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Supporting Information

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Self-Healing Microencapsulation of Biomacromolecules without Organic Solvents**

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Supporting Information

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Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA) 50:50 with an i.v. = 0.20 dl/g in hexaflouro isopropanol at 30ºC (Lot # A07-044, end-group = 1-dodecyl ester, weight average molecular weight (M_w) = 11 kDa) of Lactel Absorbable Polymers was purchased from DURECT Corporation (Cupertino, CA, USA). PLGA 50:50 with an i.v. = 0.19 dl/g in chloroform at 30 ºC (Lot # 1158-515, end-group = methyl ester, M_w = 19 kDa) and 0.57 dl/g in hexafluoroisopropanol at 30 ºC (Lot # W3066-603, end-group = lauryl ester, M_w = 51 kDa) were purchased from Lakeshore Biomaterials (Birmingham, AL, USA). α,α-Trehalose dihydrate was purchased from Pfanstiehl (Waukegon, IL, USA), zinc carbonate ($ZnCO_3$) was from ICN Biomedicals Inc., and 7-methoxycoumarin-3-carbonyl azide was from Invitrogen Corporation (Carlsbad, CA, USA). Tetrahydrofuron (THF) was purchased from Fisher-Scientific (Pittsburgh, PA, USA). Tetramethyl-rhodamine (TMR)-dextran was purchased from Invitrogen Corporation (Carlsbad, CA, USA). Tetanus toxoid (TT) (3120 Lf/mL) and equine tetanus antitoxin were received as gift samples respectively from Serum Institute of India Ltd. (Pune, India) and U.S. Food and Drug Administration (Silver Spring, USA). Human tetanus immune globulin (HyperTET^{TM} S/D, 250 units) was purchased from Talecris Biotherapeutics, Inc. (Research Triangle Park, NC, USA). Poly(vinyl alcohol) (PVA) (9-10 kDa, 80% mol hydrolyzed), Alhydrogel® aluminum hydroxide adjuvant gel ($Al(OH)_3$), bovine serum albumin (BSA) fraction V, ovalbumin (OVA), lysozyme from chicken egg white, sodium hydroxide (NaOH), magnesium carbonate ($MgCO_3$), BSA labeled with fluorescein isothiocyanate (BSA-FITC), sodium citrate, p-nitrophenyl phosphate liquid substrate, and phthaldialdehyde reagent (containing 1 mg o-phthaldialdehyde per mL solution of 2-mercaptoethanol as the sulfhydryl moiety) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Leuprolide acetate (Batch # 091203) was purchased from Shanghai Shjnj Modern Pharmaceutical Technology Co., Ltd (Shanghai, China). Male Sprague-Dawley rats were procured from Charles River Laboratories. Isoflurane was purchased from Baxter Healthcare Corporation (Deerfield, IL, USA). B-D Microtainer® blood collection and serum separation tubes were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Goat anti-human IgG-alkaline phosphatase was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). All other common salts, reagents, and solvents were purchased from Sigma-Aldrich. Size exclusion-high performance liquid chromatography (SE-HPLC) columns (TSK gel G3000SWxl and TSK gel G2000SWxl columns, Tosoh Biosciences LLC, Montgomeryville, PA, USA), Shodex Protein KW-G size exclusion chromatography guard column (Showa Denko, New York, USA), Nova-Pak C18 column (4 µm, 3.9 x 150 mm) and Bonda-Pak C18 guard column (4 µm) (Waters Corporation, Milford, MA, USA) were used.

Methods

Preparation of traditionally protein encapsulated (TM-1) and self-healing (SM) PLGA microspheres. TM-1 and blank SM PLGA microspheres were prepared by a double (w/o/w) emulsion method (see Table S1 for specific details). Briefly, initial water phase was added to the polymer solution (PLGA in 1 mL methylene chloride) and immediately homogenized (first homogenization) using a Tempest IQ^2 homogenizer (The VirTis Company) equipped with a 10 mm shaft in an ice water bath to create the first emulsion. Two mL of aqueous 5% polyvinyl alcohol (PVA) solution was added to the first emulsion, vortexed or homogenized, and the resulting emulsion was injected into 100 mL of chilled (ASM microspheres) or non-chilled (TM-1 and SM-1 to SM-5 microspheres) 0.5% PVA solution under continuous stirring. Microspheres were stirred 3 h at room temperature, and collected with sieves to

separate by size (20-63 and 63-90 µm) and washed thoroughly with distilled and deionized water (ddH_2O) to help remove residual PVA. Collected microspheres were then freeze-dried using a freeze drier (Labconco Corporation) and stored at -20 °C until further use.

***Conjugation of BSA to a pH-insensitive fluorescent coumarin*.** About 2 mL of 10 mg/mL 7-methoxycoumarin-3-carbonyl azide in dimethyl sulfoxide was added to BSA solution (1.2 g BSA in 40 mL of 0.2 M sodium bicarbonate (pH 4.5)) while stirring. After stirring the solution for 90 min in darkness, quenching of reaction was done by adding 4 mL of 1.5 M hydroxylamine hydrochloride. The solution was then extensively dialyzed using a 25,000 Da M_w cut-off membrane against degassed distilled water at 4°C.

Passive and active self-healing microencapsulation of biomacromolecules (specific details in Table S2). Blank SM PLGA microspheres of 20-63 µm size were incubated with polysaccharide/enzyme/protein/peptide solution on a rocking platform (VWR Scientific) (SM-1 to SM-5) and OVA/TT solution on a rigged rotator (ASM) for a specified period of time to load large molecules in polymer pores. Microspheres were then incubated at self-healing temperature for a specified duration on a rigged rotator to heal the polymer pores, followed by washing of microspheres for 10 times (SM-1 to SM-5) or one time (ASM) with ddH_2O. Washed microspheres were either freeze-dried and used (SM-1 to SM-5) or used without drying (ASM) for further studies.

Analysis of biomacromolecules. Protein concentrations were assessed using a modified Bradford assay method.[19] In case of SE-HPLC, samples were injected into the TSK Gel G3000SWxl column (7.8 mm i.d. x 30 cm long) column (Tosoh Biosciences LLC) and eluted by 0.05 M potassium phosphate containing 0.2 M NaCl (pH 7.0) (lysozyme and OVA) or PBS (pH 7.4) (BSA, dextran-FITC, and BSA-Coumarin) at a flow rate of 0.9 (lysozyme, BSA and BSA-Coumarin) or 0.7 (OVA) mL/min. UV detection at 215 and 280 nm and fluorescence detection with excitation and emission wavelengths of 278 nm and 350 nm for BSA, OVA and lysozyme, 490 nm and 520 nm for dextran-FITC, and 384 nm and 480 nm for BSA-Coumarin were used. RP-HPLC analysis of leuprolide acetate was accomplished by previously reported method with slight modifications.[2b] A gradient elution of acetonitrile (solvent A) and 0.05 M sodium phosphate buffer, pH 7.0 (solvent B) was used in the following manner: 0 min (20% A), 6 min (30% A), 9.5 min (37% A), 11.5 min (37% A), 16.5 min (50% A), and 19 min (20% A), followed by a 2 min recovery. Determination of total and soluble lysozyme content was performed by AAA (detailed procedure is given below). ELISA[S1] with slight modifications was used to determine antigenic TT released from the microspheres. Final step of ELISA was performed by adding p-nitrophenyl phosphate liquid substrate (Sigma-Aldrich) and incubating 96-well plate at 37 °C before measuring the absorbance at 405 nm on a Dynex II MRX microplate reader (Dynex Technology Inc.) equipped with Revelation 4.21 Software.

