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Pore Closing and Opening in Biodegradable Polymers and Their Effect on the Controlled Release of Proteins

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Abstract: The purpose of this paper was to investigate the phenomena of pore closing and opening in microspheres of poly(lactic-*co*-glycolic acid) (PLGA) and PLGA−glucose star copolymer (PLGA−Glu) and their effects on protein release. We used scanning electron microscopy (SEM) and laser scanning confocal microscopy (LSCM) to visually characterize the pore state and the uptake of dextran labeled with pH-insensitive probes by microspheres, as an indicator of pore connectivity. The effect of temperature on initial protein release from microspheres was also investigated. It was found that (1) pore closing occurs in both PLGA and PLGA−Glu; (2) pore closing can take place at later time during incubation at physiological condition (37 °C) as well as during the initial stage; (3) pore closing is much more significant at elevated temperatures; (4) previously isolated pores can become open by, for example, osmotic-mediated events; and (5) pore closing/opening correlates with the release rate of biomacromolecules from PLGA or PLGA−Glu microspheres. The pore closing/opening appeared potentially a universal event throughout the release period dictating the kinetics of protein release from PLGA microspheres. Hence, these results strongly suggest that open and isolated pores are able to toggle back-and-forth periodically during PLGA degradation while controlling protein release; these observations imply a novel new hypothesis concerning erosion-controlled release of biomacromolecules from PLGA and related polymers.

Keywords: Biodegradable microspheres; controlled-release; protein delivery; pore state; pore connectivity

1. Introduction

Poly(lactic-*co*-glycolic acid) (PLGA) remains the most commonly used biodegradable polymer to deliver drugs in a controlled-release manner.[1−3] A major challenge in designing PLGA microspheres for controlled-release delivery of

therapeutic proteins is achieving an ideal release profile.[4−7] A zero-order release would be desirable in most cases, although efforts have also been made to obtain other types

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of release profiles, for example, a pulsed-release behavior for antigen delivery to mimic booster vaccinations.[3,8,9]

It is well understood that hydrophilic macromolecules, like proteins, cannot generally partition in or diffuse through the hydrophobic polymer phase.[10,11] The release of protein drugs from PLGA requires the availability of a single water-filled pore or pore network with access to the surface of the polymer. Despite the importance of pore structure on protein release from PLGA, the state of pores/channels in PLGA and their time-dependent changes have been largely neglected.[12–14] Wang[13] recently observed that both the external and internal morphology of PLGA microspheres changed substantially during release of peptides over the first 24 h when incubated in pH 4 buffer at 37 °C. The spontaneous formation of a nonporous film at the surface of PLGA microspheres in place of an initially porous surface was correlated with a sharp decline in permeability and the cessation of the initial burst. Yamaguchi[14] found that addition of glycerol, which reduced the Tg of the polymer, could suppress the initial burst release.

The purpose of this paper was to investigate the pore closing/opening in PLGA and poly(lactic acid-*co*-glycolic acid)−glucose (PLGA−Glu), a star copolymer of PLGA and glucose, and its effects on protein release. We used scanning electron microscopy (SEM) to visually characterize the pore structure/state and their changes during incubation under physiological conditions. We also utilized laser scanning confocal microscopy (LSCM) to observe the uptake of pH-insensitive probes conjugated to dextran by microspheres

during incubation, which served as an indicator of pore connectivity to the polymer surface. The correlation between these observations and protein release, in combination with an investigation of initial protein release behavior from polymer microspheres at different temperatures, provided us direct evidence of pore closing/opening in PLGA microspheres and its importance on protein release from PLGA microspheres.

2. Experimental Section

2.1. Materials. Poly(DL-lactic-*co*-glycolic acid) 50/50, end-group capped, with an inherent viscosity of 0.17 dl/g in HFIP at 30 °C was obtained from Birmingham Polymers, Inc. (Birmingham, AL). Poly(DL-lactic-*co*-glycolic acid)-glucose (50/50, with a MW of 50 kDa) was a generous gift from Novartis Pharm AG (Basel, Switzerland). Poly(vinyl alcohol) (PVA) (80% hydrolyzed, MW 9−10 kDa) was from Aldrich Chemical Co., Inc. (Milwaukee, WI). Bovine serum albumin (BSA), magnesium carbonate, and dextran−FITC (MW = 70 kDa) were from Sigma Chemical Company (Louis, MO). 7-methoxy-coumarin-3-carbonyl-azide, dextran-bodipy (MW = 10 kDa), and BSA-bodipy were from Molecular Probes Inc. (Eugene, OR). Dextran (MW = 10 kDa) was from Polysciences, Inc. (Warrington, PA). Dextran-bodipy and dextran−FITC were dialyzed extensively before use. All other reagents were of analytical grade or higher and used as received.

2.2. Labeling of Dextran with Coumarin, a pH-Insensitive Fluorescent Probe. Dextran was labeled with a pH-insensitive probe, coumarin.[15] Briefly, 100 mg of dextran and 4 mg of 7-methoxy-coumarin-3-carbonyl-azide were dissolved in 4 mL of DMSO. The mixture was incubated at 70 °C for 3 h. After cooling down to room temperature, 12 mL of water was added to the reaction mixture followed by storage in a −20 °C freezer for 30 min. The unreacted free probe and DMSO were removed by filtration and extensive dialysis using a Spectra/Pro membrane with a MWCO of 1000 Da (Spectrum laboratory, Inc., Rancho Dominguez, CA). Finally, the dextran−coumarin conjugate was lyophilized and stored at −20 °C for future use. The degree of labeling was determined by UV absorbance of dextran−coumarin.

2.3. Encapsulation of BSA in Microspheres by the Double Emulsion-Solvent Evaporation Microencapsulation Method. PLGA microspheres were prepared by a double emulsion-solvent evaporation method. One hundreed to two hundred microliters of 300 mg/mL BSA in PBS (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.4) solution was first added to 1 mL of 700 mg/mL PLGA in CH_2Cl_2. The mixture was homogenized at 10 000 rpm with a Tempest IQ[2] homogenizer (The VirTis Company, Gardiner, NY) equipped with a 10 mm shaft in an ice/water bath for 1 min to prepare the first emulsion. Two mL of 5% (w/v) PVA solution was immediately added to the first

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emulsion, and the mixture was vortexed (Genie 2, Fisher Scientific Industries, Inc., Bohemia, NY) for 15 s to produce the w/o/w double emulsion. The resultant emulsion was poured into 100 mL of 0.5% (w/v) PVA solution under rapid stirring and hardened at room temperature for 3 h. Hardened microspheres were collected by centrifugation, washed three times with purified water, and freeze-dried. For freeze-drying, samples were flash-frozen in liquid nitrogen and placed on a Freezone 6 freeze-drying system (Labcono, Kansas City, MO) at 133×10^{-3} mbar or less vacuum at a condenser temperature of -46 °C for 48 h. Three percent $MgCO_3$ powder (w/w of polymer) was suspended in polymer solution before encapsulation when base-containing PLGA microspheres were desired.

PLGA–glucose microspheres encapsulating both BSA and dextran–FITC (MW = 70 kDa) were also prepared by the foregoing method for PLGA except as follows. The polymer concentration was 300 mg/mL of PLGA–Glu in CH_2Cl_2, and the internal phase consisted of 200 mg/mL BSA and 18 mg/mL dextran–FITC. After hardening, the microspheres with diameters of 45–90 μm were collected by sieving prior to washing and freeze-drying.

2.4. Morphology and Size Distribution of Microspheres by Scanning Electron Microscopy. Microspheres were first coated with gold for 200 s by a vacuum coater (Desk II, Denton Vacuum, Inc., Hill, NJ). Microsphere morphology was then observed by a scanning electron microscope (S3200N Variable Pressure SEM, Hitachi) with a voltage of 15 keV. For size distribution analysis, the size of more than 200 particles was measured from SEM micrographs and the weight-averaged mean radius of the microspheres was calculated. To observe the microsphere cross section, polymer specimens were pre-cut by a razor blade on a glass slide before coating.

2.5. Assay of Protein and Dextran–FITC. Protein concentration was determined either by a Coomassie Plus (Pierce, Rockford, IL) protein assay or by a size-exclusion chromatography. For the size-exclusion chromatography, a TSK 2000 SWxl column (Toso Biosep LLC, Montgomeryville, PA) equipped with a guard cartridge was used. The mobile phase consisted of 50 mM sodium phosphate and 150 mM sodium chloride and was delivered at 1 mL/min by a Waters 1525 pump. A Waters 2487 dual wavelength detector was used to monitor the protein elution at 280 nm.

