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Stabilization of Tetanus Toxoid Encapsulated in PLGA Microspheres

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Abstract: Delivery of vaccine antigens from controlled-release poly(lactic/glycolic acid) (PLGA) microspheres is a novel approach to reduce the number of antigen doses required for protection against infection. A major impediment to developing single-shot vaccines is encapsulated antigen instability during months of exposure to physiological conditions. For example, efforts to control neonatal tetanus in developing countries with a single-dose TT vaccine based on PLGA microspheres have been plagued by poor stability of the 150 kDa formaldehyde-detoxified protein antigen, tetanus toxoid (TT), in the polymer. We examined the denatured states of PLGA-encapsulated TT, revealing two primary TT instability mechanisms: (1) protein aggregation mediated by formaldehyde and (2) acid-induced protein unfolding and epitope damage. Further, we systematically identified excipients, which can efficiently inhibit TT aggregation and retain TT antigenicity under simulated deleterious conditions, i.e., elevated temperature and humidity. By employing these novel additives in the PLGA system, we report the slow and continuous release of high doses of TT for one month with retained antigen stability during bioerosion of PLGA.

Keywords: Vaccine delivery; protein aggregation; stabilization; PLGA; formaldehyde

Introduction

Since vaccine delivery from polymers was first discovered,[1] research has focused on developing polymer formulations that would be capable of reducing the required number of antigen doses for protection to as few as a single shot.[2] Efforts to this end have intensified after recognition that an effective controlled-release vaccine is among the best hope to improve vaccine coverage in developing countries.[3] To reduce injection frequency, antigens are encapsulated in biodegradable polymers such as poly(lactic/glycolic acid) (PLGA) microspheres (1−100 μm), which are easily administered through a syringe needle. PLGAs can be formulated to slowly release soluble antigens from days to several months. This release duration, for which the antigen must remain encapsulated in the polymer at physiological temperature and humidity, poses significant challenges to retain both the structural integrity and native epitopes of the antigen. Encapsulated antigen instability is considered the primary obstacle impeding the development of effective single-dose vaccines.[4–9]

Tetanus remains a major killer in developing countries, e.g., >500,000 deaths/yr[10] from neonatal tetanus alone, largely due to the logistical difficulty of delivering 2 to 3 doses of vaccine required for protection to pregnant women

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whose immunity can be passively transferred to the fetus.[11] Spearheaded by the World Health Organization in the 1990s, tetanus toxoid (TT), the formaldehyde-treated protein antigen against tetanus, was selected as one of the first antigens for single-dose vaccine development for human use based on PLGA microsphere system.[12] Despite several promising reports of neutralizing antibody responses in test animals following a single dose of TT encapsulated in PLGA microspheres,[13–19] the instability of the antigen emerged as a ubiquitous problem.[20–26] In limited cases, anti-TT antibody titers in vivo were reported to be in accordance with the in vitro release characteristics of immunoreactive TT from the particles. For example, Raghuvanshi et al. reported that anti-TT antibody titers from the stabilized particles were much better than that observed from particles made without stabilizers.[26] In addition, other formulation parameters such as polymer hydrophobicity, particle size and use of additional adjuvants may also impact the generation of immune responses in experimental animals.[16,18,19,25]

Previous attempts to stabilize encapsulated TT include the following: employing basic inorganic salts and BSA as a proton scavenger to inhibit acid-induced instability,[23,24,27] using rat serum albumin to enhance TT stability during the primary emulsification step of particle formation,[24,26] optimizing encapsulation methods,[28,29] reducing the interaction of TT with the polymer or polymer degradation products,[28,30]

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adjusting water uptake of the polymer,[23,31,32] and coencapsulating some commonly used protein stabilizers, e.g., sucrose, trehalose, cyclodextrin, dextran, heparin in PLGA microspheres to enhance the stability of TT.[24,29] Although some stability improvements have been noted,[23,24,26,29,31] few studies have clearly characterized the instability mechanisms of TT inside PLGA microspheres and systematically analyzed the effect of stabilizers on the biophysical and biochemical properties of TT. In addition, although coencapsulation of serum albumin in the polymer formulations significantly increased the continuous release of antigenically active TT,[23] potential autoimmune reactions of serum albumin may hinder its further use in humans.[33]

The purpose of this study is to investigate the instability mechanisms of TT in PLGA microspheres. By examining the denatured state of encapsulated TT and coencapsulating novel stabilizers into the polymer, we confirmed acid- and formaldehyde-based mechanisms as the two primary deleterious pathways of the encapsulated TT and more fully describe formulations that we originally reported to exhibit exceptional stability of encapsulated TT.[34,35]

Experimental Section

Chemicals. Poly(DL-lactide-*co*-glycolide) 85/15 (inherent viscosity of 0.69 and 0.86 dL/g in $CHCl_3$) was from Birmingham Polymers (Birmingham, AL). TT (specific activity of 3300 Lf mg^{-1}), was a kind gift from Chiron Corp. (San Francisco, CA). TT010 monoclonal antibody, which detects a neutralizing TT epitope, and anti-TT guinea pig IgG were generously provided by National Institute of Biological Standard Control (U.K.). Goat anti-guinea pig IgG peroxidase conjugate and 2,2′-azino-di-3-ethylbenzthiazoline-6-sulfonate (ABTS) tablets were obtained from Sigma (Milwaukee, WI). Fine $MgCO_3$ (<5 μm) powder was from Aldrich (Milwaukee, WI). All other substances used were of pharmaceutical or analytical grade and purchased from commercial suppliers.

