induces a dose dependent chain scission leading to a molecular weight reduction [12,13]. Claybourn et al. demonstrated that the concentration of free radicals formed upon exposure of PLGA to ionizing radiation increased with the radiation dose and glycolide content within the polymer [14]. More recently, Williams et al. [15] studied the effect of γ-irradiation on a range of PLGA polymers with different lactide: glycolide ratios and concluded that the higher the glycolide content the more unstable is the polymer. Several other studies have examined the effect of γ-irradiation on the release profile of microspheres, but different results have been reported depending on the active compound encapsulated. For example, the release profile of ganciclovir loaded microspheres was not altered [16], whereas Lalla and Sapna [17] reported a decrease in piroxicam release and Faisant et al. [18] reported an increase in 5-Fu release. Interestingly, these authors describe two mathematical models to predict the drug release kinetic as a function of the irradiated dose.

In the case of microspheres for vaccine development, in addition to the effect of irradiation on the polymer stability, the immunogenicity of the antigen encapsulated has to be taken into account. However, as far as we know, no information about the effect of γ-irradiation on the immune response of encapsulated antigens is available in the literature. The purpose of the present work was therefore to evaluate the effect of γ-irradiation on the biopharmaceutical properties of PLGA microspheres containing SPf66 malarial synthetic peptide. Microspheres were prepared by a double emulsion/solvent extraction technique with two different copolymers: Resomer® RG506 and RG756. Their physicochemical properties were fully characterized before and after exposure to 25 kGy $^{60}$Co γ-irradiation. Furthermore, the immunogenicity of the antigen encapsulated into irradiated and non-irradiated microspheres was evaluated in BALB/c mice.

## 2. Materials and methods

### 2.1. Materials

Poly (DL-lactide-co-glycolide) polymer (PLGA), Resomer® RG506 (50:50, lactide:glycolide ratio) (MW 102,900) and Resomer® RG756 (75:25, lactide:glycolide ratio) (MW 92,000) were supplied by Boehringer Ingelheim, Germany. Polyvinylalcohol (PVA; average MW 30,000–70,000), affinity-purified antibody peroxidase-conjugated goat anti-mouse IgG, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and Freund's complete adjuvant (FCA) were supplied by Sigma Chemicals. St. Louis, MO, USA. Antibody peroxidase-conjugated goat anti-mouse IgG1 and IgG2a were purchased from Southern Biotechnology Associates Inc., Birmingham, AL, USA. The protein assay kit (microBCA) was purchased from Pierce Co., Rockford, IL, USA. All other chemicals were of analytical grade and used as received.

### 2.2. SPf66 antigen

The SPf66 malarial antigen was produced under GMP conditions at the Fundación Instituto de Inmunología de Colombia. The molecule was synthesized using the solid-phase synthesis method described by Merrifield [19] and modified by Houghten [20], according to the *t*-Boc technique. The peptide has cysteine residues at both the amino- and carboxy-terminal ends, enabling polymerization in air conditions to obtain a final product composed of 3–5 individual peptides joined by disulfide bridges.

### 2.3. Microsphere preparation and characterization

PLGA microspheres containing SPf66 were prepared under aseptic conditions from PLGA 50:50 and PLGA 75:25 using a (w/o/w) double emulsion/solvent extraction method previously described [21]. Briefly, 250 mg PLGA was dissolved in 5 ml methylene chloride and emulsified with 250 μl of a 10% SPf66 aqueous solution by probe sonication for 30 s at 50 W (Branson® 250 sonifier, CT, USA). The resulting emulsion (w/o) was poured into 25 ml of 8% PVA aqueous solution and emulsified for 5 min at 9500 rpm using a turbine homogenizer (Ultra-turrax® T-25, IKA-Labortechnik, Staufen, Germany) to perform the double emulsion (w/o/w). Finally, 50 ml of 2% isopropanol solution was added and stirred for 1 h to extract the organic solvent and microparticle hardening. The microspheres were then collected by centrifugation at 10,000 rpm for 5 min, washed three times with distilled water to remove residual PVA, resuspended in double-distilled water and freeze-dried for 24 h. Empty microspheres containing no SPf66 peptide were prepared using the same methodology.