The concentration of dextran–FITC was determined by a FluoroMax-2 fluorometer (Instruments S.A., Edison, NJ) at $\lambda_{ex} = 495$ nm and $\lambda_{em} = 515$ nm against a linear standard curve ($R^2 > 0.999$).

2.6. Protein and Dextran Loading in Microspheres. The loading of protein and dextran in microspheres was determined following reconstitution of protein and dextran in water after removing the polymer by acetone.[16,17] The

encapsulation efficiency was calculated as the ratio of the actual to the theoretical loading.

2.7. Evaluation of Protein and Dextran Release from Microspheres. In vitro release of protein and dextran from microspheres were carried out under mild agitation conditions. 10–15 mg of microspheres were placed in 1 mL PBST (PBS, with 0.02% (w/v) Tween 80) and incubated at 4, 25, 37, or 45 °C. At predetermined time intervals, release medium was replaced. The concentration of protein and dextran in the release medium was then measured, as described in Section 2.5, to calculate the cumulative release of protein and dextran from microspheres.

2.8. Dextran Uptake Monitored by Laser Scanning Confocal Microscopy. The uptake of dextran by microspheres was monitored by LSCM during the incubation of microspheres in dextran–probe solution.

One hundred microliters of 2.5 mg/mL dextran–bodipy (MW = 10 kDa) or 20 mg/mL dextran–coumarin (MW = 10 kDa) in PBST solution was added to about 5 mg microspheres, with or without preincubation. After incubation at 4 °C or 37 °C for 5 h or 12 h, probe-conjugated dextran solution was removed. Microspheres were then briefly washed twice before observation of the detran–probe distribution by LSCM.

A Carl Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) was used to observe the probe distribution in microparticles. The instrument was equipped with four laser systems, an Ar laser (458, 488, 514 nm, 25 mW), a HeNe 1 laser (543 nm, 1 mW), a HeNe 2 laser (633 nm), an Enterprise laser (351, 364 nm, 80 mW), a photomultiplier (PMT), and a computer for image building and instrument control. The connected microscope was a Carl Zeiss inverted Axiovert 100 M that was fully motorized and could be operated via the LSM 510 software. A C-Apochromat 63 × N.A. 1.2 water immersion objective lens was used to build images. The pinhole was set at 150 μm. The laser was focused in the center of a microsphere and a 1024 × 1024 pixel image was scanned at a scan speed of 1.60 μs/pixel. The 488 nm line of the Ar-ion laser and LP 505 filter were used for dextran–bodipy, and the laser was set at 5% of 25 mW (1.25 mW). For dextran–coumarin, the 364 nm of the Enterprise laser and a BP 485–470 filter were used. The laser was set at 2.5% of 80 mW (2 mW).

2.9. Curve-Fitting and Calculation of Diffusion Coefficient. Because of low loading and high solubility, initial protein/dextran release can be regarded as drug release from a monolithic solution (or effective medium). The release medium was frequently changed to guarantee sink conditions. Crank's solution for release from spherical geometry under these conditions is as follows[18]

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$$\frac{M_t}{M_\infty} = \frac{M_t}{M_0 p} = 1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp(-D_{\mathrm{eff}} n^2 \pi^2 t / a^2)$$
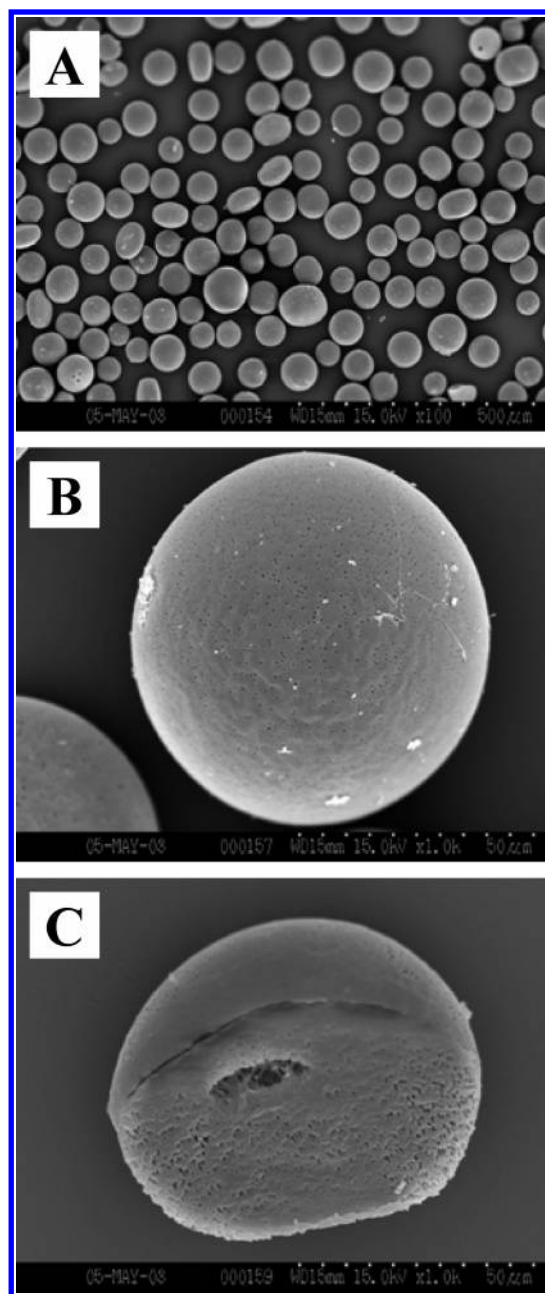
where t is time and D_{eff} is the effective diffusion coefficient in the polymer matrix. M_t, M_∞, M_0, and a are the released drug amount at time t, the releasable drug amount at infinite time, the total drug in the microspheres, and the microsphere radius, respectively. The initial releasable fraction of drug in the microspheres, p, is the ratio of M_∞ and M_0. Curve-fitting was carried out according to a least-squares nonlinear regression using $n = 12$ (DataFit, Oakdale Engineering, Oakdale, PA) to obtain the values of D_{eff}. Using values larger than $n = 12$ did not change the fitted value of D_{eff}.[19] The weight-averaged mean radius of microspheres measured by SEM was used as the radius, a, in the equation.

3. Results and Discussion

3.1. Preparation and Characterization of PLGA and PLGA−Glu Microspheres. We prepared both regular PLGA microspheres and PLGA−Glu microspheres by a double emulsion/solvent evaporation method. They were used as model microspheres to investigate the change of pore state and protein release.

BSA and dextran−FITC were co-encapsulated in PLGA−Glu microspheres. The BSA and dextran−FITC loading were $3.7 \pm 1.2\%$ (w/w) and $0.43 \pm 0.005\%$ (w/w), (mean $\pm$ SD, $n = 3$), respectively. As shown in Figure 1A−C, PLGA−Glu microspheres were porous both at the surface and in the interior.

In the case of PLGA microspheres, three microsphere formulations were prepared, as shown in Table 1. Each formulation demonstrated unique properties by carefully preselecting the microencapsulation conditions and excipients. Microsphere formulation A showed a high initial 1-day release of 61%. Burst release was eliminated by adding 5 × PBS in the hardening buffer in microsphere formulations B and C.[20,21] MgCO$_3$ (3%) was added to formulation C to neutralize the acidic microenvironment in PLGA microspheres caused by acidic PLGA degradation species.[17] As seen in Figure 2, formulation A (Figure 2A and B), which showed a larger burst release, had a relatively porous surface and interior. Formulations B (Figure 2C and D) and C (Figure 2E and F), with very limited burst release, were denser both at surface and in the interior. The higher burst release of formulation A microspheres was in accordance with its relatively more porous structure.

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Figure 1. Scanning electron micrographs of PLGA−Glu microspheres containing BSA and dextran−FITC. (A) Overview; (B) surface; (C) cross section.

In Figure 3, the distinct release profiles of PLGA microsphere formulations A−C are displayed. Formulation A released 61% BSA in the first 2 h followed by a "no release" phase for the following 2 weeks of incubation at 37 °C. Formulation B did not release BSA until 4 weeks, whereas formulation C showed a continuous release throughout 5 weeks of incubation at 37 °C.

