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Effect of WOW process parameters on morphology and burst release of FITC-dextran loaded PLGA microspheres

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Abstract

Using fluorescein isothiocyanate labeled dextran (FITC-dextran 40, FD40) as a hydrophilic model compound, microspheres were prepared by a WOW double emulsion technique. Influence of process parameters on microsphere morphology and burst release of FD40 from PLGA microspheres was studied. Internal morphology of microspheres was investigated by stereological method via cryo-cutting technique and scanning electron microscopy (SEM). Drug distribution in microspheres was observed with confocal laser scanning microscopy (CLSM). Polymer nature (RG503 and RG503H) had significant influence on the micro-morphology of microspheres. Increase in continuous water phase volume (W2) led to increased surface porosity but decreased internal porosity. By increasing PVA concentration in the continuous phase from 0.1 to 1%, particle size changed marginally but burst release decreased from 12.2 to 5.9%. Internal porosity of microspheres decreased considerably with increasing polymer concentration. Increase in homogenization speed during the primary emulsion preparation led to decreased internal porosity. Burst release decreased with increasing drug loading but increased with drug molecular weight. Drug distribution in microspheres depended on preparation method. The porosity of microspheres decreased with time in the diffusion stage, but internal morphology had no influence on the release behavior in the bioerosion stage. In summary, surface porosity and internal morphology play a significant role in the release of hydrophilic macromolecules from biodegradable microspheres in the initial release phase characterized by pore diffusion.
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Keywords: Poly(lactic-co-glycolic acid) (PLGA); Microspheres; Internal morphology; Porosity; Burst release

1. Introduction

Poly(lactic-co-glycolic acid) (PLGA), a biodegradable and biocompatible polymer, has received tremendous interest regarding the development of parenteral depot systems such as microspheres (Bodmer et al., 1992; Jain, 2000; Jalil and Nixon, 1990b). Encapsulation of drugs in PLGA matrices from which they are released at a relatively slow rate over a prolonged time allow less frequent administrations, thereby increasing patient compliance and reducing discomfort, protection of the therapeutic compound within the body, and avoidance of peak-related side effects by maintaining more-constant blood levels of the drug (Freiberg and Zhu, 2004). Furthermore, since microspheres can be administered by injection, one can also achieve localized drug delivery and high local concentration (Berkland et al., 2002), such as vaccine antigen delivery (Jiang et al., 2005).

So far, numerous investigations have been performed to encapsulate hydrophilic drug substances such as proteins and peptides, into a PLGA matrix using WOW double emulsion methods and the influence of process parameters on the burst release and drug encapsulation efficiency was extensively studied (O'Donnell and McGinity, 1997; Sinha and Trehan, 2003). Although it is clear that internal pore structure of biodegradable polymeric delivery systems plays an important role in the release characteristics of entrapped agents (Kim and Park, 2004; Wang et al., 2002; Yang et al., 2001), only limited information is available regarding the influence of process parameters on the

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internal morphology of microspheres prepared by this method (Yang et al., 2001). Therefore, using fluorescein isothiocyanate labeled dextran (FITC-dextran 40, FD40) as a model hydrophilic compound, influence of process parameters on the surface porosity, internal morphology and burst release of FD40 from PLGA microspheres was studied systematically and evolution of surface, internal morphology during release process was monitored. Internal morphology of microspheres was investigated by stereological method via cryo-cutting technique and scanning electron microscopy (SEM). Drug distribution in microspheres was studied with confocal laser scanning microscopy (CLSM). To the best of our knowledge, no similar work has been performed so far. Since the release kinetics of proteins from poly(lactic-co-glycolic acid) microspheres predominantly depend on polymer properties, internal morphology and drug distribution within microspheres (MS), this study is essential for the design of parenteral depot formulations.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate labeled dextrans (FITC-dextran, abbreviated as FD, 4000, 40,000 and 150,000 g/mol) were supplied by Sigma (Germany). Poly(lactic-co-glycolic acid) (Resomer RG503, RG503H. LA:GA 50:50) was obtained from Boehringer Ingelheim (Germany). Poly(vinyl alcohol) (PVA, 88% hydrolyzed, 130,000 g/mol, Mowiol® 18–88) was a gift from Hoechst AG (Germany). All other materials were of analytical grade.

2.2. Preparation of PLGA microspheres

Microspheres were prepared by a modified (W1/O/W2) double emulsion method as reported previously (Pistel et al., 2001). Briefly, 0.5 g of the respective polymer was dissolved in 2.5 ml methylene chloride (DCM). Into this organic phase (O), 250 μl of aqueous drug solution (W1) was emulsified using a high-speed homogenizer (Ultra-Turrax TP18/10, IKA, Germany) operating at 20,500 rpm for 30 s to form the W1/O emulsion. This primary emulsion was injected into 200 ml of an aqueous phase containing 0.1% (w/v) PVA (external phase, W2) and homogenized for 30 s (Ultra-Turrax T25, IKA) at 8000 rpm. The resulting W1/O/W2 emulsion was stirred at 200 rpm for 3 h with a propeller stirrer to allow solvent evaporation and microspheres hardening. The microspheres were then isolated by filtration, washed three times with distilled water, and freeze-dried. Final products were stored at 4 °C in a desiccator. This standard protocol was varied concerning polymer type, dispersing phase/continuous phase ratio (W1/O, W1 + O/W2), PVA content, PLGA concentration, homogenization speed, theoretical drug loading, Mw of FITC-dextran (4, 40 and 150 kDa).

In addition, for the purpose of comparison, FD40 loaded PLGA microspheres were prepared with a single emulsion method (O/W) using the following conditions: 5 mg FD40 was firstly dissolved in 250 μl DMSO and 0.5 g PLGA was dissolved in 2.5 ml DCM. Then the two solutions were mixed to form a homogeneous solution and injected into 200 ml of an aqueous phase containing 0.1% PVA and homogenized for 30 s (Ultra-Turrax T25) at 8000 rpm. The following steps were the same as described in the double emulsion method.

2.3. Characterization of microspheres

Particle size distribution of microspheres was measured using a MasterSizer X (Malvern Instruments) based on a laser light scattering technique. Weighed microspheres were suspended in 0.1% aqueous Tween 80 solution and vortexed before measurement. Results are reported as volumetric mean diameter [$D(4, 3)$]. The process yield was calculated based on the amount of isolated (lyophilized) microspheres relative to the amount of solids materials used in the process.

