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RESEARCH PAPER

Gamma Irradiation of Active Self-Healing PLGA Microspheres for Efficient Aqueous Encapsulation of Vaccine Antigens

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ABSTRACT

Purpose To investigate the effect of γ-irradiation of poly(lactic-co-glycolic acid) (PLGA)/$Al(OH)_3$/0 or 5 wt% diethyl phthalate (DEP) microspheres for active self-healing encapsulation of vaccine antigens.

Methods Microspheres were irradiated with ^{60}Co at 2.5 and 1.8 MRad and 0.37 and 0.20 MRad/h. Encapsulation of tetanus toxoid (TT) was achieved by mixing $Al(OH)_3$-PLGA microspheres with TT solution at 10–38°C. Electron paramagnetic resonance (EPR) spectroscopy was used to examine free radical formation. Glass transition temperature (T_g) and molecular weight of PLGA was measured by differential scanning calorimetry and gel permeation chromatography, respectively. Loading and release of TT were examined by modified Bradford, amino acid analysis, and ELISA assays.

Results EPR spectroscopy results indicated absence of free radicals in PLGA microspheres after γ-irradiation. Antigen-sorbing capacity, encapsulation efficiency, and T_g of the polymer were also not adversely affected. When DEP-loaded microspheres were irradiated at 0.2 MRad/h, some PLGA pores healed during irradiation and PLGA healing during encapsulation was suppressed. The molecular weight of PLGA was slightly reduced when DEP-loaded microspheres were irradiated at the same dose rate. At the 0.37 MRad/h dose rate, these trends were not observed and the full immunoreactivity of TT was preserved during encapsulation and 1-month release. Gamma irradiation slightly increased TT initial burst release. The small increase in total irradiation dose from 1.8 to 2.5 MRad had insignificant effect on the polymer and microspheres properties analyzed.

Conclusions Gamma irradiation is a plausible approach to provide a terminally sterilized, self-healing encapsulation PLGA excipient for vaccine delivery.

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KEY WORDS active self-healing encapsulation · controlled release · gamma irradiation · PLGA · vaccine antigens

ABBREVIATIONS

AAA	amino acid analysis
Active SM	active self-microencapsulating
$Al(OH)_3$	aluminum hydroxide adjuvant
BSA	bovine serum albumin
DEP	diethyl phthalate
DSC	differential scanning calorimetry
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPR	electron paramagnetic resonance
FMOC	9-fluoromethyl-chloroformate
GPC	gel permeation chromatography
HCl	hydrochloric acid
ISO	international organization for standardization
M_w	weight-average molecular weight
OPA	*o*-phthalaldehyde
PBB	phosphate buffered saline + 0.5% bovine serum albumin + 0.05% Brij® 35
PBS	phosphate buffered saline
PBST	phosphate buffered saline + 0.02% Tween 80
PLGA	poly(lactic-co-glycolic acid)
PNPP	(*p*-nitrophenyl phosphate, disodium salt) liquid substrate
PTFE filter	hydrophobic fluoropore filter
PVA	poly(vinyl alcohol)
SEM	scanning electron microscopy
T_g	glass transition temperature
THF	tetrahydrofuran
TT	tetanus toxoid

INTRODUCTION

Protection by immunization against diseases such as tetanus, diphtheria and pertussis requires multiple administrations (2–3 doses) of respective traditional vaccines with an interval of 4–6 weeks (1–4). Failure to achieve protection has been attributed to high drop-out rates (up to 70%) after the primary immunization (5). Single-dose injectable microparticulate vaccine formulations based on biodegradable poly(lactide-co-glycolide) (PLGA) have been thoroughly investigated in the past decades, which were found to provide long-term protective levels in animals with reduced number of doses (5–14).

Injectable PLGA microspheres containing vaccines are commonly prepared by double emulsion-solvent evaporation, spray-drying, and coacervation methods (1,2,9,15–18). These encapsulation methods cause damage to vaccine antigens due to harsh processing conditions (e.g., shear stresses, organic solvent/water interface, high temperature, freeze-drying) (1,2,6,7,15–17). Also, PLGA/vaccine microspheres prepared by these methods often exhibit poor encapsulation efficiency, stability and long-term *in vitro-in vivo* release of antigenic vaccines (1,2,4,6,7,15–17,19–21). Moreover, PLGA microspheres for administration by the parenteral route must be sterile.

Preparation of vaccine loaded PLGA microspheres with organic solvents under an aseptic environment involves high production costs (2,4,22–24). Therefore, terminal sterilization of the finished product is preferred since it is easier from a technological point of view and more economical than aseptic processing (25–29). The commonly employed terminal sterilization methods are by steam, dry heat, ethylene oxide gas, electron beam irradiation and γ-irradiation (15,23,25–27,29–35). Among them, dry heat and steam sterilization is carried out at high temperature and can cause significant degradation to protein antigens and hydrolysis of PLGA microparticles; and ethylene oxide is not applicable due to its toxic residues (15,23,25–27,29–32,36–38). Thus, γ-irradiation is a preferred method of terminal sterilization for injectable PLGA formulations due to its high efficiency, negligible thermal effects, and absence of post-sterilization treatment of the samples (e.g., aeration of the samples to remove toxic residues and microbial testing to verify sterility are required after ethylene oxide sterilization) (39). However, terminal γ-irradiation of finished PLGA formulations has been found to cause undesirable effects on stability of encapsulated vaccine or therapeutic protein, polymer properties, and antigen/protein release behavior (15,23,26,30–32,36,37).

Encapsulation of vaccine antigens in PLGA microspheres under mild conditions is critical to avoid the damage during preparation and to obtain controlled release. Recently, we reported a new self-healing based method by which encapsulation of vaccine antigens in PLGA microspheres was achieved by simple mixing of preformed aluminum hydroxide ($Al(OH)_3$)-PLGA-hydrophobic plasticizer microspheres with a low concentration (0.64–1 mg/mL) vaccine antigen solution at 10–38°C (22,40). The new method obviated vaccine antigen instability commonly observed with the double emulsion-solvent evaporation method; and also exhibited high loading (1–1.8 wt%) and encapsulation efficiency (~97%) of antigens. This method resulted in polymer stabilization and long-term *in vitro* release of stable/immunoreactive antigens (22,40). It was hypothesized that active self-healing encapsulation after sterilization of preformed $Al(OH)_3$-PLGA-0 and 5 wt% hydrophobic plasticizer microspheres would decrease production costs to make single-dose vaccination economically feasible. In addition, this encapsulation paradigm opens-up the possibility to encapsulate vaccine antigens at the point-of-care.