3.2. Temperature Dependency of Protein Release from PLGA−Glu Microspheres. PLGA−Glu microspheres containing both BSA and dextran (45−90 μm) were incubated in PBST at 4, 25, 37, and 45 °C. The release of both BSA and dextran was determined up to 3 days. As shown in Figure 4A, BSA release at 37 °C displayed a typical protein release

Table 1. Effect of Formulation Variables on Protein Release from PLGA Microspheres.

| formulation	internal phase ratio	base content (%)	hardening buffer	protein loading (%, w/w)[a]	encapsulation efficiency (%)	1st day release (%)[b]
A	1/5	0	0.5% PVA	4.4 ± 0.1	67	61.3 ± 0.5
B	1/10	0	0.5% PVA + 5 × PBS	3.7 ± 0.2	90	0.1 ± 0.1
C	1/5	3	0.5% PVA + 5 × PBS	6.6 ± 0.1	82	1.4 ± 0

[a] Mean ± SD, $n = 3$. [b] Mean ± SD, $n = 3$.
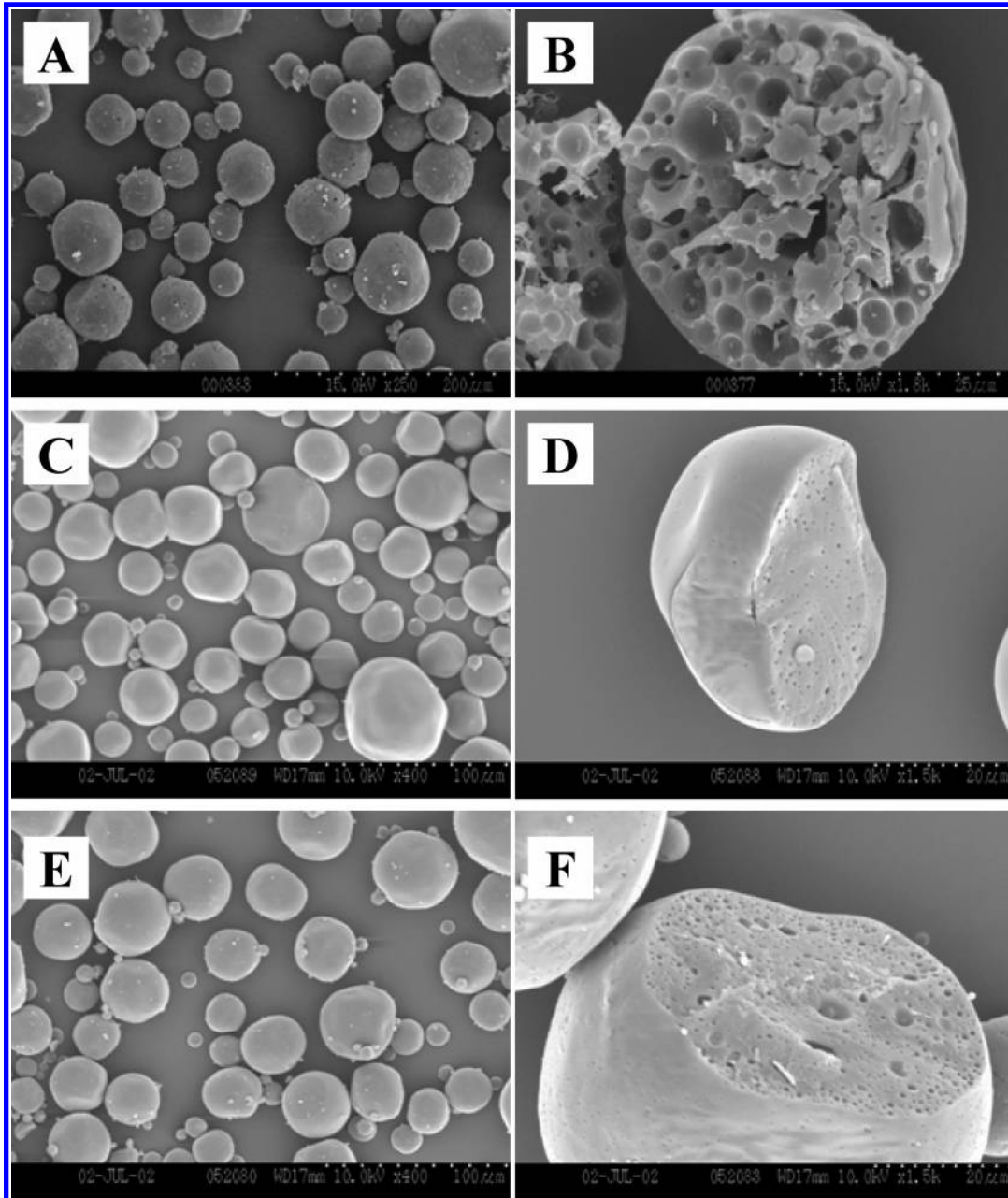
Figure 2. Scanning electron micrographs of the surface (left panel) and cross-section (right panel) of PLGA microspheres of formulation A (A and B), B (C and D), and C (E and F). The detailed formulation variables are listed in Table 1.

profile, with 18% BSA released in the first 2 h followed by minimal release. At 45 °C, BSA release showed a similar profile, but only 10% protein was released. By contrast, BSA release at 4 and 25 °C was continuous over this period, with 21% and 26% BSA released during the first 2 h, respectively. By 66 h, the cumulative BSA release from microspheres incubated at 4 and 25 °C reached 48% and 49%, respectively.
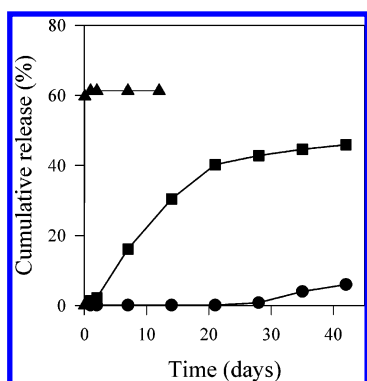
Figure 3. Cumulative BSA release from PLGA microspheres of formulation A (▲), B (●), and C (■), as listed in Table 1 (mean ± SD, $n = 3$).
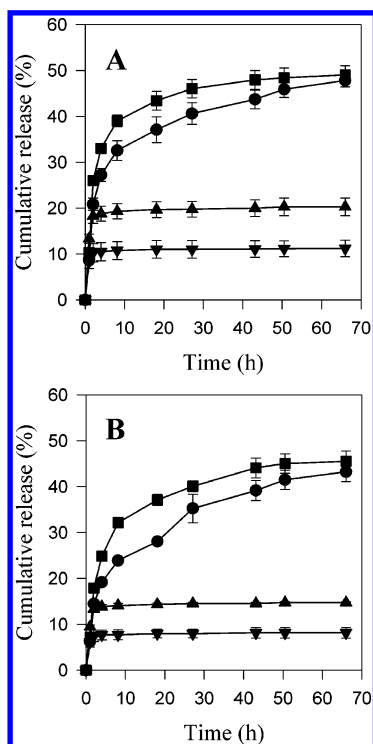
Figure 4. BSA (A) and dextran (B) release from PLGA−Glu microspheres at 4 (●), 25 (■), 37 (▲), and 45 (▼) °C (mean ± SD, $n = 3$).

As shown in Figure 4B, dextran release showed a similar temperature dependency. The cumulative release after 66 h at 4, 25, 37, and 45 °C was 43%, 46%, 15%, and 8%, respectively. Because it is well understood that macromolecules have higher diffusivity at higher temperature, we would expect opposite results (faster release at higher temperatures) if the microspheres structure and polymer properties remained the same during the release period. The faster protein release at 4 and 25 °C than at 37 and 45 °C could not be attributed to polymer degradation either because higher temperature would result in faster polymer degradation. We hypothesized that this phenomenon was a result of significant pore closing in the microspheres at $T \geq 37$ °C. At 4 and 25 °C, the polymer structure remained essentially intact, so the protein could be released continuously by
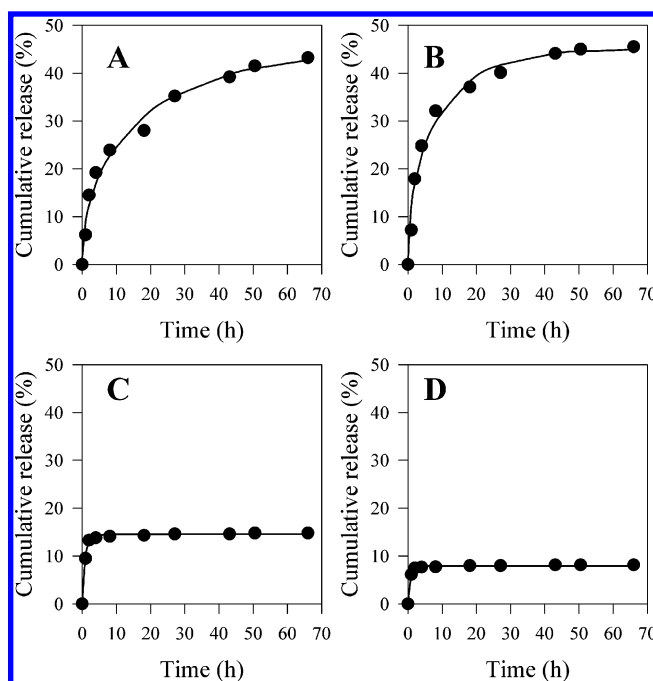
Figure 5. Curve-fitting of dextran release from PLGA−Glu microspheres at 4 (A), 25 (B), 37 (C), and 45 °C (D) by Crank's solution. The experimental data were represented by symbols and the fitted curve was by line. The adjusted coefficient of multiple determination (Ra^2) > 0.97.