Particle size distribution was determined by laser diffractometry (Coulter Counter® LS130 particle size analyzer, Amherst, MA, USA). Microparticle morphology was examined by scanning electron microscopy (SEM, Jeol® JSM-35 CF, Japan). Total peptide loading and surface associated peptide (SAP) were determined using the bicinchoninic acid assay [22] in a linear working range for peptide concentrations of 5–20 μg/ml. Total peptide loading was evaluated after microsphere disruption with 0.2 M NaOH. Surface associated peptide was measured in the supernatant after centrifuging a microparticle suspension in 20 mM PBS maintained under orbital rotation at 37 °C for 30 min.

### 2.4. γ-Irradiation of SPf66 loaded microspheres

Samples of PLGA 50:50 and PLGA 75:25 raw polymers, empty and SPf66 loaded microspheres were placed in glass aluminium sealed vials and irradiated with a $^{60}$Co source

(Ingeominas, Bogota, Colombia). A 25 kGy dose was applied following the European Pharmacopoeia recommendations for an effective sterilization [23]. The process was carried out by covering the samples with dry ice in order to maintain a low temperature during the irradiation process to prevent undesired thermal effects [24].

### 2.5. In vitro release studies

In order to carry out studies on peptide release from microspheres, 10 mg of microspheres were placed in test-tubes containing 1 ml of 20 mM phosphate buffered saline pH 7.4 and incubated at 37 °C under continuous orbital rotation. At regular time intervals up to 183 days, the samples were spun at 10,000 rpm for 10 min. The supernatant was collected and assayed for peptide quantification using the microBCA assay. The peptide release study was continued after replacement with the same volume of fresh buffer. The test was performed in triplicate for each batch of microspheres.

The similarities between *in vitro* release profiles obtained before and after γ-irradiation were assessed by a pair-wise independent-model procedure such as similarity factor ($f_2$). In this approach, recommended by the FDA [25] and EMEA [26], two dissolution profiles were declared similar if $f_2$ was between 50 and 100.

### 2.6. Peptide integrity

SPf66 integrity was analyzed after dissolving the microspheres in methylene chloride and extracting the peptide with phosphate buffered saline. The analysis was carried out by size exclusion chromatography (SEC-HPLC, Merck–Hitachi Lachrom L-7400, Merck/Hitachi, Darmstadt, Germany) on a Superdex 75 HR 10/30 column using isocratic elution with 20% acetonitrile for 40 min at a 1 ml/min flow rate, with detection at 210 nm. The same samples were also analyzed by using MALDI–TOF (matrix assisted laser desorption–time of flight) mass spectrometry (Mass Spectrometer Autoflex Bruker Daltonics LRF, Bruker Daltonics Inc., Billerica, MA, USA).

### 2.7. Differential scanning calorimetry (DSC)

DSC analysis of raw polymers, empty and SPf66 loaded microspheres was performed before and after γ-irradiation using a differential scanning calorimeter (DSC-50 Shimadzu Corporation, Kyoto, Japan). The samples were placed in sealed aluminium pans and heated from 20 °C to 200 °C at a heating rate of 10 °C/min against an empty pan as a reference. Scans were obtained and the data processed by the system software to identify glass transition temperatures ($T_g$).

### 2.8. Immunization protocol

Three groups of 10 female BALB/c mice (6–8 weeks old) (Instituto Nacional de Salud, Bogotá, Colombia) were subcutaneously immunized with a single dose of 100 μg SPf66 as detailed in Table 1. The microparticle formulation employed was a 1:2 mixture of PLGA 50:50 and PLGA 75:25 microspheres. According to our previous results [6] this was the most suitable composition to deliver a single dose vaccine since PLGA 50:50 showed a fast to moderate release profile and PLGA 75:25 provided a more sustained and slower SPf66 release, that can simulate repeated administrations of the antigen. The first group was immunized with SPf66 microencapsulated into non-irradiated microparticles. A second group was immunized with SPf66 microencapsulated into the same batch of microspheres sterilized by γ-irradiation and a third group was immunized with SPf66 solution emulsified with Freund's complete adjuvant (FCA). Two additional groups were subcutaneously injected with irradiated and non-irradiated empty microspheres. Blood samples were collected from the retroorbital plexus of mice, prior to immunization (pre-immune sera) and periodically until 27 weeks. Samples were centrifuged and sera were collected and stored at −80 °C until assayed by ELISA. Principles in good laboratory animal care were followed and animal experimentation was in compliance with the "Ethical Committee of Animal Experimentation" of the Fundación Instituto de Immunologia de Colombia (FIDIC).

