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Effect of inner pH on peptide acylation within PLGA microspheres

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ABSTRACT

Polymer degradation within the controlled-release depots comprising of lactide and glycolide (PLGA) forms an acidic microenvironment, in which severe acylation of the peptide by the polymer degradation products takes place. The aim of this study was to make out the role of the inner μpH on peptide acylation within the microspheres and how could it influence the reaction. The effects of pH on the acylation reaction within microspheres were composed of two aspects. Firstly, the inherent effect of pH on the acylation reaction itself was figured out: with the pH environment going up from acid to neutral, a model peptide (octreotide acetate) acylation became more and more serious. Then, the multivariate effect of pH on the dynamic microsphere delivery system especially the state of the acylation substrates (drug and oligomer) was investigated. When the inner pH was neutralized by $Ca(OH)_2$ to varying degrees, polymer degradation rate, drug release rate, polymer degradation mechanism and oligomer accumulation state within the microspheres all changed. These changes highly affected the mass transfer of the acylation substrates to the external release medium. Neutralization of the μpH prolonged the retention time of drug and oligomer within the microspheres. Water absorption and single microsphere swelling experiments all showed a higher retention amount of acylation substrates during the critical period for peptide acylation. Generally, when the inner μpH was neutralized, except that the neutral environment itself promoted acylation reaction, the effects of pH on the dynamic system were also highly responsible for the serious acylation within the microspheres.

1. Introduction

In recent years, there has been increasing interest in developing the peptide and protein drugs based on aliphatic polyesters, especially poly (D,L-lactide) (PLA) and poly(D,L-lactide-*co*-glycolide) (PLGA) to achieve targeted and/or sustained release profile due to their biodegradability and relative biocompatibility (Andhariya et al., 2017; Ruan et al., 2018; Chen et al., 2017; Zhang et al., 2013; García-Caballero et al., 2017; Ayoub et al., 2018). Several peptide products based on such delivery system are commercially available on the market, such as Lupron Depot® (leuprolide acetate), Sandostatin LAR® (octreotide acetate), and Zoladex® implant (goserelin acetate) (Gasmi et al., 2015; Shpigel et al., 2018). However, a very significant challenge in the development of the therapeutic peptides with PLGA depots is the modification of the actives as a result of acylation with lactic and/or glycolic units (Shirangi et al., 2016a; Kharel et al., 2018). Nucleophilic groups, particularly the amine groups present in the N-terminus and lysine residues (Na et al., 2003; Houchin et al., 2006; Shirangi et al., 2016b), would attack the electrophilic carbonyls of the ester groups in the polymer backbone (Houchin and Topp, 2008). This aminolysis reaction results in covalent addition of glycolyl or lactyl groups on the released peptide (Shirangi et al., 2015), which may pose great detrimental effects, such as loss of activity, a change of receptor specificity, immunogenicity and toxicity (Sophocleous et al., 2009).

PLGA erosion occurs via hydrolysis of the ester bonds in the polymer backbone, accompanied by accumulation of degradation products, i.e., monopolymer (lactic, glycolic acid) and their oligomers to form an acidic microclimate (μpH) (Ghassemi et al., 2012). Under this microclimate, peptide acylation takes place. The degraded oligomers have been reported to be the substrates of peptide acylation since the high solubility and small steric hindrance of which relative to the hydrophobic/long-chain polymers as received, were conducive to fully contact with the active peptide molecules (Kharel et al., 2018; Lucke et al., 2002; Wright, 2015). Similarly, when studying the acylation of human atrial natriuretic peptide (ANP) using the lactic acid solutions that mimicked the microclimate in degrading PLA, Lucke et al. found that

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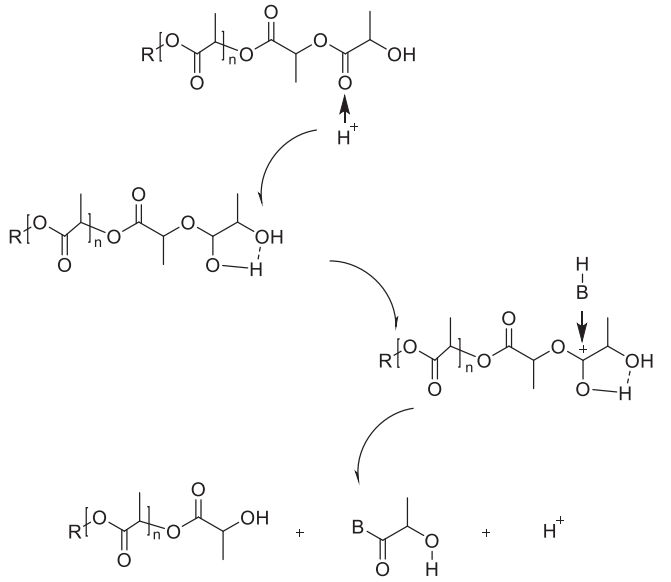

Fig. 1. Autocatalysis degradation catalyzed by acid microclimate which was generated by the accumulation of degrading oligomers (B = −NHR, −OR, −OH).

ANP underwent acylation even in diluted solutions containing only 0.05% (w/w) lactic acid oligomers (Lucke and Göpferich, 2003). Another potential source to be studied is the accompanied acidic microenvironment (μpH). The μpH in which the chemical reaction takes place is of great importance to the reaction itself. Beyond that, different from the fixed reaction kettle, acylation taking place within the microsphere depot is a more complex and multivariate system with continuous mass transfer between inner microsphere and external bulk fluid. During this process, the inner pH also highly affects the "dynamic system" especially the condition of the acylation substrates (the aforementioned degraded oligomer and the encapsulated drug). The most direct effect is on the degradation of the polymer. Autocatalysis degradation has been widely reported in early stage (Fig. 1), in which a hydrogen ion acts as the reaction partner to accelerate the degradation of polymers (Lucke et al., 2002). This leads to a typical "inside out" degradation of PLGA microspheres. By this way, the pH-dependent degradation mechanism is responsible for the production rate and mass distribution of the degraded oligomers. Moreover, the acylation reaction takes place within the microspheres depot. With the drug release and degradation proceeding, the real-time oligomers inside the microsphere are changing all the time. Their dissolution or accumulation, retention within the depot or releasing out is highly related to the inner pH as well.

In most cases, the pH environment mentioned on the peptide acylation refers to the pH of external release medium. However, what really works is the inner pH which is generated by accumulating of degradation products. For peptide acylation within the microspheres, except for the inherent pH effect on the acylation itself, it is also highly related to the pH dependent microsphere degradation and state of acylation substrates during the long-term drug release.

In the present study, based on neutralizing the inner acidic microenvironment produced by polymer degradation to varying degrees, we would like to make out the role of μpH on peptide acylation within the PLGA depot. As the inner μpH is neutralized, the degradation rate of the polymer may probably decrease since acid autocatalytic degradation mechanism is disturbed. Compared with the acidic microenvironment, the neutral condition promotes the dissolution and ionization of the degraded oligomers within the microspheres. Besides these, a series changes related to the peptide acylation will also take place in the dynamic microsphere delivery system. The aim of the present study was to figure out the inherent pH effect on the acylation itself and detailed the evidence of how it influenced the dynamic system especially the acylation substrates state. With the obtained knowledge, we hope to provide a solid basis to seek for solutions to effectively inhibit peptide acylation from the perspective of inner pH in the future study. Octreotide acetate, a most popular peptide widely used in the study of peptide acylation with PLGA side chains, was chosen as the model peptide since it had two reactive sites of N terminus (Phe1) and Lys5. An antacid, calcium hydroxide ($Ca(OH)_2$), was used to neutralize the acids produced by polymer degradation.

2. Materials and methods

2.1. Materials

Octreotide acetate (H_2N-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol; Mw = 1018.4) was purchased from Nanjing Senbeijia Biological Technology Inc. (Jiangsu, China). PLGA Resomer RG503H (Mw: 2.4–3.8 kDa, inherent viscosity (i.v.): 0.32–0.44 dl/g) was obtained from Evonik Specialty Chemicals (Shanghai, China). The fluorescent pH sensitive probe Lysosensor yellow/blue® dextran (Mw = 10 kDa) was purchased from ThermoFisher Scientific (Shanghai, China). Poly (vinyl alcohol) (PVA, Mw: 9000–10,000, 80% hydrolyzed), tetrahydrofuran (THF), methylene chloride and calcium hydroxide ($Ca(OH)_2$) were purchased from Sigma-Aldrich (Shanghai, China). All chemicals were of analytical grade as received commercially.