Fickian diffusion. However, at higher temperatures, that is, at 37 and 45 °C, the pores and channels open earlier at the surface rapidly closed and protein release stopped suddenly. BSA stability did not likely play a role here because aggregates were not detected during the initial incubation (data not shown) and dextran, which is not expected to encounter stability issues over such a time scale, also demonstrated the similar behavior as that of BSA.

The distinct release behavior of BSA/dextran from PLGA−Glu microspheres suggests a critical point between 25 and 37 °C for PLGA−glucose polymer, likely in the vicinity of the glass transition temperature (T_g) of the hydrated polymer. Although the T_g of PLGA−Glu polymer is well above 37 °C (data not shown), it is known that, upon hydration, the T_g of PLGA related polymers routinely drops below 37 °C.[22,23] This suggests that polymer chains need to possess certain flexibility for pore closing.

Crank's solution for pore-diffusional drug release from spherical geometry under sink conditions was fitted to the release of both BSA and dextran at each temperature investigated (Figures 5 and 6 and Table 2). As seen in Figures 5 and 6, both BSA and dextran release were fit adequately by the simple diffusion model (Ra^2 > 0.97). The limited

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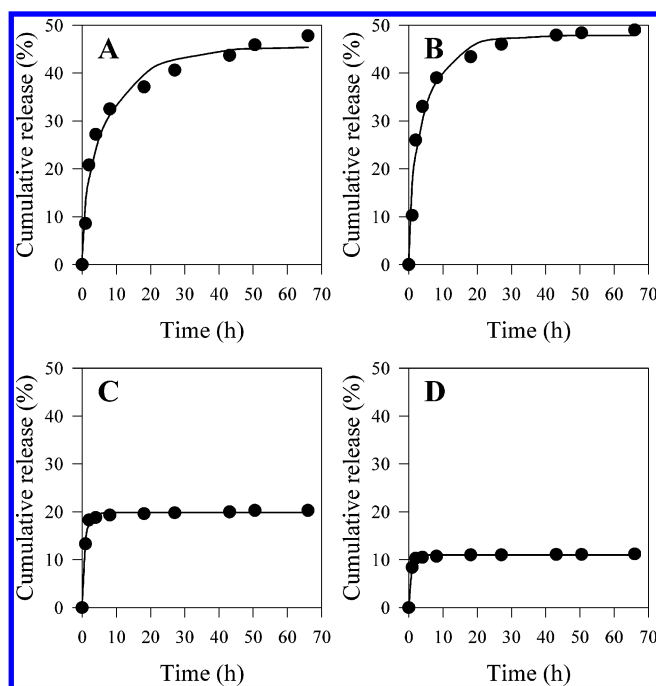
Figure 6. Curve-fitting of BSA release from PLGA−Glu microspheres at 4 (A), 25 (B), 37 (C), and 45 °C (D) by Crank's solution. The experimental data were represented by symbols and the fitted curve was by line. The adjusted coefficient of multiple determination (Ra^2) > 0.97.

Table 2. Fitted and Calculated Parameters of Macromolecular Release from PLGA−Glu Microspheres

temperature (°C)		4	25	37	45
D_{eff} ($\times 10^{-11}$ cm²/s)	BSA	2.6	4.1	22	30
	dextran (70 kDa)	1.1	2.2	24	31
	dextran (70 kDa) calculated[a]		2.2	2.9	3.5
releasable fraction p	BSA	0.45	0.48	0.20	0.11
	dextran (70 kDa)	0.45	0.45	0.15	0.08

[a] Calculated using Stokes−Einstein equation assuming D_{eff} at 4 °C is 1.1×10^{-11} cm²/s.

releasable fraction, *p*, could be explained by the hypothesis that only a limited fraction of encapsulated macromolecules had access to the surface of microspheres and, thus, were releasable. The releasable fraction of macromolecules at 4 and 25 °C was comparable, strongly suggesting the pore/channel state remained essentially the same when PLGA−Glu microspheres were incubated between 4 and 25 °C. The faster release rate of macromolecules at 25 °C than at 4 °C can be explained by the effect of temperature on the diffusion coefficient of macromolecules. As shown in Table 2, the ratio of diffusion coefficient of dextran at 25 and 4 °C, which is obtained by fitting Crank's solution to the release data, is the same as that predicted by the Stokes−Einstein equation.[24] At 37 and 45 °C, the releasable fraction of BSA decreased significantly, from 45% at 4 °C to 20% at 37 °C, and to

11% at 45 °C, indicating rapid pore closing at higher temperature. The fitted effective diffusion coefficients at 37 and 45 °C were much higher and cannot be explained by the temperature effect on aqueous diffusivity. This is apparently caused by the rapid pore closing when microspheres were incubated at higher temperatures, which yields the diffusion model inapplicable at these temperatures.

3.3. Pore State in PLGA−Glu Microspheres at Various Temperatures. As shown in Figures 1A−C, PLGA−Glu microspheres exhibited a porous surface and interior before incubation. After 2 days of incubation at 4 °C, as shown in Figure 7A and B, the porous structure of microspheres remained unaltered. However, incubation at 37 °C changed the microsphere morphology significantly, with most of the pores on the surface disappearing after 2 h of incubation (Figure 7C and D). Further incubation at 37 °C for 2 days only had minimal effect on microsphere morphology (Figure 7E and F). The interior of PLGA−Glu microspheres did not change significantly when incubated at 37 °C in contrast to the change at the surface. These data provided visual evidence for the hypothesis that pores on the PLGA−Glu surface close quickly when incubated at ∼37 °C or higher temperature, but not at 4 °C.

In addition to visual observation, uptake of dextran by microspheres was also used to characterize the pore state in PLGA−Glu microspheres. Dextran (MW = 10 kDa) was first labeled with a pH-insensitive dye, coumarin. The labeling degree was 0.1% of coumarin per glucose unit. The low labeling degree assured that the conjugation of probe molecules would not affect the hydrophilic nature of dextran. The uptake of dextran−coumarin was observed by LSCM. A higher uptake rate presumably represents higher pore connectivity, which is expected to directly correlate with protein release from microspheres because the dextran uptake and BSA release likely occur through the same pore network. As shown in Figure 8A and B, the uptake of dextran−coumarin by the preincubated (at 37 °C) microspheres was much less than that by the microspheres without preincubation, as indicated by the lower fluorescence intensity. This confirmed the pore closing on the surface of PLGA−Glu microspheres during short-term incubation at 37 °C, as observed by SEM. The result also correlated well with the minimal release of BSA/dextran from the microspheres after the initial burst phase, when incubated at 37 °C.

3.4. Pore Closing/Opening in PLGA Microspheres. PLGA microspheres (formulation A) exhibited the same structure change as PLGA−Glu microspheres when incubated at different temperatures. As shown in Figure 9, incubation at 4 °C for 2 days (Figure 9A and B) had little impact on the observed microsphere morphology. However, incubation at 37 °C for 2 h (Figure 9C and D) or 2 days (Figure 9E and F) significantly changed the pore state. All pores on the microsphere surface were either closed completely or were reduced to a size below the detection limit of the electron microscope.

