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NMR Spectroscopic Evaluation of the Internal Environment of PLGA Microspheres

Kirby C. Wong-Moon, Xin Sun, Xichdao C. Nguyen, Bin P. Quan, Kai Shen, and Paul A. Burke*

Department of Pharmaceutics, Amgen Inc., Thousand Oaks, California 91320

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Abstract: The internal environment of poly(lactide-*co*-glycolide) (PLGA) microspheres was characterized using ^{31}P and ^{13}C solid-state and solution NMR spectroscopy. Physical and chemical states of encapsulated phosphate- and histidine-containing porogen excipients were evaluated using polymers with blocked (i.e., esterified) or unblocked (free acid) end groups. Spectroscopic and gravimetric results demonstrated that the encapsulated porogen deliquesced upon hydration at 84% relative humidity to form a solution environment inside the microspheres. Dibasic phosphate porogen encapsulated in unblocked PLGA was partially titrated to the monobasic form, while in the same formulation ^{13}C NMR showed partial protonation of the histidine imidazole. Similarly, encapsulated monobasic phosphate was partially converted to phosphoric acid. Coencapsulation of monobasic and dibasic phosphate porogens resulted in a single peak on hydration, indicating chemical exchange between discrete excipient microphases. Exogenous buffer addition differentiated external from internal, nontitratable, excipient populations. Microspheres containing dibasic phosphate porogen were hydrated with fetal calf serum, incubated at 37 °C, and characterized by ^{31}P NMR through the polymer erosion phase. Within 48 h the ^{31}P chemical shift moved over 2 ppm upfield and the line width narrowed to <60 Hz; there was little additional change through day 14. This indicated complete conversion to the monobasic phosphate form throughout the polydisperse sample and that pH remained below 4 but above the phosphoric acid pK_a during matrix erosion.

Keywords: Biodegradable polyesters; polymeric drug delivery systems; stability; solid-state NMR spectroscopy; macromolecular drug delivery

Introduction

Poly(lactide-*co*-glycolide) (PLGA) is a preferred polymer for multiple biomedical applications including delivery of protein and peptide therapeutics.[1] Hydrolytic degradation of the polyester ultimately generates lactic and glycolic acids. The possible accumulation of these erosion byproducts within PLGA matrices, which could compromise the integrity of encapsulated acid-labile drugs, has been addressed by a number of research groups focusing primarily on assessment of local pH and its control. Electron paramagnetic resonance (EPR) spectroscopy using pH-sensitive spin labels was used to assess the microenvironment, including pH, of PLGA implants,[2] and combined with magnetic resonance imaging to characterize drug release and matrix erosion.[3] EPR of PLGA microspheres containing spin-labeled albumin showed a decrease in pH to 3.5 over approximately one week.[4] Visual evidence of an acidic microsphere interior was obtained by confocal fluorescence microscopy using entrapped pH-

* Address correspondence to this author at Merck & Co., Inc., WP 26-462, Sumneytown Pike, West Point, PA 19486-0004. Telephone: 805-312-5121. Fax: 215-616-2154. E-mail: pburke@alum.mit.edu.

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sensitive dyes;[5–7] under some conditions pH in the microsphere core was concluded to have dropped to around 2 after incubation. Solution ^{13}C NMR was used to assess pH in a PLGA film under accelerated storage conditions (70 °C) using ^{13}C-labeled fumarate as a reporter group.[8] Glass electrodes were used to monitor pH in PLGA films,[9] illuminating the importance of polymer phase diffusion path length and enabling development of an equilibrium mathematical model characterizing the system.[10] Below a certain length scale diffusional path lengths are thought to be too short for water-soluble degradation products to accumulate, although the aforementioned results indicate that even micron-scale systems are susceptible to pH extremes under some conditions. This conclusion is supported, albeit indirectly, by an observed increase in recovery of intact drug from microsphere formulations of acid labile proteins where basic salts were incorporated as neutralizing excipients.[11,12]

Despite this emerging understanding, unanswered questions remain. For example, PLGA-based depot formulations of gonadorelin analogues have been quite successful commercially. Several of these are known to undergo acid-mediated degradation reactions such as debutylation (of buserelin and goserelin at the *O-tert*-butyl-D-serine) and deamidation (of triptorelin at the C-terminal primary amide)[13,14] yet are available in formulations delivering active drug up to three months following a single injection. The correlation of determined local pH values with degradation reaction kinetics characterized in bulk solution at the same nominal pH is potentially complicated by microenvironment-associated changes in pK_a's, ionic strength, and surface effects. That the microenvironment is changing over time further complicates this challenge. The objective of our research program is to introduce approaches to directly monitor structure, in situ in real time, to address these challenges, and ultimately support a rational approach to microsphere formulation development.

Nuclear magnetic resonance (NMR) spectroscopy is attractive for the characterization of PLGA microspheres for several reasons. NMR is noninvasive with minimal sample preparation required, and allows direct observation of the protonation state of incorporated drugs or excipients in addition to other structural features. Thus it enables assessment of internal pH without the use of exogenous molecular probes or reporter groups which may have buffering capacity or partition into the polymer phase. Further, the technique avoids disruption of polymer matrix microstructure required by physical probes such as electrodes. NMR can be applied to samples with both solution and solid phase components, an important advantage in a mixed-phase system where rate and extent of water uptake will vary among formulations. The transition from a dry solid drug particle to a fully hydrated one has profound implications for macromolecular drug stability.[15] Solid-state NMR spectroscopy was used previously by others to characterize water uptake in PLGA.[16] More recently the technique has been used to characterize a variety of pharmaceutical solids.[17–19]

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A solution-state ^{31}P NMR study reported by one of the present authors (P.A.B.) employed endogenous inorganic phosphate in sheep serum, a model physiological fluid, to hydrate PLGA microspheres containing ribonuclease.[20] Under these conditions phosphate diffused into the microsphere (remaining in a solution state), and pH was assessed by observing the difference in ^{31}P chemical shifts between internal and external phosphate populations. Results indicated that pH stabilized around 6.4. The study did not address drug dissolution or the possibility of pH gradients, subsequently reported by others in PLGA microspheres[5] and in films of poly(orthoester) containing 30% lactic acid in the backbone.[21] The present work introduces NMR as a technique for the characterization of encapsulated drugs in matrix-style PLGA microsphere formulations. ^{31}P, a naturally abundant, sensitive spin-1/2 nucleus,[22] was used to probe physical and chemical environments. Porogen particles containing phosphate, a common buffering excipient, were encapsulated in both blocked (terminal ester, or "capped") and unblocked (free acid) PLGA's. The kinetics and extent of phosphate dissolution were assessed using solid- and solution-state NMR methods. Following hydration, dibasic, monobasic, and phosphoric acid forms were distinguished based on chemical shift analysis. Accessibility of solutes to the microsphere interior was assessed by adding exogenous buffer and observing the impact on internal phosphate protonation state. The approach was applied to address the mechanism of PLGA microsphere hydration and the evolution of the internal environment during matrix erosion.