In this study, we investigated the effect of terminal γ-irradiation sterilization on pre-formed PLGA microspheres that are used for active self-healing encapsulation of tetanus toxoid (TT) and other alum-adsorbing antigens. To test this concept, polymer properties (glass transition temperature (T_g), molecular weight, and self-healing of PLGA), active self-encapsulation kinetics (loading capacity and encapsulation efficiency), and *in vitro* release characteristics were evaluated before and after γ-irradiation of $Al(OH)_3$-PLGA-0 and 5 wt% hydrophobic plasticizer microspheres. The effect of γ-irradiation dose and dose rate on the aforementioned properties was also studied.

MATERIALS AND METHODS

Materials

Tetanus toxoid (3120 Lf/mL) was received from Serum Institute of India Ltd. (Pune, India). $Al(OH)_3$ adjuvant, bovine serum albumin (BSA), poly(vinyl alcohol) (PVA) (80% hydrolyzed), succinic acid, diethyl phthalate (DEP), and trehalose were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Step™ PNPP (*p*-Nitrophenyl phosphate, disodium salt) liquid substrate was purchased from Thermo Scientific (Rockford, IL, USA). Equine tetanus antitoxin was received from the U.S. Food and Drug Administration (Silver Spring, MD, USA). PLGA 50:50 [i. v = 0.57 *dl/g*, end group = lauryl ester] was purchased from (Medisorb, Alkermes, Cambridge, MA, USA). Human tetanus immune globulin (HyperTET™ S/D, 250 units) was purchased from Talecris Biotherapeutics, Inc. (Research Triangle Park, NC, USA). Goat anti-human IgG-alkaline phosphatase was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA,

USA). Quartz electron paramagnetic resonance spectroscopy tubes were purchased from Wilmad LabGlass (Vineland, NJ, USA). All other reagents and solvents were of analytical grade or purer and purchased from commercial suppliers.

Preparation of Active Self-Microencapsulating (Active SM) PLGA Microspheres

Blank $Al(OH)_3$-PLGA-0 or 5 wt% DEP (active SM PLGA) microspheres were prepared by a water-in-oil-in-water (w/o/w) emulsion-solvent evaporation method, as described previously (40). Briefly, 0.2 mL of inner phase containing an appropriate quantity of $Al(OH)_3$ (3.2 wt%) and trehalose (3.5 wt%) was added to 1 mL of 250 mg/mL PLGA (with or without DEP) in methylene chloride. The mixture was homogenized at 17,000 rpm with a Tempest IQ^2 homogenizer (The VirTis Company, Gardiner, NY, USA) equipped with a 10 mm shaft in an ice/water bath for 1 min to prepare the first emulsion. Two mL of 5% (w/v) PVA solution was immediately added to the first emulsion, and the mixture was vortexed (Genie 2, Fisher Scientific Industries, Inc., Bohemia, NY, USA) for 50 s to produce the w/o/w double emulsion. The resultant emulsion was poured into 100 mL of chilled 0.5%w/v PVA solution under rapid stirring and hardened at room temperature for 3 h. Hardened microspheres were collected by sieve (20–63 μm), washed repeatedly with chilled double-distilled (ddH_2O), and freeze-dried.

Gamma (γ)-Irradiation of Blank Active SM PLGA Microspheres

About 250 mg blank active SM PLGA microspheres were freeze-dried, placed in 5-mL ampoules and ampoules were sealed under vacuum. All the samples were irradiated at room temperature with ^{60}Co (Ford Nuclear Reactor, Michigan Memorial Phoenix Project, University of Michigan) at 2.5 and 1.8 MRad doses, and 0.37 and 0.20 MRad/h dose rates.

Electron Paramagnetic Resonance (EPR) Spectroscopy

About 1 cm of Quartz EPR tubes were filled with irradiated and non-irradiated active SM PLGA microspheres and sealed under vacuum. EPR analysis of active SM PLGA microspheres before and after gamma irradiation was performed at room temperature by using a Bruker EMX electron spin resonance spectrometer with the following parameters: center field, 3,480 G; sweep width, 5,000 G; microwave power, 20.5 mW; mod. amplitude, 1 G; mod. frequency, 100 kHz; time constant, 10.24 ms; and sweep time, 41.94 s.

Differential Scanning Calorimetry (DSC)

The T_g of active SM PLGA microspheres before and after γ-irradiation was measured by a differential scanning calorimeter (Perkin-Elmer® DSC7, Waltham, USA). Microspheres samples (~ 6 mg) were sealed in aluminum hermetic pans and thermograms were determined by first cooling the sample to 0°C, then heating to 100°C at a heating rate of 10°C/min. The startpoint of the second run was used for T_g calculation.

Gel Permeation Chromatography (GPC)

The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of PLGA before and after gamma irradiation was measured by GPC. The Waters 1525 GPC system (Waters, Milford, MA, USA) consisted of two Styragel® columns (HR 1 and HR-5E columns, Waters, Milford, MA, USA) connected in series (4.6 × 300 mm each), a binary HPLC pump, waters 717 plus autosampler, Waters 2414 refractive index detector and Breeze® software to compute molecular weight. Sample solutions in tetrahydrofuran (THF) at a concentration of ~ 5 mg/mL were filtered through a 0.45 μm hydrophobic fluoropore (PTFE) filter (Millipore, Bedford, USA) before 50 μL injection into the GPC system and elution with THF at 0.5 mL/min. The M_w and M_n of each sample were calculated using monodisperse polystyrene standards, M_w 2,330 Da−4.2 MDa.

Scanning Electron Microscopy (SEM)

Surface morphology of active SM PLGA microspheres before and after gamma irradiation, and active self-healing encapsulation was examined by taking SEM images using a Hitachi S3200N scanning electron microscope (Hitachi, Tokyo, Japan). Briefly, microspheres were fixed on a brass stub using double-sided adhesive tape and then were made electrically conductive by coating in a vacuum with a thin layer of gold (3–5 nm) for 100 s at 40 W. The surface view images of microspheres were taken at an excitation voltage of 8 kV.