The dextran uptake by PLGA microspheres demonstrated a dramatic effect of incubation on the pore state in micro-

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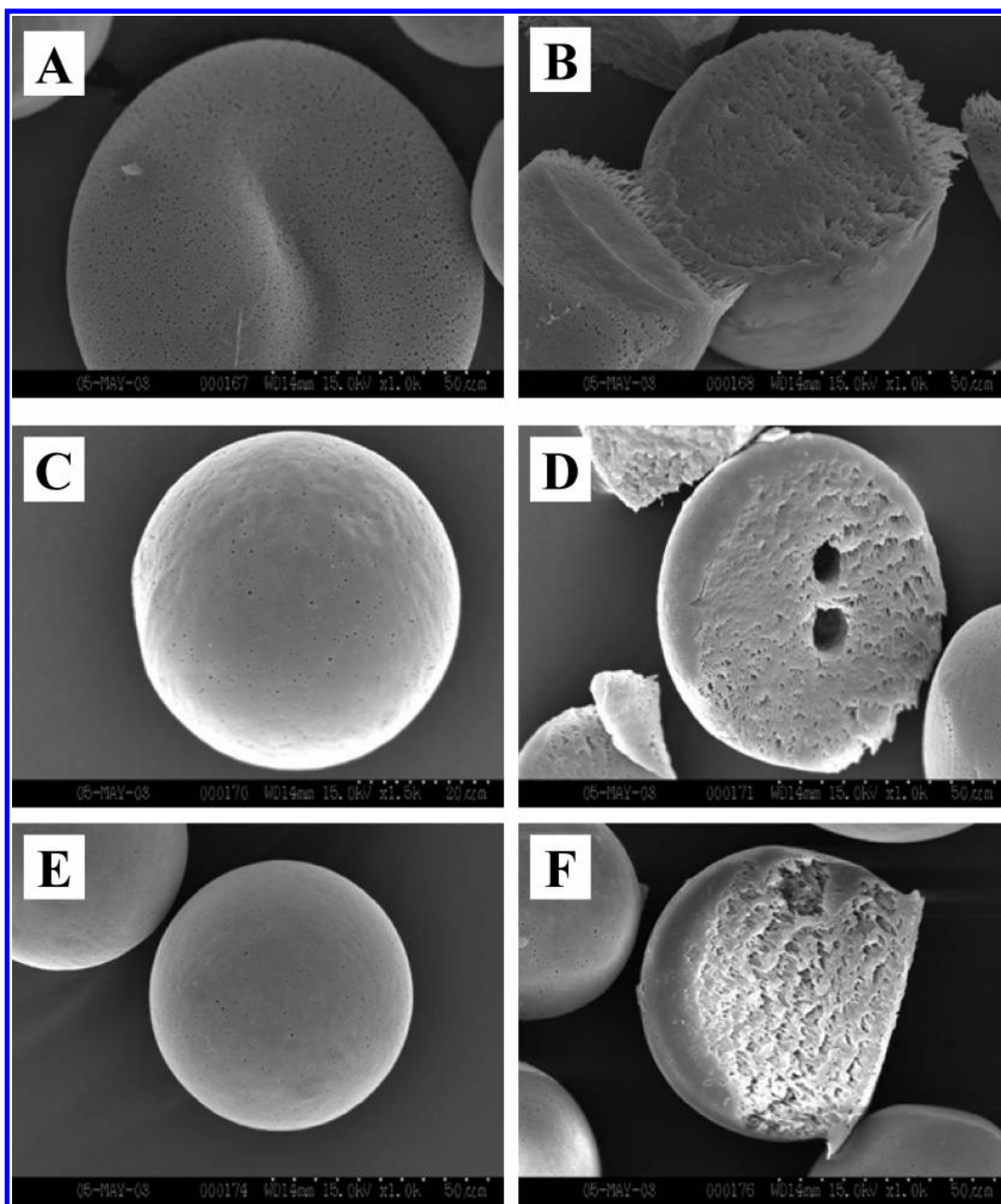
Figure 7. Scanning electron micrographs of the surface (left panel) and cross sections (right panel) of PLGA−Glu microsphere after incubation in PBST at 4 °C for 2 days (A and B) or at 37 °C for 2 h (C and D) and 2 days (E and F).

spheres. As seen in Figure 10A (no preincubation, dextran uptake at 37 °C for 12 h) and E (2 days of preincubation at 37 °C, dextran uptake at 37 °C for 12 h), PLGA microspheres without preincubation absorbed a significantly higher amount of dextran−bodipy (MW = 10 kDa). Only minimal dextran uptake could be observed by LSCM when the microspheres were preincubated at 37 °C for 2 days. Preincubation at 4 °C did not affect the dextran-uptake capability of PLGA microspheres (Figure 10C, 2 days of preincubation at 4 °C, dextran uptake at 4 °C for 12 h) as compared with microspheres without preincubation, indicative of the unaltered pore state.

After dextran uptake, microspheres were incubated in blank PBST at the same temperature as in uptake study (4 °C

for C, 37 °C for A and E) for 24 h, followed by observation of dextran−bodipy in the microspheres using LSCM, as shown in Figure 10B, D, and F (resulted from 10A, C, and E, respectively). As we expected for the microspheres without preincubation (10B), almost all of the dextran remained in the microspheres after incubation with blank medium for 24 h at 37 °C, due to the closing of pores during incubation at 37 °C. Previously open pores became isolated pores, and thus the previously absorbed dextran−bodipy was trapped in the microspheres. For microspheres incubated at 4 °C, if the pore state remained unchanged during incubation, then we would expect that all of the previously absorbed dextran−bodipy would diffuse out to the surrounding medium and essentially no dextran would be left in the
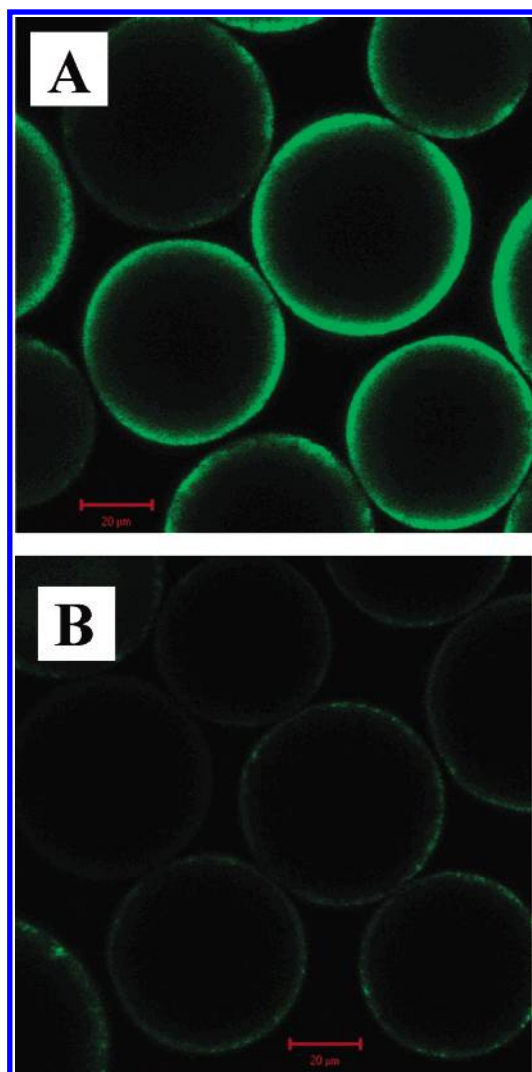
Figure 8. Confocal micrographs of the dextran uptake by PLGA−Glu microspheres without (A) or with (B) 2 days of preincubation in PBST at 37 °C. The microspheres were incubated in 20 mg/mL dextran−coumarin in PBST solution at 37 °C for 12 h. The Gain was set at 971.

microspheres. It turned out that although most previously absorbed dextran was indeed released, a small fraction of dextran was trapped in microspheres after incubation in blank medium, as seen in Figure 10D. This may be explained by that although the larger pores appeared unchanged when incubated at 4 °C, some of the smaller pores or channels connecting macropores (previously referred to as "throats" in examination of copolymers of ethylene and vinyl acetate[25]) closed, causing some previously open pores to become isolated.

3.5. Effect of Neutralizing Insoluble Base on Pore State in PLGA Microspheres. Increasingly more evidence suggests that the acidic microenvironment in PLGA microspheres is a principal factor to destabilize proteins during

long-term release.[17,26,27] We have used poorly soluble bases, such as $MgCO_3$, as neutralizing agents to counteract this harmful condition.[16,17,28,29] The effect of neutralizing insoluble base on pore state in PLGA microspheres was investigated herein by comparing the pore state in two PLGA microspheres: formulation B (without base) and formulation C (with base). The properties of the two formulations are described in Table 1.

As seen in Figure 2C−F, BSA-loaded microspheres with and without coencapsulated base exhibited similar morphology with numerous small pores in the interior. However, when incubated at 37 °C, the morphology of base-containing microspheres became significantly different from those microspheres without base. As seen in Figure 11A and C, the pore state of base-free microspheres was essentially maintained over 9 days of incubation within the length-scale of resolution of the SEM measurement. By contrast, the pores of base-containing microspheres exhibited a significantly altered structure (Figure 11B and D). Small pores that existed before incubation merged into large pores. After 42 days, the shape of base-free microspheres remained intact while a significant amount of base-containing microspheres were fractured and broken (Figure 11E and F). The increased pore connectivity, that is, pore opening, apparently resulted in the faster release rate of BSA from base-containing microspheres than that from the base-free microspheres.

Base-containing microspheres also exhibited a different dextran-uptake behavior. As seen in Figure 12, after preincubated at 37 °C for 6 days, base-containing microspheres showed significant dextran uptake, whereas dextran uptake of base-free microspheres was minimal. The greater uptake of dextran by base-containing microspheres reflected greater pore connectivity, which was in accordance with the faster release of BSA from microspheres. After uptake, the dextran-absorbed microspheres were incubated in blank PBST at 37 °C. As seen in Figure 12F, a substantial amount of dextran remained in the polymer, indicating that some of the pores in the microspheres became closed, suggesting that pore closing can occur at later bioerosion stage besides during the initial release period.