### 2.9. Antibody response evaluation

Anti-SPf66 antibodies were determined by conventional ELISA, as previously described [27]. Total SPf66-specific IgG titres were measured at all time points, while IgG1 and IgG2a isotypes were assessed at 6, 15 and 27 weeks for all mice groups. Briefly, microtitre plates (Nunc-Immuno Plate FP 96, Maxisorp, Nunc) were coated with 100 μl per well of 10 μg/ml SPf66 solution in PBS pH 7.4 and incubated overnight at 4 °C. They were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked for 1 h with 200 μl of 0.5% BSA in PBST. Serum samples were serially diluted 1:1 starting from 1:100 dilution in blocking buffer, 100 μl of each sample was added to each coated ELISA plate well and was incubated for 1 h at 37 °C. After washing three times with PBST, 100 μl horseradish peroxidase-labelled goat anti-mouse IgG, IgG1 or IgG2a isotypes diluted 1:1000 in blocking buffer was added to each well and incubated at 37 °C for 1 h. The plates were washed again and 100 μl ABTS 0.1 mg/ml solution, in 50 mM citrate buffer plus hydrogen

Table 1
Immunization protocols in BALB/c mice

| Group | Formulation | Day of immunization | Antigen dose (μg) |
|---|---|---|---|
| I | Non-irradiated MP | 0 | 100 |
| II | Irradiated MP | 0 | 100 |
| III | SPf66-FCA | 0 | 100 |
| IV | Non-irradiated empty MP | 0 | – |
| V | Irradiated empty MP | 0 | – |

peroxide, was added to each well and incubated at 37 °C for 15 min for colour development. End-point titres were expressed as $\log_2$ of the last dilution reciprocal giving an $OD_{405}$ over the mean $OD_{405}$ for the preimmune sera plus three standard deviations (SD).

### 2.10. Statistical analysis

The results were expressed as means ± standard deviation (SD) for each group of mice. Normal distribution of samples was evaluated by Shapiro–Wilk trial and differences among groups at significance levels of 95% were calculated by the non-parametric Mann–Whitney *U*-test. Pair-wise Spearman rank correlation was used to assess relation between variables. Statistical analysis was completed with the SPSS 11.0 program (SPSS®, Chicago, USA).

## 3. Results and discussion

### 3.1. Microsphere characteristics

When observed under SEM, both irradiated and non-irradiated microspheres prepared with either PLGA 50:50 or PLGA 75:25 polymers appeared smooth and spherical (Fig. 1). Granulometric analysis of different microsphere formulations by laser light scattering showed a unimodal size distribution (90% particles between 0.95 and 3.75 μm) with a mean particle size, expressed in terms of equivalent volume diameter, ranging from 1.63 to 2.06 μm, as shown in Table 2. These results showed that no morphological change due to the temperature rise, e.g. particle aggregation, took place after γ-irradiation, since samples were protected with dry ice during γ-irradiation exposure [28].