2.2. Preparation of microspheres

A traditional water-in-oil-in-water ($W_1/O/W_2$) emulsion solvent extraction/evaporation method was used for octreotide acetate microsphere formulation (Bailey et al., 2017). In brief, 20 mg octreotide acetate was dissolved in 100 μl of water. In the case of addition of the fluorescent pH sensitive probe Lysosensor yellow/blue® dextran (Mw = 10 kDa), 3 mg of which was added into the inner water phase together with the drug. 600 mg PLGA was dissolved in 2 ml of methylene chloride (with presence of 0–5% $Ca(OH)_2$ (w/w), relative to polymer materials). Then the drug and polymer solutions were mixed together and homogenized at the speed of 11,400 rpm for 2 min in the ice bath using a T10 digital basic ULTRA-TURRAX (IKA, Germany). The first water-in-oil emulsion (W_1/O) was stabilized by addition of 2 ml of 1% PVA solution and was vigorously vortexed for 30 s. The formed emulsion was immediately transferred into a 100 ml 0.5% PVA aqueous solution under stirring at 350 rpm to evaporate the methylene chloride. Then, the microspheres were washed repeatedly with deionized water and were sieved to obtain uniform particle size (45–90 μm, Test Sieve, Endecotts ASTM E11). Finally, the microspheres were lyophilized for 48 h (Freezing Dryer LGJ-10C, Four-ring Science Instrument Plant Beijing Inc.) to dry the microspheres and stored in a desiccator at 4 °C before further investigation.

For the preparation of blank microspheres with presence of 0–5% $Ca(OH)_2$ (w/w), all processes were same as the drug loaded microspheres expect that the drug was not added in the inner water phase.

2.3. Characterization of microspheres

2.3.1. Determination of the loading and encapsulation efficiency of drug and additive

The amount of octreotide acetate encapsulated in the microsphere was determined using a two-phase extraction method (Sophocleous et al., 2009). Approximately 20 mg freeze-dried microspheres were dissolved in 2 ml methylene chloride and the drug was extracted with 0.05 M acetate buffer (pH 4.0) for three times. The concentration of octreotide acetate was determined using high performance liquid chromatography (HPLC). HPLC conditions (Murty et al., 2003): octreotide acetate was analyzed by reversed phase (RP)-HPLC using a Kromasil C-18 column (4.6 mm × 250 mm) and separation of the

peptide was accomplished using a gradient elution method: mobile phase A: 0.1% (v/v) TFA in acetonitrile and B: 0.1% (v/v) TFA in water. The linear gradient of mobile phase A changed from 25% to 35% over 15 min with a flow rate of 1.0 ml/min. UV detection was set at 215 nm.

For the content of $Ca(OH)_2$, after dissolving the microspheres with methylene dichloride, 0.1 M hydrochloric acid was used to exact the calcium for three times. The concentration of calcium was determined by a Calcium Colorimetric Assay Kit. In this assay, the calcium ion concentration was determined by the chromogenic complex formed between calcium ions and *o*-cresolphthalein, which was measured at 575 nm and was proportional to the concentration of calcium ions present.

Drug loading and encapsulation efficiency was determined as:

$$\text{Drug loading} = (\text{weight of drug loaded/weight of microspheres}) \times 100\%$$

$$\text{Encapsulation efficiency} = (\text{Experimental drug loading/theoretical drug}) \times 100\%$$

For the basic additive, the algorithm of loading and encapsulation efficiency was the same as that of drug. Each sample was analyzed in triplicate.

2.3.2. Determination of the structural stability and bioactivity of octreotide acetate loaded microspheres with presence of 0–5% $Ca(OH)_2$

The bioactivity of the encapsulated peptide was determined by circular dichroism (CD) spectroscopy (Applied Photophysics Ltd., Leatherhead, U.K.). The peptide extracted from the microspheres with presence of 0–5% $Ca(OH)_2$ was transferred into a quartz cell with an optical path of 0.1 cm and detected in the wavelength range from 195 to 250 nm under a nitrogen atmosphere. Peptide concentrations were precisely determined by RP-HPLC and diluted to 80 μg/ml in PBS (pH 7.4).

2.4. Evaluation of the neutralization effect of $Ca(OH)_2$

2.4.1. Particle size of $Ca(OH)_2$

The particle size of finely ground $Ca(OH)_2$ was measured using a Zeta Sizer (Zeta Sizer Nano-ZS90, Malvern Instruments Ltd., UK). Samples were suspended in purified water and tipsonicated for 1 min before analysis.

2.4.2. The pH changes in external release medium

The pH changes in the release medium of the formulations with presence of several amount of $Ca(OH)_2$ were measured at predetermined time points with a pH meter (PHSJ-5, Shanghai). During this measurement, the release medium was not refreshed. Each sample was analyzed in triplicate.

2.4.3. Fourier Transform Infrared Spectroscopy

Samples were milled with KBr to form the uniform power, which were then compressed into a thin pellet for Fourier Transform Infrared Spectroscopy (FT-IR) analysis. The measurement was recorded in the 4000–400 cm^{-1} region at a resolution of 4 cm^{-1} and 50 scans per sample (Spectrum 100, Perkin Elmer, Inc., USA).

2.5. Inherent effect of pH on acylation reaction

Approximately 20 mg PLGA (as received) was added into 1 ml of octreotide acetate solution (300 μg/ml) in PBST release medium (7.74 mM Na_2HPO_4, 2.26 mM NaH_2PO_4, 137 mM NaCl and 3 mM KCl, 0.02 wt% Tween 80) at 37 °C. The initial pH of the solutions was adjusted to 7.4, 6, 4 and 2 respectively with hydrochloric acid. With the polymer degradation proceeding, the pH of the tested solutions will go on dropping. For each 0.5 drop in pH, then it will be adjusted with sodium hydroxide to maintain the initial pH. Samples were collected at predetermined time points. The supernatants after centrifuge were analyzed by HPLC. Each sample was analyzed in triplicate.

2.6. In vitro release and acylation test

In vitro release and acylation kinetics of peptides encapsulated in PLGA microspheres with incorporation of several amount of $Ca(OH)_2$ were examined in PBST release medium (pH 7.4). Approximately 20 mg of microspheres were placed into a 5 ml polyethylene tube containing 1 ml of PBST. The release experiment was carried out using an orbital shaker (Shaker THZ-103B, Shanghai Bluepard Instrument) where the samples were placed in it at 300 rpm and incubated at 37 °C. Release medium was replaced weekly. At preselected time points, the microspheres were collected, washed and lyophilized. After two-phase extraction, the native and acylated peptide contents were analyzed by HPLC. Each sample was analyzed in triplicate.

2.7. Investigating changes to the formulations with the neutralization of inner pH to several degrees

2.7.1. Gel permeation chromatography

The decrease of polymer molecular weight (Mw) during the drug release was determined by gel permeation chromatography (1525 separation modules, 2414 refractive index detector, Waters, USA). The mobile phase was tetrahydrofuran (THF) with a flow rate of 1 ml/min. Samples were dissolved in 2 ml THF and filtered through 0.45 μm filters for GPC measurement. Molecular weights were calculated using the Breeze GPC software based on the calibration of polystyrene standards. Each sample was analyzed in triplicate.

2.7.2. Scanning electron microscopy

The surface morphology of microspheres after freeze-drying was characterized using scanning electron microscopy (SEM). Microspheres samples were mounted on carbon taped aluminum stubs and sputter-coated with gold for analysis by SEM (Nova Nano SEM NPE 218, FEI, USA).

2.7.3. Thermal analysis

The aggregation and accumulation states of oligomers within the microspheres were characterized by differential scanning calorimetry (DSC 25, TA instruments). Samples were purged with pure and dry nitrogen and subjected to a first heating cycle, ramp from 0 °C to 80 °C at 10 °C/min, isothermal for 2 min, and cooled to 0 °C at a rate of 100 °C/min. Then to a second cycle, ramp from 0 °C to 200 °C at 10 °C/min. The melting endotherms were determined from the second heating ramp.