We hypothesized that the higher pore connectivity in base-containing microspheres was caused by the osmotic pressure building up in PLGA microspheres when magnesium car-

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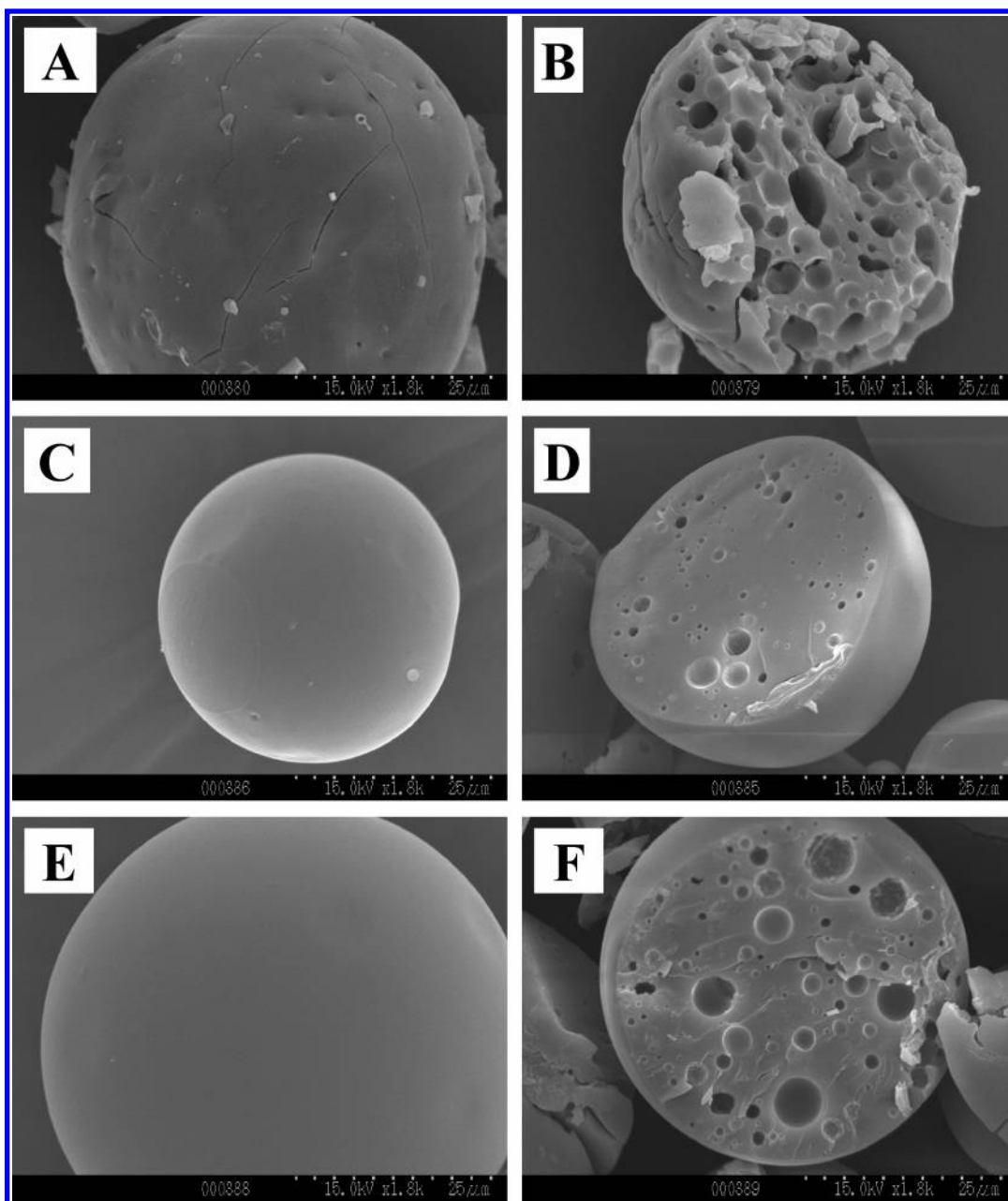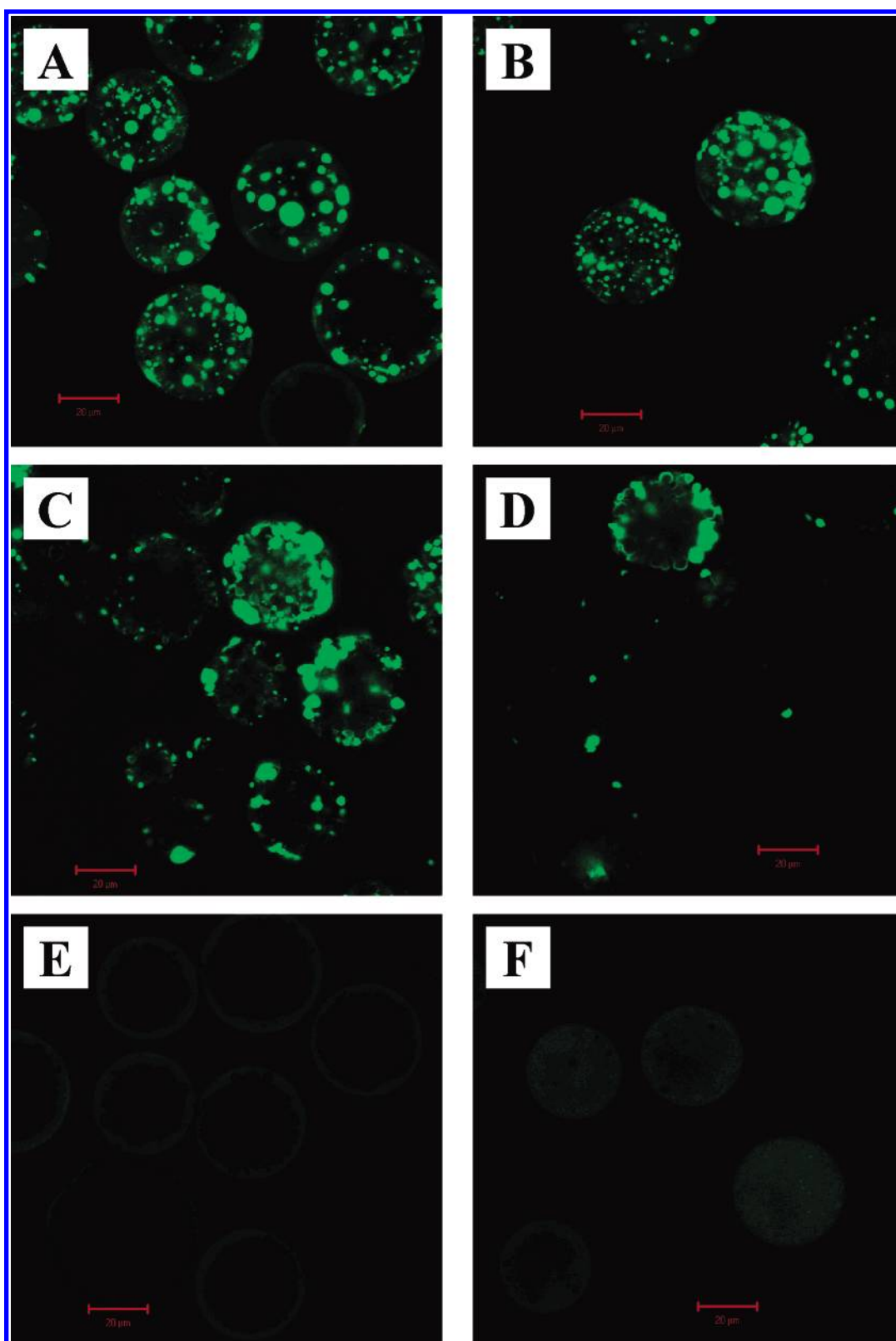
Figure 9. Scanning electron micrographs of the surface (left panel) and cross sections (right panel) of BSA-containing PLGA microspheres (formulation A) after incubation in PBST at 4 °C for 2 days (A and B) or at 37 °C for 2 h (C and D) and 2 days (E and F).

bonate neutralized water-soluble acidic polymer degradation products. To test this hypothesis, we compared the protein release from base-containing microspheres in PBST and PBST + 0.3 M NaCl. If osmotic pressure was responsible for the altered pore structure, we would expect the protein release in 0.3 M NaCl + PBST to be much slower than that in PBST because osmotic pressure would be suppressed by the high concentration of salt added in the release medium (that is, presuming little effect of the salt on solubility and stability of the protein). As shown in Figure 13, the protein release rate was indeed slower in PBST + 0.3 M NaCl than in PBST alone. Also consistent with this hypothesis, when incubated in PBST with a high concentration of NaCl, the

cross section of base-containing PLGA microspheres were significantly denser (Figure 14) than that of microspheres incubated in PBST with a low concentration of NaCl. CO_2 formation by reaction of acid with $MgCO_3$ may have also contributed to the structural disruption.