Materials

α,α-Trehalose dihydrate (high purity, low endotoxin) was from Pfanstiehl (Waukegan, IL). Terminal ester ("blocked"; RG502, inherent viscosity 0.19 dL/g in chloroform) and terminal free acid ("unblocked"; RG502H, inherent viscosity 0.16 dL/g in chloroform) PLGA 50:50 were from Boehringer Ingelheim (Ingelheim, Germany). ^{13}C-Labeled L-histidine HCl (U-^{13}C6, 98%) was from Cambridge Isotope Laboratories (Andover, MA). Dialyzed fetal bovine serum (GIBCO, Catalog No. 26400-044) was from Invitrogen (Carlsbad, CA). Dichloromethane (USP-NF grade) was from J. T. Baker (Phillipsburg, NJ). All other chemicals were of analytical grade or purer and were from commercial suppliers.

Methods

Polymer Characterization. Unblocked polymer M_n was determined by end-group titration using a Mettler-Toledo DL55 autotitrator (Mettler-Toledo, Inc., Columbus, OH). Polymer was dissolved in 80:20 v/v acetone:methanol and titrated with 0.1 N potassium hydroxide in methanol. M_w was determined relative to polystyrene standards (American Polymer Standard) by gel permeation chromatography (GPC). Samples were dissolved (10 mg/mL) in tetrahydrofuran, and analyzed using a Waters 2695 Alliance system with an HR2 column at 30 °C. In vitro polymer erosion rates were assessed, and reported as the pseudo-first-order degradation rate constant, as described.[23]

Porogen Fabrication and Encapsulation. Phosphate-containing porogen particles, with a nominal composition of 20% sodium phosphate (either dibasic or monobasic), 10% histidine (natural abundance or, where noted, ^{13}C-labeled), 20% trehalose, and 50% sodium chloride, were fabricated by spray drying pH-adjusted (to 8.0 for dibasic; to 4.0 for monobasic) 10 mg/mL aqueous solutions as described.[24] Porogen particles were encapsulated in PLGA microspheres by spray-freeze drying as described,[25] using dichloromethane as the fabrication solvent and pentane as the extraction solvent. Samples were stored at −20 °C over desiccant prior to use.

Physical Characterization. Particle size was determined by laser light scattering using a Malvern Mastersizer X (Malvern Instruments Ltd., Worcestershire, U.K.). Particles were suspended in dichloromethane (porogen) or in water containing 0.1% polysorbate 80 (microspheres) and sonicated prior to analysis. Particle size, reported as volume median diameter, was calculated using the Fraunhoffer optical diffraction model to yield the volume-based diameter parameters at 10%, 50%, and 90% cumulative volume percent ($d_{0.1}$, $d_{0.5}$, and $d_{0.9}$, respectively). Batch-to-batch variability was <15%. Particle morphology was characterized by scanning electron microscopy (SEM) using a Philips XL30ESEM (FEI Company, Hillsboro, OR). Samples were mounted on a carbon adhesive tab and sputter coated (10−15 nm) with gold palladium (60/40 alloy).

Water content was determined in duplicate (20 mg per sample) using a Mettler DL37 Karl Fischer Coulometer

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(Mettler-Toledo, Inc., Columbus, OH) with the Aqua Star Coulomat single solution reagent (EM Science, Darmstadt, Germany).

Modulated differential scanning calorimetry (DSC) measurements were performed using a TA Instruments DSC Q100 (TA Instruments, New Castle, DE). The temperature was ramped at 3 °C/min from 10 to 80 °C, with a modulation of 0.5 °C every 40 s.

X-ray powder diffraction (XRPD) was performed using a Philips X'Pert PW3040-Pro equipped with an X'Celerator wide area detector (PANalytical Inc., Natick, MA). Samples were loaded on a silicon zero background holder and rotated at 60 rpm. The following instrument parameters were used: Cu Kα source, 45 kV, 40 mA, and continuous $\theta-\theta$ scan from 3.0° to 40.0° 2θ at 0.070°/s.

Microsphere Hydration and Sample Preparation. Microsphere samples were hydrated by placing 100 mg of sample in closed chambers containing saturated solutions of potassium chloride at 20 °C, providing a relative humidity (RH) of 84%.[26] Hydrated samples were lightly packed in 7 mm solid-state NMR rotors. NMR signal acquisition was completed within 4 h of sample removal from the hydration chamber. Gravimetry before and after data acquisition confirmed that moisture content remained constant.

Equilibrium water uptake levels were measured by dynamic vapor sorption using a DVS Advantage (Surface Measurement Systems, Allentown, PA) or equivalent. After equilibration at 50% RH and 20 °C, the humidity was increased to 85% RH and water uptake was monitored as a function of time. Equilibrium was defined as <0.005% weight change over 10 min; this condition was satisfied within 24 h for all samples tested.

Exogenous Buffer Addition. The osmolarities of porogens deliquesced at 84% RH and 20 °C were measured to be 7000 mOsm/kg (dibasic phosphate porogen) and 6800 mOsm/kg (monobasic phosphate porogen) using an Advanced Osmometer 2020 (Advanced Instruments, Inc., Norwood, MA). In order to eliminate osmotic pressure difference-mediated loss of the phosphate analyte (which would adversely affect signal strength) a solution of 1.0 M glycine HCl was adjusted to iso-osmolarity with the deliquesced dibasic phosphate porogen by adding sodium chloride, with a resulting pH of 0.8. A solution of tricine base (1:1 molar ratio of tricine and NaOH) was similarly adjusted to iso-osmolarity with the deliquesced monobasic phosphate porogen. The molarity of tricine base solution for the monobasic porogen was 2.1 M for blocked PLGA and 3.2 M for unblocked PLGA, with resulting pH values of 9.4 and 9.7 respectively. Buffer (20 μL) was added to the solid-state NMR rotor containing the hydrated microsphere sample (previously spun at 5 kHz to obtain a spectrum before buffer addition). NMR signal acquisition was completed within 4 h of buffer addition.