Microsphere Preparation. TT was encapsulated by an oil-in-oil emulsion and solvent extraction method. To avoid TT aggregates during grinding, TT with or without stabilizers was micronized by adding 150 μL of aqueous antigen solution into 1.2 mL of 300 mg/mL PLGA (0.86 dl/g) in acetonitrile. The suspension was homogenized at 15,000 rpm (Model IQ^2, Virtis Co., Gardiner, NY) for 3 min at 4 °C and dropwise added to 100 mL of cottonseed oil containing 1.6 g of Span 85 with stirring at 700 rpm. After 5 h, petroleum ether (bp 50−110 °C) was poured into the cottonseed oil bath to extract the acetonitrile from the polymer. After an additional 15 min of stirring, the microspheres were sieved (120 μm screen), washed with 250 mL of petroleum ether, collected and lyophilized. The loading of antigen was determined by both destructive and nondestructive methods (see below). $MgCO_3$ was incorporated in microspheres by suspension in the polymer solution before addition of antigens. All microspheres were well formed with a mean diameter of 80−90 μm for TT/PLGA microspheres.

Determination of Antigen Loading. The amount of TT encapsulated in microspheres was determined by two methods. The first was to extract the protein antigen from the microspheres by removing the polymer and determine the protein content by modified Bradford assay (Coomassie brilliant blue plus protein assay, Pierce, Rockford, IL) and ELISA. The second was to determine encapsulated protein content by amino acid analysis after direct acid hydrolysis of microspheres (see Amino Acid Analysis below). In the former, acetone was added in microspheres at room temperature to dissolve the polymer. The mixture was vortexed and centrifuged, and then the supernatant was removed. After removal of polymer was repeated 3 times, the remaining protein pellet was reconstituted in phosphate buffer saline containing 0.02% Tween 80 (PBST) at 37 °C for 2 h and protein content was determined by modified Bradford assay and ELISA.

Evaluation of Antigen Release from Microspheres and Aggregation within Microspheres. Microspheres (15−20 mg) were incubated with mild agitation at 37 °C in 0.5 mL PBST, or in PBST containing 0.2% BSA (PBSTB) to inhibit potential TT adsorption to the polypropylene eppendorf tube.[36] Release medium was collected periodically by centrifugation and replaced with new medium. The soluble and antigenic protein content collected from PBST was determined by using a modified Bradford assay and ELISA, respectively. For the release medium collected from PBSTB, only the amount of antigenic TT was determined by ELISA. Soluble and antigenic protein content during release were normalized by the antigen content from the loading measurement (modified Bradford and ELISA) to determine total and antigenic release kinetics, respectively.

At the end of release, incubated microspheres were collected, dried and dissolved in acetone. After centrifugation and removal of the polymer solution, the remaining protein pellet was reconstituted in phosphate buffer saline containing 0.02% Tween 80 (PBST) at 37 °C for 2 h, and soluble protein

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content was determined by modified Bradford assay and ELISA. If any insoluble aggregates were observed in the reconstituted solution, aggregates were collected, washed with distilled water and then freeze-dried. These insoluble aggregates were reconstituted in denaturing agent (6 M guanidine-HCl (GnCl)). Determination of any aggregate soluble in denaturing agent gave the amount of noncovalently bonded aggregates. With the further addition of reducing agent (10 mM dithiothreitol, 1 mM EDTA), any disulfide-bonded aggregates were dissolved. The total dissolved portion in denaturing and reducing agents gave the total amount of noncovalent and disulfide-bonded aggregates. Any insoluble aggregates, which were insoluble in both denaturing and reducing agents, were further subjected to amino acid analysis or counted as formaldehyde-mediated aggregates.

Amino Acid Analysis. Dry microspheres or insoluble protein aggregates were weighed and dissolved in 6 N HCl/TFA (1:1) and hydrolyzed under 6 N HCl vapor at 125 °C for 24 h. An Applied Biosystems (ABI, Foster City, CA) 420 H was used to derivatize hydrolyzed amnio acids with phenylisothiocyanate (PITC) at alkaline pH to form PTC-amino acid derivatives, which were injected onto an ABI model 130A HPLC and detected at 269 nm.

Solid State Stability of Antigens. For solid-state stability, TT was diluted to ~1 mg/mL and dialyzed (10 kDa cutoff) against 1 mM sodium phosphate buffer pH 7.3 at 4 °C. Stabilizers were added to TT solutions, flash frozen in liquid N_2 and lyophilized for 48 h on a Freezezone 6 freeze-drying system (Labconco, Kansas City, MO) at 133×10^{-3} mbar or less with a condenser temperature of −46 °C. Lyophilized antigen samples were incubated at 37 °C and 80% relative humidity (RH). Incubated samples were reconstituted in 1 mM phosphate buffer and assessed for soluble protein. Any protein aggregates were further analyzed with the addition of denaturing and reducing agent described in the section Evaluation of Antigen Release from Microspheres and Aggregation within Microspheres.

