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Acidic pH-Stimulated Tiotropium Release from Porous Poly(lactic-*co*-glycolic acid) Microparticles Containing 3-Diethylaminopropyl-Conjugated Hyaluronate

Sol Kim†, Dong Sup Kwag†, Dong Jin Lee, and Eun Seong Lee*

Department of Biotechnology, The Catholic University of Korea, 43-1 Yeokgok 2-dong, Wonmi-gu, Bucheon, Gyeonggi 14662, Korea

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Abstract: In this study, we developed porous poly(lactide-*co*-glycolide) (PLGA) microparticles (PM) exhibiting pH-activated drug release properties. The PMs were prepared *via* the water-in-oil-in-water ($W_1/O/W_2$) multi-emulsion method using PLGA, 3-diethylaminopropyl amine (DEAP)-conjugated hyaluronate (HA) (HA-DEAP), and an anti-cholinergic model drug (tiotropium). Here, HA-DEAP was incorporated into the PMs; it acted as a drug release activator, accelerating drug release. *In vitro* drug release studies revealed that the tiotropium was released from the PMs as their pores were destabilized by eletrostatic interactions between the carboxyl groups (negatively charged) of the HA molecules and the tertiary amine groups (positively charged) of the DEAP moieties under acidic environments. This PLGA microparticle system, which contains a HA-DEAP, could provide unique advantages in treating chronic obstructive pulmonary disease (COPD) with chronic respiratory acidosis.

Keywords: porous microparticles, poly(lactide-*co*-glycolide), tiotropium, 3-diethylaminopropyl, respiratory acidosis.

Introduction

Recently, the therapeutic benefits of inhalant drug delivery systems have gained tremendous attention. Such systems are convenient and non-invasive, and they can be used for administrating drugs that are useful for treating lung diseases such as chronic obstructive pulmonary disease (COPD).[1-8] The major advantage of inhalant drug formulations is that they allow systemic exposure to be reduced by delivering the drug directly to the target organ,[9] resulting in improved drug permeability and the easy access of the drug to the human lung epithelium.[6-10] Therefore, there has been increasing interest in developing inhalers or drug carriers that achieve enhanced drug inhalation efficiencies[8-13] and therapeutic activities. In particular, inhalant drug formulations involving particles that are between 5 and 20 μm in size and that have low particle mass densities (~0.5 g/cm^3, likely due to their porous structures) have exhibited increased inhalation efficiencies and decreased phagocytic clearances from the lung periphery.[14-20] These structures can potentially be utilized as effective drug carriers for the treatment of respiratory diseases.[19,20]

In this study, we developed a porous poly(lactide-*co*-glycolide) (PLGA) microparticle (PM) with pH-activated drug release properties (Figure 1). It is known that COPD is associated with the chronic inflammation of the bronchi and emphysema; patients with COPD often have obstructed airways, a condition that results in acute or chronic respiratory acidosis (*e.g.*, 6.0<pH<7.35, because of the increase in alveolar carbon dioxide tension).[18-21] In this respect, these PMs may respond to such an acidic environment, providing a potential opportunity to engineer these structures in a functional inhalant drug formulation. Here, we prepared 3-diethylaminopropyl (DEAP)-conjugated hyaluronate (HA) as a pH-sensitive drug release activator.[22-28] It has been reported that DEAP is non-ionic at pH 7.4 and ionic at pHs lower than 7.0.[29-31] In addition, it has been well-known that biocompatible and biodegradable HA has multiple functional chemical groups (such as hydroxyl groups and carboxylic acid groups) useful for biomedical applications.[25] The synthesized DEAP-conjugated HA (HA-DEAP) was incorporated into the PMs. The HA-DEAP in the

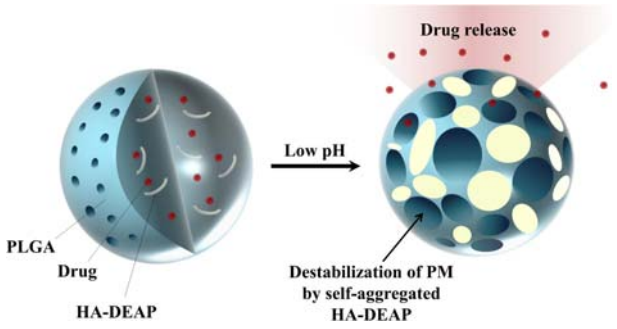

Figure 1. Schematic concept for a porous poly(lactic-*co*-glycolic acid) (PLGA) microparticle (PM) containing HA-DEAP.