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Kinetic Study of Hydrated Microsphere Internal Environment at 37 °C. Fetal bovine serum was osmotic-pressure adjusted to match that of the deliquesced dibasic phosphate porogen by adding sodium chloride; the pH was 6.8. Dibasic porogen-containing unblocked PLGA microspheres (250 mg) were combined with serum (5 mL, equilibrated to 37 °C), followed by incubation at 37 °C for up to 22 days. The medium was exchanged every one to two days to maintain supernatant pH. Prior to NMR analysis, samples were washed with fresh medium twice to ensure removal of phosphate released from the microspheres. There was no contribution to the observed ^{31}P signal from endogenous phosphate in the medium.

Following incubation for the designated time, microspheres were collected by aspiration with a transfer pipet or with a spatula, and loaded into the sample rotor. Samples were packed with intermittent spinning (1−2 kHz) followed by removal of excess supernatant and further sample addition. This procedure was repeated to maximize the packed sample mass. Samples were dried under vacuum prior to analysis by GPC.

NMR Spectroscopy. Solid-state NMR measurements were performed using a Varian Unity INOVA 400 spectrometer (Varian NMR Instruments, Palo Alto, CA) at resonance frequencies of 162 MHz for ^{31}P and 100 MHz for ^{13}C. All spectra were acquired at room temperature with magic angle spinning (MAS) at 5 kHz using a Varian 7 mm, or 6 mm T3, CPMAS probe.

^{31}P spectra were acquired using a single ^{31}P pulse followed by ^{1}H decoupling at an rf field strength of 40 kHz. ^{31}P chemical shifts were referenced to 85% phosphoric acid (0.0 ppm) using a secondary standard (brushite, δ1.4 ppm). Recycle delays of 5 s were used for the porogen samples, and 1 s for microsphere samples. Signal averaging was performed using 5120 scans for dry samples and 1024 scans for hydrated samples. Exponential line broadening (25 Hz) was applied prior to Fourier transformation. ^{31}P NMR spectra were cropped to highlight the region of interest and normalized to the maximum peak intensity; full spectral width versions appear in Figure S2.

^{13}C spectra were acquired using a single ^{13}C pulse followed by ^{1}H decoupling. Recycle delays of 1 s were used, and signal averaging performed using 5120 scans. ^{13}C chemical shifts were referenced to tetramethylsilane (0 ppm) using a secondary standard (hexamethylbenzene; methyl shift at δ17.3 ppm).

Results

Characterization of Solid Porogen and Encapsulated Porogen. Solid porogen particles were characterized for moisture level, particle size, and crystallinity. Moisture levels ranged from 1.1% to 1.3%, and particle size ranged from 2.3 to 2.7 μm. The porogen particles were shown to be amorphous by XRPD (Figure S1), with the exception of a signal corresponding to a trace amount of crystalline sodium chloride. No increase in crystallinity was observed on storage.

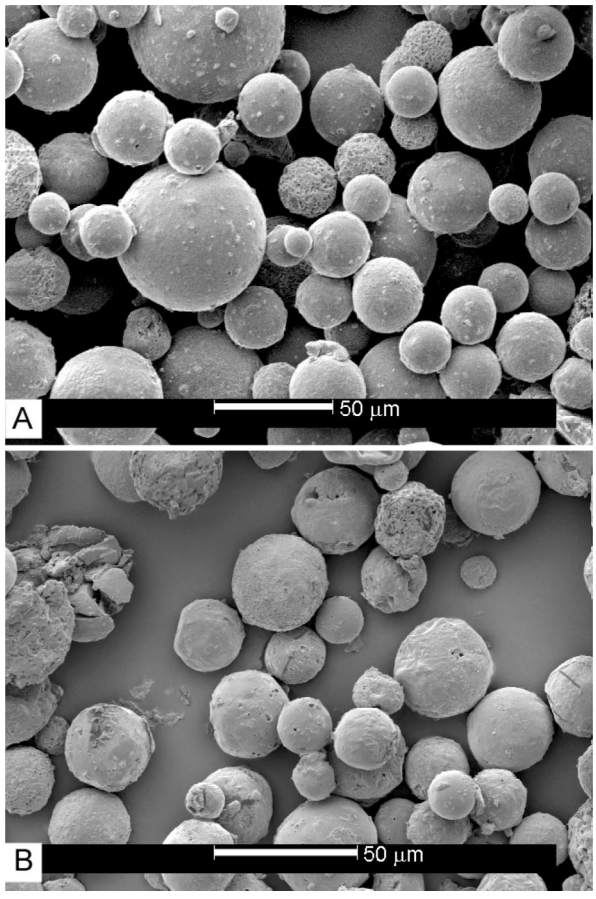

Figure 1. Scanning electron micrographs of microspheres made from unblocked (A) and blocked (B) PLGA containing dibasic phosphate porogen.

The unblocked PLGA M_n was determined to be 3.8 kDa by end-group titration. The 20% sodium phosphate porogen content, with a 10% porogen load in the microspheres, corresponded to a slight molar excess of unblocked polymer to phosphate (1.7-fold for dibasic and 1.4-fold for monobasic). M_w was determined to be 12 kDa and 17 kDa for unblocked and blocked polymer, respectively. The encapsulated porogen particles were characterized for moisture level, particle size, and morphology by SEM. Moisture levels were <0.1%. Mean particle size for all microsphere batches was 29 μm; the $d_{0.1}$ and $d_{0.9}$ were 16 and 52 μm, respectively. SEM images of dibasic phosphate-containing microspheres (Figure 1) showed slight surface porosity for both unblocked and blocked polymer. Similar results were obtained with monobasic phosphate-containing microspheres for both polymers (not shown).

Anhydrous salts, porogens and encapsulated porogens were characterized by ^{31}P MAS NMR spectroscopy (Table 1; Figure 2). The ^{31}P chemical shifts for the porogen particles moved slightly upfield from their respective monobasic and dibasic sodium phosphate anhydrous salts (Figure 2A,B; 2E,F). Upon encapsulation, the chemical shifts moved further upfield for both blocked (Figure 2C,D) and unblocked (Figure 2G,H) polymer. Line widths for the porogens approximated those of their respective phosphate salts but decreased significantly upon encapsulation.