Content of FD in microspheres was analyzed by an extraction method described previously (Pistel et al., 2001). Briefly, approximately 10 mg microspheres were dissolved in 0.5 ml DCM, followed by addition of 4 ml PBS buffer pH 7.4 and agitated in an orbital shaker maintained at 37 °C for 15 h at 30 rpm. The FD concentration in the aqueous phase was determined fluorimetrically (Excitation: 493 nm, Emission: 515 nm, LS 50B Luminescence Spectrometer, Perkin-Elmer, Germany) using standard control (Pistel et al., 2001). Each sample was measured in triplicate. Actual drug loading and drug encapsulation efficiency (EE) were calculated using the following equations:

$$\text{Theoretical drug loading} = \frac{\text{drug(tot.)}}{\text{drug(tot.)} + \text{polymer}}$$

$$\text{Actual drug loading} = \frac{\text{drug(enc.)}}{\text{drug(tot.)} + \text{polymer}}$$

$$\text{Encapsulation efficiency} = \frac{\text{actual drug loading}}{\text{theoretical drug loading}} \times 100\%$$

In vitro release of FD40 from microspheres was measured by suspending 15 mg microspheres in 4 ml PBS-buffer pH 7.4 containing 0.05% NaN_3 and 0.01% Tween 20 (Pistel et al., 2001). The samples were placed in an orbital shaker maintained at 37 °C and shaken at 30 rpm. At prederimined time intervals the samples were taken out of the shaker and centrifuged at 2000 rpm for 5 min and 4 ml medium was taken for analysis and fresh medium of the equal volume was added in the meantime. The precipitated microsphere pellets were resuspended in the medium and placed back in the shaker. Burst release was defined as the relative drug release obtained within 4 h of incubation. Each batch was studied in triplicate.

2.4. External and internal morphology of microspheres

For morphology studies, freeze-dried microspheres were dusted on a double-sided adhesive tape applied previously to an aluminum stub and attached onto graphite surface. Excess samples were removed and stub sputter coated with a carbon layer at 4–6 A for 30 s then with a gold layer at 2 A for 30 s (Edwards Auto 306 Vacuum Coater, Edwards, Germany). Morphology of microspheres was analyzed by scanning electron

microscopy (The CamScan Series 4 Scanning Electron Microscopes, Cambridge Scanning Company Limited, England) at a voltage of 9–15 kV. Cross-sectional morphology of microspheres was obtained by embedding the microspheres in an aqueous solution containing 30% gelatin and 5% glycerin, as described previously (Ehtezazi et al., 1999). The SEM images were analyzed using Image J software [developed at the U.S. National Institutes of Health and available on the internet at: http://rsb.info.nih.gov/nih-image/download.html]. Porosity was employed to describe the internal morphology of the microspheres, which is defined as the area of total pores divided by the area of cross-sections of microspheres. At least six representative microspheres were used for the calculation.

2.5. FD distribution in microspheres

Drug distribution within PLGA microspheres was observed by confocal laser scanning microscopy (Carl Zeiss LSM 5100). The microspheres were re-dispersed in distilled water and placed onto a glass slide, and the image was taken. FITC was detected using an argon laser with an excitation wavelength of 488 nm and a 505–550 nm band-pass emission filter. All the images were obtained under the same resolution.

2.6. Differential scanning calorimetry (DSC)

Thermal characterization of microspheres was performed with a Perkin-Elmer DSC 7 (Perkin-Elmer, Wellesley, MA). The equipment was calibrated with indium. The sample (approximately 5 mg) was heated twice from −10 to 160 °C at 20 °C/min in a nitrogen atmosphere (flow rate 61 ml/min). The melting temperature (T_m) was determined from the endothermic peak of the DSC curve recorded in the first heating scan. The glass transition temperature (T_g) was determined from the DSC curve recorded in the second heating scan. Reported glass transition temperatures are midpoint values.

2.7. Calculations and statistics

Results are depicted as mean ± S.D. from at least three different batches of micropheres prepared at the specified conditions. Significance between the mean values was calculated using ANOVA one-way analysis (Origin 7.0 SRO, Northampton, MA, USA). Probability values $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Influence of polymer nature

Two types of PLGA polymers (RG503 and RG503H) with the same molecular weight were investigated in this study. PLGA RG503 bears capped end groups as ester while PLGA RG503H bears uncapped carboxylic terminus as acid and therefore it is more hydrophilic. Properties of the microspheres are listed in Table 1. The particle size and EE decreased slightly for microspheres prepared with RG503H compared to that with RG503. Similar results were observed for DNA microspheres (Capan

Table 1
Characteristics of FD-loaded PLGA microspheres prepared at different conditions

Variables	Parameters	Actual drug loading (%)	EE (%)	$D(4, 3)$ (μm)	Drug burst (%)	Yield (%)
Polymer nature	RG503	0.93 ± 0.04	93 ± 4	37.9 ± 0.8	4.5 ± 0.1	71
	RG503H	0.88 ± 0.04	88 ± 4	33.3 ± 0.3	13.0 ± 0.6	70
Internal phase volume	60 μl	1.24 ± 0.04	124 ± 4	36.7 ± 0.7	1.2 ± 0.1	77
	250 μl	1.02 ± 0.05	102 ± 5	42.4 ± 0.7	2.9 ± 0.1	84
	800 μl	0.84 ± 0.04	84 ± 4	43.8 ± 1.3	3.8 ± 0.2	81
Continuous phase volume	100 ml	0.92 ± 0.02	92 ± 2	33.7 ± 0.4	1.7 ± 0.1	77
	200 ml	0.91 ± 0.05	91 ± 5	44.0 ± 1.3	13.5 ± 0.4	85
	400 ml	0.73 ± 0.01	73 ± 1	43.6 ± 0.8	10.5 ± 0.1	85
PVA concentration	0.1%	0.83 ± 0.03	83 ± 3	35.6 ± 0.3	12.1 ± 0.4	80
	0.5%	0.88 ± 0.04	88 ± 4	36.8 ± 0.2	8.1 ± 0.2	86
	1%	0.90 ± 0.07	90 ± 7	30.8 ± 0.4	5.9 ± 0.3	83
PLGA concentration	8%	0.62 ± 0.02	62 ± 2	24.2 ± 0.1	21.8 ± 0.3	77
	20%	1.01 ± 0.01	101 ± 1	45.4 ± 0.4	5.3 ± 0.2	83
	32%	1.23 ± 0.03	123 ± 3	65.0 ± 1.3	1.0 ± 0.1	69
Homogenization speed	20,500 rpm	0.91 ± 0.04	91 ± 4	34.3 ± 0.2	7.9 ± 0.2	77
	13,500 rpm	0.81 ± 0.04	81 ± 4	28.6 ± 0.1	17.4 ± 0.5	78
Theoretical drug loading	1%	0.81 ± 0.04	81 ± 4	38.5 ± 0.8	8.7 ± 0.3	72
	5%	3.75 ± 0.29	75 ± 6	49.3 ± 0.2	7.6 ± 0.5	75
	10%	6.29 ± 0.17	63 ± 2	43.4 ± 1.4	2.1 ± 0.1	80
FDMW	4 kDa	0.84 ± 0.03	84 ± 3	43.0 ± 0.7	3.6 ± 0.1	71
	40 kDa	0.84 ± 0.08	84 ± 8	50.7 ± 0.3	8.0 ± 0.1	86
	150 kDa	0.75 ± 0.04	75 ± 4	42.0 ± 0.3	12.8 ± 0.4	76
Preparation method	O/W	0.55 ± 0.03	55 ± 3	53.8 ± 1.6	0.9 ± 0.1	70
	W/O/W	0.91 ± 0.05	91 ± 5	44.0 ± 1.3	13.5 ± 0.4	85