Determination of soluble and insoluble lysozyme loading by amino acid analysis (AAA). Determination of total and soluble lysozyme content in microspheres and soluble lysozyme content in other solution-based samples was performed by AAA. Briefly, microspheres (~ 4 mg), soluble protein solutions, and standard samples were weighed into clear glass ampoules in a total volume of 1.5 ml 6 N HCl. Ampoules were then sealed under light vacuum and incubated at 110 °C for 25 h. The hydrolyzate from each vial was completely emptied into microcentrifuge tubes and each vial was rinsed with 250 µL water and emptied into respective microcentrifuge tube. The resulting solution was then evaporated under vacuum at room temperature. About 1 mL of 1.0 M sodium bicarbonate buffer (pH 9.5) was weighed

into each tube to neutralize the remaining acid. For individual amino acid analysis, a weighed amount of 350 µL of hydrolyzed protein solution and 350 µL of o-phthaldialdehyde reagent solution were combined in a microcentrifuge tube, vortexed for 15 s, and immediately injected onto a C18 column fitted with a guard column (total time from mixing to injection < 1 min).[S2] Samples were eluted (gradient elution) with mobile phases of methanol:water (65:35) (A) and methanol:THF:50 mM phosphoric acid (20:20:960) (titrated to pH 7.5 with 10 N NaOH) (B) at a flow rate of 1.4 mL/min. Gradient elution conditions were 40% A for 0.5 min, 17 min concave gradient to 50% A, 15 min linear gradient to 100% A, 5 min isocratic elution with 100% A, 7.5 min linear gradient to 40% A, and isocratic 40% A for 5 min. Amino acids were detected by measuring fluorescence respectively at an excitation and emission wavelength of 350 and 455 nm. Protein and standards were quantified using the average of the 3 individual amino acids standards found to be stable under hydrolysis conditions: alanine, phenylalanine, and lysine.

Determination of biomacromolecule loading in PLGA microspheres. Extraction of protein or dextran-FITC from the microspheres after removing the polymer was accomplished as described previously.[19] The recovered protein/dextran-FITC was then dissolved in 10 mM potassium phosphate buffer, pH 7.0 (lysozyme) or PBS, pH 7.4 (BSA, dextran-FITC, and BSA-Coumarin). Determination of soluble protein fraction/ dextran-FITC and insoluble protein fraction were performed by SE-HPLC and modified Bradford assay, respectively. Two-phase extraction[2b] and RP-HPLC was employed for leuprolide acetate. To monitor active protein (OVA or TT) loading in $Al(OH)_3$-PLGA microspheres (ASM), analysis of remaining protein in loading solution as a function of time and temperature of incubation was performed by modified Bradford assay. In order to determine OVA content in $Al(OH)_3$-PLGA microspheres, removal of polymer[19] was performed using ethyl acetate. One milliliter of 190 mM sodium citrate was added to the residue, mixed thoroughly, and incubated at 37 ºC for 3 days with constant agitation to elute OVA from $Al(OH)_3$ completely. Fraction of OVA monomer and insoluble aggregates were analyzed by SE-HPLC and modified Bradford assay, respectively.

Scanning electron microscopy (SEM). Surface morphology of microspheres was examined by taking SEM images using a Hitachi S3200N scanning electron microscope (Hitachi, Tokyo, Japan). Briefly, microspheres were fixed previously 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 (approximately 3 to 5 nm) for 60 s at 40 W. The surface view images of microspheres were taken at an excitation voltage of 5-10 kV.

Confocal microscopy. Distribution of self-healing microencapsulated protein in the PLGA microspheres was observed by taking confocal images of BSA-coumarin loaded PLGA microspheres. Briefly, 1 mg of BSA-coumarin loaded PLGA microspheres were suspended in 80 µL of water. A droplet of this suspension was placed on a clean glass slide, a glass cover slip placed on top of the droplet, and excess of water was removed. Samples were imaged at an excitation/emission wavelength of 384/480 nm using a confocal microscope (Olympus America Inc., Center Valley, PA, USA).

Determination of polymer matrix porosity of SM microencapsulating microspheres. Measurement of polymer matrix porosity of blank SM PLGA microspheres was done by Porous Materials, Inc. (Ithaca, NY, USA) using an AMP-60K-A-1 mercury porosimeter, generating pore volume versus pressure data. The pore volume was reported as volume per gram microspheres (cc/g). Total microsphere volume was calculated as the sum of the pore

volume and the polymer volume, where the polymer density (1.25 g/cc for 51 kDa PLGA 50:50, provided by manufacturer) and weight of the porosimetry sample were used to calculate the pore volume. Percent porosity was calculated from the fractional pore volume of the microspheres. Pressure associated with microspheres' packing and surface wetting, before mercury intrusion into the pores had taken place, was not calculated into the final pore volume as has been reported previously.[S3] The method of determination of porosity utilized large amount of microspheres sample (~250 mg) and there was no significant difference (p>0.05) among the different measurements (three measurements) of the same formulation. Hence, only one test was run for the measurement of polymer matrix porosity of various SM microspheres formulations.

Determination of lysozyme activity. Lysozyme was extracted from PLGA microspheres as per the method described in loading assay and the enzyme was reconstituted in PBST (pH 7.4). For analysis, 0.15 mL of soluble lysozyme solution was combined with 0.15 mL of 1.5 mg/mL *Micrococcus lysodeikticus* (prepared in 1X PBS, pH 7.4) and the absorbance at 450 nm was monitored every 30 s for a period of 5 min. The activity was calculated using the decrease in absorbance in the linear region (between 0.5 and 3.0 min) assuming one unit of enzyme activity reduces the ΔA_{450nm} by 0.001/min. Specific activity is defined in units of activity per mg of protein and is given as % of the specific activity of the native, standard lysozyme. The actual amount of soluble monomeric lysozyme in the solution was determined via SE-HPLC and was used for the specific activity calculations.

Kinetics of self-healing microencapsulation. About 50 mg of SM PLGA 50:50 (M_w = 51 kDa) microspheres were placed into separate tubes of 65 mg/mL dextran-FITC (10,000 M_w). Microspheres were incubated at 4 °C for 20 h, and then transferred to 42.5 °C for 72 h, with approximately 10% of microspheres removed at preset time points while replacing the volume post sampling with fresh dextran-FITC solutions. Sample microspheres were washed 10-fold with distilled water, with centrifugation at 3200 rpm for 10 min to collect the microspheres after each wash. The dextran-FITC was extracted using acetone to dissolve the PLGA and concentrating the insoluble dextran-FITC using centrifugation (10,000 rpm at 10 min), and repeating 3-fold. Dextran-FITC was dissolved in PBS, pH 7.4, and loading was determined via HPLC with fluorescence (without column separation) using 20 or 40 μL injection volume and a 1 mL/min PBS, pH 7.4 mobile phase. The fluorescence of the dextran-FITC was measured respectively at an excitation and emission wavelength of 490 and 520 nm.

Assessment of in vitro biomacromolecule release from PLGA microspheres. Studies were conducted by incubating 4-20 mg microspheres in either phosphate buffered saline (PBS) + 0.02% Tween 80 (PBST, pH 7.4)[19] (BSA, lysozyme, and leuprolide acetate release) or both PBS and 190 mM sodium citrate (OVA release) or PBST + 0.2% BSA[S1] (TT release) at 37°C under constant agitation (100 rpm/min). Fraction released vs. incubation time was calculated by quantifying either the amount released into the release medium (BSA, lysozyme, TT, and OVA) or remaining in the polymer (leuprolide acetate).