Table 1. ^{31}P Chemical Shifts of Sodium Phosphate Salts

salt	^{31}P chemical shift (ppm)
dibasic	
anhydrous	7.1[a]
dihydrate	5.4[b]
heptahydrate	3.7[a]
monobasic	
anhydrous	2.4[a]
monohydrate	0.9[b]

[a] Determined experimentally. [b] From Hartmann et al.[27]

Moisture Uptake by Porogen and Encapsulated Porogen. Equilibrium moisture uptake data for porogen and encapsulated porogen appear in Table 2. Porogen samples were observed to deliquesce. Microsphere samples appeared moist, remaining as discrete particles but no longer free flowing. Moisture uptake for porogen-free microsphere controls was <0.1% for both polymer types tested. The extent of polymer hydrolysis over the time frame of hydration and sample characterization was assessed by monitoring the time-dependent decrease in the T_g, measured following drying. A modest drop in T_g (1.1 $\pm$ 0.4 °C) over the course of the experiments was observed.

^{31}P NMR Spectroscopy of Encapsulated Phosphate Porogens Following Hydration. Following hydration of the encapsulated porogens at 84% RH and 20 °C, line widths narrowed significantly (Figure 3A−D). In addition, the spinning sidebands present in the dry samples (Figure S2) were not observed in the hydrated samples. Hydrated monobasic phosphate porogen microspheres were analyzed further, and results (Figure S4) compared with Figure 3D. Proton decoupling was omitted from the pulse sequence; signal intensity and line width remained unchanged. The rotation angle was adjusted away from the magic angle; again, the line width remained unchanged. A ^{31}P cross polarization pulse sequence was applied; this resulted in complete loss of signal. Similar results were obtained with encapsulated dibasic phosphate.

^{13}C NMR was employed to assess the effect of microsphere hydration on the porogen histidine. Dibasic phosphate-containing porogen was prepared using ^{13}C-labeled histidine, and encapsulated using either blocked or unblocked PLGA. Prior to hydration the histidine resonances were poorly resolved in the ^{13}C MAS NMR spectrum (not shown) with significant interference from PLGA natural abundance peaks. In contrast ^{13}C histidine peaks were well-resolved after sample hydration (Figure 4). To assess protonation by the polymer, histidine imidazole (p$K_a \sim 6$) chemical shifts were considered. The chemical shift of C-3 changed from 131.7 ppm (blocked PLGA) to 129.4 ppm (unblocked PLGA). In

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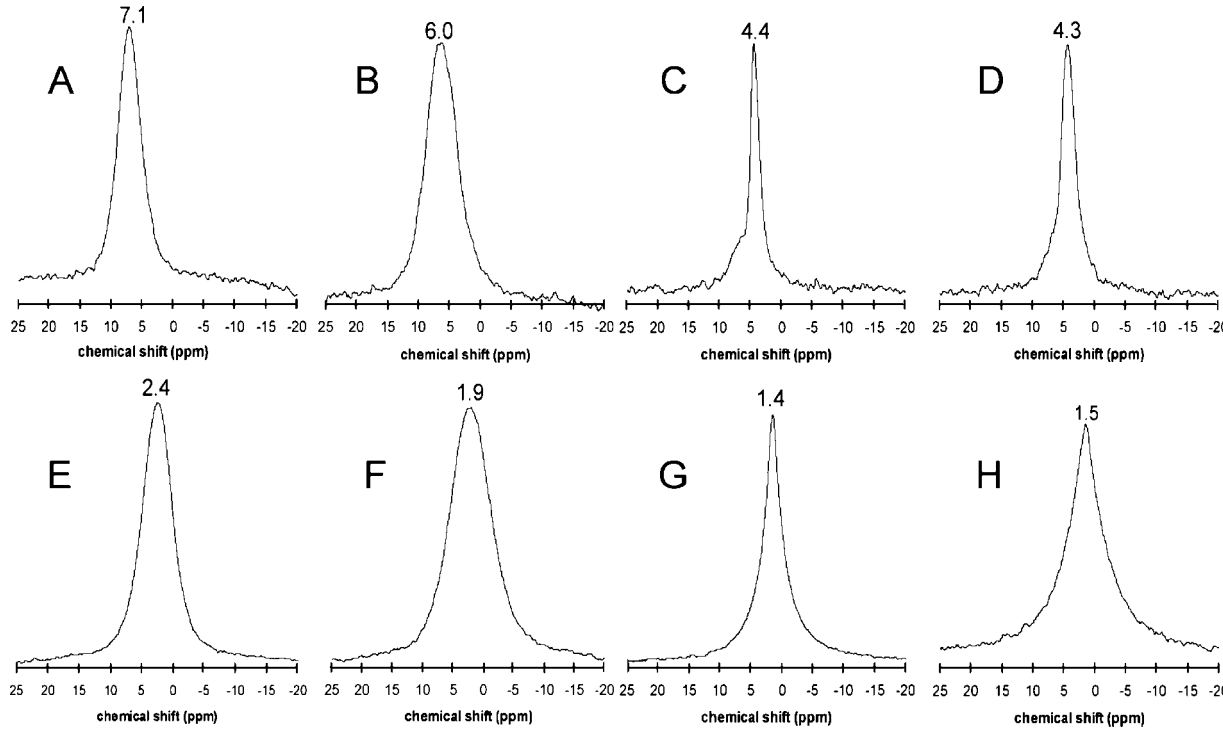

Figure 2. Proton decoupled ^{31}P MAS NMR spectra of dibasic (A−D) and monobasic (E−H) sodium phosphates. Anhydrous salts (A, E) were formulated as micron-sized porogen particles (B, F) and then encapsulated in blocked (C, G) or unblocked (D, H) PLGA.

Table 2. Equilibrium Moisture Uptake of Phosphate-Containing Porogens

	water uptake, %[a]	
sample	monobasic sodium phosphate	dibasic sodium phosphate
porogen	227	235
porogen encapsulated (10% w/w) in		
blocked PLGA	25.0	23.4
unblocked PLGA	25.0	23.4

[a] At 84% RH, 20 °C (see *Methods*).

addition, the chemical shift of C-2 changed from 137.2 ppm (blocked PLGA) to 135.6 ppm (unblocked PLGA).