Structural Analysis of TT. Far-UV circular dichroic (CD) spectra were taken with a J-810 Jasco spectropolarimeter (Hachioji, Japan) at room temperature to assess secondary structure of TT. Tertiary structural changes were monitored with a fluorimeter (Fluoromax-2, ISA Instrument Inc., NJ). TT solutions were excited (λ_{ex}) at 280 nm. The ratio of emission intensity at 350 nm over that at 329 nm (I_{350}/I_{329}) was monitored. Both changes in emission wavelength maximum (λ_{max}) and I_{350}/I_{329} are sensitive indices of conformational changes.[27,37]

ELISA for TT. Microtiter plates were coated with 100 μL of 1 μg mL^{-1} of the monoclonal anti-TT in coating buffer overnight at 4 °C. After blocking, and washing with PBST, serial dilutions of reference tetanus toxoid and samples in PBSTB were added and incubated for 2 h at 37 °C. After PBST washing, plates were incubated with purified guinea pig polyclonal anti-TT IgG 5 μg/mL for 2 h at 37 °C, followed by incubation with goat anti-guinea pig IgG-horseradish peroxidase (HRP) conjugate in PBSTB (1 in 2000) for 1 h at 37 °C. After adding the substrate solution containing 0.5 mg mL^{-1} of ABTS and 0.04% H_2O_2 in 0.05 M citric acid, pH 4.0 plates were read after 25−30 min at room temperature at 405 nm.

Results and Discussion

Encapsulation of TT in PLGA. To investigate TT stability in the polymer, we encapsulated TT in PLGA 85/15 microspheres (1.5% w/w TT) (Figure 1A, Table 1) by an oil-in-oil emulsion and solvent extraction method. Figure 1A shows the size and morphology of TT microspheres. Microsphere particles were spherical but not smooth. Small pores were observed on micropshere surfaces, possibly formed after water and acetonitrile removal during freeze-drying.

Theoretical TT loading in PLGA microspheres was 3%. We determined the actual TT loading value by quantification of total protein and antigenically active protein. Total protein content encapsulated in PLGA microspheres was determined by two methods: (1) modified Bradford assay following TT extracted from PLGA micropsheres and (2) amino acid analysis after complete acid digestion of the TT microsphere.

As seen in Table 1, TT loading determined by modified Bradford assay was 1.5%, indicative of 50% encapsulation efficiency obtained by the O/O encapsulation method. For the extracted soluble protein, the antigenicity was further determined as 0.8%. This result suggested that ~50% of encapsulated TT lost antigenicity, likely due to rapid precipitation and micronization of the protein in the polymer solution during microsphere preparation.

TT loading estimated by amino acid analysis is 1.6% after complete acid hydrolysis (Table 1). The amino acid analysis method can accurately determine the levels of total protein entrapped in PLGA microspheres; however, it leads to the inactivation of toxoid. For TT microspheres, similar loading values were obtained when determined by modified Bradford assay and amino acid analysis, indicating that modified Bradford assay after extraction process can have an accurate estimation of the encapsulated TT content.

Identifying Instability Mechanisms of TT in PLGA. We monitored TT release kinetics in a phosphate buffer and recovery of the antigen from the polymer after the release interval. TT release was brief (<7 days) and incomplete (only 12% and 17% of total and antigenic TT released, Figure 1C). The remaining unreleasable TT residue was removed from the microspheres and found mostly insoluble in phosphate buffer solution. In Table 2, it was shown that 2% of encapsulated TT remained soluble, whereas 10% and 9% of encapsulated TT formed noncovalent and disulfide-bonded aggregates, respectively. This low TT recovery indicates that ~60% of encapsulated TT formed aggregates which were

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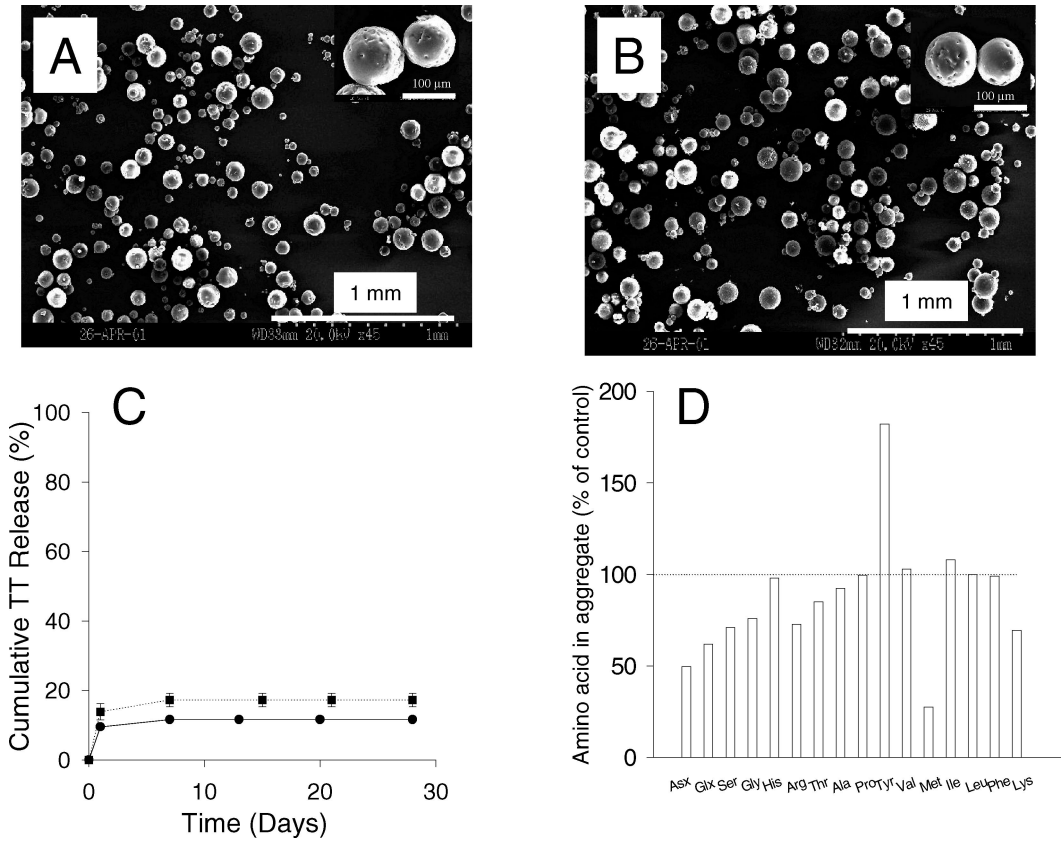