Evaluation of in vivo testosterone suppression ability of leuprolide acetate microencapsulated in self-healing PLGA microspheres. The treatment of experimental animals was in accordance with University of Michigan animal care guidelines, and all NIH guidelines for the care and use of laboratory animals were observed. Study was conducted in male Sprague–Dawley rats according to the reported method[2b,18] with slight modifications. Animals were anesthetized with 2-4% isoflurane and subcutaneously injected with leuprolide

acetate microencapsulated by self-healing (1X 2-month dose), leuprolide acetate solution (1X 1-month dose), and blank SM PLGA microspheres without drug (1X dose), and commercial 1-month Lupron Depot® (Abbott Laboratories) (2X dosing at days 0 and 28) were (6 animals/study group). Average body weight of 425 g at midpoint (day 28) of the study was used for leuprolide acetate dose (100 µg/kg/day) calculation. Blood samples were collected via jugular vein stick before (day -7 and 0 for baseline testosterone level) and after (every week) injection of preparations and the serum testosterone levels were assayed by radioimmunoassay using a TESTOSTERONE Double Antibody-125I RIA Kit (MP Biomedicals LLC.). Lowest detection limit of testosterone was 0.1 ng/mL. In case of samples which exhibited testosterone level below the detection limit, a 0.1 ng/mL value was used for statistical evaluation and plotting the curve.

Sterilization of active self-healing microencapsulating (ASM) ($Al(OH)_3$-PLGA) PLGA microspheres with gamma irradiation. ASM PLGA microspheres were irradiated by using ^{60}Co as irradiation source (Michigan Memorial Phoenix Project, University of Michigan) at 2.5 MRad dose and 0.37 MRad/h dose rate. Briefly, about 250 mg ASM PLGA microspheres were freeze-dried, placed in 5-mL ampoules and then ampoules were sealed under vacuum. All the samples were irradiated at room temperature.

Statistical Analysis. The results are expressed as mean ± standard error of mean (s.e.m.) (n = 3 or 5 or 6). An unpaired Student's t-test was used to assess statistical significance between numerous SM PLGA microsphere formulations with respect to polymer porosity, protein and peptide loading, stability, and in vitro release, and in vivo testosterone level. Results were considered statistically significant if $p<0.05$.

Table S1. Formulation conditions for preparing traditional (lysozyme encapsulated) and various types of self-healing microencapsulating PLGA (50:50) microspheres.

Formulation code	Initial water phase (WP) composition	PLGA 50:50		WP volume (µL)	First homog.	Second homog./ vortexing
		M_w (kDa)	Conc.(mg polymer in 1 mL CH_2Cl_2) + excipient			
SM-1	500 mg trehalose in 1 g PBS	51	320	175	20,000 rpm for 1.5 min	Homog. at 6000 rpm for 45 s
SM-2	300 mg/mL BSA in PBS	19	700	150	10,000 rpm for 1 min	Vortexing for 15 s
SM-3	0 or 500 mg trehalose in 1 g PBS	51	320 + 0, 4.8, 14.4, and 39.5 mg $MgCO_3$	25, 100, 200, and 350	17,000 rpm for 1 min	Homog. at 6000 rpm for 25 s
SM-4	500 mg trehalose in 1 g PBS	11	1100	175	20,000 rpm for 1.5 min	Homog. at 6000 rpm for 45 s
TM-1	200 mg/mL lysozyme with or without 0.45 M sucrose in water	51	320	110	20,000 rpm for 1.5 min	Homog. at 6000 rpm for 45 s
SM-5	1 g PBS or 500 mg trehalose in 1 g PBS	51	320 + 0, 3.5, and 10 mg $ZnCO_3$	200	10,000 rpm for 1 min	Vortexing for 15 s
ASM	200 µL of 25 mM succinate buffer (pH 4.0) containing 8.3-8.5 mg $Al(OH)_3$ gel and 9.6-9.9 mg trehalose	51	250 + 0, 7, and 14.2 mg DEP	200	17,000 rpm for 1 min	Vortexing for 50 s

SM: self-healing microencapsulating PLGA microsphere formulation; TM: traditional PLGA microsphere formulation; ASM: active self-healing microencapsulating PLGA microsphere formulation; CH_2Cl_2: methylene chloride; M_w: molecular weight; PBS: phosphate buffered saline (pH 7.4); $MgCO_3$: magnesium carbonate; $ZnCO_3$: zinc carbonate; DEP: diethyl phthalate; Conc.: concentration.

Table S2. Conditions for investigating passive and active self-healing microencapsulation of enzyme/protein/peptide by various types of self-healing microencapsulating PLGA (50:50) microspheres

FC	Enzyme/protein/ peptide microencapsulated	SM/ASM microspheres added (mg)	Concentration of loading solution: volume (mg/mL: mL)	Incubation temperature/duration (°C/h)	
				Loading	Self-healing
SM-1	Lysozyme	200 or 225	200 or 230:1 or 1.4	4/48	42.5/44
SM-2	BSA-coumarin	125	43 or 79 or 115 or 157 or 175 or 204:0.8 or 1	4/24 or 48	37 or 42/18 or 24
SM-3	BSA or Lysozyme or TMR-Dextran or FITC-Dextran	50 or 100 (FITC-Dextran/ TMR-Dextran) 80 or 150 (BSA/ Lysozyme)	1 (TMR-Dextran or FITC-Dextran) or 65 (FITC-Dextran) or 200 or 300 (BSA or Lysozyme with or without 0.45 M sucrose):1	4/24 or 48 (TMR-Dextran or FITC-Dextran) or 4/16 or 72 (BSA or Lysozyme)	42.5/48 or 72 (TMR-Dextran or FITC-Dextran) or 43/46 or 48 (BSA or Lysozyme)
SM-4	Lysozyme	200	250 with or without 0.45 M sucrose:1	4/24	37/12
SM-5	Leuprolide acetate	1000	127:4	4/42	43/48
ASM-1 to ASM-3	OVA or TT	20	0.5 or 1.0:0.4 (OVA) or 0.8:0.5 (TT)	10/48 + 25/24 (OVA) or 10/24 + 25/24 (TT)	37/30 or 43/48 (OVA) or 38/40 (TT)

FC: formulation code; SM: self-healing microencapsulating PLGA microsphere formulation; ASM: active self-healing microencapsulating PLGA microsphere formulation; OVA: ovalbumin; TT: tetanus toxoid.

Table S3. OVA mass loss kinetics as a function of incubation time during active self-healing microencapsulation from an OVA/$Al(OH)_3$-PLGA 50:50 (M_w = 51 kDa) microspheres mixture

Temp. and t		IM[a] (μg)	Formulation code							
			Remaining OVA mass in solution[a] (μg)*				OVA loaded (μg)*			
°C	h		Blank	ASM-1	ASM-2	ASM-3	Blank	ASM-1	ASM-2	ASM-3
10	3	404	415 ± 5	325 ± 3	317 ± 6	319 ± 3	-11 ± 5	79 ± 3	87 ± 6	85 ± 3
	6	404	415 ± 3	321 ± 4	310 ± 4	310 ± 1	-11 ± 3	83 ± 4	94 ± 4	94 ± 1
	24	404	416 ± 2	315± 3	300 ± 10	301 ± 6	-12 ± 2	89 ± 3	104 ± 10	103 ± 6
	48	404	425 ± 3	285 ± 3	285 ± 7	284 ± 4	-21 ± 3	119 ± 3	119 ± 7	120 ± 4
25 + 37	24 + 30	404	419 ± 6	203 ± 4	212 ± 5	197 ± 3	-15 ± 6	201 ± 4	192 ± 5	208 ± 3

Temp and t: incubation temperature (°C) and duration (h); IM: initial OVA mass in solution
[a]: volume = 0.4 mL; *: Mean ± SE, n = 6; ASM-1: 3.2 %w/w $Al(OH)_3$/3.5 %w/w trehalose/0 %w/w diethyl phthalate (DEP)/PLGA microspheres; ASM-2: 3.2 %w/w $Al(OH)_3$/3.5 %w/w trehalose/2.5 %w/w DEP/PLGA microspheres; ASM-3: 3.2 %w/w $Al(OH)_3$/3.5 %w/w trehalose/5 %w/w DEP/PLGA microspheres

Table S4. OVA loading and self-healing microencapsulation efficiency of $Al(OH)_3$-PLGA 50:50 (M_w = 51 kDa) microspheres at different initial OVA loading concentrations.