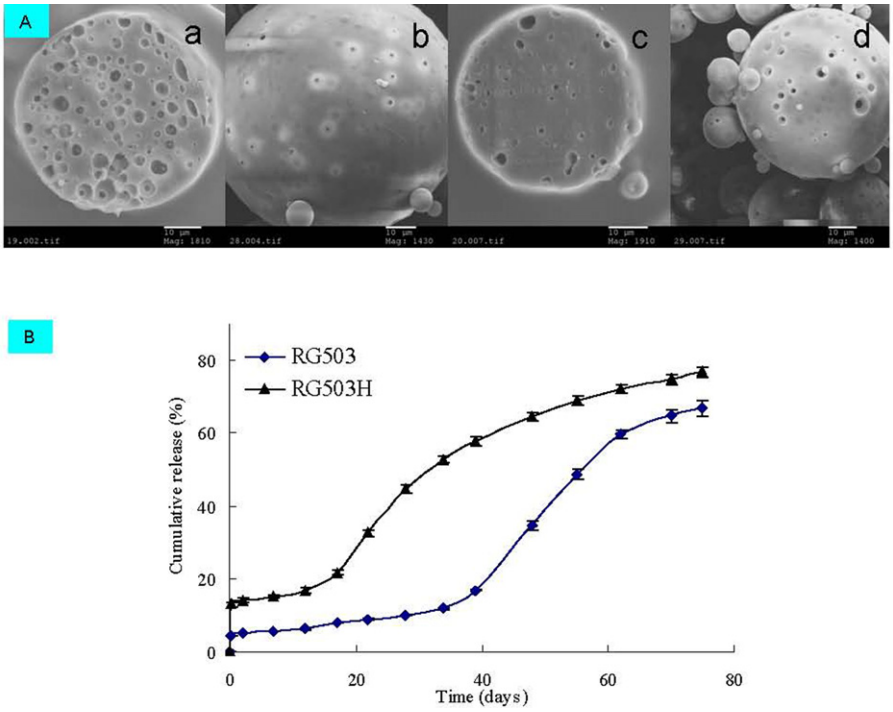

Fig. 1. (A) Internal and surface morphology of microspheres prepared with RG503 (a and b) and RG503H (c and d) under same conditions. (B) Influence of polymer nature on the release of FD40 from PLGA microspheres.

et al., 1999), which was attributed to the lower viscosity of the polymer solution and the longer time required for RG503H to solidify as compared to RG503.

Fig. 1A shows the external and internal morphology of microspheres prepared with PLGA RG503 and RG503H with a theological drug loading 1%. All the microspheres are spherical with porous surface and the porosity is polymer property dependent. Microspheres prepared with RG503 had very high internal porosity with pore size approximately 3 μm, but surface porosity was low, with pore size less than 1 μm. In contrast, microspheres prepared with RG503H revealed a low internal porosity with less pores of approximately 1.5 μm, but many pores in the range of 2–4 μm were formed on the surface. The bursts were 4.5 and 13.0%, for RG503 and RG503H microspheres, respectively, implying that the burst was mainly controlled by the surface porosity in this case.

In vitro release of FD40 from different microspheres is shown in Fig. 1B. During the first 15 days, the release profiles were similar when bursts effects were neglected. However, after 18 days, significantly faster release rates were observed for RG503H microspheres, which can probably be explained by the more rapid degradation of RG503H than RG503 due to increased hydrophilicity. It has been reported that the decrease in molecular weight correlated well with increased water uptake and therefore the degradation rate is higher for the hydrophilic polymer. Also, auto-catalytic degradation due to the carboxylic acid groups may be decreased by blocking the end groups (Rothen-Weinhold et al., 1997). Despite the high internal porosity of RG503 microspheres, the release rate was quite low and the diffusion process lasted for 38 days. This implies that the difference in hydrophility of polymers and surface porosity overweigh the difference in internal porosity in this case. Therefore, RG503 was chosen for the following process parameter studies for ease of comparison.

3.2. *Influence of internal aqueous phase (W1)/continuous phase (W2) volume*

Keeping other conditions constant, effect of internal aqueous phase volume on properties of microspheres was investigated (Table 1). The particle size increased slightly when W1 volume increased from 60 to 250 μl. Further increase of W1 to 800 μl caused no significant change of the particle size ($P > 0.05$). However, drug loading and EE decreased significantly with increasing internal aqueous phase volume. This is in agreement with the results reported previously (Schlicher et al., 1997). The fact that EE is larger than 100% could be attributed to the loss of PLGA at higher concentrations. The initial drug bursts were 1.2, 2.9 and 3.8% at W1 volume 60, 250 and 800 μl, respectively. Low volumes of W1 phase resulted in a minimal microporosity of the polymer matrix, whereas high W1 volumes caused an increasing number of micropores and channels thereby facilitating the rapid initial diffusion of encapsulated drug (Schlicher et al., 1997; Ghaderi et al., 1996). On the other hand, small internal phase is surrounded by a thicker organic phase and the diffusion rate of FD40 into the continuous phase decreased in the solvent evaporation step. Anyhow, when W1 was less than 800 μl, its influence on the initial drug burst was marginal.

On the other hand, the influence of the continuous phase volume on properties of microspheres was studied (Table 1). Particle size increased considerably with increasing W2 volumes from 100 to 200 ml and Further increasing W2 from 200 to