2.8. Mass transport between microspheres and release medium

2.8.1. The swelling of individual microsphere

The swelling of individual microsphere after water absorption was carried out using a 96-well plate monitored by Olympus microscopy (IX51, Japan). Small amount of microspheres were put into each well with addition of 250 μl PBST (pH 7.4). The plates were kept at 37 °C in the orbital shaker (Shaker THZ-103B, Shanghai Bluepard Instrument) and agitated at 50 rpm. In order to minimize water evaporation, the plates were closed and covered with parafilm. Fresh PBST was added to each well at least once a week to ensure the 250 μl liquid during the entire observation period. At predetermined time points, pictures were captured using an Olympus camera (DP71, Japan).

2.8.2. Measurement of water uptake in PLGA microspheres

After incubation in PBST at 37 °C for several days, at predetermined time points, the microspheres were collected and blotted with tissue paper, and the wet weight (W1) was recorded. Then the microspheres were freeze-dried to a constant weight and the dry weight (W2) was recorded. The water uptake in PLGA microspheres were calculated by:

Water uptake (%) = (W1 − W2)/W2 × 100%

2.8.3. The pH map inside the microspheres

The pH map inside the microspheres was delineated by a classic pH sensitive probe, Lysosensor yellow/blue® dextran (Mw = 10 kDa), using an Olympus FV 3000 confocal laser scanning microscope (Olympus Corporation, Shinjuku Monolith, Tokyo, Japan). The probe co-encapsulated in the microspheres with the drug was excited at 364 nm and emission at two wavelengths (450 and 520 nm) as described in previous study (Liu and Schwendeman, 2012). The images were scanned at 8.0 μs/pixel.

2.9. Statistical analysis

Results were analyzed using student's *t*-test and GraphPad Prism 5 software and presented as means of three replicates ± standard deviation (SD). Statistical comparisons were assessed using an unpaired *t*-test and one-way ANOVA. Differences with a value of $P < 0.05$ were considered to be statistically significant. SPSS 11.0 software was used for the analysis.

3. Results and discussion

3.1. Preparation and characterization of microspheres

To investigate the role of inner pH microclimate on peptide acylation and how could it influence the reaction, small amount of $Ca(OH)_2$ (0–5% w/w, relative to polymer materials) was selected as the antacid to neutralize the acid μpH generated by polymer degradation to varying degrees. This poorly water-soluble inorganic base ($Ca(OH)_2$) has been reported to successfully inhibit the insoluble noncovalent aggregation and instability of the acid-labile protein (BSA) within the PLGA microspheres by neutralizing such severely acidic microenvironment in previous studies (Zhu and Schwendeman, 2000; Zhu et al., 2000; Kang et al., 2008; Jiang and Schwendeman, 2008).

Octreotide acetate and $Ca(OH)_2$ were co-encapsulated in the PLGA matrix using a modified emulsion solvent extraction/evaporation method with the drug dissolved in the inner water phase and the additives suspended in the oil phase. Since the peptide was highly soluble in the water, the inner water phase containing drug was susceptible to migrate to the outside under severe mechanical force conditions (i.e., vigorous vortex and homogenizing) during the process of emulsion. Thus, the calculated encapsulation efficiency of drug was relatively low (around 40%) as shown in Table 1. The formed microspheres with or without presence of $Ca(OH)_2$ showed a similar drug entrapment efficiency (43.81%, 42.85% and 48.64% respectively), which meant that corporation of the poorly water-soluble base had ignorable impact on drug encapsulation. As expected, the encapsulation efficiency of the $Ca(OH)_2$ was high (over 90%) due to its poor solubility both in water and oil phase, in which case, no osmotic gradient was formed for escape of such additive.

The structural stability and bioactivity of the peptide extracted from the microspheres with presence of 0–5% $Ca(OH)_2$ was determined using a classic CD spectroscopy method. The CD spectrum of original octreotide acetate (Fig. S1) and the drug extracted from microspheres with addition of 0–5% $Ca(OH)_2$ were nearly superimposable in the range of 195 to 250 nm. This result suggested that the secondary structure of octreotide acetate encapsulated in the PLGA microspheres was well maintained.

3.2. Evaluation of the neutralization effect of $Ca(OH)_2$ in PLGA microspheres

Since the basic additive was poorly water/oil-soluble, which can only be suspended in the emulsion before polymer solidified. To facilitate diffusion of the $Ca(OH)_2$ to the acidic pores inside the microspheres uniformly, it was finely ground and sieved beforehand. The achieved mean size of $Ca(OH)_2$ particle was 3.9 μm (Fig. S2).

The changes of pH in external release medium indirectly reflected the neutralization effect of $Ca(OH)_2$ inside the microsphere as the only reason for pH drop was originated from the release of acidic oligomers to the outer phase. When the degrading oligomers were partly or totally neutralized, released out or entrapped within the microspheres before got dissolved, it would be going to show up in the pH changes of external medium.

Without presence of base (Fig. 2, 0%B-MSs), the pH value of external release medium kept dropping to 2.26 during the whole incubation time. In presence of 3% $Ca(OH)_2$ (3%B-MSs), the dropping rate was slowed down and came to an acidulous environment (4.4). When the amount of the additive increased to 5% (5%B-MSs), the pH remained unchanged throughout the incubation time (7.2). This result indicated that the inner acidic μpH can be totally neutralized during the incubation period with presence of 5% $Ca(OH)_2$.

To confirm the neutralization effect of $Ca(OH)_2$, a Fourier Transform Infrared Spectroscopy experiment was carried out. In hybrid orbital theory, the carboxylic acid, produced by polymer degradation, was planar structure with asymmetric carbon-oxygen single bond (−C−O) and double bond (−C═O) as shown in Fig. S3a. When it was neutralized by $Ca(OH)_2$, the carboxyl group (−COOH) would change into carboxylate ($-COO^-/Ca^{2+}$). It would be easier for O^- to be conjugated with carbonyl group. In the structure of O═C−O^- ($^-$O−C═O), three atoms each provided a P orbital and formed an extended π bond, which was three-center molecular orbital with four electrons (Fig. S3b). For carboxylate, the stretching vibration of carbon-oxygen bonds was same. Thus, the infrared characteristic absorption peaks of carboxylic acid and carboxylate were different (1758.24 cm^{-1} and 1624.21 cm^{-1} respectively, Fig. 3).

At the end of incubation, relatively complete degradation, the microspheres were collected and measured to evaluate the neutralizing effect. The characteristic absorption peaks of carboxylic acid (1758.24 cm^{-1}) and carboxylate (1624.21 cm^{-1}) were both observed in 0%B-MSs (Fig. 3). Small carboxylate peak appearing in this formulation resulted from the reversible ionization of carboxylic acid and small amount of which may be combined with salt (Na^+, K^+/Ca^{2+}) in the releasing medium. For 3%B-MSs and 5%B-MSs, the peak of carboxylate grew up gradually. In presence of 3% $Ca(OH)_2$, the carboxylic acid and carboxylate peaks were both obvious, which indicated part

Table 1
Loading and encapsulation efficiency of octreotide-loaded microspheres with presence of several amount of $Ca(OH)_2$[a].

Formulation #	$Ca(OH)_2$ content	L_T^D (w/w%)	L_A^D (w/w%)	EE^D (%)	L_T^B (w/w%)	L_A^B (w/w%)	EE^B (%)
1	0%	3.22	1.41 ± 0.07	43.81 ± 2.24	–	–	–
2	3%	3.13	1.34 ± 0.10	42.85 ± 3.20	2.82	2.65 ± 0.07	93.81 ± 2.51
3	5%	3.08	1.50 ± 0.17	48.64 ± 5.50	4.61	4.24 ± 0.13	91.87 ± 2.82

t-Test for drug loading and encapsulation efficiency: compare with non-additive formulation in each group; for additive loading and encapsulation efficiency: compare with 3%-additive formulation, P* < 0.05, P** < 0.01. There was no significant difference among each group.

[a] Definitions: L_T^D and L_T^B were the theoretical loading of drug and inorganic base. L_A^D and L_A^B were the actual loading of drug and base. EE^D and EE^B were the drug and base encapsulation efficiency.