Figure 1. Scanning electron micrographs of TT/PLGA micropsheres (A) and TT/PLGA micropsheres containing lysine, sorbitol and $MgCO_3$ (B). In vitro release of TT from TT/PLGA microspheres without stabilizers: protein content (mean ± SD, $n = 2$) (●), and antigenic TT (mean ± SD, $n = 2$) (■) (C). Changes in the amino acid composition of TT aggregates formed in PLGA microspheres after 28 days of release (D). The amino acid composition of the acid hydrolysate of the aggregated TT is given relative to the unencapsulated TT standard control. All amino acid concentrations were normalized for the concentration of leucine.

Table 1. Encapsulation of TT

	TT loading (%)		
formulations	soluble[a,e]	antigenic[b,c]	total (AAA)[c,d]
no stabilizers	1.5 ± 0.1	0.82 ± 0.02	1.6
lysine + $MgCO_3$[f]	1.7 ± 0.4	1.4 ± 0.1	1.7
lysine + sorbitol + $MgCO_3$[g]	2.1 ± 0.2	1.1 ± 0.2	2.3
lysine + trehalose + $MgCO_3$[g]	2.5 ± 0.1	1.5 ± 0.4	2.8

[a] Extracted TT detected by modified Bradford assay [b] Extracted TT determined by ELISA [c] TT loading % determined by amino acid analysis (AAA). [d] Encapsulation efficiency was 54−93% as determined by AAA loading/theoretical loading. [e] Mean ± SD ($n = 2$). [f] The weight ratio of TT:lysine was 1:3, and $MgCO_3$ was loaded at 3%. [g] The weight ratio of TT:lysine:sorbitol (or trehalose) was 1:1.5:1.5, and $MgCO_3$ was loaded at 3%.

held together by covalent, non-disulfide bonds.[38,39] To further assess the aggregation mechanism, the aggregate residue was subjected to amino acid analysis. As seen in Figure 1D, much higher levels of the strongly formaldehyde-interacting tyrosine[40,41] were recorded and a noticeable loss of lysine and other amino acids were observed, consistent with previously reported solid-state aggregation of TT via formaldehyde-mediated aggregation pathway.[39] Hence, the analysis revealed two salient features of the instability of TT in the PLGA system: the formation of insoluble, non-disulfide bonded covalent aggregates and alterations in strongly formaldehyde-interacting amino acid content.

The denatured state of the unreleased TT is characteristic of insoluble aggregation mediated by formaldehyde reversibly bound to the antigen following formaldehyde detoxification of the dangerous tetanus toxin.[39] According to the formaldehyde-mediated aggregation pathway (FMAP), during exposure of the solid antigen to intermediate moisture levels, reactive formaldehyde-bound species (e.g., Schiff base of lysine residues) become exposed and react with another strongly formaldehyde-interacting side chain on a second TT molecule (e.g., another lysine or tyrosine) to form intermo-

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Table 2. Stability of Controlled-Released TT

stabilizers	released soluble (%)[a]	released antigenic (%)[b]	soluble residue (%)[c]	noncovalent aggregate (%)[d]	noncovalent + disulfide-bonded aggregate (%)[e]	recovery (%)[f]
no stabilizers	12 ± 2[g]	17 ± 2	2 ± 1	10 ± 3	19 ± 6	33 ± 6
lysine + $MgCO_3$[h]	60 ± 1	52 ± 1	1 ± 1	25 ± 3	40 ± 1	100 ± 3
lysine + sorbitol + $MgCO_3$[i]	66 ± 1	64 ± 2	1 ± 1	10 ± 2	20 ± 1	87 ± 2
lysine + trehalose + $MgCO_3$[i]	64 ± 2	78 ± 1	2 ± 1	11 ± 1	18 ± 3	84 ± 3

[a] Cumulative released soluble TT detected by modified Bradford assay. % normalized by actual drug loading based on soluble protein value. [b] Cumulative released soluble antigenic TT detected by ELISA after 28 days. % normalized by actual drug loading based on antigenic protein value. [c] TT extracted from microspheres after 28 days soluble in PBST, in GnCl but not in PBST, and in GnCl/DTT but not in PBST, respectively. % normalized by actual drug loading based on soluble protein value. [d] TT extracted from microspheres after 28 days soluble in PBST, in GnCl but not in PBST, and in GnCl/DTT but not in PBST, respectively. % normalized by actual drug loading based on soluble protein value. [e] TT extracted from microspheres after 28 days soluble in PBST, in GnCl but not in PBST, and in GnCl/DTT but not in PBST, respectively. % normalized by actual drug loading based on soluble protein value. [f] Recovery accounted for the total of % soluble residue, % noncovalent and % disulfide bonded aggregates. % normalized by actual drug loading based on soluble protein value. [g] Mean ± SD ($n = 2$). [h] The weight ratio of TT:lysine was 1:3, and $MgCO_3$ was loaded at 3%. [i] The weight ratio of TT:lysine:sorbitol (or trehalose) was 1:1.5:1.5, and $MgCO_3$ was loaded at 3%.

lecular cross-linking, further leading to the formation of insoluble aggregates.[39]

Besides formaldehyde-mediated aggregation, like most proteins, TT is susceptible to irreversible acid denaturation, and loses higher order structure and antigenicity rapidly below pH ∼ 4.[27,37] PLGA microspheres are known to commonly develop an acidic microclimate (e.g., pH < 3[42,43]) during incubation at physiological conditions, which has been hypothesized to cause damage to TT.[37] *Therefore, we hypothesized that formaldehyde- and acid-induced instability were the primary instability mechanisms during release from PLGA microspheres.* Other potential stresses of instability such as reactions initiated by the surface-active water-soluble oligomers of PLGA or by adsorption to the polymer surface are expected to be insignificant. For example, losses of antigenic TT were found to depend solely on pH (3−7.4) and be independent of the presence of either PLGA (MW 2000 Da, 100% L-lactide) oligomers or microspheres,[44] also shown in our studies described below.