Concerning antigen loading and adsorbed to the microparticle surface (SAP), irradiation did not seem to have an effect, since no significant differences were observed between irradiated and non-irradiated microspheres ($p < 0.05$, Mann–Whitney *U*-test). Similar results have been previously reported in the literature by Calis et al. [29]. As shown in Table 2, peptide loading and SAP were about 80% and 20%, respectively, for PLGA 50:50 microspheres, whereas the corresponding values for PLGA 75:25 formulations were around 70% and 40%, respectively. The difference in the amount of peptide accumulated at the surface of PLGA 50:50 and PLGA 75:25 microparticles might have been due to the more hydrophobic nature

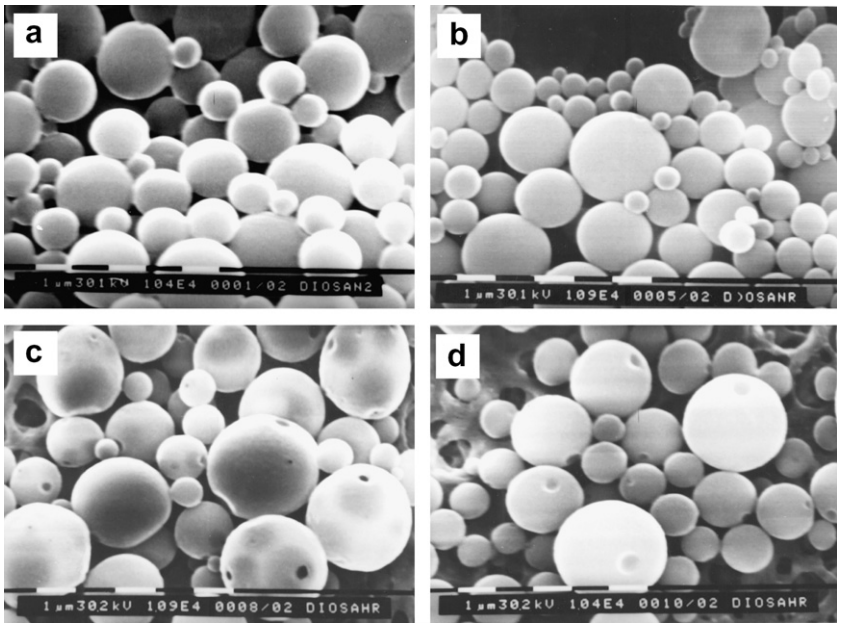


Fig. 1. SEM photomicrographs of SPf66-loaded microspheres. (a) Non-irradiated PLGA 50:50 MP; (b) γ-irradiated PLGA 50:50 MP; (c) non-irradiated PLGA 75:25 MP; (d) γ-irradiated PLGA 75:25 MP.

Table 2
Microparticle characterization

| Formulation[a] | Size[b] (μm) | Surface adsorbed peptide (SAP %) | Peptide loading (μg/mg MP) |
|---|---|---|---|
| Non-irradiated PLGA 50:50 MP | 1.63 (90% 0.96–2.82) | 23.57 | 83.07 |
| Irradiated PLGA 50:50 MP | 1.63 (90% 0.95–2.83) | 24.31 | 83.65 |
| Non-irradiated PLGA 75:25 MP | 2.06 (90% 1.10–3.75) | 41.97 | 69.11 |
| Irradiated PLGA 75:25 MP | 2.02 (90% 1.08–3.60) | 42.57 | 68.90 |

[a] Three individual batches of either PLGA 50:50 or PLGA 75:25 were prepared with similar results. The results shown are from the batches used *in vivo*.
[b] Size results are expressed in terms of equivalent volume diameter.

Table 3
Values of glass transition temperature ($T_g$) for raw polymer, empty microspheres and SPf66 loaded microspheres before and after γ-irradiation

| | $T_g$ before irradiation (°C) | $T_g$ after irradiation (°C) |
|---|---|---|
| PLGA 50:50 | 48.31 ± 0.96 | 46.48 ± 0.95 |
| PLGA 50:50 empty MP | 54.47 ± 1.07 | 51.92 ± 0.93 |
| SPf66-PLGA 50:50 MP | 54.96 ± 0.94 | 53.32 ± 1.15 |
| PLGA 75:25 | 56.75 ± 1.14 | 53.48 ± 1.20 |
| PLGA 75:25 empty MP | 59.30 ± 1.06 | 58.74 ± 0.98 |
| SPf66-PLGA 75:25 MP | 59.22 ± 1.16 | 55.99 ± 1.14 |

Mean ± SD for $n = 3$.

of PLGA 75:25. This would result in greater peptide diffusion to the external aqueous phase when the double emulsion is formed.