FC	Initial OVA mass[a] (µg)	Remaining OVA mass[a] (µg)*	OVA loaded (µg)*	OVA recovered from polymer (µg)*	Encapsulation efficiency[b] (%)*	OVA Loading[b] (% w/w)*
ASM-1	202	7 ± 3	195 ± 3	194 ± 3	96 ± 2	1.00 ± 0.01
	404	203 ± 5	201 ± 5	198 ± 4	49 ± 1	0.98 ± 0.02
ASM-2	202	4 ± 1	198 ± 1	196.2 ± 0.3	97.1 ± 0.2	0.97 ± 0.01
	404	212 ± 6	192 ± 6	191 ± 6	47 ± 2	0.90 ± 0.03
ASM-3	205	3 ± 2	203 ± 2	202 ± 2	98 ± 2	1.00 ± 0.05
	404	197 ± 3	208 ± 3	204 ± 4	50 ± 2	1.00 ± 0.03

FC: formulation code; [a]: volume = 0.4 mL; *: Mean ± SE, n = 6; [b]: based on the OVA content in the polymer; ASM-1: 3.2 %w/w $Al(OH)_3$/3.5 %w/w trehalose/0 %w/w diethyl phthalate (DEP)/PLGA microspheres; ASM-2: 3.2 %w/w $Al(OH)_3$/3.5 %w/w trehalose/2.5 %w/w DEP/PLGA microspheres ASM-3: 3.2 %w/w $Al(OH)_3$/3.5 %w/w trehalose/5 %w/w % DEP/PLGA microspheres

Table S5. Effect of gamma irradiation of active self-healing microencapsulating (ASM, Table S1) PLGA 50:50 (M_w = 51 kDa) microspheres on active loading and encapsulation efficiency of tetanus toxoid (TT).

	Initial TT mass[a] (µg)	Remaining TT mass[a] (µg)*	TT loaded (µg)*	TT Loading[b] (%w/w)*	Encapsulation efficiency[b] (%)*
Unencapsulated $Al(OH)_3$[c]	400	0.0	400 ± 0	-	-
Before irradiation					
ASM-1	400	54 ± 2	346 ± 2	1.62 ± 0.02	86.6 ± 0.5
ASM-2	400	52 ± 2	348 ± 2	1.66 ± 0.03	86.9 ± 0.4
After irradiation[d]					
ASM-1	400	53 ± 2	347 ± 2	1.64 ± 0.03	86.8 ± 0.3
ASM-2	400	53 ± 1	347 ± 1	1.61 ± 0.03	86.7 ± 0.2

[a]: volume = 0.5 mL; *: Mean ± SE, n = 3; [b]: based on the TT mass loss from solution; [c]: mass of $Al(OH)_3$ = 0.6 mg; ASM-1: 3.2 %w/w $Al(OH)_3$/3.5 %w/w trehalose/0 %w/w diethyl phthalate (DEP)/PLGA microspheres; ASM-2: 3.2 %w/w $Al(OH)_3$/3.5 %w/w trehalose/5 %w/w DEP/PLGA microspheres ; [d]: irradiation dose and dose rate were 2.5 MRad at 0.37 MRad/h.

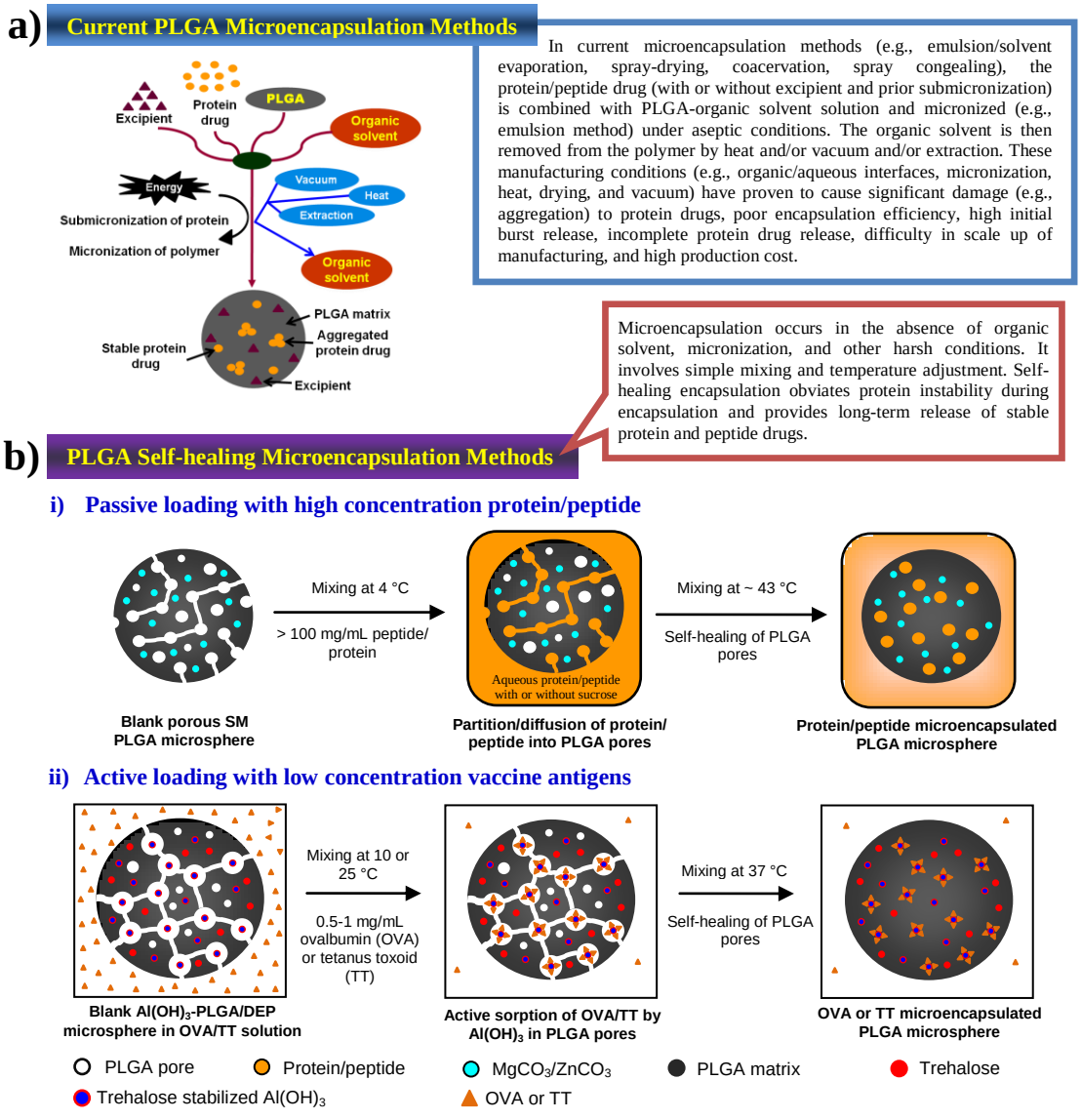

Figure S1. Schematic representation of current (**a**) and self-healing (**b**) PLGA microencapsulation methods for protein and peptide drugs.