Active Self-Healing Encapsulation of TT in PLGA Microspheres

Active self-healing encapsulation of TT in $Al(OH)_3$-PLGA-0 or 5 wt% DEP microspheres before and after sterilization was accomplished as described previously (22,40). Briefly, ~20 mg active SM PLGA microspheres were placed into microcentrifuge tubes and then TT solution (0.5 mL of 0.8 mg/mL TT in normal saline (pH 6.8)) was added and mixed. The microcentrifuge tubes were incubated under

constant rotation on a rigged rotator (Glas-Col, Terre Haute, IN, USA) first at 10 and 25°C for 24 h to load TT, and then at 38°C for 40 h to heal the PLGA pores (see Fig. 1). After completion of active self-microencapsulation, the tubes were centrifuged at 8,000 rpm for 10 min and the supernatant was analyzed by a modified Bradford assay to quantify remaining TT. Residual TT encapsulated PLGA microspheres were washed one time with ddH_2O. Washed microspheres were immediately (without freeze-drying) used to conduct *in vitro* TT release or freeze-dried and used to examine the surface morphology of microspheres and perform amino acid analysis.

Modified Bradford Protein Assay

A modified Bradford assay was used to determine the remaining TT in loading solution. Briefly, appropriate volume of standard or sample was mixed with Coomassie Plus® reagent (Thermo Fisher Scientific, Rockford, IL, USA) in a 96-well plate (Nalge Nunc International, Rochester, NY, USA). Then, the absorbance was read at 595 nm within 30 min using a Dynex II MRX microplate reader (Dynex Technology Inc., Chantilly, VA, USA).

Amino Acid Analysis (AAA)

Tetanus toxoid content in self-encapsulated PLGA microspheres was quantified by AAA at the Protein Chemistry Laboratory of Texas A & M University. Briefly, samples (microspheres, standards, and human serum albumin (control) with two internal standards (norvaline and sarcosine respectively for primary and secondary amino acids) were weighed into ampoules and 100 μL of 6 M HCl solution was added, followed by incubation at 110°C for 18 h. Hydrolysates were then taken to dryness and reconstituted in 0.4 N borate buffer to bring the eventual pH to 10 for optimum derivitization and then transferred to the autosampler for automated pre-column derivitization with *o*-phthalaldehyde (OPA) and 9-fluoromethyl-chloroformate (FMOC) and loading. The separation of derivatized amino acids was accomplished on a Hewlett-Packard AminoQuant II chromatography system (Hewlett-Packard Co., Palo Alto, CA, USA) using a Hypersil AA-ODS reverse phase column (2.1× 200 mm, 5 μm; Agilent Technologies, Inc., Santa Clara, CA, USA). Elution of derivatized amino acids was achieved using solvent (A) 20 mM sodium acetate containing 0.018%v/v triethylamine, 0.05 mM EDTA and 0.3%v/v tetrahydrofuran (pH 7.2) and (B) 100 mM sodium acetate: acetonitrile: methanol (20:40:40). The solvent gradient begins at 0 min at 100% A at 0.45 mL/min and goes to 60% B over 17 min. Primary (tagged with OPA) and secondary (tagged with FMOC) amino acids were respectively detected at excitation/emission wavelength of 340/450 and 266/324 nm by a fluorescence detector. The observed peak area for each amino acid was compared to an internal and external standard and the resulting concentration values in nano moles were recorded. Analysis was performed in triplicate for each sample.

Evaluation of *In Vitro* Antigenic TT Release from PLGA Microspheres

After completion of active self-microencapsulation, the residual TT encapsulated PLGA microspheres were washed once with ddH_2O. To the washed TT/PLGA microspheres, 1 mL of release buffer (phosphate buffered saline + 0.02% (v/v) Tween 80 (PBST) + 0.2% BSA, pH 7.4) was added and incubated at 37°C under constant agitation (240 rpm/min). At different incubation times (1, 3, 7, 14, 21, and 28 days), the mixture was centrifuged at 8,000 rpm for 10 min and supernatant was taken out, followed by the addition of fresh medium (1 mL). Analysis of released antigenic TT was performed by enzyme-linked immunosorbent assay.

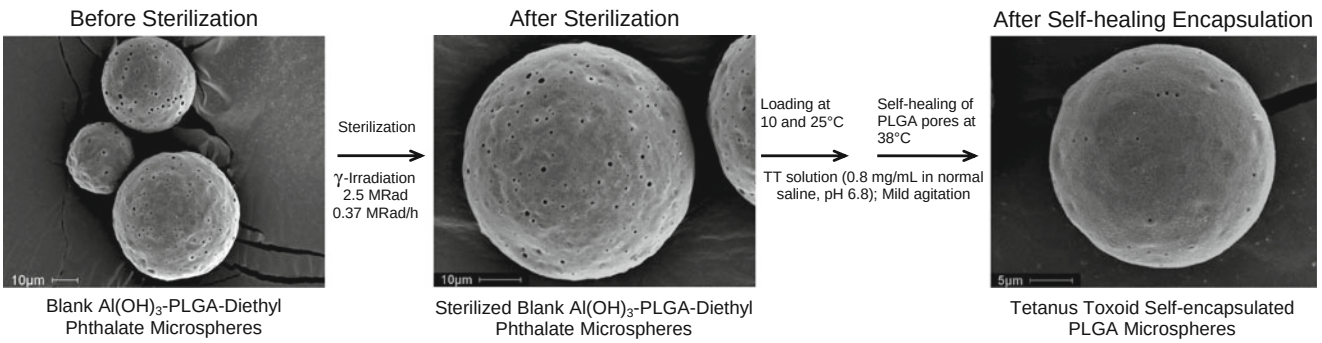


Fig. 1 Effect of γ-irradiation of pre-formed PLGA microspheres on microsphere morphology after self-healing encapsulation of tetanus toxoid (TT). Representative scanning electron microscopic images of 3.2 wt% $Al(OH)_3$−3.5 wt% trehalose-PLGA-5 wt% diethyl phthalate microspheres before sterilization, after sterilization (2.5 MRad dose at 0.37 MRad/h dosing rate) and after self-healing encapsulation of TT.