Identifying Inhibitors of FMAP and the Acidic Microclimate in PLGA. TT was found to form aggregates by FMAP during release from microspheres (see above). Previously, to investigate FMAP, we studied a model formalized antigen, f-BSA, by treating bovine serum albumin (BSA) with formaldehyde under routine detoxification conditions. We discovered that strongly formaldehyde-interacting amino acids such as histidine and lysine could efficiently inhibit FMAP in the solid state by scavenging the reactive species in FMAP.[38] Coencapsulating the f-BSA with histidine and a sugar/polyol (trehalose) provided complete inhibition of FMAP in PLGA microspheres for one month.[45] We note that both trehalose[23] and sorbitol[46] have also been identified to improve TT stability in the solid state when exposed to moisture and in PLGA microspheres.

Table 3. TT Aggregation as a Function of pH before Lyophilization

pH	soluble TT[a] (%)
2	48 ± 1
5	48 ± 8
7	15 ± 1
10	7 ± 1

[a] After 6-day incubation at 37 °C and 80% RH, soluble TT content was determined by modified Bradford assay.

To determine if FMAP inhibitors identified in studies with model formalinized antigen f-BSA would be effective for TT, we compared the aggregation behavior of TT, BSA, and f-BSA in both solid state and PLGA microspheres. When exposed to intermediate moisture levels, solid state f-BSA exhibits nearly identical aggregation kinetics as TT and such aggregates are insoluble in 10 mM DTT/1 mM EDTA/6 M urea.[38] The pH-aggregation profile of TT (Table 3) and f-BSA[38] followed the same trend: aggregation is accelerated with increasing pH. Similarly, both f-BSA aggregates and TT aggregates formed in PLGA microspheres were characterized as covalent and non-disulfide bonded.[38,45] By contrast, the BSA control under either neutral or acidic conditions in the solid state, or in acidic PLGA microspheres, forms exclusively noncovalent and/or disulfide-bonded aggregates that are completely soluble in combined denaturing and reducing solvents.[38,45,47,48] The distinct difference in aggregation behavior of f-BSA and BSA in both the solid state and PLGA microspheres proved the existence of a FMAP

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in the polymer. Furthermore, close similarity of TT and f-BSA aggregation behavior indicates that those covalent, non-disulfide bonded aggregates are not TT-specific, but formaldehyde-specific, which suggests that the stabilization approaches developed with f-BSA may be also efficient to inhibit FMAP in TT.

As for the second critical deleterious instability pathway for TT, namely, acid denaturation in PLGA, earlier studies in our laboratory have demonstrated that inorganic basic additives ($MgCO_3$ or $Mg(OH)_2$)[48,49] or pore-forming agent, poly(ethylene-glycol) (PEG),[50] can prohibit the large microclimate pH drop in the PLGA induced by the water-soluble acidic polymer degradation products.[51] These excipients directly neutralized acids and/or increased the acid diffusion out of the polymer during bioerosion of PLGA, resulting in successful inhibition or prevention of acid-induced hydrolysis and aggregation of proteins such as BSA, basic fibroblast growth factor and several other proteins.[52]

Therefore, we selected three classes of stabilizers to inhibit acid- and formaldehyde-induced damage of TT encapsulated in PLGA: (1) strongly formaldehyde-interacting amino acids such as free histidine and lysine;[38,45] (2) polyols and sugars such as sorbitol and trehalose;[23,45,46] and (3) poorly soluble bases such as $MgCO_3$.[48,52] Any highly soluble base, such as sodium bicarbonate used in TT microsphere formulation by Katare et al., was not selected considering its fast diffusion and short duration in the polymer system.[24]

Effect of Stabilizers on Heat and Moisture-Induced TT Instability. Before coencapsulation in PLGA system, putative stabilizing excipients including $MgCO_3$, histidine, lysine, trehalose and sorbitol were examined for their effects on the physicochemical properties of TT when exposed to heat or moisture. $MgCO_3$ was selected in this study relative to $Mg(OH)_2$ because the carbonate salt is a better neutralizer of microclimate pH throughout PLGA microspheres.[53]

The excipients were first added to TT solution at a weight ratio of 10:1 (excipients:protein) and incubated at 45 °C for 22 days. At the end of incubation, TT was taken out for CD, fluorescence and antigenicity analysis. In Table 4, the effect of excipients on fluorescent properties and antigenicity of TT is displayed. As seen in Table 4, when excited at 280 nm, the maximum emission wavelength (λ_{max}) of TT standard was 329 nm and I_{350}/I_{329} was 0.75. For TT at pH 7.3 (1 mM phosphate buffer), with no excipients added, after incubation at 45 °C for 22 days, the λ_{max} of TT was shifted to a longer wavelength, 339 nm, and the I_{350}/I_{329} was increased to 0.99. Also, the antigenicity of TT was decreased to 28%. With the addition of most excipients, TT antigenicity was significantly improved. For example, TT coincubated with histidine, lysine and sorbitol retained above 80% of antigenicity after 22 days of incubation (Table 4). These samples similarly showed the least alterations in λ_{max} and I_{350}/I_{329} (Table 4). Their CD spectra were almost identical to that of TT standard (Figure 2). In contrast, fluoresecent properties of TT incubated with trehalose showed a significant change. The λ_{max} and I_{350}/I_{329} exceeded 330 nm and 0.80, respectively (Table 4). Similarly, the CD spectra deviated largely from that of TT standard (Figure 2) and the antigenicity of TT with trehalose only retained 44% (Table 4).