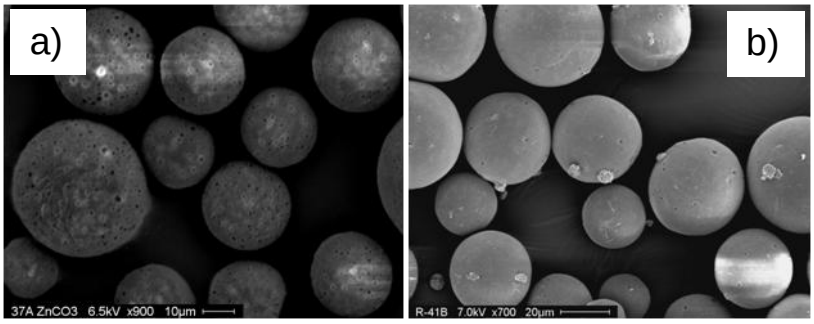

Figure S2. Self-healing microencapsulation of leuprolide acetate in PLGA 50:50 (M_w = 51 kDa) microspheres (SM-5, Tables S1 and S2). Scanning electron microscopy image of SM PLGA microspheres before (a) and after (b) self-healing microencapsulation of leuprolide acetate. Actual amount of leuprolide acetate microencapsulated by this formulation was 3.0 ± 0.2 (mean ± s.e.m.; n = 3) % (w/w peptide/polymer matrix).

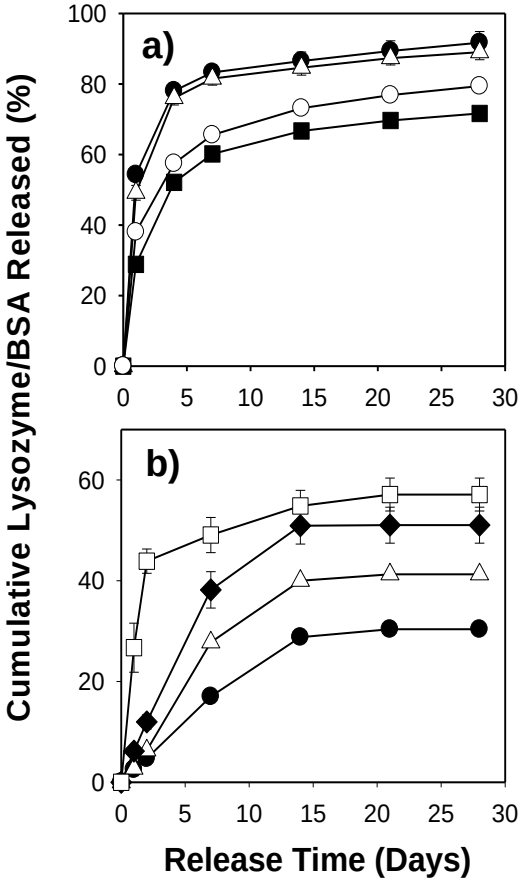

Figure S3. Protection of protein (BSA and lysozyme) against acid-induced instability during release from self-healing PLGA microspheres (SM-3, Tables S1 and S2). a) Effect of $MgCO_3$ (porosigen/stabilizer) loading (3 (●) and 4.5 (Δ) %w/w) and addition of sucrose (0 (■) and 0.45 (○) M) in protein loading solution on BSA release. Actual BSA loading in ●, Δ, ■, and ○ was 4.25 ± 0.05, 5.65 ± 0.06, 7.26 ± 0.09, and 5.54 ± 0.04 % (mean ± s.e.m.; *n* = 3), respectively. b) Effect of $MgCO_3$ loading (0 (●), 1.5 (Δ), 4.5 (♦), and 11 (□) %w/w) on lysozyme release. Actual lysozyme loading in ●, Δ, ♦, and □ was 4.2 ± 0.2, 6.4 ± 0.1, 9.8 ± 0.3, and 8.7 ± 0.4 % (mean ± s.e.m.; *n* = 3), respectively. *S*ymbols represent mean ± s.e.m.; *n* = 3.

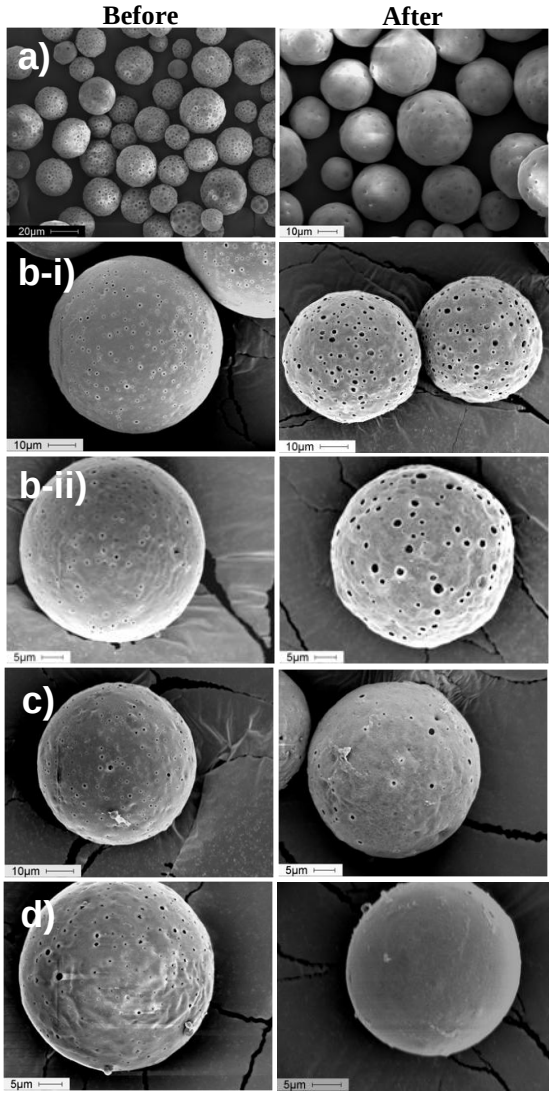

Figure S4. Effect of introduction of $Al(OH)_3$ into PLGA pores and blending of hydrophobic plasticizer (diethyl phthalate (DEP)) on self-healing of PLGA 50:50 (M_w = 51 kDa) microspheres. Surface morphology of blank (a; 3.5 %w/w trehalose loaded), and 0 (b-i and b-ii), 2.5 (c), and 5 (d) %w/w DEP loaded $Al(OH)_3$-PLGA microspheres before and after self-healing microencapsulation of OVA. b, c, and d corresponds to ASM-1, ASM-2, and ASM-3 in Tables S2-S4. Formulations a) and b-i) were incubated at 25 and 43 ºC for 48 h. Formulations b-ii), c) and d) were incubated for 48, 24, and 30 h at 10, 25, and 37 ºC, respectively.