Enzyme-Linked Immunosorbent Assay (ELISA)

Antigenically active TT was determined by an ELISA (41). Except for the final incubation step with the PNPP liquid substrate, all initial ELISA steps were performed at room temperature. Briefly, 100 μL of 2–3 international units (IU) /mL of equine tetanus antitoxin in PBS (pH 7.2) was added to 96-well microtitration plates (Nalge Nunc International, Rochester, NY, USA) and incubated overnight. The plates were washed 3 times between all steps with PBS containing 0.05% Tween® 20 (pH 7.2). Phosphate blocking buffer (PBB, PBS/0.5% BSA/0.05% Brij® 35, pH 7.4) was used as a diluent for all TT samples and antibodies (except equine tetanus antitoxin above). Standard TT with known concentration and test samples were diluted at 2-fold steps in coated plates using PBB as a diluent. The plates were held for 2 h and washed. Then, 100 μL of human anti-TT IgG (HyperTET^{TM} S/D, 1:5000 dilution) was added and allowed to react for 2 h followed by 100 μL of goat anti-human IgG-alkaline phosphatase diluted 1:20000 in PBB for another 2 h. The plates were washed and 100 μL of PNPP liquid substrate was added. After 30 min incubation at 37°C, the absorbance was read at 405 nm on a Dynex II MRX microplate reader (Dynex Technology Inc., Chantilly, VA, USA) equipped with Revelation 3.2 Software. Log/Logit curve fitting model was used to plot the standard curve and calculate unknown concentration of TT in test samples.

RESULTS AND DISCUSSION

Evaluation of the Feasibility of Sterilization of Active SM PLGA Microspheres Before Antigen Encapsulation

It is apparent from our previous studies (22,40) that three properties of active SM PLGA microspheres are critical for active self-healing encapsulation of vaccine antigens in PLGA microspheres. These include: a) an inter-connected porous network in the polymer phase for penetration of antigen solution so that $Al(OH)_3$ present in PLGA pores could adsorb antigen from surrounding solution, b) adsorption capacity in self-healing PLGA microspheres, and c) self-healing of PLGA pores at modest temperature > T_g of the polymer. Since the active self-healing encapsulation of vaccine antigens occurs after preparing blank microspheres, this new method provides an opportunity to sterilize PLGA microspheres prior to antigen encapsulation. Such sterilization would be desirable for injectable PLGA microsphere formulations, particularly for delivery of vaccine antigens at the point-of-care. In this study, we investigated the influence of γ-irradiation sterilization of active self-microencapsulating PLGA microspheres on the critical attributes of these particles, which include: T_g, molecular weight and pore-closing/self-healing of PLGA, antigen loading capacity and efficiency, and *in vitro* release characteristics of active SM PLGA microspheres (see below). According to the International Organization for Standardization specification (ISO 11137-2: Sterilization of health care products-Radiation-Part 2: Establishing the sterilization dose) (42,43), a gamma irradiation dose of 15 (1.5 MRad) or 25 (2.5 MRad) KGy at room temperature is required to achieve a sterility assurance level of 10^{-6}. In this study, we employed 1.8 and 2.5 MRad doses to evaluate the effect of total dose above this threshold. Dosing rate was evaluated at 0.20 and 0.37 MRad/h.

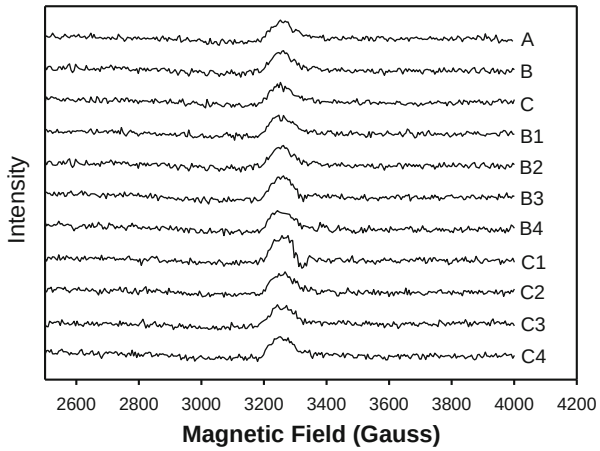
EPR Spectroscopic Analysis of Active SM PLGA Microspheres

It has been documented that γ-irradiation of PLGA produces free radicals in the polymer which in turn convert into peroxidic radicals (24,30). This undesired effect, however, depends upon: a) chemical composition of the polymer system, b) structure of polymer, c) dose and dose rate of radiation, d) sample size, and e) environmental parameters (e.g., presence or absence of oxygen, temperature) (24,30). EPR spectroscopy is a sensitive and specific technique that is commonly used to characterize the formation of free radicals in the polymer. The presence of oxygen in the sample during γ-irradiation leads to formation of free radicals in the polymer (24,30). Therefore, before irradiation of the active SM PLGA microspheres we evacuated and sealed the microspheres in ampoules.

To find out whether sterilization of active SM PLGA microspheres produced any free radicals in the polymer, microspheres before and after γ-irradiation were characterized by EPR spectroscopy. The EPR spectra of active SM PLGA microspheres obtained after γ-irradiation at 1.8 and 2.5 MRad doses and 0.20 and 0.37 MRad/h dose rates was no different from those of non-irradiated samples (see Fig. 2), indicating that sterilization method did not result in residual free radicals in the polymer matrix. This observation is in agreement with a report by Dorati *et al.*(30), who studied γ-irradiation of PEG-d,l-PLA and PEG-PLGA multiblock copolymers.

Effect of γ-Irradiation on T_g and Molecular Weight of PLGA

The interaction of high-energy γ-radiation with PLGA has been described with subsequent effects on polymer chain scission occurring as a random rupturing of bonds, which reduces the molecular weight of polymer (33,44). This scission process has been found to occur typically through free radical formation (24,30,33,36,44). Since the polymer chain



A: Control (no microspheres)

Before irradiation
B: 0 wt% DEP microspheres
C: 5 wt% DEP microspheres

After irradiation

	Dose (MRad)	Dose Rate (MRad/h)
B1 & C1	2.5	0.37
B2 & C2	1.8	0.37
B3 & C3	2.5	0.20
B4 & C4	1.8	0.20

Fig. 2 Effect of γ-irradiation on EPR spectra of 0 and 5 wt% diethyl phthalate (DEP) loaded 3.2 wt% $Al(OH)_3$−3.5 wt% trehalose-PLGA microspheres. A: Control (no microspheres). B: 0 wt% DEP microspheres before irradiation. C: 5 wt% DEP microspheres before irradiation. B1–B4: 0 wt% DEP microspheres after irradiation at 2.5 and 1.8 MRad doses and 0.37 and 0.20 MRad/h dose rates. C1–C4: 5 wt% DEP microspheres after irradiation at 2.5 and 1.8 MRad doses and 0.37 and 0.20 MRad/h dose rates.