Table 4. Effect of Excipients on Fluorescent Properties and Antigenicity of TT after Exposure to Heat (45 °C and 22 Days)

	fluorescent properties		
excipients	λ_{max}	I_{350}/I_{329}	antigenicity (%)
TT standard	329	0.75	100
none	339	0.99	28 ± 1
histidine	329	0.76	96 ± 22
lysine	329	0.78	82 ± 12
trehalose	331	0.82	44 ± 10
sorbitol	330	0.78	83 ± 6
monomers (pH 2.9)	335	0.88	23 ± 1
$MgCO_3$/monomers, pH 5.7	329	0.78	88 ± 5
$MgCO_3$/monomers, pH 8	331	0.82	56 ± 6

We also investigated the effect of PLGA degradation products (lactic acid and glycolic acid monomers) and $MgCO_3$ on the structure of TT. As seen in Figure 2, when TT was incubated with 22 mM lactic acid and 24 mM glycolic acid (pH 2.9) at 45 °C for 22 days, both 208 and 222 nm, characteristic peaks of TT α helix were diminished in its CD spectra. For fluorescent properties, the λ_{max} and I_{350}/I_{329} of this sample were changed to 335 nm and 0.88, respectively (Table 4), corresponding to an antigenicity decrease to 23%. When the pH of the TT solution was adjusted to 6.7 with $MgCO_3$, a CD spectrum identical with that of standard TT was exhibited (Figure 2). The λ_{max} and I_{350}/I_{329} of TT approached that of TT standard, and we also observed that 88% of TT antigenicity was retained (Table 4). When the pH of the TT solution was further increased to 8 by the addition of $MgCO_3$, the whole CD spectrum curve was shifted up (Figure 2). The λ_{max} and I_{350}/I_{329} of TT also shifted away from that of TT standard and only 56% of TT antigenicity was retained after 22 days of incubation (Table

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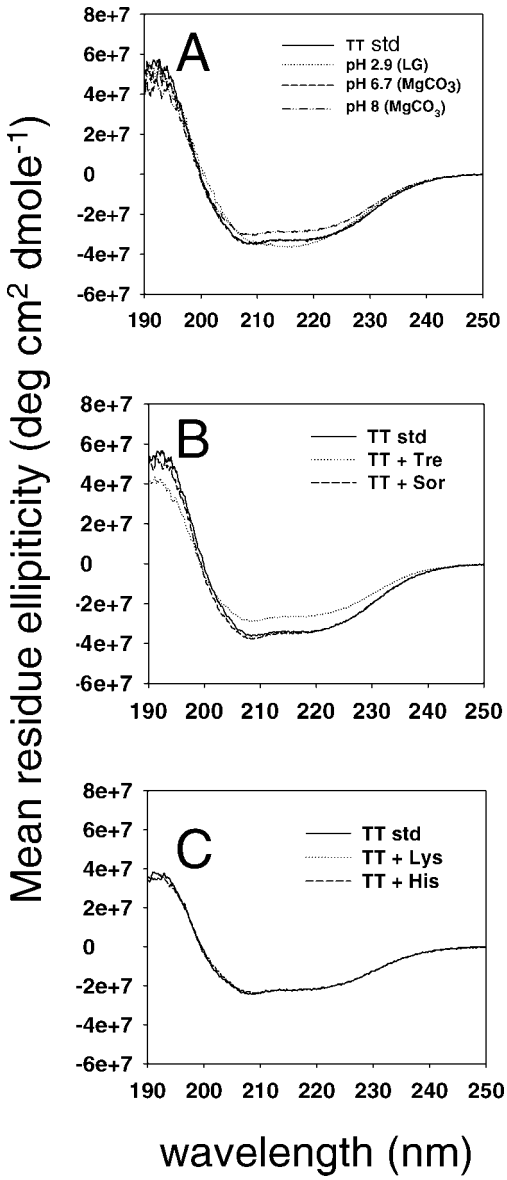

Figure 2. (A) Far UV−-CD spectra of TT standard (—), TT incubated with lactic and glycolic acid monomer at pH 2.9 (•••), monomers adjusted by $MgCO_3$ to pH 6.7 (- - -) and pH 8 (—••) at 45 °C for 22 days. (B) TT standard (—), TT incubated with trehalose (•••) and sorbitol (- - -) at 45 °C for 22 days. (C) TT standard (—), TT incubated with lysine (•••) and histidine (- - -) at 45 °C for 22 days.

4). The above results suggested a good correlation of high-order structural loss of TT with antigenicity loss. These changes are easily monitored by CD and fluorescence spectra. Excipients, which improved the structural integrity of TT, such as lysine, sorbitol, histidine, significantly improved TT antigenicity upon exposure to heat. As expected, low solution pH with the addition of lactic acid and glycolic acid monomers largely deteriorated the secondary and tertiary structure and antigenicity of TT. With pH neutralization by the addition of $MgCO_3$, the secondary and tertiary structure of TT was recovered, corresponding to the antigenicity recovery of TT.