*Corresponding Author. E-mail: eslee@catholic.ac.kr
†These authors equally contributed to this work.

PMs may undergo aggregation because of the self-electrostatic interactions between the carboxylic acid groups (negatively charged) of the HAs and the tertiary amine groups (positively charged) of the DEAPs at acidic pHs. The aggregates in the PMs will destabilize their porous structure, activating drug release (Figure 1). To evaluate the pH-sensitivity of these microparticles, their morphologies, porosities, and drug release profiles were preferentially investigated.

Experimental

Materials. Hyaluronic acid (HA, Mw=40 kDa) was purchased from Bioland (Republic of Korea). Dichloromethane (DCM), sodium hydroxide (NaOH), hydrochloric acid (HCl), 3-diethylaminopropyl amine (DEAP), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), triethylamine (TEA), dimethylsulfoxide (DMSO), sodium tetraborate, sodium chloride (NaCl), ammonium bicarbonate (AB), sodium azide, acetonitrile, ammonium acetate, and polyvinyl alcohol (PVA; MW=12-23 kDa) were purchased from Sigma-Aldrich (USA). PLGA (RG503H; lactide:glycolide=50:50, MW=48 kDa) was purchased from Boehringer-Ingelheim (USA). Tiotropium was kindly provided by the Dongkook Pharm. Corporation (Republic of Korea).

HA-DEAP Synthesis. The carboxylic acid groups of HA (2 mM) were reacted with excess DEAP (4 mM) at room temperature for 3 days, in the presence of DCC (10 mM), NHS (10 mM), and TEA (1 mL) in DMSO (10 mL). This process produced DEAP-conjugated HA (HA-DEAP).[25] The resulting solution was then purified *via* dialysis (using a Spectra/Por® MWCO 50 K membrane) against fresh DMSO (for 2 days) and fresh deionized water (for 2 days) to remove any unconjugated chemicals. The obtained solution was freeze-dried for 2 days.[25,30]

Acid/Base Titration Curves. HA-DEAP (30 mM) or NaCl (30 mM, as a control) was dissolved in deionized water and adjusted to a pH of 13.0 or 1.0 using 1 M NaOH or 1 M HCl, respectively. The solutions were titrated *via* the stepwise addition of 1 M HCl or 1 M NaOH to obtain the pH profiles.[25,30]

Zeta-Potential Analysis. The zeta potential of the HA-DEAP solution (1 mg/mL) at different pH values (pH 7.4-5.0, PBS 150 mM) was measured with a Zetasizer 3000 (Malvern Instruments, USA). Prior to these tests, the HA-DEAP solutions were stabilized at room temperature for 2 h.[31]

Preparation of PMs. PMs were fabricated using the conventional $W_1/O/W_2$ multi-emulsion method.[22,27] Tiotropium (20 mg) dissolved in 10 mM sodium tetraborate aqueous solution (1 mL; W_1 phase) containing AB (80 mg, as a porogen for preparing porous microparticles[27]) and HA-DEAP (between 0 and 100 mg) was vigorously emulsified with PLGA (RG503H; 200 mg) dissolved in DCM (3 mL; O phase). The resulting solution was injected into a 1.0 wt% PVA and 0.9 wt% NaCl aqueous solution (W_2 phase). The $W_1/O/W_2$ emulsification was performed for 5 min, using a homo-mixer (Primix Corp, Japan) at 4,000 rpm, and was hardened by mild stirring for 1 h at 50 °C. The particles were collected by centrifugation (3,000 rpm for 2 min) and were washed three times with 0.9 wt% NaCl aqueous solution. The final particles were then freeze-dried for 3 days.[22,27] According to the initial feed amount of HA-DEAP, we denoted each formulation as follows: H0-PM (0 mg of HA-DEAP), H20-PM (20 mg of HA-DEAP), H50-PM (50 mg of HA-DEAP), and H100-PM (100 mg of HA-DEAP). Here, we confirmed that the H100-PM formulation did not reliably produce PMs likely due to the high feed amount (100 mg) of HA-DEAP (data not shown).