stability and mobility is critical in self-healing of PLGA pores, we investigated the γ-irradiation sterilization effect on the molecular weight and T_g of PLGA (see Table I). The γ-irradiation of DEP-free $Al(OH)_3$-PLGA microspheres had little effect on the molecular weight of PLGA. For example, the M_w of PLGA before and after γ-irradiation at 1.8 and 2.5 MRad doses and 0.20 and 0.37 MRad/h dose rates was 53.8 and 52.2–53.1 kDa, respectively. Further, the polydispersity index before and after γ-irradiation was found to be 2.2 and 2.1–2.3, respectively (see Table I). These results strongly suggest that sterilization did not induce significant random PLGA chain scission in the absence of hydrophobic plasticizer. In the case of 5 wt% DEP-loaded $Al(OH)_3$-PLGA microspheres, the molecular weight of PLGA was slightly reduced when irradiated at 0.20 MRad/h dose rate (M_w of PLGA before and after γ-irradiation at 0.20 MRad/h dose rate was 51.3 and 42.5–46.7 kDa, respectively) (see Table I). The polymer molecular weight decline at 0.20 MRad/h was further influenced by total irradiation dose as was the polydispersity index increased (from 2.5 to 3.2–3.8). These results strongly suggest mild PLGA scission when DEP-loaded PLGA microspheres were irradiated at the lower 0.2 MRad/h dose rate. It is noteworthy to mention here that the hydrophobic plasticizer (DEP) was co-incorporated in PLGA to overcome PLGA self-healing suppression induced by $Al(OH)_3$ (to improve polymer chain mobility by reducing the T_g) and facilitate self-healing of PLGA pores at modest temperature (37/38° C) (22,40). At the higher irradiation dose rate, the polymer molecular weight reduction was again marginal (M_w of PLGA before and after γ-irradiation at 0.37 MRad/h dose rate was 51.3 and 49.3–50.7 kDa, respectively), suggesting that the higher dose rate may be suitable for optimal microsphere performance. Interestingly, γ-irradiation of both the active SM PLGA formulations (0 and 5 wt% DEP loaded $Al(OH)_3$-PLGA microspheres) did significantly affect the T_g of the dry polymer (see Table I).

Effect of γ-Irradiation of Active SM PLGA Microspheres on Pre-encapsulation Morphology of Microspheres

The effect of sterilization of blank active SM PLGA microspheres (0 and 5 wt% DEP loaded $Al(OH)_3$-PLGA microspheres) by γ-irradiation on the morphology of microspheres before active self-encapsulation is shown in Fig. 3. γ-irradiation at 2.5 and 1.8 MRad doses and 0.37 and 0.20 MRad/h dose rates appear to have indistinguishable effect on initial PLGA pores (absence of noticeable pore-closing) in the polymeric matrix of DEP-free $Al(OH)_3$-PLGA microspheres. Similarly, initial pores in the polymeric matrix of 5 wt% DEP loaded $Al(OH)_3$-PLGA microspheres also appear to be unaltered when these microspheres were sterilized at the 0.37 MRad/h dose rate. However, noticeable initial pores were "healed" or "closed" when DEP-loaded $Al(OH)_3$-PLGA microspheres were irradiated at 0.20 MRad/h, indicating that the polymer chains were undesirably mobilized during irradiation exposure. Note that the M_w of PLGA was also reduced at the 0.20 MRad/h dose rate. Therefore, 0.37 MRad/h was found to be an acceptable dose rate to avoid pre-encapsulation pore-closing in hydrophobic plasticizer-loaded active SM PLGA microspheres.

Effect of γ-Irradiation of Active SM PLGA Microspheres on the Kinetics of Active Self-Encapsulation

Crystallinity and surface area of $Al(OH)_3$ are inter-related key factors that govern the adsorption of antigen/protein to $Al(OH)_3$ (45–47). A change in these physical properties has been found to reduce antigen/protein adsorption capacity

Table I Effect of γ-Irradiation of Blank Active SM PLGA Microspheres on Weight-Average Molecular Weight (M_w), Number-Average Molecular Weight (M_n), Polydispersity Index (PI), and Glass Transition Temperature (T_g) of PLGA

Formulation			M_w (kDa)*	M_n (kDa)*	PI*	T_g (°C)
Before irradiation						
− DEP			53.8 ± 0.8	24.9 ± 1.1	2.2 ± 0.1	42.8
+ DEP			51.3 ± 0.7	20.8 ± 1.4	2.5 ± 0.1	38.1
After irradiation						
	Dose (MRad)	Dose rate (MRad/h)				
− DEP	2.5	0.370	52.8 ± 0.5	24.4 ± 1.5	2.2 ± 0.2	41.5
	1.8	0.370	53.1 ± 0.4	25.6 ± 1.1	2.1 ± 0.1	42.1
	2.5	0.20	52.3 ± 0.7	22.7 ± 2.2	2.3 ± 0.2	42.2
	1.8	0.20	52.7 ± 0.5	23.6 ± 0.5	2.2 ± 0.1	42.1
+ DEP	2.5	0.370	49.4 ± 0.4	19.3 ± 2.3	2.6 ± 0.3	38.4
	1.8	0.370	50.7 ± 0.8	21.5 ± 1.5	2.4 ± 0.2	38.3
	2.5	0.20	46.8 ± 1.1	12.8 ± 2.6	3.8 ± 0.7	38.2
	1.8	0.20	42.6 ± 1.3	13.5 ± 2.1	3.2 ± 0.6	38.5

− DEP 3.2 wt% $Al(OH)_3$/3.5 wt% trehalose/0 wt% diethyl phthalate (DEP)/PLGA microspheres

+ *DEP* 3.2 wt% $Al(OH)_3$/3.5% trehalose/5 wt% DEP/PLGA microspheres

PI polydispersity index = M_w/M_n; * Mean ± SEM, $n = 2$

of $Al(OH)_3$ (45–47). Thermal and other stresses, and pressure may affect the crystallinity of $Al(OH)_3$ (45–47). In order to obtain optimal active loading of vaccine antigen after γ-irradiation sterilization of active SM PLGA microspheres, it is critical that antigen sorbing capacity of $Al(OH)_3$ adjuvant is retained after exposure to γ-rays for a considerable duration. Therefore, we investigated the effect of γ-irradiation sterilization on active antigen loading characteristics of 0 and 5 wt% DEP loaded $Al(OH)_3$-PLGA microspheres. The adsorption capacity of $Al(OH)_3$ was not adversely affected by γ-irradiation sterilization. For example, TT loading capacity of 0 and 5 wt% DEP loaded 3.2 wt% $Al(OH)_3$-PLGA microspheres before and after γ-irradiation at 1.8 and 2.5 MRad doses and 0.20 and 0.37 MRad/h dose rates was found to be between 1.4–1.6 and 1.5–1.7 wt%, respectively (see Table II). A similar finding was also observed with encapsulation efficiency of both the active SM microsphere formulations.