3.6. Methodologies Used in Characterization of Pore Behavior in Biodegradable Microspheres. The purpose of this study was to qualitatively investigate the phenomenon of pore closing/opening in PLGA microspheres and its implication on protein release instead of a quantitative measurement of pore transition. Several different microsphere formulations with distinct properties, such as polymer type, burst release rate, with or without neutralizing salts, were

Figure 10. Confocal micrographs of the dextran uptake by BSA-containing PLGA microspheres (formulation A) without (A) or with (C and E) 2 days of preincubation in PBST at 4 °C (C) or 37 °C (E). The uptake was carried out at 4 °C (C) or 37 °C (A and E) in 2.5 mg/mL dextran-bodipy solution for 12 h. After uptake, A, C, and E were incubated in blank PBST for 24 h at the same temperature as in the uptake study and observed by LSCM (B, D, and F correspond to A, C, and E, respectively). Gains were set at 820 except for F, which was taken with a gain of 907 to observe the faint fluorescence.
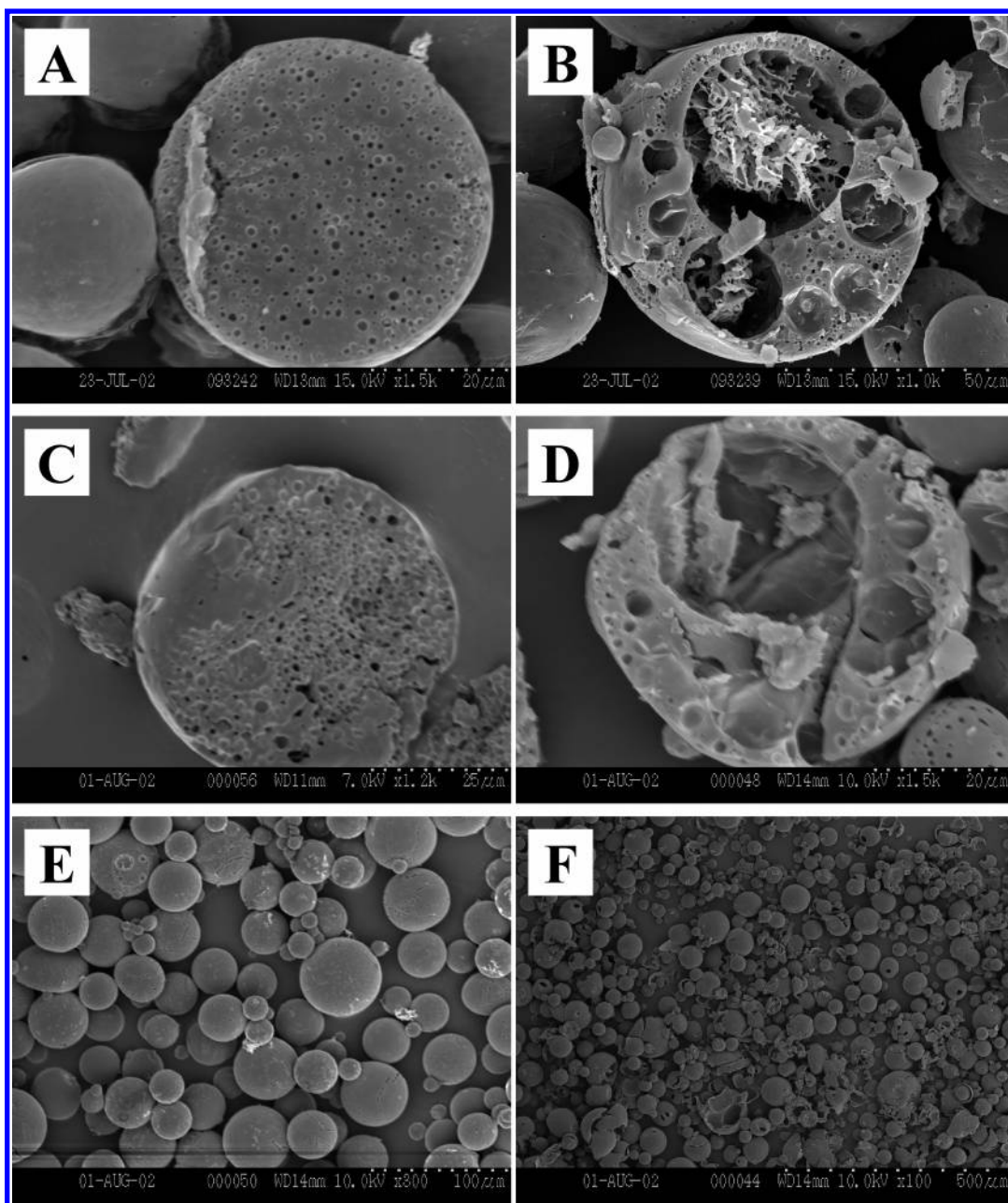
Figure 11. Scanning electron micrographs of the cross section of PLGA microspheres containing either BSA only (formulation B) (left panel) or both BSA and MgCO₃ (formulation C) (right panel) after 9 days (A and B) and 42 days (C, D, E, and F) of incubation in PBST at 37 °C.

used in this study to test the generality of the pore change in PLGA microspheres.

We used three techniques to characterize the pore transition in biodegradable PLGA and PLGA−Glu microspheres: (1) release of both BSA and dextran from same microspheres at different temperatures; (2) visual observation of pore state on the surface and in the interior of microspheres by SEM before and after incubation at different temperatures; and (3) investigation of dextran−probe uptake by microspheres with or without preincubation at different temperatures using LSCM. The results from these experiments provided direct evidence of the critical role of pore closing/opening on protein release from biodegradable microspheres.

LSCM was used previously to observe the drug distribution[30,31] and uptake[13] in PLGA microspheres. However, the results of these observations were often compromised because pH-sensitive fluorescent probes, such as fluorescein,