The effect of γ-irradiation on the glass transition temperature ($T_g$) of the samples studied is shown in Table 3. First of all, we can observe that PLGA 50:50 and 75:25 microsphere samples had a significantly higher $T_g$ than the corresponding raw polymers (changes in the $T_g$ of samples were defined as significant if they were more than three times of the standard deviation). This could be attributed to the loss of the residual hydrosoluble oligomers that can plasticize the PLGA raw polymer, during the microencapsulation process, which involves exposing the microspheres to a large volume of water [15].

It can also be seen that γ-irradiation caused only a small reduction in the $T_g$ values. Both PLGA 50:50 and 75:25 raw polymers and microsphere samples showed a decrease in the $T_g$ values after γ-irradiation, without significant differences between irradiated and non-irradiated samples (changes in $T_g$ lower than three times of the standard deviation). This is likely to be due to slight changes in the polymer occurring as a consequence of radiolytic events such as chain scissions. This observation is in agreement with previous studies by Montanari et al. [30].

### 3.2. SPf66 antigen release profile

The effect of γ-irradiation on the antigen release from PLGA 50:50 and PLGA 75:25 γ-irradiated microspheres was evaluated, taking the same non-irradiated microsphere formulations as reference. Fig. 2 illustrates the release profile of SPf66 from microspheres in PBS 7.4 before and after sterilization. As shown in the figure, PLGA 50:50 microspheres presented a similar triphasic release pattern in both irradiated and non-irradiated samples. However, the irradiation of microspheres causes a faster release of the microencapsulated peptide, probably due to a reduction of the polymer molecular weight which provokes a faster degradation and therefore a faster release of the antigen from the γ-irradiated microspheres. These *in vitro* results agreed with those previously reported by Montanari et al. [31] who describe that the release rate from γ-irradiated microspheres was slightly faster than from the non-irradiated ones.

In the case of PLGA 75:25 microspheres a biphasic profile was observed with an initial burst followed by a constant, approximately zero-order release phase. Irradiation has no significant effect on the percentage of antigen released until day 144, where the amount released from γ-irradiated microspheres increased gradually. Thus, the percentages released by the end of the study were 92% and 84% for irradiated and non-irradiated microspheres, respectively.

Similarity factor ($f_2$) was calculated in order to compare the *in vitro* release profiles obtained before and after sterilization, as proposed by the FDA and EMEA. According to this criterion, the sterilization procedure employed did not almost modify the SPf66 *in vitro* release and the profiles behaved similarly before and after γ-irradiation, $f_2 = 52.73$ and $f_2 = 80.45$ for PLGA 50:50 and PLGA 75:25 microparticles, respectively.

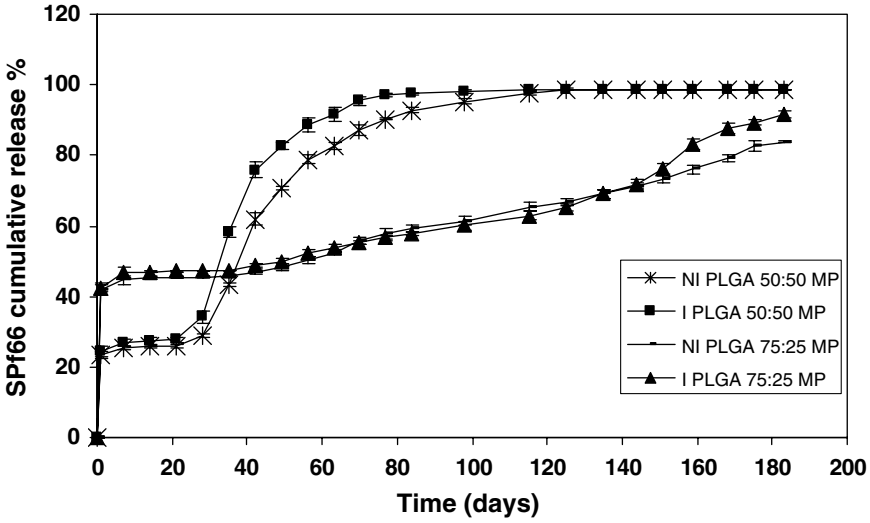


Fig. 2. *In vitro* release profile of SPf66-loaded microspheres before (NI) and after (I) irradiation at 25 kGy.