Effect of γ-Irradiation of Active SM PLGA Microspheres on Post-encapsulation Morphology of Microspheres

The influence of γ-irradiation of 0 and 5 wt% DEP loaded $Al(OH)_3$-PLGA microspheres at 1.8 and 2.5 MRad doses and 0.20 and 0.37 MRad/h dose rates on post active self-encapsulation morphology of microspheres is shown in Fig. 4. There was no noticeable change in self-healing of PLGA pores in the polymeric matrix of plasticizer-free

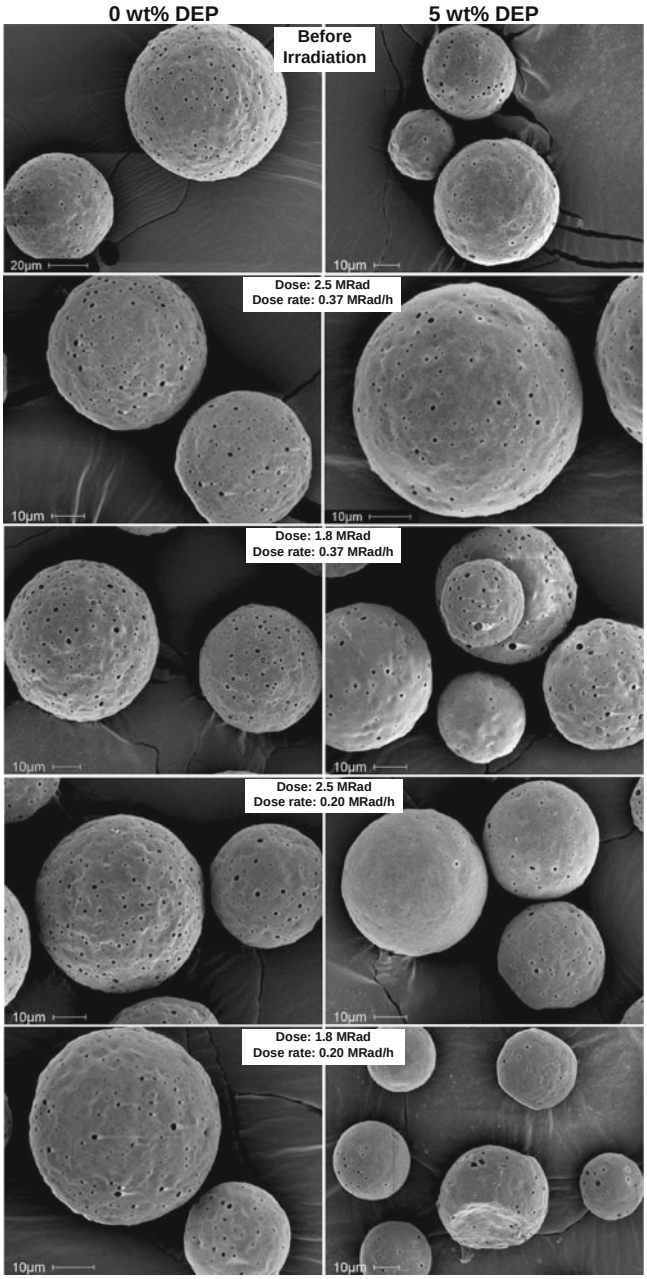


Fig. 3 Effect of γ-irradiation of 0 and 5 wt% diethyl phthalate (DEP) loaded 3.2 wt% $Al(OH)_3$−3.5 wt% trehalose-PLGA microspheres at 2.5 and 1.8 MRad doses and 0.37 and 0.20 MRad/h dose rates on pre-encapsulation morphology of microspheres. Scanning electron microscopic image of 0 and 5 wt% DEP loaded blank $Al(OH)_3$-PLGA microspheres before and after γ-irradiation.

$Al(OH)_3$-PLGA microspheres after γ-irradiation at both doses and dose rates. In the case of 5 wt% DEP loaded $Al(OH)_3$-PLGA microspheres, PLGA pore-closing/self-healing after γ-irradiation at 0.37 MRad/h was not significantly affected. However, some pores were visible in the polymeric matrix of 5 wt% DEP loaded $Al(OH)_3$-PLGA microspheres irradiated at the lower 0.20 MRad/h dose rate. It is possible that this slight change in PLGA pore-

Table II Effect of γ-Irradiation of Pre-formed PLGA Microspheres on Active Self-Healing Microencapsulation of TT

		Initial TT mass[a] (μg)	Remaining TT mass (μg)*	TT loaded (μg)* based on		TT loading (wt%)* based on		EE (%)* based on	
				Mass loss from LS[b]	Polymer content[c]	Mass loss from LS[b]	Polymer content[c]	Mass loss from LS[b]	Polymer content[c]
Before irradiation									
− DEP[d]		400	53 ± 2	347 ± 2		1.60 ± 0.02	–	86.6 ± 0.5	
+ DEP[d]		400	71 ± 3	330 ± 3	308 ± 43	1.50 ± 0.01	1.4 ± 0.2	82.5 ± 0.6	77.0 ± 10.7
After irradiation									
	Dose (MRad)	Dose rate (MRad/h)							
− DEP	2.5	0.370	400	53 ± 3	347 ± 2		1.60 ± 0.03	–	86.8 ± 0.3
	1.8	0.370	400	52 ± 1	348 ± 1		1.70 ± 0.02	–	87.1 ± 0.3
	2.5	0.20	400	51 ± 3	349 ± 3		1.70 ± 0.03	–	87.1 ± 0.7
	1.8	0.20	400	53 ± 1	347 ± 1		1.60 ± 0.03	–	86.8 ± 0.2
+ DEP	2.5	0.370	400	78 ± 2	322 ± 2	349.3 ± 62.5	1.50 ± 0.04	1.6 ± 0.3	80.6 ± 0.3	87.3 ± 15.6
	1.8	0.370	400	54 ± 2	346 ± 2		1.60 ± 0.04	–	86.5 ± 0.5	
	2.5	0.20	400	67 ± 5	333 ± 5		1.50 ± 0.04	–	83.4 ± 1.2	
	1.8	0.20	400	82 ± 1	318 ± 1		1.50 ± 0.02	–	79.5 ± 0.2	