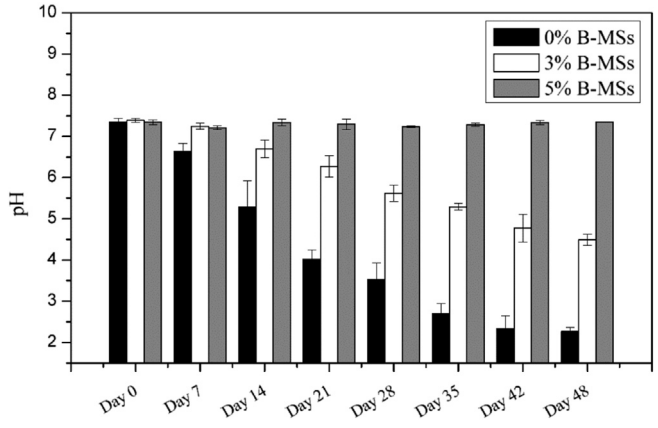

Fig. 2. Changes of pH value of formulations with presence of different amount of $Ca(OH)_2$.

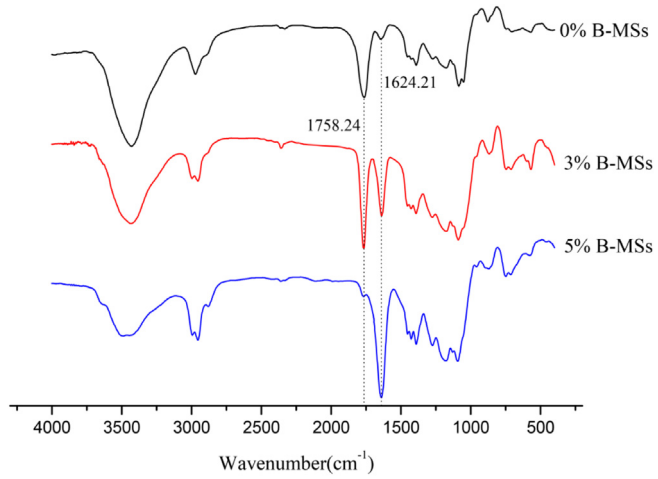

Fig. 3. Fourier Transform Infrared Spectroscopy of drug loaded microspheres with 0–5% $Ca(OH)_2$ at the end of incubation.

neutralization of μpH. When the amount of $Ca(OH)_2$ reached to 5%, the carboxylic acid peak was negligible and replaced with a sharp characteristic peak of carboxylate. This was consistent with the aforementioned result of external pH changes and indicated that the μpH produced by degrading products can be totally neutralized with presence of 5% $Ca(OH)_2$ during the whole release period of microspheres. The infrared spectra of the unloaded microspheres were also determined to eliminate the encapsulated drug interference to the infrared vibration of carboxylic acid and carboxylate in PLGA carriers. A similar trend was obtained in the blank microspheres (Fig. S4, 0–5%B-BMSs).

3.3. Determination the inherent effect of pH on acylation reaction

The effect of μpH on peptide acylation within the microsphere delivery system was multivariate and complex since it was a "dynamic system". Upon the μpH was partly or totally neutralized, a serious of associated changes would correspondingly take place such as drug release, polymer degradation, oligomers accumulation and mass transfer between the inner microspheres and outside bulk fluid. Before characterizing the pH effect on this "dynamic system" that was highly related to peptide acylation, the inherent effect of pH on the reaction itself should be investigated first. Here we called it "static system".

The acylated peptide content with time (area percentage relative to the sum of parent and acylated peptide peaks) was calculated as shown in Fig. 4. In the pH 2.0 environment, there was almost no acylation reaction taking place between peptide and polymer (Fig. 4). As the pH

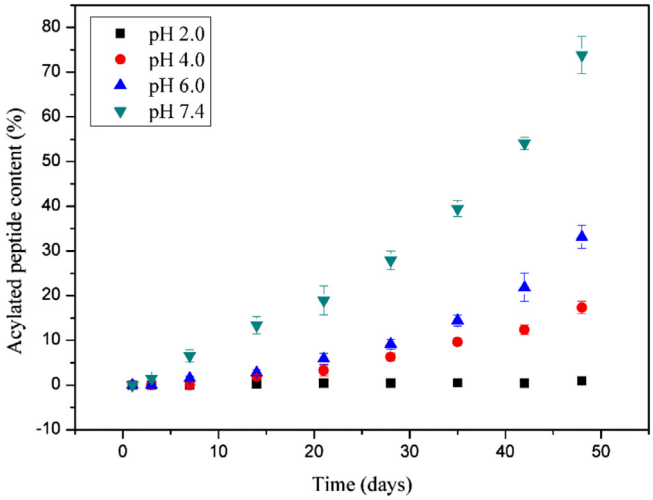

Fig. 4. The inherent effect of pH on acylation reaction itself under "static system".

increased to neutral gradually, the acylation progressively increased. Under pH 7.4, the acylation peptide content was up to 73.83% within 48 days' incubation. Thus, the inherent effect of pH on the acylation reaction itself can be seen: with the pH environment going up from acid to neutral, it would be more susceptible for peptide to react with the polymer.

3.4. The inner pH effect on the peptide release and acylation within the microsphere system

Within the microsphere delivery system, peptide acylation and drug release were concomitant. Assuming that the molar absorptivity coefficient under Beer's Law was the same for the parent peptide and the acylated byproduct, the cumulative release was calculated based on the sum of each peak area (Zhang et al., 2009) (Fig. S5). Drug release of the three formulations all followed a typically triphasic release profile (Zolnik et al., 2006) (Fig. 5, initial burst release, followed by a lag phase and a secondary apparent-zero order phase). Incorporation of additives did not affect initial burst from PLGA microspheres (around 15%) because the insoluble $Ca(OH)_2$ would not let an increase in the water-uptake capacity to the formulation. Whereas, the followed lag phase and apparent-zero order phase were extended. With addition of 3% and

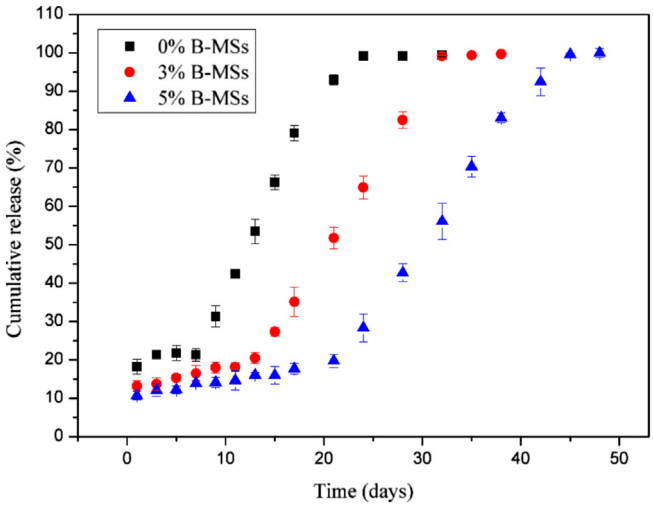

Fig. 5. Cumulative release of octreotide acetate from PLGA microspheres with presence of several amount of $Ca(OH)_2$ incubated in PBST.

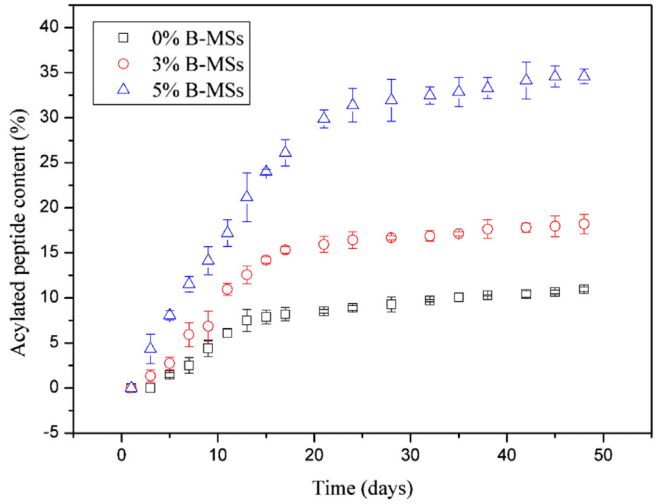

Fig. 6. Peptide acylation within the microspheres with presence of several amount of $Ca(OH)_2$.