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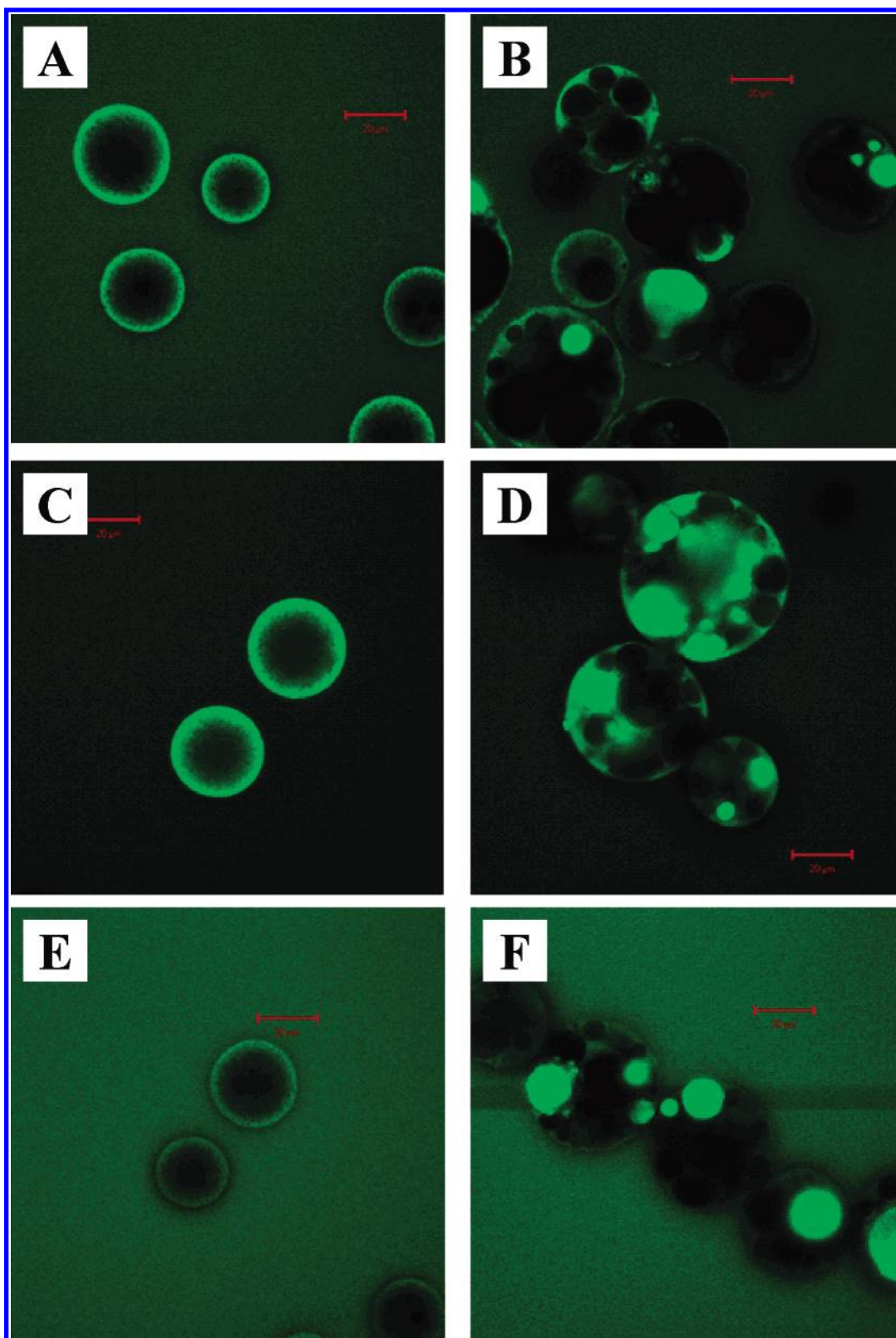
Figure 12. Confocal micrographs of the dextran−bodipy uptake by PLGA microspheres containing either BSA only (formulation B) (left panel) or both BSA and MgCO$_3$ (formulation C) (right panel) after preincubation in PBST at 37 °C for 1 week. The uptake was carried out at 37 °C in 2.5 mg/mL dextran−bodipy solution for 5 (A and B) and 12 h (C and D). After 12 h uptake, the microspheres were incubated in blank PBST for another 12 h at 37 °C and then observed by LSCM (E and F). Gain was set at 950 (A, B, E, and F) or 680 (C and D).
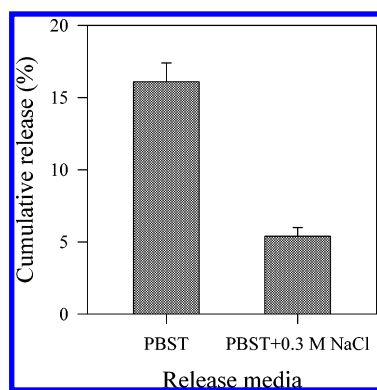
Figure 13. Effect of release medium on protein release from PLGA microspheres containing 3% (w/w of polymer) MgCO$_3$ after 1 week of incubation at 37 °C (mean ± SD, $n = 3$).
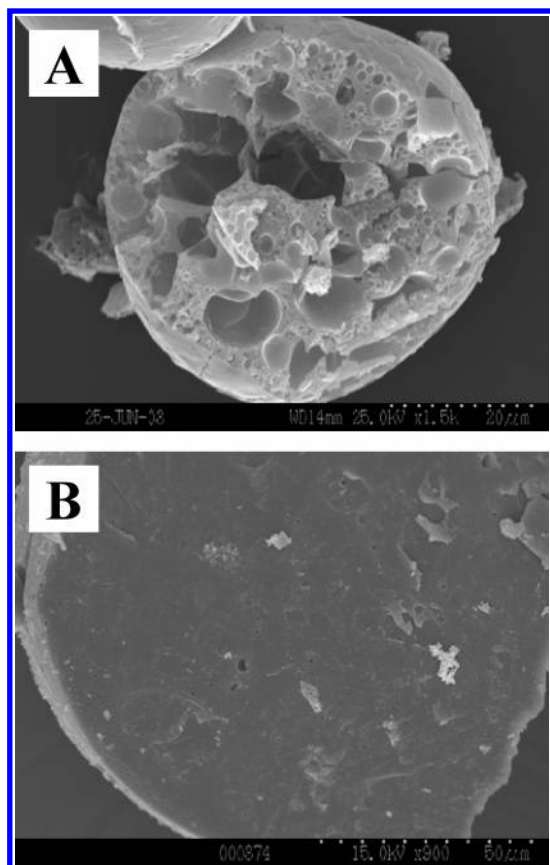
Figure 14. Scanning electron micrographs of PLGA microspheres containing both BSA and base following incubation at 37 °C for 1 week in PBST (A) or PBST + 0.3 M NaCl (B).

were used. Evidence from different labs and by a variety of methods indicates that an acidic microenvironment often exists in PLGA microspheres.[26,27] The fluorescence of pH-sensitive probes would be altered in the acidic microenvironment. For example, we found that fluorescein could be completely quenched by the acidic pH in some PLGA microspheres (data not shown). To avoid the potential artifact caused by the pH-sensitive probes in the heterogeneous microenvironment in PLGA microspheres, we used two pH-

insensitive probes, bodipy[32] and coumarin,[33] to observe the uptake and distribution of dextran in PLGA microspheres. For PLGA−Glu microspheres, dextran−coumarin was used to avoid the interference of dextran−FITC (FITC and bodipy have similar fluorescence spectrum), which was encapsulated in PLGA−Glu microspheres to characterize dextran release.

BSA and probe-conjugated dextran were coencapsulated in PLGA−Glu microspheres. It has been suggested that different protein molecules have different protein-release characteristics, which was attributed to the interaction between polymers and proteins.[34,35] Because dextran is a highly hydrophilic macromolecule, presumably without interaction with PLGA, its release from the polymer would not be affected by the potential drug/polymer interaction.

3.7. Implications of Pore Closing/Opening in Biodegradable Microspheres on Protein Release. Numerous models have been proposed for protein drug release from PLGA microspheres[11,21,34,36] Ehtezazi et al.[36] assumed that the polymer maintained its structure during the whole release period and the limited exit holes on the exterior surface dictated the first-order release rate of macromolecules, a static model. Batycky et al.[11] highlighted the importance of the pore change on protein release, but assuming that only isolated pores can become open, not vice versa, a one-way valve model.

In this study, we found that (1) pore closing occurred in both PLGA−Glu and PLGA polymers; (2) pore closing can occur at later bioerosion stages at physiological condition (37 °C) as well as during initial incubation; (3) pore closing is much more significant at elevated temperatures; (4) previously isolated pores can become open by, for example, osmotic-mediated events; and (5) pore connectivity correlates well with the release rate of biomacromolecules from PLGA or PLGA−Glu microspheres. The pore closing/opening is potentially a universal event throughout the release period, dictating the protein release kinetics from PLGA-related polymers.

On the basis of the above findings, we propose that open and isolated pores are able to toggle back and forth periodically during PLGA degradation. This two-way valve

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mechanism dictates the release profile of protein drugs from biodegradable polymer microspheres. Although still speculative, we make this proposal at this stage because of its potential significance to the field of PLGA biomaterials and related polymers.

At the beginning of the incubation, only a fraction of the pores in microspheres are open. Once in contact with aqueous medium, water will penetrate rapidly into the polymer matrix and dissolve any soluble protein and excipients, if sufficiently water soluble.[2] All of the protein in open pores will be released rapidly. Pores on the surface of the microspheres and those connecting pores inside microspheres can be closed rapidly depending on polymer properties, which will decrease the release rate and, in many cases, completely stop the release of protein. During the polymer degradation, the mechanical strength of the thin polymer membranes, which form the walls of pores, will be affected. The dissolved proteins, excipients, and polymer degradation products also cause increased osmotic pressure in pores.[37] The decreased polymer mechanic strength and increased osmotic pressure may lead to polymer rupture so that previously isolated pores become open and previously unreleasable protein molecules are now released. The pore opening and closing are in a dynamic transition, which dictates the release of proteins from polymers. Because of the small size and thus the short diffusion pathlength in microspheres ($1-100\ \mu$m), even with the high tortuosity, the release of protein would be rapid once in open pores. Therefore, the release of protein drugs from PLGA microspheres is primarily controlled by the state of pores and can be regarded as "quantized." The macroscopically observed continuous release profile consists of numerous pulsatile releases of protein from open pores.

In conclusion, SEM and LSCM observation of microsphere structure during incubation, together with temperature-dependent release profile of macromolecules, provided direct evidence of pore closing/opening in PLGA microspheres and highlighted its importance on protein release. The pore closing/opening appeared to be a potentially universal event throughout the release period, dictating protein release from PLGA microspheres. We propose that open and isolated pores can toggle back and forth periodically during polymer incubation, and this two-way valve mechanism primarily controls the release profile of protein drugs during erosion-mediated release from PLGA microspheres.

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