Loading Content of Tiotropium and HA-DEAP. The PMs (1 mg) were dissolved in immiscible solvents such as DW (1 mL) and DCM (1 mL). Tiotropium dissolved in DW was analyzed using a HPLC (Agilent 1100 series, USA) equipped with a CAPCELL PAK C18 column (2504.6 mm, 5 mm, Shiseido Co. Ltd., Japan) at ambient temperature. The samples in the mobile phase (60 vol.% 10 mM ammonium acetate buffer/40 vol.% acetonitrile, a flow-rate of 0.8 mL/min) were analyzed at 235 nm. The tiotropium loading content (%) was calculated as the weight percent ratio of tiotropium in the PMs. The tiotropium loading content (%) in the PMs was found to be approximately 5-11 wt%.

The HA-DEAP loading content (%) in the PMs was measured using an extraction method.[27] The PMs (1 mg) were dissolved in immiscible solvents, such as DW (1 mL) and DCM (1 mL). The HA-DEAP dissolved in the DW was characterized with an UV/visible spectrophotometer at a test wavelength of 660 nm (corresponding to the light absorbance of the HA-DEAP).[27] The HA-DEAP loading contents of H20-PM and H50-PM were 11.3 and 27.46 wt%, respectively.

Particle Size and Morphology of PM. The particle size of the HA-DEAPs and PMs was analyzed using a Zetasizer 3000 (Malvern Instruments, USA) equipped with a He-Ne laser beam at a wavelength of 633 nm and a fixed scattering angle of 90°.[30] The samples (0.1 mg/mL) were exposed to different pHs (PBS 150 mM, pH 5.0-7.4) for 4 h before their particle sizes were measured. The morphologies of the HA-DEAPs and PMs (0.1 mg) were evaluated using a field emission scanning electron microscopy (FE-SEM, Hitachi S-4800, USA).[23]

BET Analysis of PM. The Brunauer-Emmett-Teller (BET) method was utilized to evaluate the specific surface areas and pore diameters of the PMs. The BET plots $\{1/[V(P/P_0-1)]$ *vs.* P/P_0, where P/P_0 is the relative pressure and V is the volume of N_2 gas adsorbed$\}$ were obtained using a high performance surface area and porosity analysis instrument (BELSORP-max, USA). Before BET analysis, the PMs (200 mg) were exposed to different pHs (PBS 150 mM, pH 7.4 or 6.0) for 4 h. Then, the samples were degassed under vacuum at 60 °C for 6 h and their N_2 adsorption was analyzed at 77 K.[24]

HA-DEAP and Tiotropium Release. PMs (10 mg) were added into a dialysis membrane (Spectra/Por, MWCO 100 kDa, Spectrum Lab. Inc., Rancho Dominguez, USA), and then they were immersed in a vial containing fresh PBS pH 7.4-6.0

(20 mL, ionic strength: 0.15, 0.01% sodium azide) at 37 ºC. The outer phase of the dialysis bag was withdrawn and replaced with fresh PBS (pH 7.4-6.0) solution at predetermined time intervals. The polymer concentration in each solution (1 mg/mL) was monitored using a HPLC (Agilent 1100 series, USA).[30]

Results and Discussion

Synthesis and Characterization of HA-DEAP. The carboxylic acid groups of HA were reacted with DEAP in the presence of DCC and NHS to produce HA-DEAP conjugates.[25] We used HA-DEAP as a drug release activator. The degree of DEAP substitution of the HA-DEAP moieties (defined as the number of DEAP moieties per one repeat unit of HA) was 0.20 as estimated from the ^{1}H NMR spectra (500 MHz, DMSO-d_6) using the integration ratio of the peaks at δ 1.01 ppm (-C**H**$_3$ in the DEAP block of HA-DEAP) and δ 3.04 ppm (-C**H** in the repeat unit of HA) (data not shown).[25] In addition, Figure 2 shows the acid-base titration profile of HA-DEAP. The pK_a and pK_b of HA-DEAP was found to be approximately 2.30 and 7.04, respectively, as estimated from the variation points in the acid and base titration profiles.