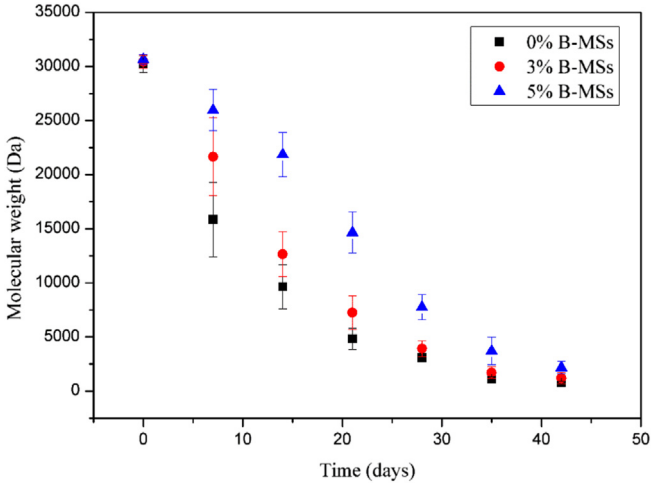

Fig. 7. The Mw change of formulations with presence of different amount of $Ca(OH)_2$ incubated in PBST.

5% $Ca(OH)_2$, the lag phase increased from 8 days to 14 and 21 days and reached a plateau in 23, 32 and 45 days separately.

For peptide acylation, the acylated byproducts increased steadily at the beginning, followed by an obvious transition appearing at day 13, 17 and 22 respectively for all of the three formulations (Fig. 6). Then the increment of acylation byproducts was negligible. At the end of incubation, with presence of 3% and 5% $Ca(OH)_2$, the acylated peptide was risen from 10.97% to 18.20% and 34.61% respectively.

From the acylation result, it can be concluded that: a) peptide acylation within the microspheres was also susceptible to take place in a neutral environment; b) different from the static system, the fast growth period for the acylation byproducts within the dynamic microsphere system was the premetaphase what we called it here "critical period". Moreover, the significant differences of acylation among the three tested formulations were also formed during this period. To make out the role of the μpH on the dynamic system and detail the evidences of how could it influence the acylation reaction, a series of specific characterization studies were then carried out.

3.5. Characterization the direct influence of the inner μpH change on the microsphere system

3.5.1. Polymer degradation

Generation of acidic oligomers within the microspheres was responsible for the autocatalysis of polymer, followed by the accelerated polymer degradation and oligomers formulation (Ford Versypt et al., 2013; Zolnik and Burgess, 2008). When the acidic μpH was partly or totally neutralized, the liberation rate of the oligomers known as the sources/substrates of peptide acylation would change accordingly. The Mw change was monitored by GPC as shown in Fig. 7. In formulation 0%B-MSs or 3%B-MSs, the reduction of Mw was in accordance with the classic first order kinetics with the apparent rate constants of 0.089 and 0.081 day^{-1} respectively. In presence of 3% $Ca(OH)_2$ slightly slowed down the degradation of polymer. Whereas, for 5%B-MSs, the degradation rate was further dropped off and followed a linear dynamics.

In addition, heterogeneous degradation was found in 0%B-MSs at day 7 (Fig. 8, a). During this time, it exhibited a bimodal degradation profile with a wide molecular weight distribution (the inverted peak at the end of the curve was the solvent peak, Fig. S6). This was corresponding with the previously reported "inside-out" degradation mechanism (Shen and Burgess, 2012; Shen et al., 2016; Zolnik and Burgess, 2007). While in presence of 3% and 5% $Ca(OH)_2$, the polymer degradation exhibited a more homogeneous pattern (unimodal molecular weight distribution) throughout the incubation time (Fig. 8, b and c, the GPC results were related to the retention time of samples rather than the peak areas).

3.5.2. Morphological changes of microspheres

Microspheres with or without addition of $Ca(OH)_2$ all showed a spherical shape and smooth surface initially (Fig. 9). For 0%B-MSs, as the degradation proceeded, surface erosion was observed at day 7. By day 21, the erosion was further aggravated and microspheres were agminated. Till day 48, a large block was found.

Similar degradation behavior was observed both in 3%B-MSs and 5%B-MSs over a later time. The spherical structure began to erode at day 21 for 3%B-MSs whereas most of microspheres were still individual and degradation was not evident at this time point for 5%B-MSs. Until day 48, all of the three formulations became a large block. The SEM results further illustrated that polymer degradation was slowed down at a relatively neutral environment.

3.5.3. Thermal analysis of microspheres

For 0%B-MSs, small endothermic peaks appearing at around 112 °C (111.97 and 112.3 °C) were separately observed at day 3 and day 7 (Fig. 10, a). But no peak was observed in 3%B-MSs or 5%B-MSs during the whole incubation time. The endothermic peak occurred at day 3 or day 7 in 0%B-MSs was owing to the crystallization of the acidic oligomers due to their low solubility at the acidic μpH (the pKa of carboxyl group for both glycolic and lactic acid of PLGA was 3.83), which was similarly reported in previous study (Zolnik and Burgess, 2007). When the acidic μpH was neutralized, facilitating the dissolution of the degradation products, no crystallization peak was observed in 3%B-MSs and 5%B-MSs. The thermograms of the unloaded microspheres were also determined to eliminate the encapsulated drug interference to the thermal results. A similar trend was obtained in the blank microspheres (Fig. S7, 0–5%B-BMSs).

3.6. Characterization the indirect influence of the inner μpH on the mass transfer process in the "critical period"

The real-time retention amount of the drugs and the degraded oligomers (both of which were acylation substrates) within the microspheres determined the actual acylation reaction that was to occur. Thus the mass transfer process between microspheres and the external bulk fluid needed to figure out. Since the most critical period for the acylation byproducts growth within the PLGA microsphere was the premetaphase, the mass process during such period was investigated.

Dissolution of the peptides and the degraded oligomers would induce an increase in the osmotic pressure inside the microspheres. In this

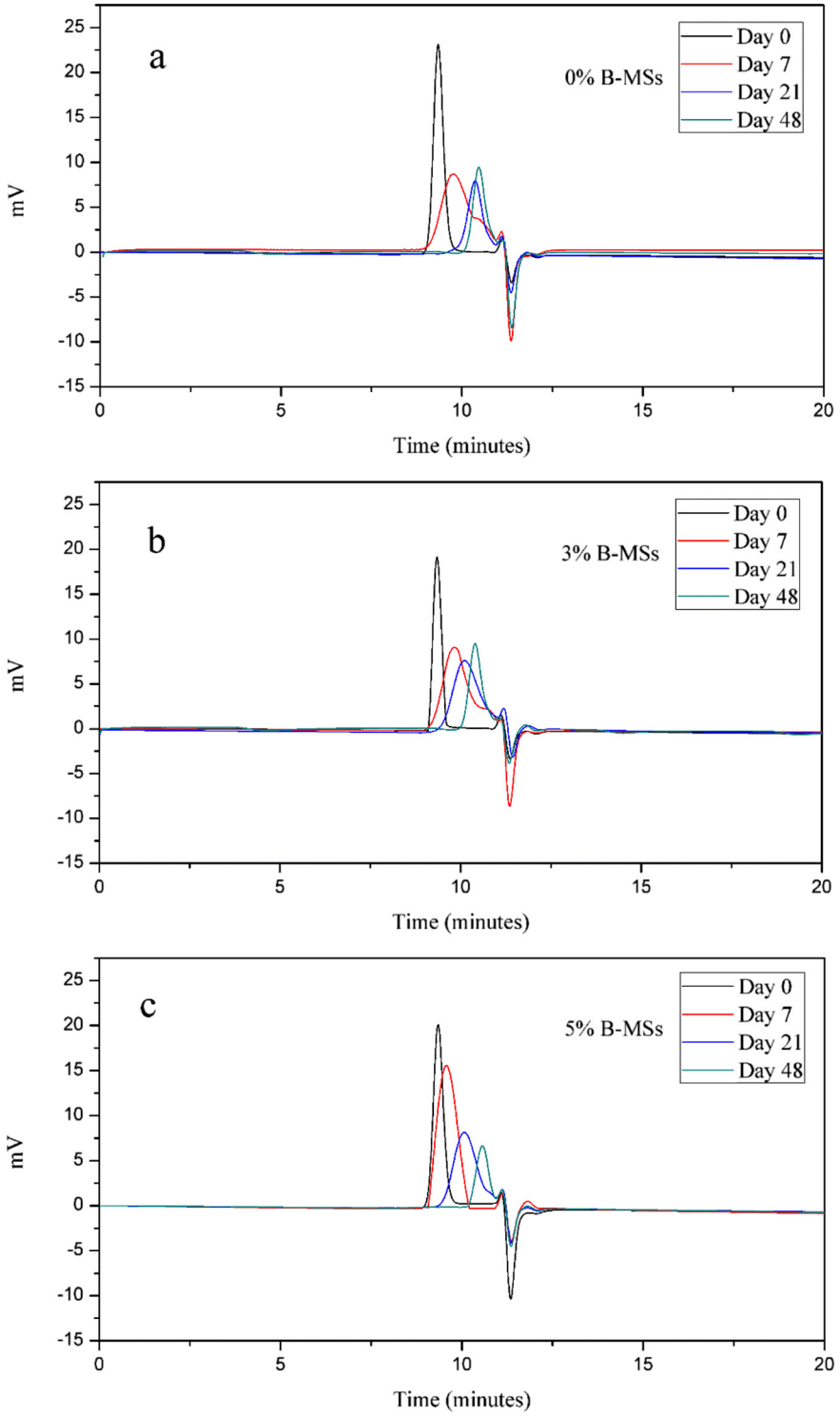

Fig. 8. Molecular weight distribution of the degrading microspheres with presence of several amount of $Ca(OH)_2$ (a, b and c) determined by Gel permeation chromatography.