LS loading solution; [a] volume = 0.5 mL; *TT* tetanus toxoid; * Mean ± SEM, $n = 3$; [b] determined by modified Bradford assay; [c] determined by amino acid analysis; *EE* encapsulation efficiency; [d] − DEP: 3.2 wt% $Al(OH)_3$/3.5 wt% trehalose/0 wt% diethyl phthalate (DEP)/PLGA microspheres, and + DEP: 3.2 wt% $Al(OH)_3$/3.5% trehalose/5 wt% DEP/PLGA microspheres

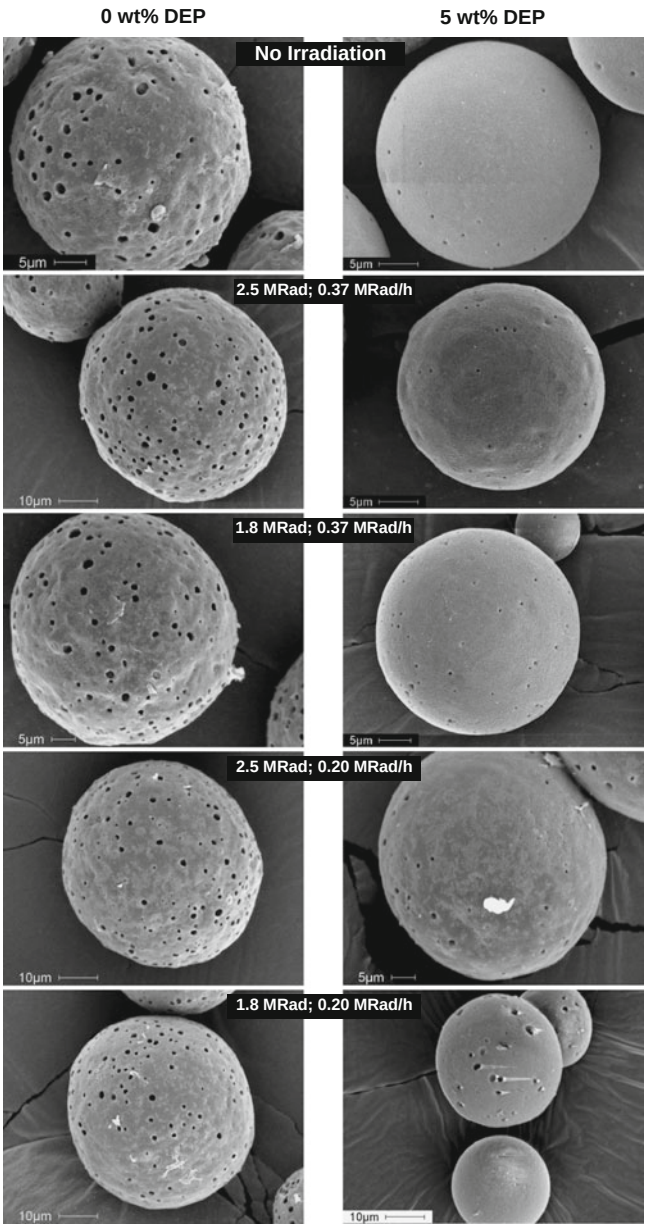


Fig. 4 Effect of γ-irradiation of 0 and 5 wt% diethyl phthalate (DEP) loaded 3.2 wt% $Al(OH)_3$−3.5 wt% trehalose-PLGA microspheres at 2.5 and 1.8 MRad doses and 0.37 and 0.20 MRad/h dose rates on post-encapsulation morphology of microspheres. Scanning electron microscopic image of non-irradiated and irradiated $Al(OH)_3$-PLGA-0 and 5 wt% DEP microspheres after active self-healing encapsulation of tetanus toxoid.

closing/self-healing may have been caused by increased polymer chain mobility/reduced chain entanglement, which could be due to a decline in M_w observed after γ-irradiation at 0.20 MRad/h.

Effect of γ-Irradiation of Blank Active SM PLGA Microspheres on *In Vitro* Antigenic TT Release

A key drawback of terminal sterilization of finished PLGA microspheres is that stability of both encapsulated