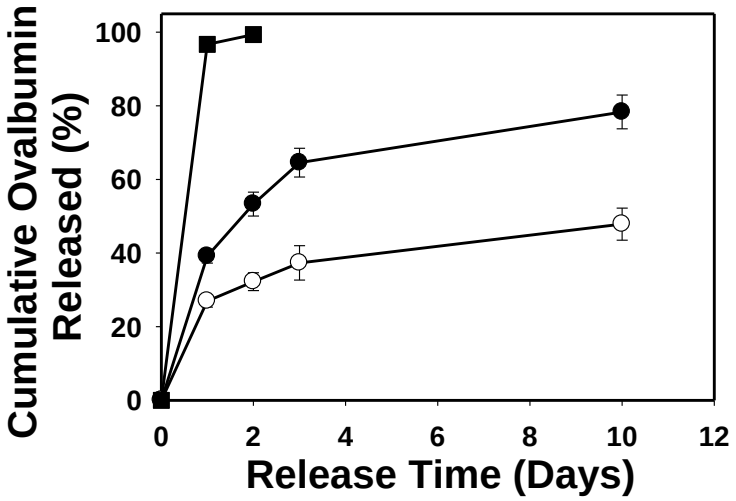

Figure S5. Evaluation of quality of protein microencapsulation by active self-healing microencapsulating PLGA (ASM PLGA) ($Al(OH)_3$-PLGA 50:50 (M_w = 51 kDa)) microspheres in $Al(OH)_3$ gel-dissolving 190 mM sodium citrate solution. Cumulative ovalbumin monomer released as a function of time from unencapsulated $Al(OH)_3$ gel (control) (■) and with (○) (ASM-3, Tables S3 and S4) and without (●) (ASM-1, Tables S3 and S4) 5 %w/w DEP in $Al(OH)_3$-PLGA microsphere formulations. In vitro release studies were conducted at 37 ºC and symbols represent mean ± s.e.m.; n = 3.

Results and Discussion

Limitations on molecular size—Self-healing microencapsulation of dextran blue. As suggested by the open pore distribution in Figure 1 before and after loading lysozyme in SM microspheres very large molecules can penetrate the polymer pore network and become encapsulated. Therefore, we tested the virtual upper limit of molecular size of biomacromolecules by encapsulating TMR-dextran (M_w = 2 MDa) relative to low molecular weight 4 kDa FITC-dextran in SM-PLGA 50:50 (M_w = 51 kDa) microspheres (SM-3, Tables S1 and S2). At a constant solution concentration of 1 mg/mL, which is just below the solubility of the TMR-dextran, both dyes were successfully encapsulated at very similar levels as indicated by the following data determined experimentally: SM PLGA microspheres encapsulated 0.30 ± 0.01 and 0.22 ± 0.01 (mean ± s.e.m.; n = 5) %w/w TMR-dextran and FITC-dextran 4 kDa, respectively.

Protection of protein during microencapsulation—Improved stability of lysozyme through self-healing microencapsulation. As lysozyme is a useful marker for testing protein damage during microencapsulation,[S4-S7] we microencapsulated this enzyme by PLGA healing and monitored its loading in terms of monomeric, total, and enzymatically active protein content. The stability of lysozyme encapsulated via self-healing microencapsulation (SM-3 and SM-4, Tables S1 and S2) was compared with the enzyme encapsulated via a traditional w/o/w process (TM-1, Tables S1). Combined soluble and insoluble lysozyme encapsulated was measured via AAA, and the soluble fractions were determined by SE-HPLC.

Addition of sucrose either to inner water phase (WP) in the double emulsion (traditional encapsulation (TE) method) or loading solution (self-healing microencapsulation method) resulted in reduced lysozyme loading (% w/w lysozyme/polymer matrix). For example, the

lysozyme loading into medium M_w PLGA (M_w = 51 kDa) microspheres prepared by TE method with and without sucrose in WP was 0.65 ± 0.01 and 4.86 ± 0.04 (mean ± s.e.m.; n = 3) %, respectively. By contrast, lysozyme loading into medium M_w PLGA (M_w = 51 kDa) microspheres by the self-healing with and without sucrose in loading solution was 3.39 ± 0.03 and 5.17 ± 0.08 (mean ± s.e.m.; n = 3) %, respectively.

The percentage (%w/w) of loaded lysozyme that exists as intact soluble monomer was slightly higher for the self-healing microsphere (SM-3, Tables S1 and S2) formulations than the TM-1 formulations. In the absence of sucrose, TM-1 and SM PLGA (M_w = 51 kDa) microspheres had 85 ± 2 and 90 ± 2 (mean ± s.e.m.; n = 3) % intact monomer, respectively. In the presence of sucrose in the loading solution, SM PLGA (M_w = 51 kDa) microspheres had 98 ± 2 (mean ± s.e.m.; n = 3) % of the loaded protein as intact monomer. Importantly, the fraction of total lysozyme loaded as insoluble aggregates was significantly less (p<0.05) by self-healing technique when compared to the TE method. For example, lysozyme loaded via double emulsion-based method (TM-1, Tables S1 and S2) underwent 40 ± 4 % and 12 ± 2 (mean ± s.e.m.; n = 3) % insoluble aggregation with or without sucrose in the inner water phase, respectively (formulation F and E in Figure 2b). By contrast, the self-healing technique respectively showed 8 ± 3 and 1 ± 2 (mean ± s.e.m.; n = 3) % insoluble aggregation without and with sucrose in loading solution (formulation C and D in Figure 2b) for the SM PLGA (M_w = 51 kDa) microsphere formulation (SM-3, Tables S1 and S2).

Protection of protein (BSA and lysozyme) against acid-induced protein instability during release from self-healing PLGA microspheres. The success of controlled protein delivery vehicles based on PLGA significantly depends upon the ability of the polymer to retain and release the stable form of proteins under physiological conditions over extended release times. Co-encapsulation of $MgCO_3$ along with proteins in PLGA 50:50 by the TE method has been used to stabilize proteins against the acidic polymer microclimate and released the proteins in a stable form compared to non-base control formulations.[19] As BSA is a useful marker for assessing protein damage due to acid produced by the PLGA polyester during long-term controlled release,[19] we microencapsulated the protein into SM-PLGA 50:50 microspheres containing $MgCO_3$ as an acid-neutralizer and porosigen with and without trehalose as an additional porosigen (SM-3, Tables S1 and S2) and monitored its loading, release from microspheres and acid-induced aggregated protein content in the polymer. Blank SM PLGA 50:50 (M_w = 51 kDa) microspheres containing 3 and 4.5 %w/w ($MgCO_3$/polymer matrix) $MgCO_3$ microencapsulated 4.25 ± 0.05 and 5.65 ± 0.06 (mean ± s.e.m.; n =3) %w/w (protein/polymer matrix), respectively. Both the healed microsphere formulations exhibited a high initial burst (49-54 % BSA release after 1-day), which was then substantially reduced by co-encapsulation of trehalose along with $MgCO_3$ during the preparation of SM microspheres (Figure S3a). Importantly, analysis of the remaining protein in the microspheres after 28 days of in vitro release provided a mass balance (total recovery) between 109 ± 4 to 121 ± 2 (mean ± s.e.m.; n =3) % and out of which < 2 % BSA was aggregated (i.e., below detection limit).