case, the drug and degraded oligomers tended to be released out while water to be absorbed in according to the osmotic gradient. However, with complex and tortuous structure of the micro-channel, the polymer matrix acting as mass diffusion barrier was reported to be semi-permeable (Qi et al., 2013). Before the polymer degraded to a certain degree, it would be retained within the microspheres with continuous absorption of water from the release medium to balance the osmotic differential.

Neutralization of the μpH of the microspheres caused associated changes which have been characterized above such as polymer degradation rate (the drug release and oligomer production rates), the degradation mechanism (homogeneous/heterogeneous, the molecular weight distribution of oligomers) and the accumulation state of oligomers within the microspheres (dissolution or crystallization). These changes would greatly influenced the mass transfer process of the acylation substrates (drug and oligomers) within the microspheres to the outside and determined the real-time amount of substrates inside the “dynamic system”.

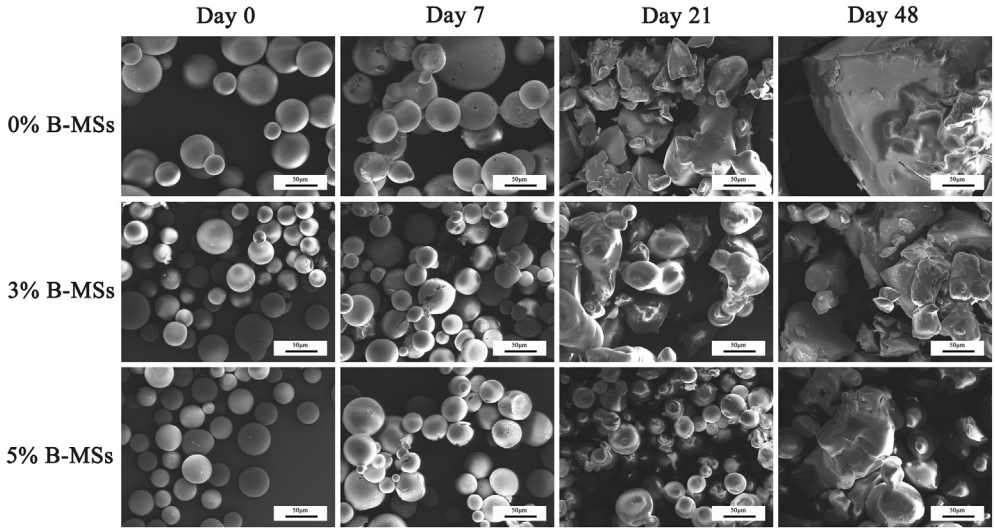

Fig. 9. Scanning electron micrographs of octreotide acetate-loaded microspheres with addition of 0–5% $Ca(OH)_2$ at predetermined time points.

3.6.1. Single microsphere swelling

The single microsphere swelling experiment was one of the most classical methods developed in recent years to investigate the mass transfer process especially the water uptake from the incubation medium to the microspheres (Gasmi et al., 2015). The real-time retention amount of the water-soluble drugs and oligomers within the formulation would be reflected in its ability to absorb water from the external release medium. This method allowed monitoring the dynamic changes in the size of the same microspheres after they absorbed water, offering highly valuable insight into the underlying mass transfer process. Samples were monitored using a normal optical microscope (Olympus, IX 51).

For all the three formulations, the spatial location of the microspheres in the 96 wells can be followed individually without agglomeration over 14 days' incubation (Fig. 11). In 0%B-MSs, during the incubation time, no significant swelling was observed (red reference line helped to analyze the increment in particle size of microsphere, Fig. 11, a). But in 3%B-MSs (Fig. 11, b), the individual microspheres all showed great volume expansion. In 5%B-MSs (Fig. 11, c), the swelling of the single microsphere was even more evident.

These results indicated that the real-time retention amount of the drugs and oligomers in the 3%B-MSs and 5%B-MSs were much more than the 0%B-MSs. The almost invisible swelling of 0%B-MSs was partly owing to the release out of the drugs after a short lag phase (8 days, Fig. 5).

3.6.2. Water uptake

During the critical period, water absorption of the three formulations at different time points was quantitatively determined. For all of the three formulations, the uptake of water increased gradually and reached a balance at day 7, 10 and 14 separately (Fig. 12). The equilibrium water uptake amounts of 0%B-MSs, 3%B-MSs and 5%B-MSs were 53%, 105% and 166% separately. This further confirmed that the real-time retention amount of the drugs and oligomers in 0%B-MSs, 3% B-MSs and 5%B-MSs gradually increased.

During the progressive neutralization of the much, the drug transfer (drug release, Fig. 5) slowed down and the lag phase was extended. The prolonged retention time of the drugs within the microspheres would be more or less responsible for the increased water absorption and swelling of microspheres. However, in addition to the effects induced by drug release, the different production and retention of the oligomers within the three tested formulations may also play an important role in their differences of water uptake and microsphere swelling. Evidences were as followed: For 3%B-MSs and 5%B-MSs, within 14 days incubation, drug release was negligible (lag phase) after an initial burst (Fig. 5, about 15% for both formulations). This meant that the remaining drugs within the microspheres for 3%B-MSs and 5%B-MSs were similar (about 85%) within 14 days. The water uptake difference was highly attributed to the different retention amount of water-soluble oligomers produced by continuous degradation of long-chain polymer. The significant difference of the water absorption between the two formulations (97.12% and 162.60%) reminded us that the oligomers production, accumulation, retention or transfer to external bulk fluid may quite different when the inner pH got changed.

3.6.3. The oligomers transfer-pH map

To directly visualize the transportation and accumulation of the oligomers and the μpH details inside the three formulations, a classic pH sensitive probe, Lysosensor yellow/blue® dextran was chosen to image this process. Lysosensor was sensitive to acidic pH changes as a result of its pyridyl group. The protonation of this group under low pH let a shift of the emission wavelength to a short case. Moreover, the conjunction of such dye with high molecular weight dextran (10 kDa) facilitated a long-term tracking of the pH changes and mass transfer process of oligomers (Liu and Schwendeman, 2012; Ding and Schwendeman, 2008).

In 0%B-MSs, the microenvironment was always acidic during the whole incubation time (Figs. 13 and S8, 0%B-MSs). At day 3, it was highly acidic with strong fluorescence intensity inside the microspheres. Obvious “black hole” in the center of the microsphere was observed at day 7, which was consistent with the previous reported “inside-out” degradation theory that the polymer degradation took place first in the center of microsphere and followed by releasing out of the first and fully degraded oligomers (Shen and Burgess, 2012). It was ascribed to the autocatalysis and heterogeneous degradation of this formulation (Figs. 1, 7 and 8) that a great amount of the first and fully degraded oligomers transferred out and left the “black holes” during the acylation critical period. As the degradation proceeded, the fluorescence intensity decreased gradually with more and more “black hole” observed at day 14 and 21. This indicated the rapid release of the oligomers to the external medium.