Assessment of Mechanism of Microsphere Hydration. The mechanism of microsphere hydration is hypothesized to involve a combination of water transport (i) through interconnecting pores or cracks, (ii) through transient fissures due to osmotic pressure differences, or (iii) through the polymer phase. For a matrix-style microsphere, mixing of contents of individual entrapped particles following hydration and dissolution will depend on the mechanism of water uptake. For (i) and (ii), mixing and consequent chemical exchange are expected, while for (iii) mixing is not expected for polymer-impermeable solutes. To probe the mechanism of water uptake, microspheres were prepared containing 5% of each of the two porogen formulations (corresponding to a 1.2:1 molar ratio of monobasic:dibasic), and hydrated as above. For both blocked (Figure 3E) and unblocked (Figure 3F) PLGA microspheres, the resulting ^{31}P NMR spectra showed single peaks, with chemical shifts approximating the mean of those observed for the respective pure porogen formulations (2.1 ppm for blocked; 1.6 ppm for unblocked).

To verify that the resolution was sufficient to detect different populations of phosphate within the same sample, a physical blend (50:50 by weight) of microspheres containing dibasic or monobasic phosphate porogen was prepared and hydrated as above. Peaks for dibasic and monobasic phosphate populations are clearly visible for both blocked (Figure 3G; 3.2 and 0.9 ppm respectively) and unblocked (Figure 3H; 2.9 and 0.7 ppm respectively) microspheres. In addition, in both cases a third population is visible, with a chemical shift midway between the other two (2.2 ppm for blocked; 1.6 ppm for unblocked).

Addition of Exogenous Buffer to Hydrated Porogen-Containing Microspheres. The above result suggested the presence of separate exchanging and nonexchanging phosphate populations. This was further assessed by adding exogenous buffer to hydrated samples. To minimize loss of phosphate from the microsphere interior, and consequent loss of signal, buffer osmotic pressure was adjusted to match that of the respective deliquesced porogen solution (see Methods). Buffer pH was adjusted to 0.8 for evaluation of dibasic phosphate porogen microspheres, to fully convert phosphate in the external population to phosphoric acid (δ 0.0 ppm). For evaluation of monobasic phosphate porogen microspheres, buffer pH was 9.4 (blocked microspheres) or 9.7 (unblocked microspheres), to fully convert external phosphate

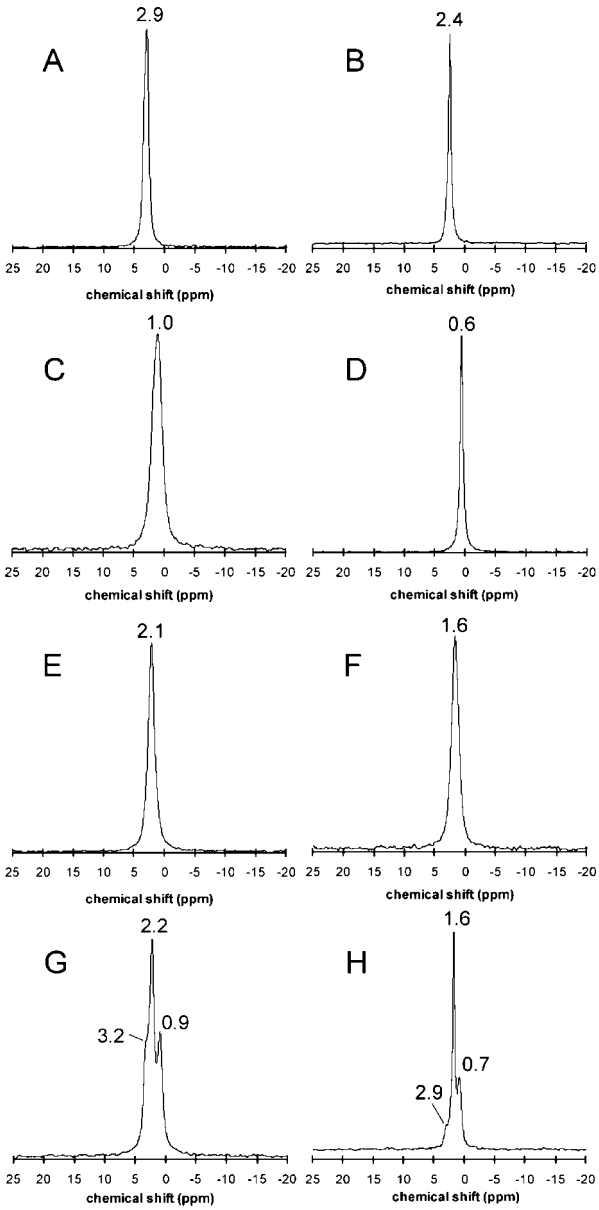

Figure 3. Proton decoupled ^{31}P MAS NMR spectra of phosphate-containing porogens encapsulated in blocked (A, C, E, G) or unblocked (B, D, F, H) PLGA, following hydration at 84% RH. Microsphere samples contained 10% dibasic (A, B); 10% monobasic (C, D); or 5% dibasic/5% monobasic (E, F) sodium phosphate porogen. Physical blends (50:50 w/w) of microsphere formulations were run in G (blend of samples A and C) and H (blend of samples B and D).

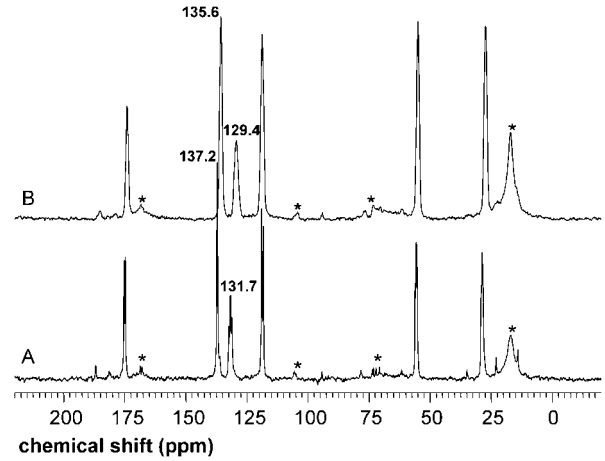

Figure 4. Proton decoupled ^{13}C MAS NMR spectra of dibasic phosphate-containing porogen encapsulated in blocked (A) or unblocked (B) PLGA, following hydration at 84% RH. The porogen was prepared using ^{13}C-labeled histidine. Resonances from natural abundance trehalose and polymer are indicated with an asterisk (*).