Interestingly, the HA-DEAPs aggregated as a result of the electrostatic interactions between the carboxyl groups (negatively charged) of the HA units and the tertiary amine groups (positively charged) of the DEAP moieties at acidic pHs. As shown in Figure 3(a), the soluble HA-DEAP at pH 7.4 exhibited

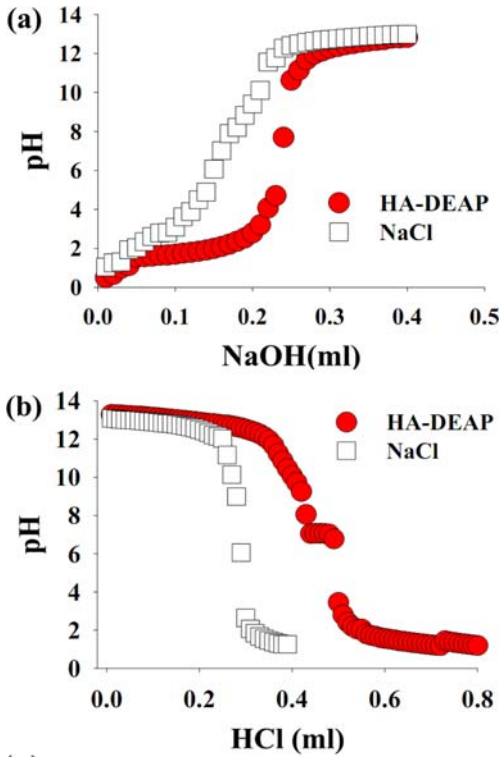

Figure 2. pH profiles of HA-DEAP and NaCl (control) measured by (a) acid or (b) base titration.

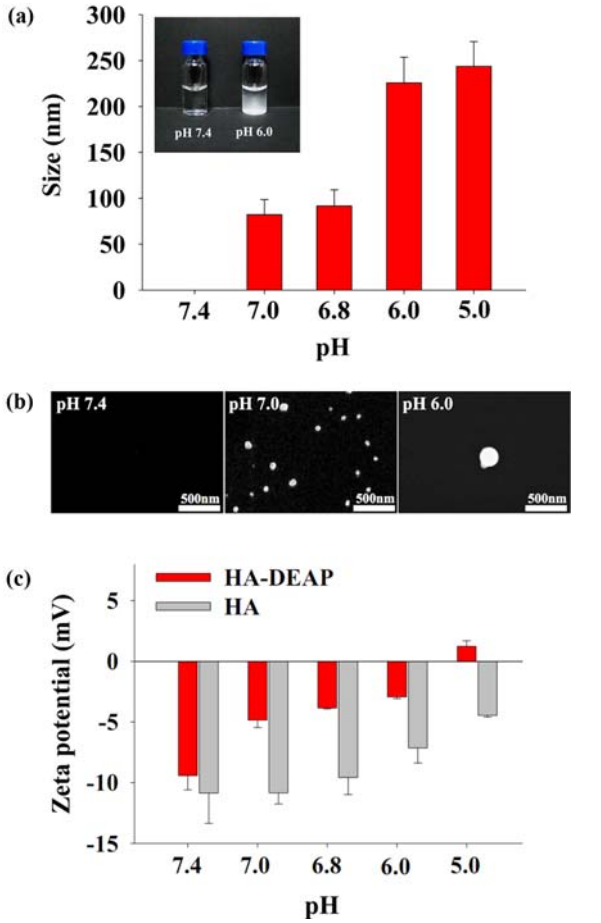

Figure 3. (a) Particle size of HA-DEAP after incubation at pH 7.4-5.0 (PBS 150 mM) for 1 h (the inner image shows a relative change in transmittance of HA-DEAP solution at pH 7.4 or 6.0 (PBS 150 mM) for 1 h). (b) FE-SEM images of HA-DEAPs (0.1 mg/mL) at pH 7.4, 7.0, and 6.0. (c) Zeta potential of HA-DEAP and HA at pH 7.4-5.0 (PBS 150 mM) (n=3).