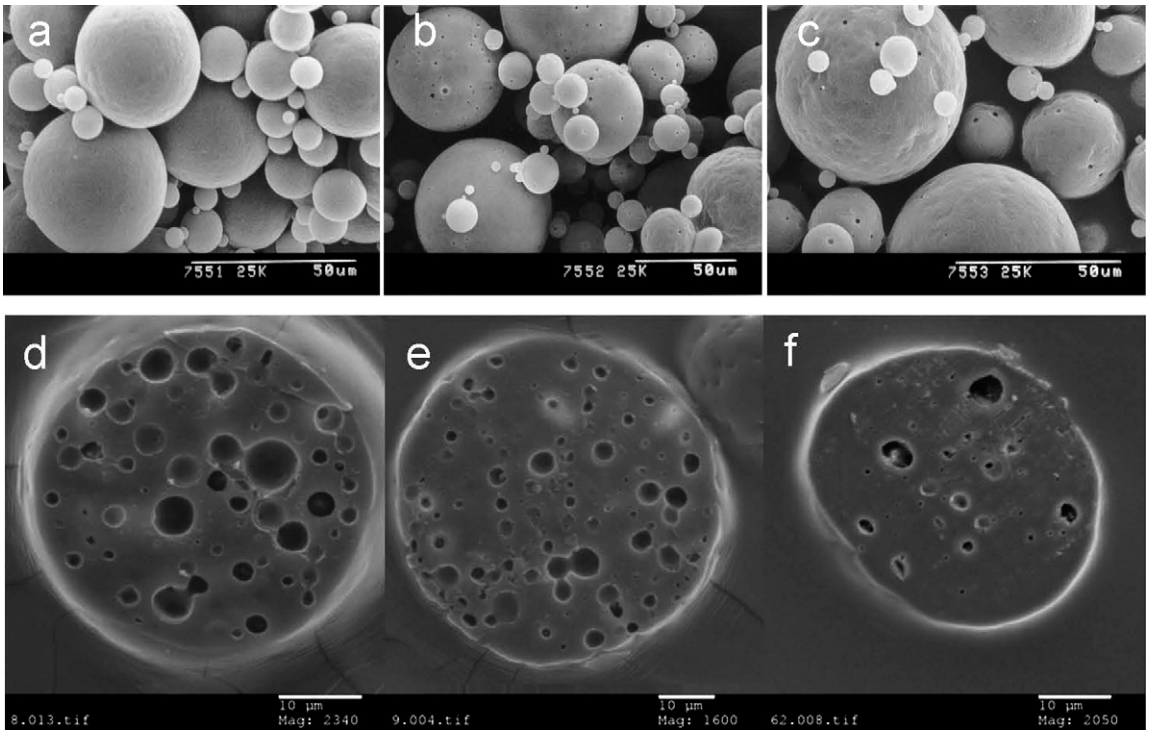

Fig. 2. Surface and internal morphology of microspheres prepared at continuous phase volume 100 ml (a, d), 200 ml (b, e) and 400 ml (c, f).

400 ml caused no remarkable change in particle size ($P > 0.05$). In contrast, no statistical difference in EE was found by increasing W2 from 100 to 200 ml. However, EE decreased to 73% at W2 volume 400 ml compared to 90% at W2 volume 100 or 200 ml, which can probably be attributed to drug loss during the hardening stage. As shown in Fig. 2, at W2 volume 100 ml, spherical microspheres with smooth surface without pores were formed. In contrast, the surface became porous along with the increase in W2 volume. Internal morphology analysis demonstrated that internal porosity of microspheres decreased slightly with increasing W2 volume, it was 15.83, 13.09 and 11.81%, respectively, at W2 volume 100, 200 and 400 ml. It has been reported that the porosity in a system of microspheres is determined during microspheres hardening as the organic solvent evaporates during preparation (Yang et al., 2000). Li et al. (1995) indicated that the continuous phase containing the large amount of water resulted in faster polymer precipitation and therefore less porous spheres were formed. This does not seem to be the case in our study and the external surface was smoother at W2 100 ml. This can probably be explained by the fact that the solubility of DCM in water is about 2% (v/v) and due to some loss of DCM in the first emulsion step (total volume 2.5 ml), 100 ml W2 volume might be sufficient for DCM to distribute into the aqueous phase immediately. Further increase in W2 will adversely cause drug loss or drug redistribution on the microsphere surface in the hardening stage due to the increased concentration gradient. This explanation is in good agreement with initial drug burst data. High burst release (>10%) was found at W2 200 and 400 ml compared to 1.7% at W2 100 ml. Since the microspheres had comparable internal porosity, the difference in initial burst release can be attributed to pores on the surface of microspheres, which provide channels for water penetration. Also different drug distributions in microspheres could cause higher initial drug bursts when the hydrophilic drug substance is mainly located close to the surface of microspheres.

3.3. Influence of polyvinyl alcohol concentration in the external water phase

PVA concentrations in the external water phase are known to be a key factor influencing the particle size of microspheres. Jalil and Nixon (1990a) observed a sharp drop in diameter within the optimal emulsifier concentration beyond which little change was found due to saturated emulsifier packing at the surface of spheres. Keeping other conditions constant, the effect of PVA concentration in the external water phase on the properties of microspheres was studied (Table 1). The sizes of microspheres fabricated at 0.1, 0.5 and 1% PVA were 35.6, 36.8 and 30.8 μm, respectively. Increasing PVA concentration from 0.1 to 1% caused only approximately 16% particle size decrease. It is well known that the presence of PVA in the external phase stabilizes emulsion droplets against coalescence. The stabilization effect is dominant at higher PVA concentrations and leads to the decrease in the size of microsphere. Based on our experimental data, 0.1% PVA seems to be sufficient to prepare stable FD40 loaded microspheres. Interestingly, it was noticed that PVA concentration in the external phase had a significant effect on the burst release. Bursts decreased from 12.1 to 5.9% by increasing PVA concentration from 0.1 to 1%. Probably a higher PVA concentration yields a more stable emulsion that hinders the mass transfer of FD40 with surroundings. Similar observations were reported previously (Yang et al., 2001).

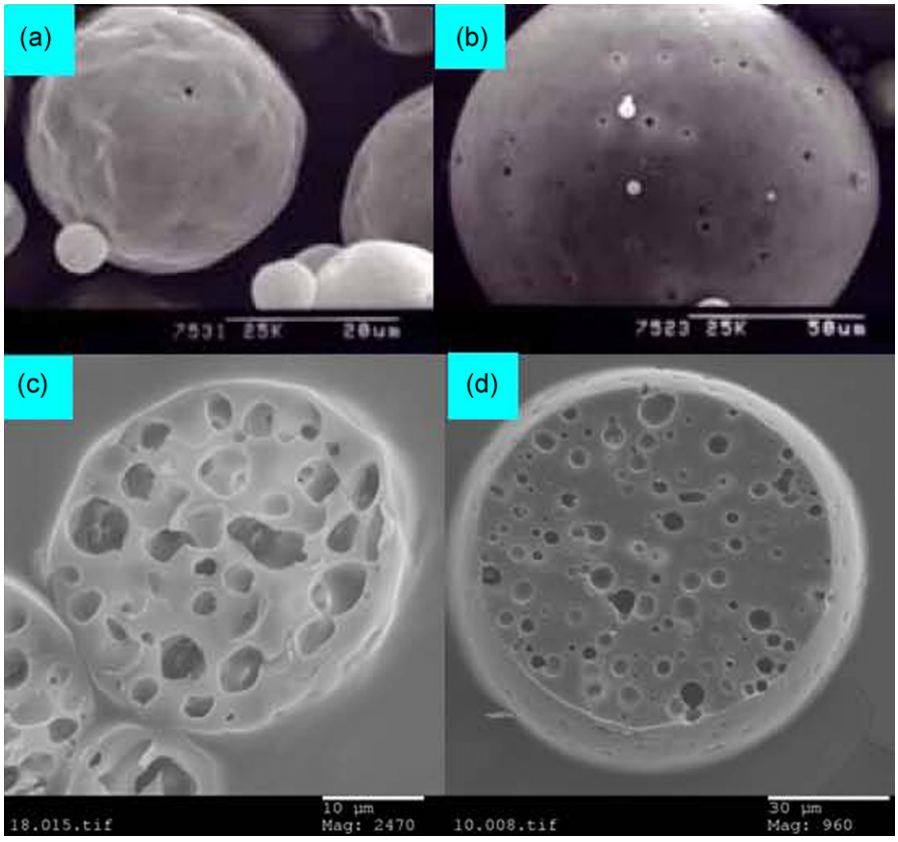

Fig. 3. Surface and internal morphology of microspheres prepared with 8% PLGA (a, c) and 32% PLGA (b, d).