For 3%B-MSs, with the μpH partly neutralized, the transfer of the degraded oligomers to the outside was delayed and the retention time of which was prolonged. During the critical period, the high fluorescence intensity and rare “black hole” indicated the high retention amount of the oligomers. In 5%B-MSs, the retention of the oligomers further improved, which was highly related to the slow and homogeneous degradation behavior when the inner μpH was totally

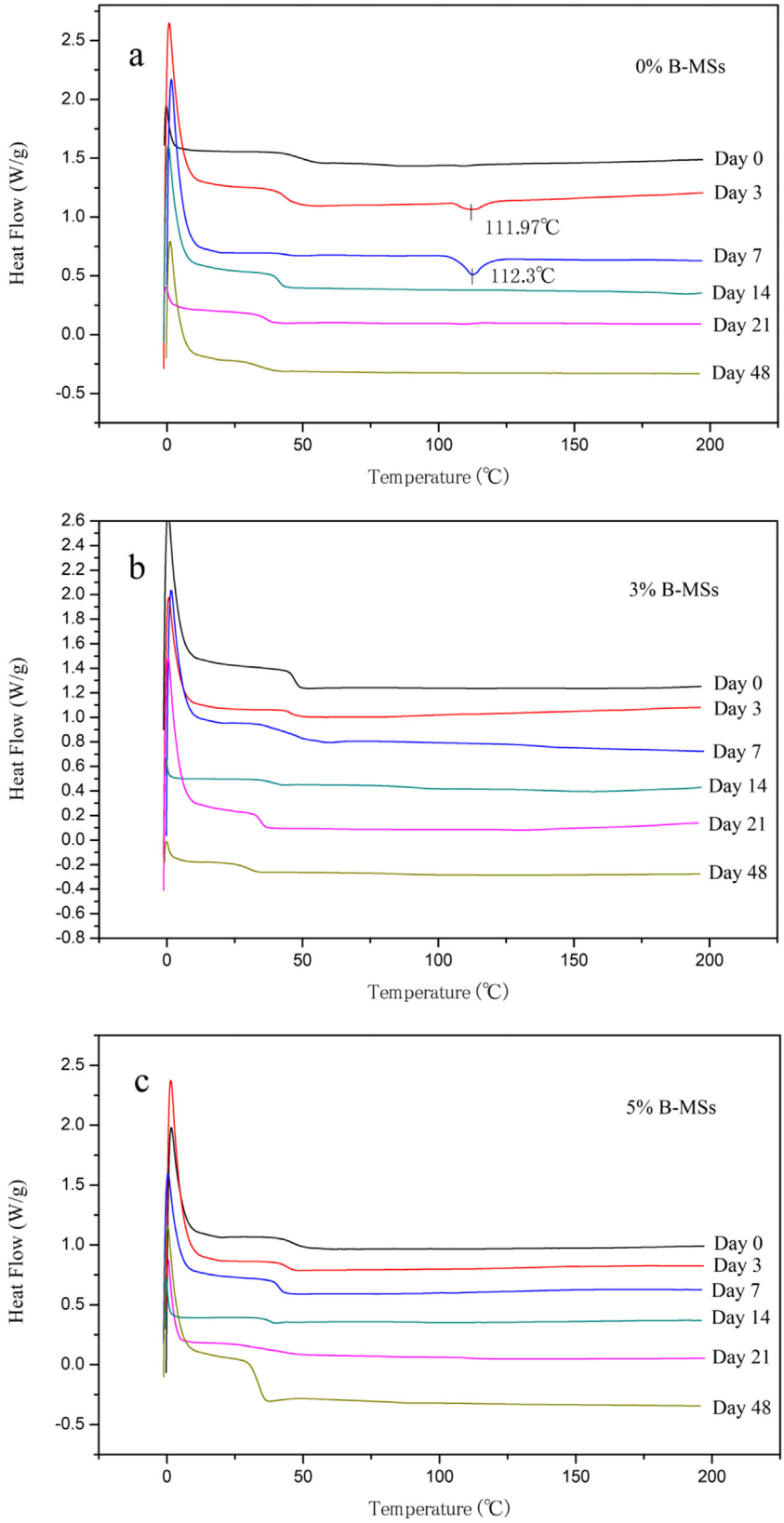

Fig. 10. Thermal analysis of the accumulation state of the oligomers inside the drug loaded microspheres with addition of 0–5% $Ca(OH)_2$ (a, b and c) at pre-determined time points.

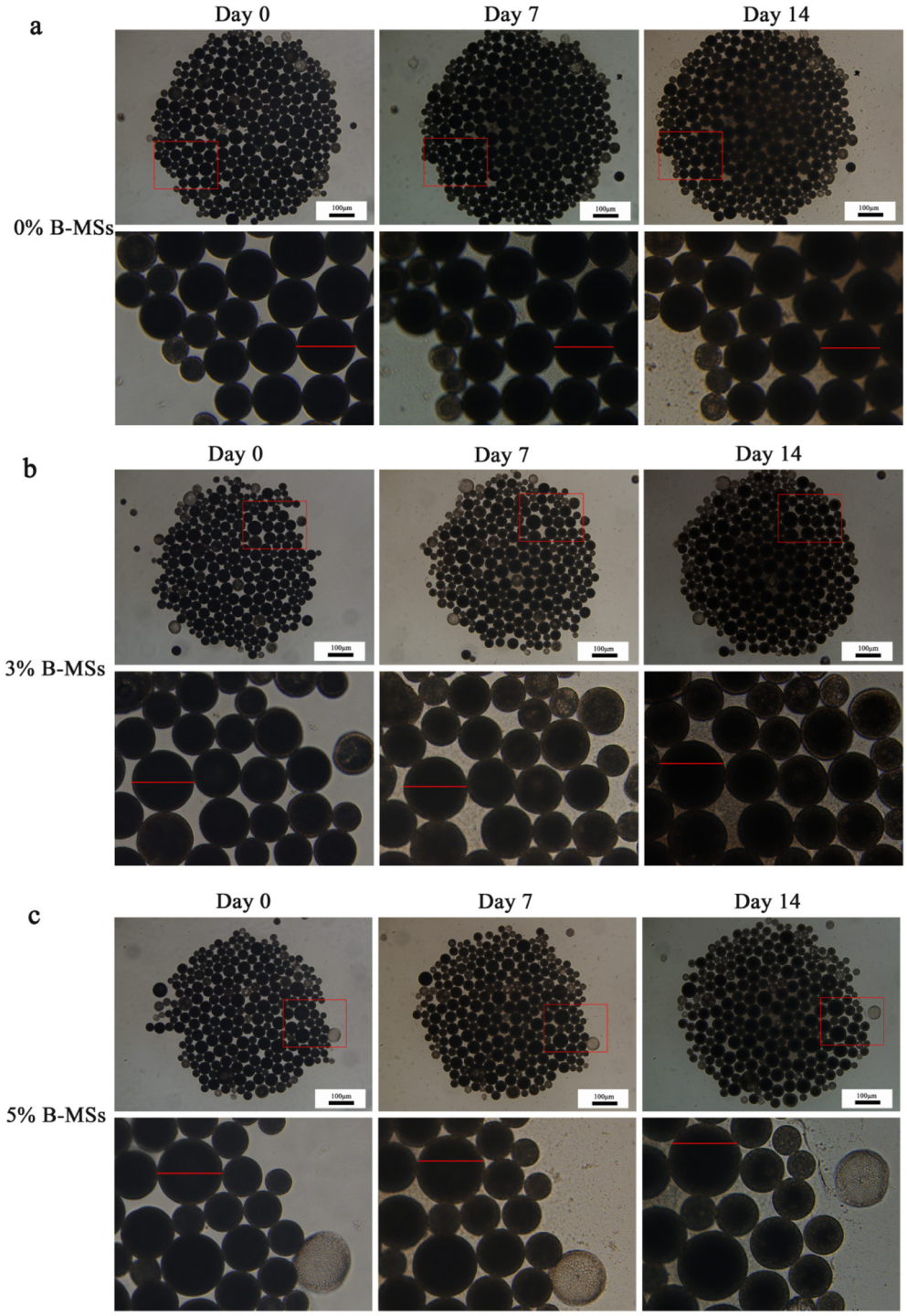

Fig. 11. Microscopic observation of single microsphere swelling for formulations with presence of 0–5% $Ca(OH)_2$ (a, b and c) during the critical time for peptide acylation.

neutralized.