Another model protein, lysozyme, was similarly microencapsulated in blank SM PLGA 50:50 (M_w = 51 kDa) microspheres (SM-3, Tables S1 and S2) containing 0, 1.5, 4.3, 11.0 % w/w ($MgCO_3$/polymer matrix). The amount of lysozyme microencapsulated by healing in 0, 1.5, 4.3, 11.0 %w/w $MgCO_3$ loaded blank PLGA 50:50 microspheres was 4.5 ± 0.2, 6.4 ± 0.1, 9.8 ± 0.3, and 8.7 ± 0.4 (mean ± s.e.m.; n =3) %w/w (lysozyme/polymer matrix), respectively. The release rate of lysozyme from above mentioned formulations was directly related to the amount of base loaded into the blank PLGA 50:50 microspheres (Figure S3b). For example, after 28 days, the cumulative amount of lysozyme released from the

microsphere formulations was 30 ± 1, 41 ± 1, 51 ± 4, and 57 ± 4 (mean ± s.e.m.; n =3) % respectively for 0, 1.5, 4.3, and 11.0 %w/w $MgCO_3$ initial loading. The amount of protein remaining in the microspheres after 28 days of release, including soluble monomer, soluble aggregates, and insoluble aggregates, was similarly quantified and >90 % mass balance (total recovery) was achieved for all four formulations. For example, total amount of lysozyme recovered from self-microencapsulated microspheres formulations was 92 ± 2, 91 ± 1, 99 ± 4, and 110 ± 4 (mean ± s.e.m.; n =3) % respectively for 0, 1.5, 4.3, and 11.0 %w/w $MgCO_3$ initial loading. Importantly, the total amount of soluble protein, both released over 28 days and recovered as residual soluble monomer, was significantly higher (p<0.05) for 4.3 and 11.0 %w/w $MgCO_3$-based SM microsphere formulations. For example, total (released + residual) amount of soluble protein recovered for 0, 1.5, 4.3, and 11.0 %w/w $MgCO_3$ loaded PLGA 50:50 microspheres was 83 ± 2, 85.0 ± 1, 95 ± 4, and 107 ± 4 (mean ± s.e.m.; n =3) %, respectively. Furthermore, the specific activity of the residual lysozyme remaining in the self-healing microsphere preparations after 28 days of release was analyzed. The specific activity, calculated based upon the total amount of soluble protein analyzed, both monomer and aggregated, was 102 ± 6, 116 ± 19, 100 ± 5, and 97 ± 5 (mean ± s.e.m.; n =3) % respectively for 0, 1.5, 4.3, and 11.0 %w/w $MgCO_3$ loaded PLGA 50:50 microspheres. Thus, the soluble lysozyme retained in the self-healing microspheres after 28 days of release was still completely active within experimental error for all formulations.

Kinetics of PLGA self-healing microencapsulation. In order to determine the time required for self-healing of PLGA to encapsulate large molecules, SM PLGA microspheres (SM-3, Table S1) were loaded with FITC-dextran (SM-3, Table S2) and the loaded biomacromolecular dye was analyzed at various times before and after initiating self-healing microencapsulation by increasing temperature from 4 ºC ($T < T_g$) to 42.5 ºC ($T > T_g$). At each time point, microspheres were washed extensively with ddH_2O to remove any unencapsulated dye. As shown in Figure 2a, only background levels of dextran were loaded when at the low temperature for 20 h. However, after increasing temperature to 42 ºC (at time 0) loading of two separate, but equivalent, formulations both increased steadily over 12 h reaching a maximal and steady loading value for 72 h. Hence, without any manipulation of the polymer (e.g., by plasticization) self-healing time for this formulation was on the order of 12 h. In addition, loading was completely reproducible for the two identical formulations.

PLGA self-healing microencapsulation provides long-term delivery of bioactive large molecules in vivo—Long-term testosterone suppression in rats after single injection of healed leuprolide acetate/PLGA microspheres. Leuprolide acetate (M_w = 1209.6 Da) is commonly used in the treatment of hormone-dependent cancers (e.g., prostate cancer) and gynecologic disorders (e.g., endometriosis and precocious puberty). Commercially available injectable PLGA microsphere-based formulation of leuprolide (Lupron Depot®) is prepared by the traditional (water-in-oil-water) encapsulation method. The ability of self-assembly materials to provide long-term in vivo release of biomacromolecule was evaluated by assessing long-term testosterone suppression in male Sprague-Dawley rats (six rats/study group) in comparison with commercial Lupron Depot® and negative controls (Figure 2d). The initial serum testosterone level (1-2 ng/mL) increased to ~5 ng/mL after one day of subcutaneous injection of Lupron Depot®, self-healing microencapsulated leuprolide acetate/PLGA microspheres, and leuprolide solution. This initial elevation is typical to treatment with luteinizing hormone-releasing hormone (LHRH) agonists (e.g., leuprolide acetate) resulting from initial stimulation of pituitary LHRH receptors and increased release of luteinizing hormone (LH), thereby stimulating testicular steroidogenesis and release of gonadotropins.[2b,18,S8] The testosterone levels fell below the castration level (0.5 ng/mL)

within a week and remained under that level for 6-7 weeks following a single injection of self-healing PLGA microspheres containing leuprolide and 8 weeks after two injections (day 0 and 28) of Lupron Depot®. Importantly, there was no significant serum testosterone levels difference (p<0.05) between healed PLGA microspheres and commercial Lupron Depot® over a period of 6 weeks, indicating equivalent potential of new microencapsulation paradigm to provide long-term in vivo delivery of peptide drugs. In contrast, leuprolide solution control because of the short serum half-life of the peptide, failed to provide testosterone suppression below the castration level during the whole study duration. However, there was a significant serum testosterone levels difference (p>0.05) between negative controls (blank SM PLGA microspheres and leuprolide solution) and long-term leuprolide delivery vehicles (healing PLGA microspheres and Lupron Depot®) over a period of 6-8 weeks.

Active self-healing microencapsulation of protein with high encapsulation efficiency by $Al(OH)_3$-PLGA microspheres—Effect of hydrophobic plasticizer. The encapsulation efficiency of SM PLGA microspheres by the passive self-healing microencapsulation method is typically low (1.5-13 %). In addition, as partitioning of the proteins between polymer pores and external solutions should be close to unity when loading by the passive process, high external protein concentrations (e.g., > ~50 mg/mL) were required previously to achieve loading >1%. Therefore, to increase the encapsulation of efficiency of SM PLGA microspheres and to reduce external protein concentration, we added a well-known protein sorbing and stabilizing substance, $Al(OH)_3$ adjuvant gel in SM PLGA microspheres (ASM PLGA microspheres, Table S1) and tested their potential to actively microencapsulate different vaccine antigens (ovalbumin (OVA) and tetanus toxoid (TT)), from low aqueous protein concentrations (0.5 or 0.8 or 1.0 mg/mL) (Tables S2-S5).

As shown in Table S3, all the three ASM (ASM-1, ASM-2, and ASM-3) PLGA microspheres sorbed the OVA from surrounding protein solution within the incubation time compared blank porous PLGA microspheres, suggesting active self-healing microencapsulation of protein by ASM PLGA microspheres. Negative loading values were observed with porous blank (no $Al(OH)_3$) microspheres, which can be attributed to the uptake of small amounts of water by the polymer leaving behind more concentrated protein solution. With 400 µg initial incubation mass of OVA from 1 mg/mL OVA, the ASM PLGA microspheres microencapsulated about 200 µg (self-healing microencapsulation capacity = 1 %w/w (OVA/polymer matrix)) thereby exhibiting a microencapsulation efficiency of about 50% (Table S4).