3.4. Influence of polymer concentration

Polymer concentration is another key factor influencing burst release and internal morphology of microspheres. As shown in Table 1, the actual drug loading, EE and size of microspheres increased with increasing PLGA concentration significantly due to the increased viscosity of the organic phase, which prevented out-flow of drug during the hardening phase. Similar results have been previously reported (Ghaderi et al., 1996; Painbeni et al., 1998; Schlicher et al., 1997; Sturesson et al., 1993).

Influence of polymer concentration on the external and internal morphology of microspheres is shown in Fig. 3. At PLGA concentration 8%, microspheres with wrinkled surface and less external pores of approximately 1 μm were formed. However, the internal porosity was extremely high (52.7%) with pore diameter of ca. 5 μm. Probably the internal water droplets in the low polymer concentrated solution tend to coalesce more easily, leading to bigger pores and a less tortuous network. In contrast, the surface of microspheres became smoother at PLGA concentration 32% but with more small pores in the range of 1–1.5 μm on the surface. Similarly, Yang et al. (2000) investigated the influence of preparation temperature on the morphology of microspheres and found rough surface at higher temperature (low viscosity) and porous surface at lower temperature (high viscosity). Also, the diameter of internal pores decreased to 2.5 μm and the internal porosity was calculated to be 1.6%. The initial burst decreased from 21.8 to 1.6% by increasing PLGA concentration from 8 to 32% despite the increased external pores at higher PLGA concentration. The high viscosity might restrict the diffusion of the drug in the matrix and compromise the contribution of external channels. On the other hand, the decrease in surface area/volume ratio with increasing size could be another cause for the decreased burst at higher polymer concentration. Moreover, it is clear that high internal porosity leads to much easier and deeper water penetration upon incubation and shortens the diffusion time and path of the loaded drug, leading to higher burst release. Similarly, Yang et al. (2000) observed that porosity has an important effect on drug release characteristics and a large number of pores may greatly increase the rate of drug release. By using mercury intrusion porosimetry, it was also demonstrated that the internal porosity of microspheres decreased with increasing polymer concentration (Schlicher et al., 1997). Additionally, our in vitro release data demonstrated that the porosity of microspheres affected mainly the initial drug burst but not the release behavior at later stages (data not shown).

3.5. Influence of homogenization speed

During the primary emulsion preparation, two homogenization speeds, 13,500 and 20,500 rpm, were investigated. The particle size increased slightly at higher homogenization speed but with remarkable increase in EE ($P < 0.05$) (Table 1). It has been reported that increased stirring rate resulted in the formation of finer primary emulsion (Jalil and Nixon, 1990b) and therefore higher EE was obtained. As shown in Fig. 4, difference in homogenization speed caused no significant difference

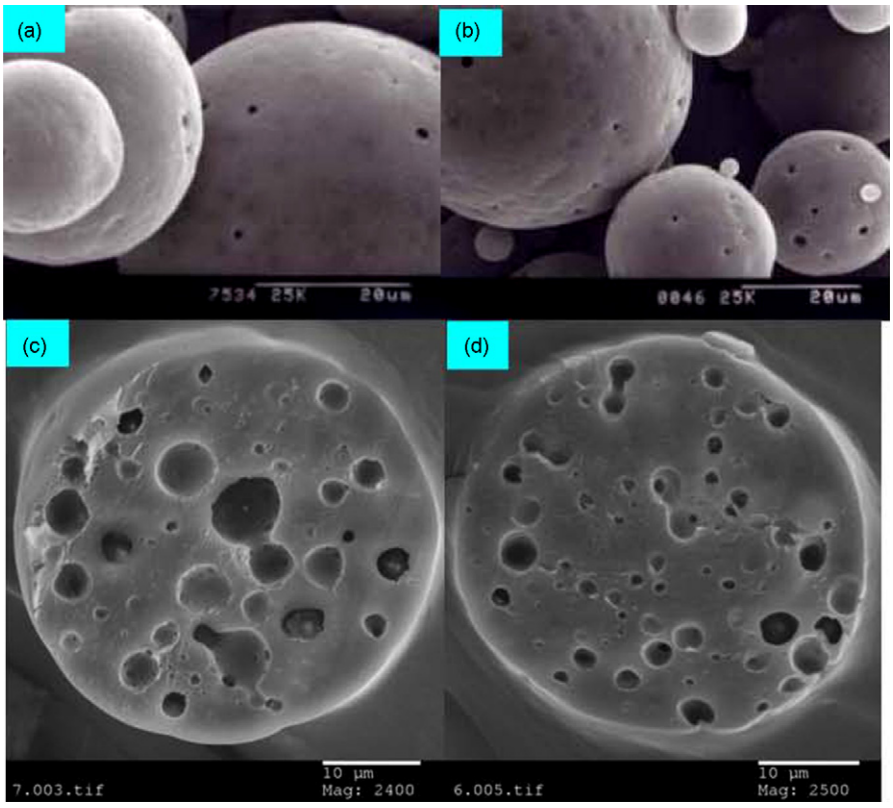

Fig. 4. Surface and internal morphology of microspheres prepared at homogenization speed 13,500 rpm (a and c) and 20,500 rpm (b and d).

in surface morphology and all microspheres are spherical with some 1 μm diameter pores on the surface. A remarkable influence of this process parameter on the internal morphology of microspheres was observed (Fig. 4). The diameter of internal pores was approximately 7 μm at stirring rate 13,500 rpm compared to 3 μm at stirring speed 20,500 rpm and the porosity was calculated to be 21.89 and 13.80%, respectively. Similarly, lower porosity was observed in microspheres prepared at higher homogenizing speed (Ehtezazi et al., 2000). Low stirring rates resulted in smaller size (Table 1), high porosity and therefore higher burst release. The burst was 17.4% at stirring rate 13,500 rpm whereas 7.9% at 20,500 rpm implying that internal morphology plays a critical role for initial drug burst.