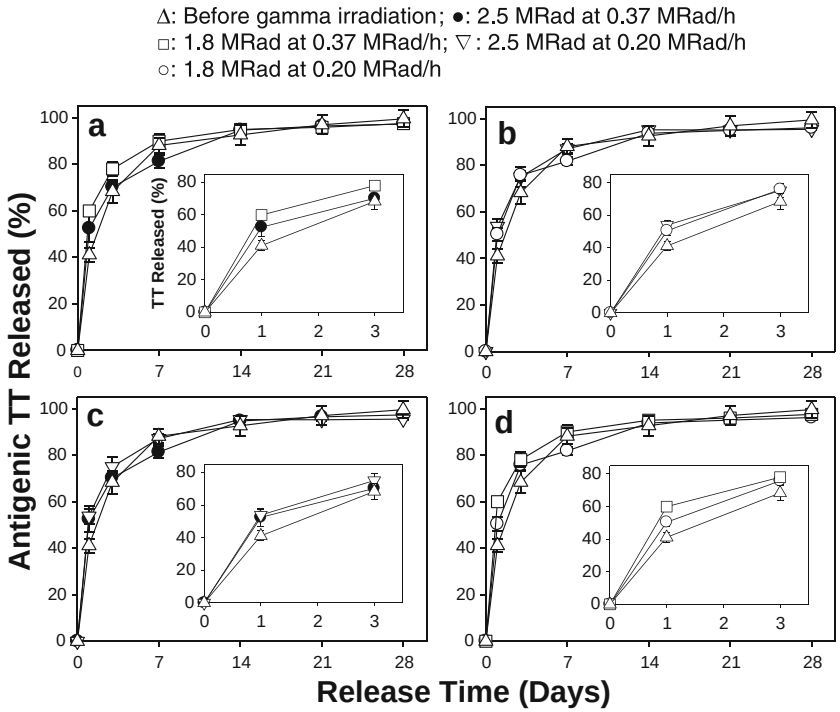


Fig. 5 Effect of pre-encapsulation γ-irradiation of 3.2 wt% $Al(OH)_3$−3.5 wt% trehalose-PLGA-0 wt% diethyl phthalate microspheres at 2.5 and 1.8 MRad doses and 0.37 and 0.20 MRad/h dose rates on *in vitro* release of tetanus toxoid (TT). Cumulative amount of antigenic TT released as a function of incubation time from self-encapsulated PLGA microspheres. (**a** and **b**)—Effect of irradiation dose at fixed dose rates on TT release. (**c** and **d**)—Effect of irradiation dose rate at fixed doses on TT release. Average TT loading (determined on the basis of TT mass loss from loading solution) in all the self-encapsulated PLGA microsphere formulations was found to be 1.6–1.7 wt%. *In vitro* release was conducted in PBST + 0.2% BSA at 37°C. Symbols represent mean ± SEM, $n = 3$.

macromolecule and polymer is adversely affected by the γ-irradiation (15,23,25,26,31,44,48). As a result, a significant change in release characteristics of PLGA microspheres has been observed. For example, terminal sterilization of PLGA microspheres containing macromolecules such as TT, ovalbumin or insulin-like growth factor-I by γ-irradiation has been found to adversely affect release of these macromolecules (15,25,31). The influence of γ-irradiation of 0 and 5 wt% DEP loaded $Al(OH)_3$-PLGA microspheres at 1.8 and 2.5 MRad doses and 0.20 and 0.37 MRad/h dose rates on *in vitro* antigenic TT release is shown in Figs. 5 and 6, respectively. There was little difference in TT release from plasticizer-free $Al(OH)_3$-PLGA microspheres before and after γ-irradiation at both the doses and dose rates (see Fig. 5); only a slightly higher initial burst was found in the irradiated samples. In the case of 5 wt% DEP-loaded $Al(OH)_3$-PLGA

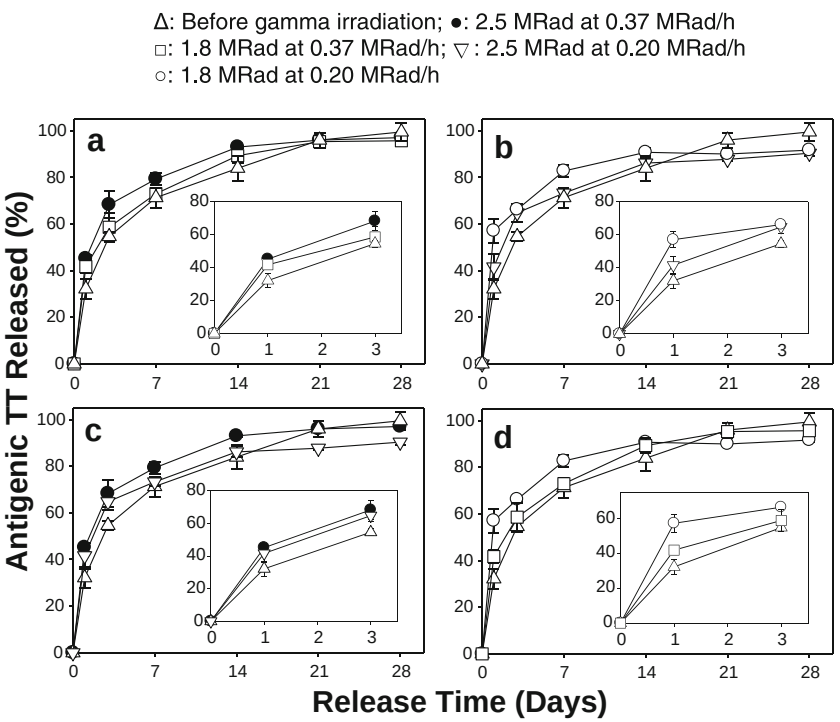


Fig. 6 Effect of pre-encapsulation γ-irradiation of 3.2 wt% $Al(OH)_3$−3.5 wt% trehalose-PLGA-5 wt% diethyl phthalate microspheres at 2.5 and 1.8 MRad doses and 0.37 and 0.20 MRad/h dose rates on *in vitro* release of tetanus toxoid (TT). Cumulative antigenic TT released as a function of incubation time from self-encapsulated PLGA microspheres. (**a** and **b**) Effect of irradiation dose at fixed dose rates on TT release. (**c** and **d**) Effect of irradiation dose rate at fixed doses on TT release. Average TT loading (determined on the basis of TT mass loss from loading solution) in all the self-encapsulated PLGA microsphere formulations was found to be 1.5–1.6 wt%. *In vitro* release was conducted in PBST + 0.2% BSA at 37°C. Symbols represent mean ± SEM, $n = 3$.

microspheres, γ-irradiation at 2.5 and 1.8 MRad dose respectively at 0.37 and 0.20 MRad/h dose rate exhibited slightly higher antigen release over a period of 7 days relative to non-irradiated microspheres (see Fig. 6). As reported before (22,40), the initial burst release was reduced in the plasticizer-containing microspheres (i.e., Fig. 6 *vs.* Fig. 5 without plasticizer) owing to the reduction in PLGA T_g and improved healing of pores during encapsulation. Importantly, slow and nearly complete release of antigenic TT over the 1 month release period was exhibited by sterilized $Al(OH)_3$-PLGA microspheres.

CONCLUSIONS

The objective of this study was to evaluate the effect of sterilization of active SM PLGA microspheres on the key attributes of these microspheres. With optimal gamma irradiation conditions, sterilization of blank active SM PLGA microspheres and effective active self-healing encapsulation was achieved in the current study without significantly or adversely affecting antigen sorbing capacity of $Al(OH)_3$, T_g, molecular weight and self-healing of PLGA, and stability and release of antigen. This potential feasibility may obviate any γ-irradiation related antigen instability and poor controlled-release behavior. This also opens-up the possibility to achieve encapsulation of vaccine antigen in a single sterile syringe by simple of mixing of sterile active SM PLGA microspheres and antigen solution at the point-of-care. Further studies are in progress to: a) investigate other protein trapping agents; b) develop active self-healing PLGA encapsulation method for other important therapeutic proteins; c) optimize the stability and long-term release kinetics; and d) expand the method to other carriers.

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