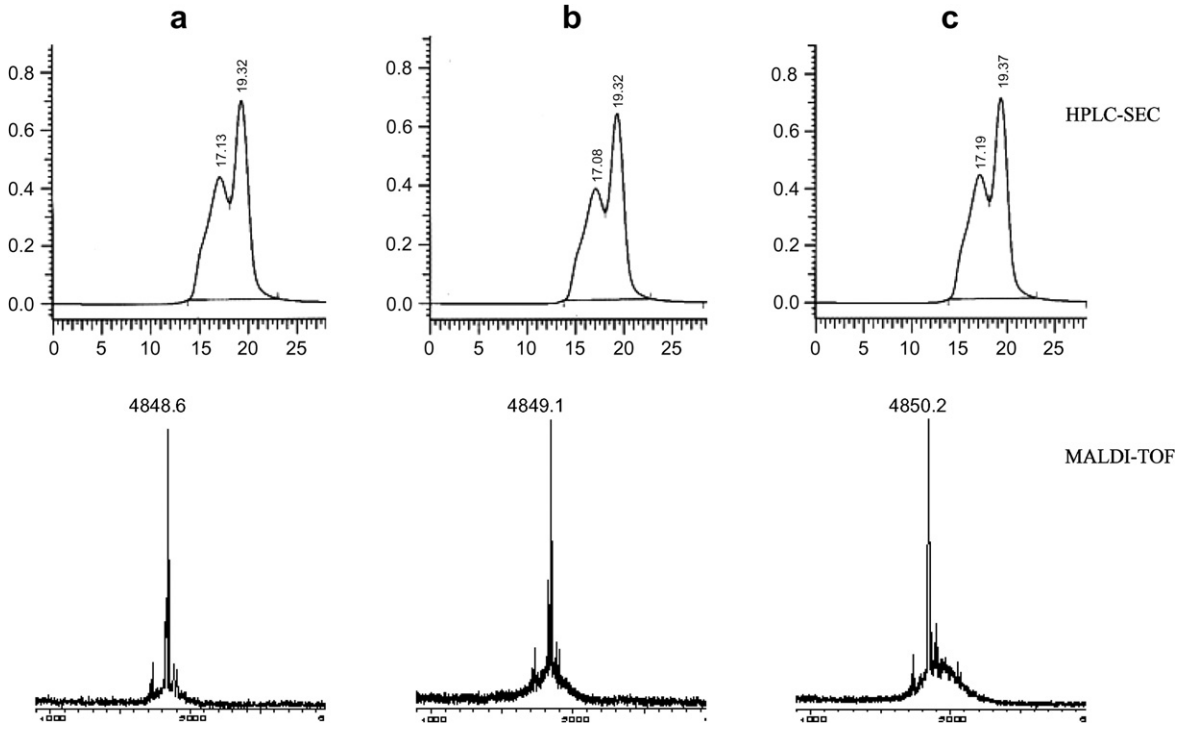


Fig. 3. Size exclusion chromatography (HPLC-SEC) and mass spectrometry (MALDI–TOF) of different samples of SPf66 antigen. (a) Native peptide; (b) peptide extracted from non-irradiated PLGA 50:50 MP; (c) peptide extracted from γ-irradiated PLGA 50:50 MP.

### 3.3. SPf66 antigen integrity

The results obtained from size exclusion chromatography (HPLC-SEC) and MALDI–TOF mass spectrometry of native SPf66 peptide, peptide extracted from irradiated microspheres and extracted from non-irradiated ones demonstrated that there was no apparent effect of γ-irradiation on microencapsulated antigen integrity. As can be seen in Fig. 3, the chromatographic profile of the peptide extracted from irradiated microspheres was similar to those of native peptide and peptide extracted from non-irradiated microspheres and did not reveal the presence of additional signals due to the degradation or formation of aggregates. Furthermore, mass spectrometry analysis revealed that SPf66 antigen monomeric unit molecular weight was maintained in all samples analyzed (around 4850 Da), leading us to conclude that there was no degradation of the active molecule after γ-irradiation. These results should be later on confirmed by performing *in vivo* immunization studies.