3.6. Influence of theoretical drug loading

Keeping other conditions constant, properties of microspheres prepared with different theoretical drug loading are described in Table 1. The initial drug loading did not influence either the mean particle size or size distribution significantly, as demonstrated previously (Painbeni et al., 1998; Yang et al., 2001). No remarkable difference in drug EE was found at theological drug loading 1 and 5%. However, the EE decreased considerably with increasing drug loading further to 10%. A higher drug loading caused a higher FD40 concentration in the emulsion droplets and this increase in concentration gradient led to a loss of drug into the W2 phase. The distribution of FD40 in the microspheres was studied with DSC. The T_g of RG503 was 41.9, 41.9 and 40.4 °C, respectively, for 1, 5 and 10% FD40 loaded microspheres, compared to 44.5 °C for pure RG503. No melting temperature of FD40 was found in the thermograms of all samples, implying that FD40 was physically dispersed in the polymer matrix and not dissolved in the PLGA.

As shown in Table 1, the initial drug bursts decreased with increasing drug loading. Since it is assumed that burst release is normally caused by the drug near the surface of microspheres, this implies that most of the drug substance was distributed in the internal matrix of the polymer instead of near the surface. The release profiles of microspheres with different drug loading are shown in Fig. 5. During the first 20 days, no apparent difference in release was found between microspheres with theoretical drug loading 1 and 5% but slower release was noticed for 10% microspheres. The release profile was not influenced by drug

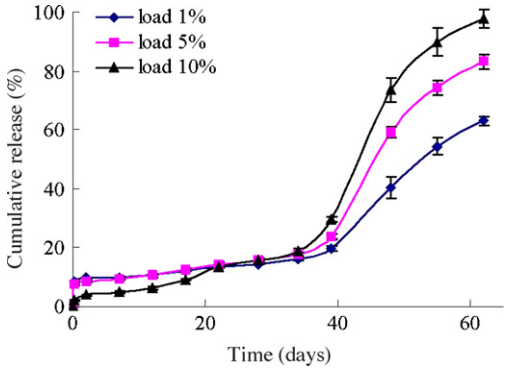

Fig. 5. Influence of drug loading on the release profiles of FD40 from PLGA microspheres.

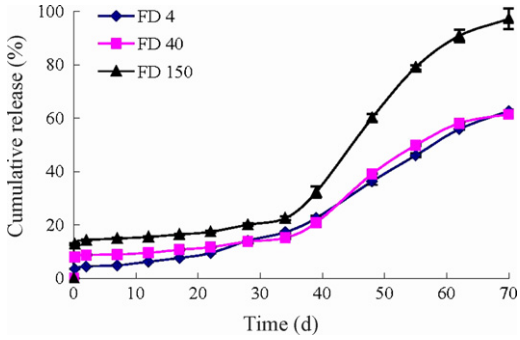

Fig. 6. Influence of FD molecular weight on the release profiles from PLGA microspheres.

loading during 20–35 days. It was noted that FD release rate increased remarkably with drug loading in the bioerosion stage, it was 2.17, 3.19 and 3.81% per day at theoretical drug loading 1, 5 and 10%, respectively. Similar phenomenon was observed for rhodamine-loaded microspheres (Pistel et al., 2001). Higher release rate was also reported for protein-loaded microspheres (Sandor et al., 2001). Probably the microparticles with higher initial drug loading has higher water uptake capacity during the diffusion stage than microparticles with lower drug loading due to the high hydrophilicity of FD40, leading to faster polymer degradation (Rothen-Weinhold et al., 1997).

3.7. Influence of FD molecular weight

Keeping other conditions constant, effect of FD molecular weight on the properties of microspheres was investigated and the results are listed in Table 1. Only slight differences in particle size were observed. EE was comparable for FD4 and FD40 ($P > 0.05$), and the value decreased remarkably for FD150 loaded microspheres ($P < 0.05$). Probably due to the long chain length of FD150, the diffusion channel could be formed easily, leading to increased drug loss during DCM evaporation stage. Similarly, burst release increased with increasing FD molecular weight, and it was 3.6% for FD4 compared to 12.8% for FD150. Surface and internal morphology of microspheres were observed and however no apparent difference was found (data not shown), all the microspheres are spherical with some 1 μm pores on the wrinkled surface.

The release profiles are shown in Fig. 6. No apparent difference in release was found in the diffusion stage except burst. In the bioerosion stage, the release behavior of FD 4 and 40 kDa loaded microspheres was similar, with release rate 1.37 and 1.63% per day, respectively, and only approximately 60% drug substance was released in 65 days. In contrast, FD150 loaded microspheres had a higher release rate of 2.58% per day and almost all the encapsulated drug was released in 65 days. Due to its long chain length, FD150 was probably entrapped in polymer matrix like network and channel forming agent, with quicker water uptake and therefore faster degradation rate (Rothen-Weinhold et al., 1997). Moreover, DSC was employed to investigate the existing state of different molecular weight FD in the microspheres. Encapsulation of different molecular weight FD in RG503 caused no obvious change of its glass transition temperature, indicating that no interaction between each other. A melting peak was observed in microspheres containing FD 150 kDa, implying that FD 150 kDa was distributed in a crystalline state in the microspheres at least partly, whereas FD4 and FD40 were distributed in a molecular state. It is well known that drug substance in a crystalline state will be released slower compared to that in the molecular state while a dissolution process is involved, but it is not the case in our study, implying that the channel forming function of FD150 plays a critical role in release and FD150 dissolution was not a rate-limiting process.

3.8. Influence of microspheres preparation method

Compared to microspheres prepared with double emulsion method, microspheres prepared with single emulsion method had larger particle size and considerably decreased EE (Table 1). Morphology of microspheres prepared with single emulsion method showed a very smooth surface without pores. In contrast, microspheres prepared with double emulsion method had many ca. 1 μm pores on the surface (Fig. 7). As a consequence, microspheres prepared by double emulsion method exhibited a burst of 13.5%, whereas no sign of initial drug burst from microspheres prepared by O/W method was noted. Internal porosity was 9.01 and 13.09%, respectively, for microspheres prepared by O/W method and double emulsion method (Fig. 7).

CLSM was employed to visualize the distribution of FD40 throughout microspheres prepared with different method, as shown in Fig. 8. In microspheres prepared by the WOW method, FITC-dextran tended to aggregate and drugs were preferentially distributed near the surface of microspheres (Fig. 8a–c), which was indicated clearly by the three dimensional images of FD40 intensity (Fig. 8c). In contrast, FD distribution was quite uniform in microspheres prepared by O/W method (Fig. 8d–f). Due to the absence of apparent external pores on the surface of microspheres prepared by O/W method and its homogeneous distribution, no burst was found. On the other hand, drug aggregation near the surface and higher external porosity contributed to the high burst of FD from microspheres prepared by WOW double emulsion method.