The effect of excipients on moisture-induced instability of TT is shown in Table 5 (excipient to protein ratio is 10:1). TT samples after incubation were first analyzed for soluble protein by visual observation, modified Bradford assay and A280 absorbance determinations. For TT samples which retained significant solubility after incubation, their higher order structure and antigenicity were further evaluated. As seen in Table 5, TT lyophilized with lactic acid and glycolic acid (pH 2.9) formed aggregates before incubation. When pH was adjusted to 6.7 and 8 by $MgCO_3$, still significant amounts of TT aggregates were formed. TT co-lyophilized with histidine and lysine remained 100% soluble after incubation, whereas TT incubated with trehalose and sorbitol formed a small amount of aggregates.

We further analyzed the higher order structure and antigenicity properties of the soluble TT fraction. Similar CD spectra were observed for TT samples incubated with excipients and TT standard, indicating similar secondary structure among these samples (data not shown). As for fluorescent properties (see Table 5), for TT just after lyophilization, the λ_{em} and I_{350}/I_{329} were shifted to 327 nm and 0.73 respectively. However, TT antigenicity remained the same as standard. Fluorescent properties of TT lyophilized with sorbitol and lysine were closest to the TT standard, and similarly the antigenicity in these samples was highly retained after incubation. Although TT lyophilized with histidine and trehalose also had similar fluorescent properties with untreated TT, their antigenicities differed from untreated TT substantially. We speculate that incubation of these excipients with TT may not alter the higher order structure but may alter the chemical compositions of their epitopes (i.e., incorporation of excipients in the TT molecule), therefore causing large differences in antigenicity.

Overall, lysine and sorbitol were excellent inhibitors of aggregation and antigenicity losses during exposure to moisture in the solid state and heat in solution. The stabilization effect of lysine on TT was consistent with the finding that the presence of lysine, of all tested amino acids, imparted superior antigenicity during formaldehyde detoxification of tetanus toxin.[54] It was proposed that the high antigenicity and relative stability of TT with lysine may be associated with the presence of side chains derived from the incorporation of lysine into protein molecules.[54] TT antigenicity was also largely improved after monomers were neutralized by magnesium salts. Although trehalose and histidine can significantly inhibit TT aggregation but TT antigenicity was compromised in the presence of these species. CD and fluorescence spectroscopy appear to be useful techniques to predict TT antigenicity losses in most instances.

Stabilization of TT in PLGA. Based on the above studies, we coencapsulated with TT in PLGA microspheres putative stabilizers against formaldehyde- and acid-induced inactiva-

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Table 5. Effect of Excipients on Fluorescent Properties and Antigenicity of TT after Exposure to Moisture (37 °C and 80% RH for 30 Days)

excipients	soluble protein (%)	fluorescence properties		antigenicity (%)
		λ_{max}	I_{350}/I_{329}	
TT standard		329	0.75	100
lyophilized TT standard		327	0.73	100
none	15 ± 1 (15 days)	*a*	*a*	*a*
histidine	101 ± 5	329	0.72	24
lysine	103 ± 1	329	0.76	81 ± 12
trehalose	92 ± 4	328	0.73	47
sorbitol	87 ± 1	329	0.75	90 ± 8
monomers, pH 2.9	aggregates formed before incubation	*a*	*a*	*a*
$MgCO_3$/monomers, pH 5.7	9 ± 1	*a*	*a*	*a*
$MgCO_3$/monomers, pH 8	4 ± 2	*a*	*a*	*a*

[a] Not determined.