The similar loading of the three preparations (ASM-1, ASM-2, and ASM-3) also indicated that the protein loading was governed by the effective capacity of the $Al(OH)_3$ for sorbing OVA when the external OVA content exceeded the $Al(OH)_3$ sorbing capacity available in the polymer. Therefore, the encapsulation efficiency was further increased by decreasing external OVA content to roughly that encapsulated (~200 µg) previously, that is, by incubating microspheres with 200 µg OVA from 0.5 mg/mL OVA. As expected, ASM-1, ASM-2, and ASM-3 microspheres microencapsulated almost the entire OVA mass, thereby exhibiting extraordinary efficiency (96-98 %, Table S4). The potential of ASM PLGA microspheres to actively microencapsulate the very sensitive vaccine antigen (TT) after sterilization of blank microspheres with gamma radiation was investigated and compared with the results obtained prior to irradiation (Table S5). There was no significant difference in active loading of TT before and after sterilization of ASM PLGA microspheres, strongly suggesting the effectiveness of novel strategy to microencapsulate vaccine antigens after terminal

sterilization of microspheres. For example, with 400 µg initial incubation mass of TT from 0.8 mg/mL loading solution, all the ASM PLGA microsphere formulations microencapsulated TT equivalent to about 1.6 %w/w polymer loading and 87% encapsulation efficiency (n = 3). It is noted that the $Al(OH)_3$ gel appeared to suppress self-healing as pores still appeared after incubation of the ASM PLGA microspheres at 43 ºC as compared to full healed microspheres prepared without $Al(OH)_3$ gel (Figure S4). Hence, we sought to increase PLGA mobility by incorporating a hydrophobic plasticizer in the polymer. For example, diethyl phthalate (DEP) was blended with the polymer while preparing blank $Al(OH)_3$-PLGA microspheres. As expected, with increasing amount of hydrophobic plasticizer in PLGA, pore-closing (self-healing) at 37 ºC was clearly visible in Figure S4, indicating that any suppression of self-healing by $Al(OH)_3$ could be overcome by the plasticizer.

The protein/peptide loading capacity and initial burst release exhibited by passive self-healing process was highly desirable in certain formulations described here (e.g., >5% w/w and <20%, respectively). By contrast, the active loading strategy illustrated with OVA and TT exhibited lower loading capacity and at times higher initial burst release. Although we expect the performance for the active SM preparations to be sufficient for antigen delivery, these values may need to be improved when applying the active encapsulation approach to protein pharmaceuticals. We anticipate that alternative protein-trapping agents, which binds protein at higher excipient: protein ratios than does $Al(OH)_3$, will allow an increase in the active loading capacity from 1-2% for $Al(OH)_3$/PLGA to easily 5-10%, which is desired for current commercial peptide controlled release depots and protein controlled release formulations development.

It can be emphasized that SM PLGA microspheres with respectively 3.2, 3.5, and 5 %w/w of $Al(OH)_3$, trehalose and DEP were found to be an optimal formulation for active self-healing microencapsulation of protein at physiological temperature (37 ºC) with high encapsulation efficiency. Moreover, the successful employment of DEP to reduce the required temperature for self-healing opens-up the door to microencapsulate temperature-sensitive molecules in higher M_w PLGA at or below physiological temperature.

Evaluation of quality of active self-healing microencapsulation of protein in $Al(OH)_3$-PLGA microspheres. Evaluation of quality of active self-healing microencapsulation of protein in $Al(OH)_3$-PLGA (M_w = 51 kDa) (ASM PLGA) microspheres was tested first in 190 mM sodium citrate solution, a buffer commonly used to elute protein antigens completely from $Al(OH)_3$ adjuvant within 3 days.[S9] The release of OVA from ASM microspheres was significantly different (p<0.05) compared to unencapsulated $Al(OH)_3$ (Figure S5). For example, ASM-3 PLGA microspheres (3.2 %w/w $Al(OH)_3$/3.5 %w/w trehalose/5 %w/w DEP/PLGA microspheres) largely retained OVA (i.e., 60-73 %) after 1-day of exposure to the citrate buffer, whereas unencapsulated $Al(OH)_3$ released all the protein (97 ± 1 (mean ± s.e.m.; n = 3) % release). In addition, ASM-3 PLGA microspheres released OVA slowly in a controlled manner over a period of 10 days (48 ± 5 (mean ± s.e.m.; n = 3) % release after 10 days), indicating an effective active microencapsulation of protein in $Al(OH)_3$-PLGA microspheres. After 10 days of release duration, 47 ± 7 (mean ± s.e.m.; n = 3) % OVA monomer and 5.8 ± 0.8 (mean ± s.e.m.; n = 3) % insoluble aggregate (covalent and non-covalent) was recovered from ASM-3 PLGA microspheres with a total recovery of 100.8 ± 0.8 (mean ± s.e.m.; n = 3) %.

Long-term controlled release of proteins from $Al(OH)_3$-PLGA microspheres after active self-healing microencapsulation. The potential of active self-healing microencapsulation of

protein in $Al(OH)_3$-PLGA microspheres to provide long-term release of stable proteins was evaluated by assessing OVA monomer and antigenic TT release from unencapsulated $Al(OH)_3$ and ASM microspheres without (ASM-1) and with (ASM-3) 5 %w/w DEP (Tables S3-S5) in PBS (pH 7.4) (Figure 3c) or PBS + 0.02% Tween® 80 + 0.2% BSA (Figure 3d) at 37 ºC. The release of OVA monomer or antigenic TT from ASM-3 PLGA microspheres (3.2 %w/w $Al(OH)_3$/3.5 %w/w trehalose/5 %w/w DEP/PLGA microspheres) was also significantly different (p<0.05) than unencapsulated $Al(OH)_3$ (Figure 4c and d). For example, OVA-$Al(OH)_3$ control gel exhibited ~ 76, 90, and 99 % OVA monomer release and TT-$Al(OH)_3$ control gel exhibited ~ 87, 95, and 98 % antigenic TT release respectively after 1, 3, and 7 days. In contrast, ASM-3 PLGA microspheres exhibited much lower initial burst (17 % OVA monomer or 32 % antigenic TT release after 1 day) and provided slow and continuous release of OVA monomer or antigenic TT over a period of 28 days (~ 49 and 68 % OVA monomer or 83 and 99 % antigenic TT release respectively after 14 and 28 days). After 28 days of release, 19 ± 3 (mean ± s.e.m.; n = 3) % soluble OVA monomer and 10 ± 2 (mean ± s.e.m.; n = 3) % insoluble OVA aggregate (covalent and non-covalent) was recovered from ASM-3 PLGA microspheres with a total recovery of 98 ± 3 (mean ± s.e.m.; n = 3) %.

TT self-encapsulated PLGA microspheres exhibited higher initial burst and overall release compared to OVA encapsulated microspheres. This can be attributed to the presence of BSA in TT release medium, which was added to stabilize released TT. As BSA is also known to bind strongly to $Al(OH)_3$,[S10] it is likely that external BSA eluted TT from $Al(OH)_3$ due to competitive binding,[S11] thereby leading to higher TT release from self-encapsulated PLGA microspheres relative to OVA. However, we cannot rule out additional contribution from $Al(OH)_3$, which suppressed the self-healing of PLGA pores. Therefore, we are currently examining alternative trapping agents, which do not affect polymer healing.

As the microencapsulation of vaccine antigens in $Al(OH)_3$-PLGA microspheres can be easily performed by simple of mixing of vaccine antigens/$Al(OH)_3$-PLGA microspheres and heating the mixture to physiological temperature (no harsh manufacturing conditions), this new approach opens-up a new mode for sustained vaccine delivery similar to commercial injectable peptide controlled release depots, thereby improving the stability and efficacy of vaccine antigens. Note that injectable PLGA microspheres represent an exciting approach to control the release of vaccine antigens to reduce the number of doses in the immunization schedule, reduce neonatal tetanus, and optimize the desired immune response via selective targeting of antigen to antigen presenting cells.[S12]

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