3.7. Comprehensive analysis of the pH effect on the dynamic microsphere delivery system

The critical period for acylation byproducts growing within the microsphere system was premetaphase. After then, the byproducts increased at a significantly lower rate. Such acylation kinetics was the common characteristic for all of the three tested formulations. In the dynamic microsphere delivery system, this kinetics was reasonable since the secondary apparent-zero order phase was onset after the critical period that peptides released at a rapid speed (Fig. 5). Considerable degraded oligomers also began to transfer outside at a significant rate (Figs. 13 and S8, the oligomers within the microspheres were sharply decreased after the critical period).

When the acidic environment inside the microsphere was neutralized, the degradation of the polymer slowed down (Figs. 7 and 9) and the lag time of drug release was prolonged (Fig. 5), which extended the retention time of the drug (one of the acylation substrate) in the microsphere. For the other acylation substrate oligomer, the retention

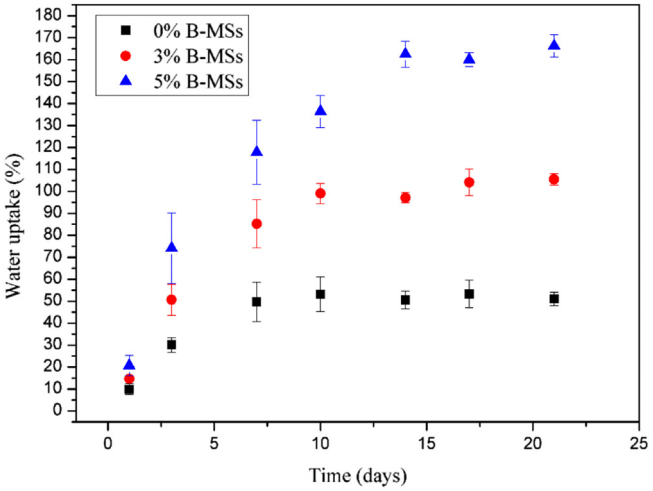

Fig. 12. Water uptake of PLGA microspheres with addition of 0–5% $Ca(OH)_2$ during the critical period.

time of the oligomer was also prolonged (Fig. 13). Degradation mechanism changed from the heterogeneous autocatalytic degradation to the homogeneous degradation (Fig. 8). The loss of first and fully degraded oligomers produced by heterogeneous degradation in the critical period has been greatly decreased. High retention amount of the oligomers was obtained under homogeneous degradation (Fig. 13). Moreover, no crystallization of oligomers was observed partly because the presence of $Ca(OH)_2$ promoted the dissociation and dissolution of oligomers (Fig. 10). To summary, when the μpH was neutralized, the oligomer within the dynamic microsphere system kept at a higher concentration in the critical period. It was supported by the results of water absorption, swelling of microspheres and mass transfer map of oligomers (Figs. 11–13).

It was worth noting that addition of $Ca(OH)_2$ to neutralize the μpH prolonged the lag phase of drug release from the microspheres (Fig. 5, lag phase was 8, 14 and 21 days for 0–5%B-MSs). The critical period for peptide acylation within the microspheres was also prolonged (13, 17 and 22 days). It was speculated that there may be some underlying relationship between the lag period and the critical period of acylation since both of the acylation substrates kept at high concentrations in lag phase. A little delay for critical period compare to the lag phase was also easy to imagine: although the drugs and oligomers were onset to release after the lag phase, it took time for drugs/oligomers

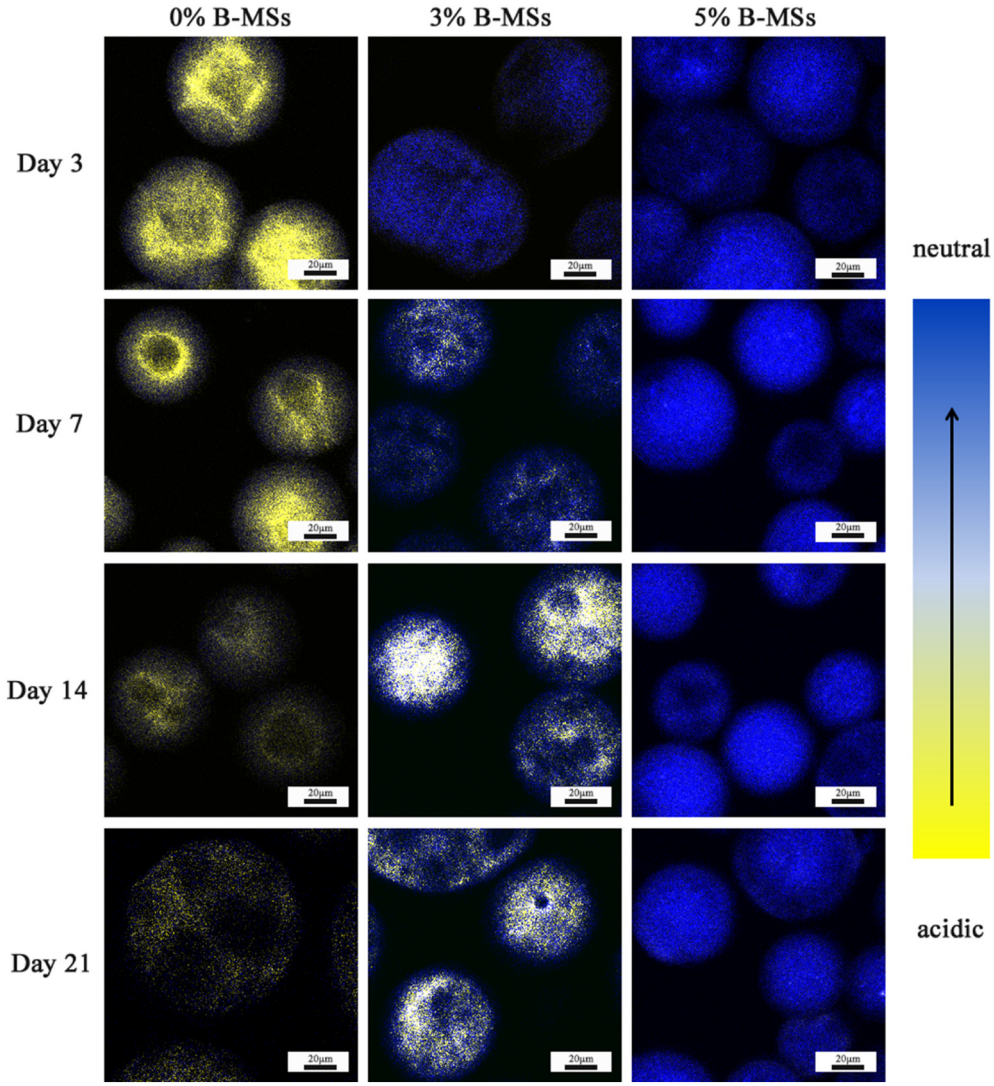

Fig. 13. Confocal laser scanning microscope of the oligomers transportation and pH changes in formulations with presence of 0–5% $Ca(OH)_2$ during the critical time for peptide acylation.

concentration to drop to a low level. We may test this hypothesis in a separate study since numerous experiments are required.

4. Conclusion

In the present study, it was found that peptide acylation within the microspheres was highly dependent on the inner μpH. Different from the fixed reaction kettle, acylation taking place within the microsphere depot was a more complex and multivariate system with continuous mass transfer between inner microspheres and external bulk fluid. Firstly, the inherent effect of pH on the acylation reaction itself was figured out: with the pH environment going up from acid to neutral, peptide acylation became more and more serious. Then, the effect of inner pH on the dynamic system especially the acylation substrates (drug and oligomer) was investigated. The critical period for peptide acylation within the microspheres was the premetaphase. When the inner pH was changed from acid to neutral gradually, a series of associated changes were taking place: a) the retention time of the drug and oligomers which meant the critical time for peptide acylation within the microspheres was prolonged (polymer degradation slowed down); b) the oligomer concentration kept during the critical period was much higher (homogeneous degradation resulted in less mass transfer of oligomer to the outside and oligomer did not crystallize under neutral conditions).

When the inner μpH was neutralized, except that the neutral environment itself promoted the acylation reaction, the effects of pH on the dynamic system were also highly responsible for the serious acylation within the microspheres (prolong the reaction time and increase the substrates concentration). Generally, to effectively inhibit peptide acylation, improvements aiming at the critical time (shorten the critical time and reduce the oligomer retention) or inner μpH (keep a lower pH condition) provided a new perspective for the further study.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejps.2019.04.017.

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