3.9. Visual evidence of morphology change during incubation

In order to investigate the influence of internal morphology on the release behavior, microspheres with PLGA concentrations 8, 20 and 40% were chosen in this study and internal morphology change during incubation was monitored. After 15 days incubation microspheres showed obviously swelling, as indicated by the decreased internal porosity shown in Fig. 9A. Fifteen days later the porosity decreased to 30.78, 12.04 and 0.64%, respectively, for microspheres prepared with PLGA 8, 20 and 40%, compared to 52.73, 26.58 and 1.58% before release study. As shown in the cross-section images, a large amount of small pores existed in microspheres after 38 days incubation (Fig. 9A) irrespectively of polymer concentration.

On the other hand, taking PLGA 20% microspheres as an example, evolution of surface morphology during incubation

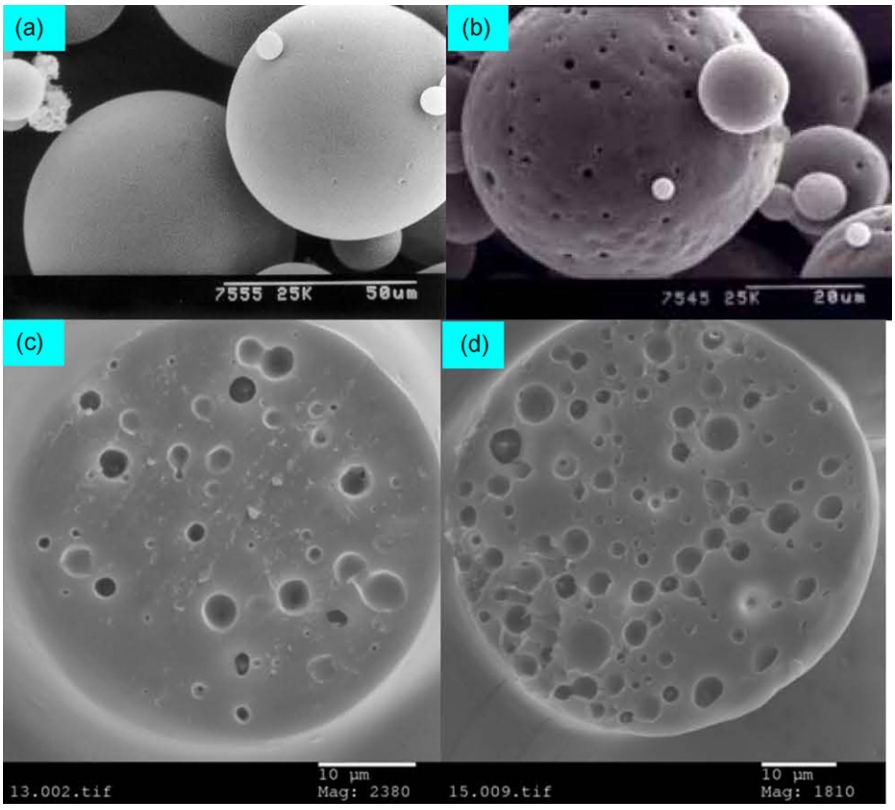

Fig. 7. Surface and internal morphology of microspheres prepared with O/W single emulsion method (a and c) and W/O/W double emulsion method (b and d).

was investigated as well and is shown in Fig. 9B. The surface pores were approximately 1 μm before incubation and no pore size change was found after 4 h incubation. Slightly decreased pore sizes (0.6 μm) were observed after 2 and 7 days incubation. In contrast, the pore size increased two-fold after 15 days incubation and this can probably be attributed to a compensation of the decreased internal porosity and internal pressure release at this time point, but the spherical morphology of microspheres

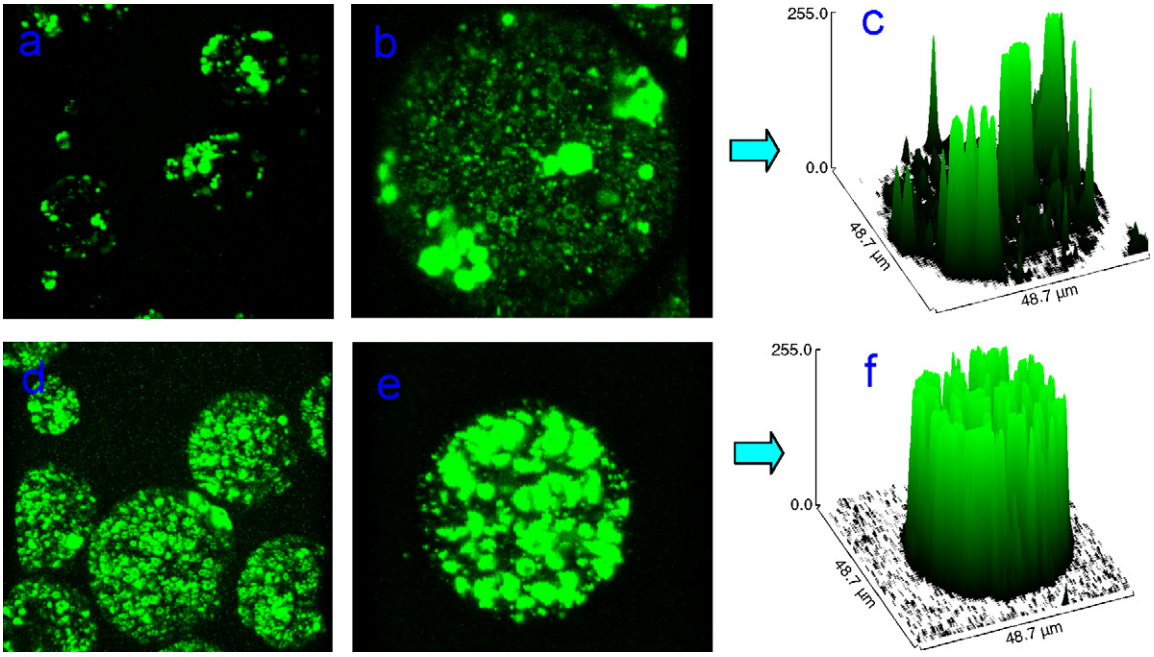

Fig. 8. Distribution of FD40 in microspheres prepared with different methods observed by CLSM. Microspheres prepared by double emulsion method: (a) overview, (b) detail, (c) FD40 distribution with surface intensity plot. Microspheres prepared by single emulsion method: (d) overview, (e) detail, (f) FD40 distribution with surface intensity plot.