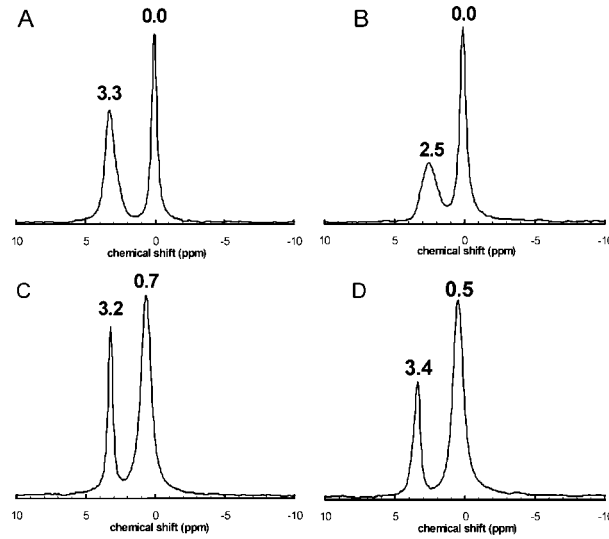

Figure 5. Proton decoupled ^{31}P MAS NMR spectra of phosphate-containing porogens encapsulated in blocked (A, C) or unblocked (B, D) PLGA, following hydration at 84% RH and subsequent addition of exogenous buffer. Acidic buffer was added to dibasic porogen microspheres (A, B), and basic buffer to monobasic porogen microspheres (C, D).

to the dibasic form ($\delta \sim 3$ ppm; Figures 3A, S3). In both cases buffering capacity was sufficient to fully titrate all of the phosphate and histidine contained in the sample (see Methods). Two peaks were observed in the resulting NMR spectra, indicative of two phosphate populations (Figure 5). The fraction in each population was estimated by integrating the respective peaks. For dibasic phosphate microspheres, the internal population corresponded to 50% (blocked PLGA; Figure 5A, 3.3 ppm) or 37% (unblocked PLGA; Figure 5B, 2.5 ppm) of total encapsulated phosphate, while for monobasic phosphate the results were 69% (Figure 5C, 0.7 ppm) and 72% (Figure 5D; 0.5 ppm). The possible impact of the exogenous buffer pH extremes (which could accelerate polymer hydrolysis, resulting in changes to the spectra) was assessed by reanalysis of the samples depicted in Figure 5 following an extended (>1 day) exposure time; no significant peak movement was observed.

Evolution of Internal Microsphere Environment during Matrix Erosion at 37 °C. The above conditions represent the initial microsphere environment within hours of hydration at room temperature, similar to conditions under

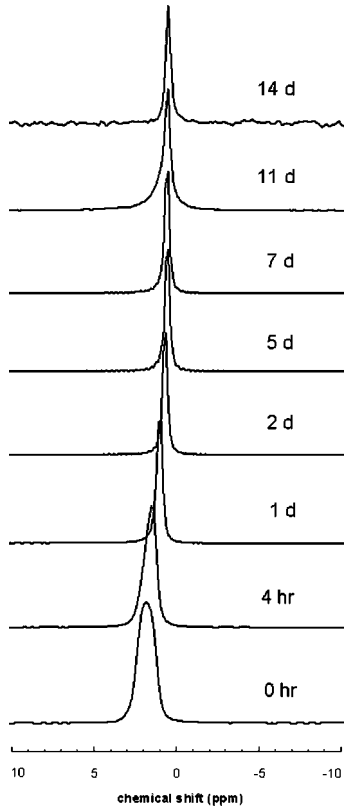

Figure 6. Proton decoupled ^{31}P MAS NMR spectra of dibasic phosphate-containing porogen encapsulated in unblocked PLGA, following hydration with fetal calf serum and incubation at 37 °C for the time indicated.

which the dosage form might be prepared for injection in a medical setting. Under in vivo conditions postinjection, the microsphere internal environment will evolve as the polymer hydrolyzes, the matrix erodes, and drug is released. This situation was studied using dibasic phosphate porogen encapsulated in unblocked PLGA. Microspheres were hydrated in osmotic pressure-adjusted fetal calf serum, incubated at 37 °C, and examined periodically by ^{31}P NMR spectroscopy (Figure 6). At $t = 0$ h the chemical shift was 1.9 ppm, slightly upfield from that seen for the same formulation hydrated at 20 °C (2.4 ppm; Figure 3B); the line width was significantly increased (205 Hz compared with 55 Hz in Figure 3B). Between 4 and 48 h the signal moved further upfield to 0.7 ppm, and remained at 0.5−0.6 ppm through day 14. Line widths remained relatively constant between days 2 and 14, ranging from 50 to 71 Hz. At day 14, the signal diminished significantly, attributed to release of encapsulated phosphate. Extensive matrix erosion prevented packing and NMR analysis of samples collected beyond day 14; the study was terminated at day 22. GPC analysis indicated that M_w dropped to 2.4 kDa by day 14 (and to 1.5 kDa by day 22) with a pseudo-first-order degradation rate constant of 0.10 day^{-1}.[23]

Discussion

XRPD results (Figure S1) show that the phosphate salts in the porogen particles are in an amorphous state. Although this rules out formation of crystalline hydrates, local hydration of phosphate is possible. The porogen moisture content of <1.5% allows an estimation of the phosphate hydration level following spray drying. Partitioning of all of the residual moisture to the porogen phosphate would result in a water: phosphate molar ratio of 0.5−0.6 based on the 20% porogen phosphate content. As the porogen contains an equal amount by mass of trehalose, known to absorb up to 10% moisture in an anhydrous form prior to crystallizing as a dihydrate,[28] this is considered to be an overestimate. Most of the phosphate in the porogen samples is concluded to be in the anhydrous form. This conclusion is supported by the porogen particle ^{31}P chemical shifts (Figure 2B,F). Solid-state ^{31}P chemical shifts of mono- and dibasic phosphates vary with form, whether anhydrous or hydrate (of which several are known; Table 1). The correlation of upfield chemical shift movement with increasing hydration observed in crystalline phosphates is expected to obtain in amorphous samples. Prior to encapsulation the porogen particle ^{31}P chemical shifts (Figure 2B,F) fall between the values for their respective anhydrous salts and the lowest hydrate forms (monohydrate for monobasic and dihydrate for dibasic; Table 1), consistent with the above conclusion.