### 3.4. Effect of γ-irradiation process on immune response

In order to study the possible effect of γ-irradiation on the immune response *in vivo* immunization studies were performed in BALB/c mice. Fig. 4 shows serum antibody responses (IgG) induced by a single subcutaneous administration of 100 μg SPf66 encapsulated in non-irradiated PLGA microspheres compared to the response obtained when administering the same antigen dose in sterilized microspheres or the peptide solution emulsified with Freund's complete adjuvant. The results obtained for anti-SPf66 IgG antibody levels induced by γ-irradiated microspheres were slightly lower than those induced by non-irradiated microspheres and FCA, however these differences were not statistically significant ($p > 0.05$) at any point of the study, confirming that the immunogenic activity of the antigen is maintained after γ-irradiation.

We then measured the IgG isotype secretion at 6, 15 and 27 weeks in order to indirectly evaluate the type of T helper cells (Th1 versus Th2) elicited. In general, Th1 immune responses, capable of activating cellular immune response which is crucial for malaria, promote the production of IgG2a antibody in mice; whereas Th2 immune responses promote the production of IgG1 antibody. As expected according to previous studies [32], subcutaneous administration of irradiated and non-irradiated microspheres into mice induced a balanced IgG1/IgG2a response. Furthermore, there were few differences among groups at the same time point for either IgG1 or IgG2a isotype levels. These data are relevant because persistence of a Th1-like response is associated with protection in murine models of asexual blood stage malaria [33].

## 4. Conclusions

In the present work the effect of γ-irradiation on the biopharmaceutical properties of PLGA microspheres containing SPf66 malarial antigen has been evaluated. *In vitro* characterization of microspheres did not reveal a marked effect of γ-radiation on either the microparticle system or

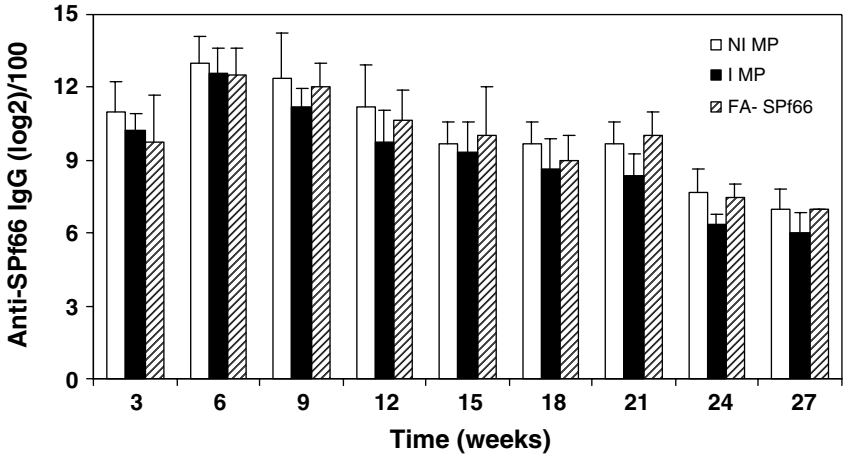


Fig. 4. Anti-SPf66 IgG antibodies (mean ± SD) of BALB/c mice after a single subcutaneous immunization with 100 μg SPf66 in different formulations. NI MP, non-irradiated MP; I MP, γ-irradiated MP; FA-SPf66, SPf66 emulsified with Freund's adjuvant. (MP: 1:2 mixture of PLGA 50:50 and PLGA 75:25 microspheres.)

the microencapsulated antigen. Furthermore, *in vivo* immunogenicity results suggest that the antigen remains immunogenic after γ-irradiation. From the experimental results obtained, γ-irradiation can be proposed as a method for terminal sterilization of microparticulate delivery systems based on chemically synthesized antigens encapsulated into biodegradable PLGA microspheres.

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