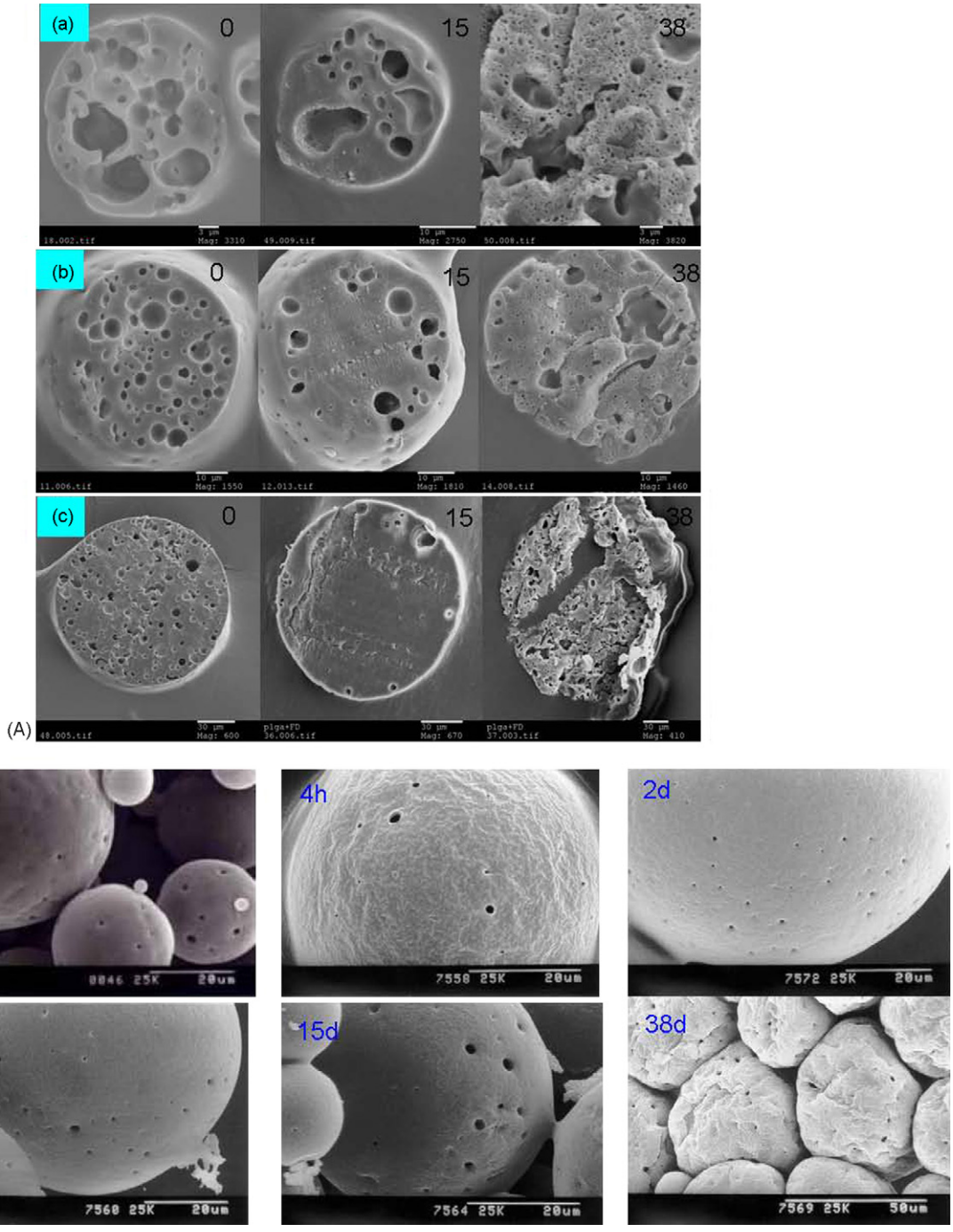

Fig. 9. (A) Internal morphology evolution of microspheres prepared with different concentrated PLGA at different incubation stage: (a) PLGA 8%, (b) PLGA 20% and (c) PLGA 32%. (B) Surface morphology evolution of microspheres prepared with 20% PLGA at different incubation stages.

was preserved at this stage. After 38 days incubation, microspheres lost their round shape and the surface was uneven, with some irregular pores extending to the inside of microspheres, also some micropores on the surface.

It was clear that the release of FD40 from PLGA microspheres is a combination of diffusion and bioerosion (Bittner et al., 1999). During the diffusion stage, the release of FD40 occurs by diffusion through aqueous channels in microspheres. These aqueous channels could be the external pores on the surface or were generated via the leaching of the drug at or near the surface, leading to initial burst release. After that the release profiles exhibit a plateau period and during this period microspheres swell and internal porosity decreases sharply as shown in Fig. 9A, with slight surface morphology change (Fig. 9B). During this period of time, the polymer matrix presents itself as a dense netlike structure and fewer drugs could diffuse out due to entanglement between the polymer and the drug. The second release stage involves the degradation of PLGA and is

associated with generation of micropores in the degrading polymer. The large amount of micropores after 38 days incubation (Fig. 9B) leads to a loose structure and allows easy water penetration into the matrix. However, no distinct difference in internal morphology between PLGA 8% and PLGA 40% microspheres were found after 38 days incubation. It is known that during the degradation of PLGA, acidic oligomers and monomers will be produced leading to an acidic microenvironment in the aqueous pores of the matrix. Since the degradation process of PLGA is catalyzed by protons and different internal morphology of microspheres will probably influence the distribution of protons in the microclimate, it was assumed that different internal morphology of microspheres might lead to different degradation speed and therefore different microstructure of eroding microspheres (Burke, 1996; Li and Schwendeman, 2005; Brunner et al., 1999). However, as shown in Fig. 9, no apparent difference in the microstructure of microspheres was found after 38 days incubation despite of their remarkable different internal morphology at the initial stage, implying that internal morphology has negligible influence on the degradation of PLGA.

4. Conclusions

Using fluorescein isothiocyanate labeled dextran as a model hydrophilic compound, influence of process parameters on the morphology and burst release of FD40 from PLGA microspheres was studied. Polymer type (RG503 and RG503H) had significant influence on the micro-morphology of microspheres. Microspheres prepared with RG503 had a high internal porosity but low external porosity. In contrast, microspheres prepared with RG503H revealed a low internal porosity but high external porosity. Increase in internal phase volume (W1) led to slightly increased burst release. In contrast, increase in the volume of continuous phase (W2) caused increased surface porosity but decreased internal porosity in the range studied. Increasing PVA concentration in the continuous phase changed particle size marginally but burst release decreased remarkably. Polymer concentration has considerable influence on the internal morphology of microspheres. At PLGA concentration 8%, microspheres with large porosity (52.7%) and high burst release (21.8%) was formed. In contrast, the porosity (1.6%) decreased significantly at PLGA concentration 32% and the burst was 1%. Homogenization speed during the primary emulsion preparation showed remarkable influence on internal morphology of microspheres as well. The internal pores were larger when lower stirring rate were employed and subsequently the burst is higher. Burst release decreased with increasing drug loading but increased with drug molecular weight. In microspheres prepared by double emulsion method, FITC-dextran tended to aggregate and drug was preferentially distributed on the surface of microspheres. In contrast, FD distribution was quite uniform in microspheres prepared by O/W method. The porosity of microspheres decreased upon incubation and no significant difference in internal morphology was found in the bioerosion stage. In summary, surface porosity and internal morphology play a significant role in the release of hydrophilic macromolecules from biodegradable microspheres in the initial release phase characterized by pore diffusion.

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