Encapsulation using blocked PLGA resulted in upfield shifts of 1.6 and 0.5 ppm for di- (Figure 2C) and monobasic (Figure 2G) porogens, respectively. That similar shifts were observed with unblocked PLGA (Figure 2D,H) indicates that this is attributable to the altered chemical environment when the porogen is entrapped in the polymer, and rules out proton transfer from the unblocked polymer during microsphere fabrication. The reason for the decrease in line width on encapsulation is not clear, but could result from partial crystallization of the amorphous porogen during processing. Barich et al. reported a decrease in anisotropic bulk magnetic susceptibility (ABMS), and consequent reduction in line width,[29] upon dilution of crystalline ibuprofen with various excipients.[17] A small second component suggested in the encapsulated dibasic porogen spectra (Figure 2C, and to a lesser extent in Figure 2D) is possibly due to a minor amount of incompletely encapsulated porogen.

Equilibrium microsphere moisture levels were 10% to 11% of those observed for unencapsulated porogens (Table 2), corresponding to the 10% nominal porogen weight percentage and indicating a negligible impact of the polymer on the extent of encapsulated porogen water uptake. (Note that this is a pseudoequilibrium; PLGA water uptake increases dramatically on polymer erosion.[23]) Essentially all the absorbed water is concluded to have partitioned to the encapsulated porogen. Rapid water penetration has been reported with PLGA-encapsulated albumin, characterized by microscopy and by EPR,[4] and is consistent with the reported

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rapid drop in T_g on PLGA hydration.[30] The encapsulated porogens were hypothesized to have deliquesced, as was observed for the unencapsulated porogens. Deliquescence was attributed to the formulation's high sodium chloride content. While the rapidity and completeness of hydration will be formulation dependent, stability of encapsulated proteins is expected to be optimal when these properties are maximized.[15] Thus it was of interest to probe porogen deliquescence spectroscopically.

^{31}P NMR demonstrated unequivocally that the encapsulated porogen changed from a solid to a solution environment inside the microspheres upon hydration. First, removing the proton decoupling neither diminished signal intensity nor increased line width (Figure S4A), as would have been expected for a solid environment due to incompletely averaged ^{31}P−^{1}H dipolar interactions. Second, deviating from the magic angle had no impact on line width (Figure S4B), demonstrating that spinning was not required to average chemical shift anisotropy as expected in a solid. Third, performing a ^{31}P cross polarization experiment resulted in no signal (Figure S4C), indicating a solution environment. (Solid samples give a cross polarization signal, whereas solution samples do not.)

Compared with the corresponding dry microsphere samples (Figure 2C,D;G,H), upon hydration ^{31}P chemical shifts moved upfield 1.5−1.9 ppm for dibasic sodium phosphate porogen (Figure 3A,B), and 0.4−0.9 ppm for monobasic sodium phosphate porogen (Figure 3C,D). For both dibasic and monobasic sodium phosphate porogen, shift changes observed with blocked polymer were attributed to the change in chemical environment on hydration. The incrementally greater shifts observed with unblocked polymer were attributed to protonation by the polymer acid end-groups, which likely occurred prior to acquisition of the spectra. ^{31}P chemical shifts were time-invariant, demonstrating that no significant phosphate titration occurred during the course of data acquisition. The T_g drop observed to occur between initial sample hydration and the completion of spectral acquisition indicates only modest polymer hydrolysis during the course of the experiment, and is consistent with similar results reported at 23 °C.[30]

With rapid chemical exchange in a liquid the observed chemical shifts reflect the fraction protonated, which is estimable if the shifts of pure phosphate forms are known.[20] The solution ^{31}P chemical shifts of pure dibasic (2.9 ppm, Figure 3A) and monobasic (1.0 ppm, Figure 3C) phosphate forms encapsulated in blocked PLGA were within 0.7 ppm of their shifts in bulk solution (Figure S3), with a 1.9 ppm range between the two forms. Dibasic porogen encapsulated in unblocked PLGA shows an upfield shift of 0.5 ppm (Figure 3A to 3B), indicating that a fraction of the dibasic phosphate salt was titrated. Similarly, monobasic porogen encapsulated in unblocked PLGA shows an upfield shift of 0.4 ppm (Figure 3C to 3D), suggesting partial titration to form phosphoric acid (δ 0.0 ppm; p$K_a \sim 2$), the pK_a of which is within 1 pH unit of the polymer's carboxylic acid end group.

The correlation between pH and the ratio of phosphate forms, related under bulk aqueous conditions by the Henderson−Hasselbalch equation, is complicated in the current system by the impact of elevated ionic strength and possible surface effects on the pK_a's. The porogen histidine afforded an opportunity for independent assessment of the ability of the polymer to titrate encapsulated contents in the same formulation. The monobasic phosphate and histidine imidazole pK_a's move in opposite direction as ionic strength increases; the movement of the former is substantially greater than that of the latter due to the dianionic character of its conjugate base. Thus it was expected that in the dibasic phosphate sample similar fractions of the two solutes may be titrated by the unblocked PLGA. Upon protonation, C-3 and C-2 imidazole chemical shifts move approximately 4 and 2 ppm, respectively.[31] Compared with the blocked polymer, histidine imidazole resonances in the unblocked PLGA microspheres shifted 2.3 ppm (C-3) and 1.6 ppm (C-2) upfield, demonstrating that a significant fraction (but not all) of the histidine was protonated by the polymer. Based on the observed partial titration of phosphate and histidine solutes, the dibasic phosphate/unblocked PLGA formulation was considered appropriate for characterizing the evolution of the microsphere interior during incubation.