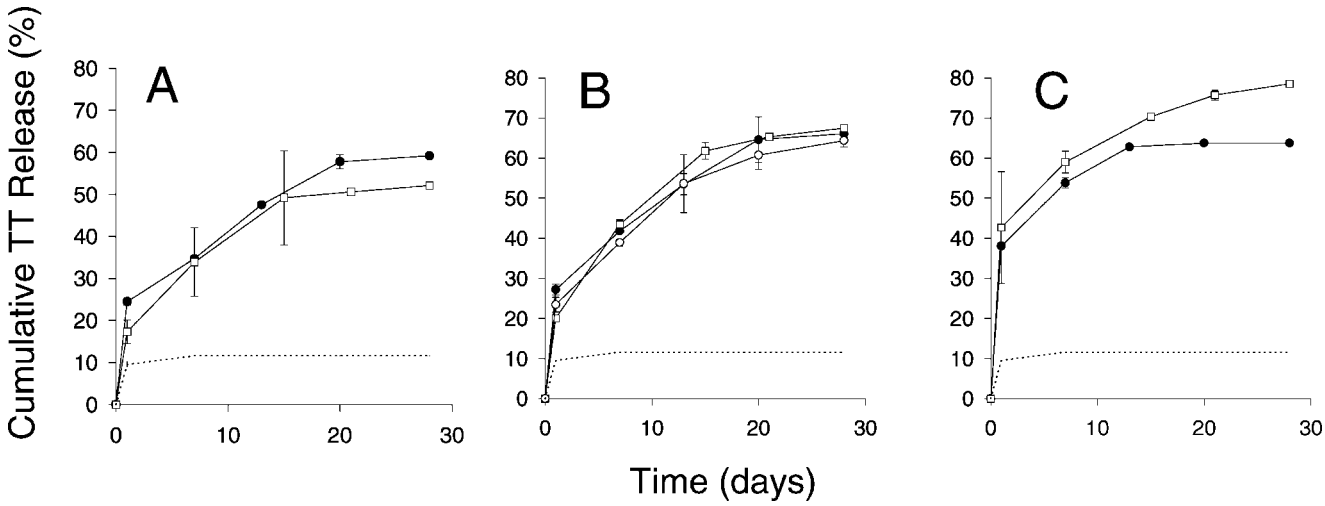

Figure 3. Release kinetics of TT from PLGA 85/15 (iv = 0.86 dL/g) microspheres coencapsulated with lysine + $MgCO_3$ (A), lysine + sorbitol + $MgCO_3$ (B), and lysine + trehalose + $MgCO_3$ (C). Protein content determined by modified Bradford assay (mean ± SD, $n = 2$) (●) and antigenic TT (mean ± SD, $n = 2$) (○) when release medium contained no BSA; antigenic TT (mean ± SD, $n = 2$) (□) released from microspheres when release medium contained 0.2% BSA. Dashed line represents release kinetics of TT (determined by modified Bradford assay) from PLGA microspheres containing no stabilizers.

tion uncovered by our mechanistic analysis (Tables 4 and 5), lysine, lysine/sorbitol, or lysine/trehalose and $MgCO_3$. Lysine was chosen over histidine as the inhibitor of FMAP because of large histidine-induced losses of TT antigenicity during solid-state stability assessment of FMAP.

Figure 1B shows the morphology of microspheres coencapsulated with lysine, sorbitol and $MgCO_3$. Similar SEM image of this formulation to TT microspheres without any stabilizer indicated that coencapsulation of these excipients did not change microsphere morphology.

As shown in Table 1, the antigenic TT loading for each formulation with stabilizers ($MgCO_3$/lysine, $MgCO_3$/lysine/sorbitol, and $MgCO_3$/lysine/trehalose) was >1% w/w with an encapsulation efficiency of 54−93%. Whereas negligible TT solubility was lost during encapsulation, some noticeable antigenicity losses of the antigen were noted. These losses appeared not to affect the protein during release, as indicated by the similarity between antigenic and soluble TT release profiles (e.g., Figure 3B). We also note that no antigenicity loss was observed if solid protein powder was homogenized in acetonitrile at 15,000 rpm for 1 min (data not shown). In future formulation, to minimize antigencity loss during encapsulation, micronized solid TT powder can be used or precipitation/micronization of TT from aqueous concentrate should be performed in organic solvent alone.

TT loading data in Table 1 indicated that coencapsulation of the stabilizers influenced the TT encapsulation efficiency favorably. When $MgCO_3$/lysine was coencapsulated, TT loading based on soluble protein content was 1.7% and 80% of encapsulated TT retained antigenicity. When sorbitol/trehalose is further added, a substantial increase of TT loading was noted, especially with the coencapsulation of trehalose. Johansen et al. also reported that coencapsulation of trehalose increased the encapsulation efficiency and exerted a dominant increase in the initial burst.[23] The underlying mechanism by which trehalose increased encap-

sulation efficiency and burst release is unclear and warrants further investigation.

In Figure 3, release kinetics of formulations with and without stabilizers is displayed. As predicted, with stabilizers added, slow and continuous antigenic TT release was observed for the 1 month incubation. For lysine/sorbitol/$MgCO_3$ and lysine/trehalose/$MgCO_3$ PLGA microspheres (Figure 3B,C), 64−78% antigenic release was observed after a ∼25−40% initial burst without any signs of antigenic losses in the released protein. Equally compelling was the recovery data, as shown in Table 2. Without stabilizers, TT aggregation via FMAP was extensive, resulting in only 33% recovery. With lysine and $MgCO_3$ added, 60% of TT was released and the remaining TT (40%), although aggregated, was now soluble in DTT + GnCl. The 100% recovery confirms the complete inhibition of FMAP by the addition of lysine, allowing secondary aggregation mechanisms involving disulfide scrambling and hydrophobic interactions to become dominant.[38,39] Further addition of either sorbitol or trehalose to the $MgCO_3$/lysine formulation slightly decreased FMAP inhibition (i.e., <100% recovery) but improved the antigenic release (i.e., 64−78% with sugar/polyol vs 52% without), indicating that the sugars/polyols, while not as effective as lysine to inhibit FMAP, are necessary to retain TT antigenicity during release. The absence of antigenicity losses in the released TT from the sugar/polyol-containing samples also indicates that pH was effectively neutralized in the polymer.

Previously Johanson et al. reported that experiment setup exerts a great effect on the release of encapsulated TT from PLGA microspheres due to strong TT adsorption to hydrophobic surfaces such as glass.[36] In our study, when quantified by ELISA, similar TT release kinetics was observed in the release medium with and without 0.2% BSA (Figure 3B), indicating that TT adsorption was not substantial during release evaluation. The apparent discrepancy with this literature report is possibly due to higher TT loading in our microsphere formulation, causing greater protein concentration to mask the adsorption artifacts. Moreover, a large burst release may potentially block the adsorption sites in the release vessel and make TT adsorption less significant. In addition, some TT samples during later release were poorly quantitated by the modified Bradford assay, which has a higher quantitation limit than the ELISA, causing an artificial flattening in some of the release curves.

In closing, there are two principal mechanisms of TT instability during release from PLGA microspheres, acid- and formaldehyde-induced inactivation. We used stabilizers to neutralize acidic microclimate and inhibit FMAP and systematically evaluated their effect on TT stability under elevated high temperature and humidity. This strategy uncovered potent additives for stabilizing encapsulated TT, i.e., lysine, sorbitol, and trehalose to inhibit FMAP and $MgCO_3$ to bypass acid-induced damage. Coencapsulation of these additives resulted in an unparalleled stability of TT encapsulated in PLGA microspheres, and slow and continuous release of the antigen.

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