Coencapsulation of poorly soluble bases, such as $Mg(OH)_2$, has been reported to be effective in stabilizing encapsulated drugs against acid conditions.[8,11] Optimally with this approach acid neutralization in the vicinity of the buffer salts would benefit entrapped drugs which, depending on the formulation type, may be present in a separate, distinct phase. In one study, imaging data suggested a localized effect under some conditions.[6] Results with the mixed-porogen microsphere formulations reported herein indicate that hydration effects rapid exchange between the phosphate populations stemming from the two porogen types within each microsphere, consistent with mixing. This may have resulted from moisture transport through internal, interconnecting pores or cracks in the matrix, or through transient fissures due to osmotic rupture. While water transport also occurs through the polymer phase, this mechanism is not expected to result in chemical exchange of polymer-impermeable phosphate solutes.[32] Figures 3G and 3H demonstrate that mixing of deliquesced porogen between individual microspheres (i.e., interparticle mixing) occurs only to a limited extent. The center peaks in Figures 3G and 3H are attributable to rapid chemical exchange in a subpopulation of the

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total phosphate in the samples, presumably stemming from surface-accessible liquid in the visibly moist samples. This study was conducted near room temperature, and thus the samples were likely near, or slightly below, their wet T_g's;[30] the absence of complete interparticle mixing may be attributable to time-dependent microsphere plasticization and loss of surface porosity.[33] Exogenous buffer addition allowed differentiation of internal and external microsphere phosphate populations. ^{31}P chemical shifts observed for the former (Figure 5A−D) closely approximated those for the original samples (Figure 3A−D), clearly demonstrating that the internal population was not titrated by external buffering capacity on the time frame of the experiment. Following initial microsphere hydration, either phosphate is fully titrated by the external buffer or it remains trapped inside in its original protonation state.

The kinetic study (Figure 6) showed significant further titration of incorporated phosphate by the eroding polymer upon microsphere incubation. At $t = 0$ the phosphate signal was 0.5 ppm upfield compared to that observed in Figure 3B, possibly indicating a higher initial fraction of conversion to the monobasic form due to the higher temperature (37 °C). The movement of the phosphate signal from 2.9 ppm (dibasic phosphate in blocked PLGA; Figure 3A) to 0.7 ppm at 48 h indicates complete conversion to monobasic phosphate (and possibly some phosphoric acid formation), with little additional change in shift or line width through day 14. The data demonstrate that the environment seen by the entrapped phosphate was homogeneously acidic (i.e., below pH 4 but above the phosphoric acid pK_a; see below) throughout the polydisperse sample and within individual spheres.

The microsphere formulations studied herein are representative of those prepared by similar spray-freeze drying processes. The size range approximates that for PLGA microspheres prepared by a double emulsion process and reported to result in pH gradients[5] or discontinuities[6] during the course of incubation at 37 °C; in these studies pH was assessed by confocal microscopy of individual microspheres. The diffusion of oligomeric degradants has been implicated in gradient formation.[34] The spectra for days 1−14 (Figure 6) are not consistent with the formation of a neutral region near the surface, which would have resulted in a second phosphate peak if present in a significant fraction of the microspheres, nor consistent with a pH gradient, which would have resulted in line widths spanning the range of phosphate protonation states. The homogeneous pH environment observed here may be attributable to the encapsulation process (spray-freeze drying can result in higher porosity). Alternatively, the macromolecular dye conjugates used in the confocal studies may have been retained preferentially in the surface eroding region (where their signal would persist) compared with the small phosphate anion studied here. The broad lines observed in Figure 6 at $t = 0$ and, to a lesser extent, at 4 h likely resulted from time-dependent phosphate dissolution in the sample, which was analyzed immediately after serum addition. The sharper signal in Figure 3B was obtained after equilibration under conditions of elevated humidity (24 h at 20 °C).

The complete conversion of the encapsulated phosphate to the monobasic form during incubation indicates that internal pH drops below 4, consistent with previous reports[2–7,9,10] of significant acidification in eroding PLGA microspheres and films. The present results contrast, however, with our previous report of ^{31}P NMR characterization of microspheres prepared by a similar process.[20] This incongruity is attributed to differences in phosphate distribution within the samples. The previous study probed the microsphere interior region accessible to an exogenous phosphate label (i.e., phosphate originating from the in vitro release medium). Phosphate is expected to be polymer impermeable, and thus the signal observed likely derived only from the phosphate-accessible region. The estimated pH may therefore have applied to a drug-depleted region. In the present study residual phosphate in the serum medium (the subject of the previous work) contributed negligibly to the ^{31}P signal. The present results are considered representative of the microsphere internal environment seen by encapsulated drugs in vivo. Notably, the measured polymer degradation rate under the conditions of the kinetic study closely approximated that reported for a polymer of similar composition in vivo.[23]

Controlling hydration rate is critical in maintaining protein integrity in polymeric drug delivery systems;[11] stabilization has been observed with its acceleration (through incorporation of water-sorbing excipients[15]) and through its retardation (through entrapment in hydrophobic polyanhydrides with decreased water penetration rates[35]). The time required to hydrate a solid PLGA microsphere was estimated by Batycky et al., using a literature water diffusivity value, to be 8 min.[36] Pore wetting and dissolution of entrapped particles is expected to be slower—due to expected low water flux through hydrophobic polymers with low equilibrium moisture content—and to vary both with formulation and with the encapsulation process, which can impact pore morphology. The combination of solid-state and solution NMR spectroscopy offers the ability to observe hydration rate directly: both the disappearance of solids and the appearance of solutes. Optimization of hydration rate may help establish a more

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direct link with protein stability, and ultimately with the integrity of released protein. The ability to directly monitor the titration of acidic micropores by coencapsulated basic excipients may similarly help further optimize that stabilization strategy.[12]

Salt bridges play a critical role in maintenance of native conformation in proteins. The ability to assess protonation states within microspheres, independent of microenvironment impact on pH and amino acid pK_a's, suggests that it may be possible to monitor these critical electrostatic interactions in situ during the course of drug release without the need to determine proton activity. To date, secondary structure of encapsulated human growth hormone was evaluated in situ by FTIR; native structure was shown to be recovered on microsphere hydration when zinc was incorporated as a stabilizer.[37] The direct observation of structural features of encapsulated substances is relevant to a major challenge of microsphere formulation development. Specifically, degradant formation may be obscured by preferential release of intact drug (especially important if recovery is <100%); degradants may be released long after their formation, and may never be recovered (or detected) at all. The correlation of identified degradants with the environment producing them is essential to the development of rational stabilization approaches. The ability to apply NMR spectroscopy, which has been widely used to characterize native protein structure, to this challenge could prove to be an important advance.

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Supporting Information Available: X-ray powder diffractograms, proton decoupled ^{31}P MAS NMR spectra, plot of pH vs ^{31}P NMR chemical shift, and ^{31}P NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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