an average diameter of 80 nm at pH 7.0 and 86 nm at pH 6.8. The particle size of the HA-DEAP moieties significantly increased between pH 6.8 (average diameter of HA-DEAP: 86 nm) and pH 6.0 (average diameter of HA-DEAP: 224 nm); at pH 6.0, the HA-DEAP moieties aggregated significantly. The digital image reveals that the pH 6.0 PBS solution of HA-DEAP exhibits low light transmittance comparable to that of the pH 7.4 PBS solution of HA-DEAP. The images obtained from the FE-SEM also reveal that particles did not form in the soluble HA-DEAP solution at pH 7.4, but that aggregation occurred at pHs between 7.0 and 6.0 (Figure 3(b)). In addition, Figure 3(c) shows that as the pH of the solution decreased from 7.4 to 6.0, the zeta potential of the HA-DEAP sample increased from -9.2 to -2.7 mV. These data suggest that the zeta potential, which originates from the carboxylic acid groups of the HA, remarkably increases due to the protonation of the DEAP moieties at acidic pHs.

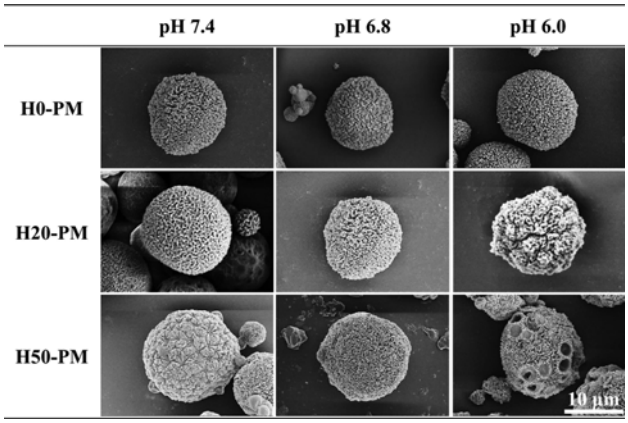

Figure 4. FE-SEM images of PMs at pH 7.4, 6.8, and 6.0.

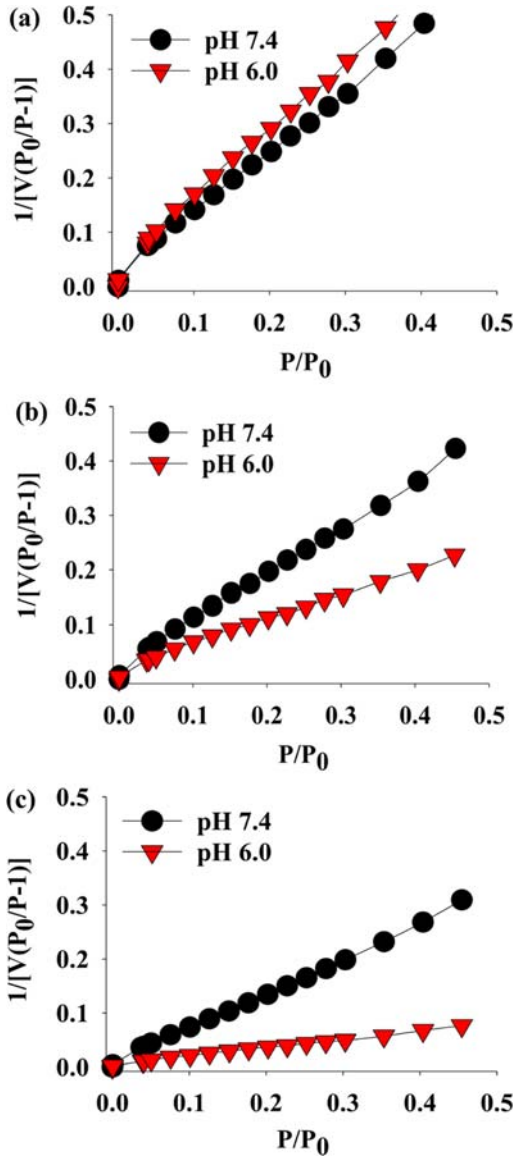

Figure 5. BET plots of the N_2-adsorption of the porous surfaces of (a) H0-PM, (b) H20-PM, and (c) H50-PM.

Preparation and Characterization of PMs. The porous PLGA microparticles (PMs) were prepared *via* the $W_1/O/W_2$ multi-emulsion method using PLGA (as a polymeric matrix), AB (as a porogen),[27] HA-DEAP (as a drug release activator), and tiotropium (as a model drug). The average particle size of the PMs was approximately 10 μm as observed by FE-SEM (Figure 4). The aggregation of the HA-DEAP moieties at acidic pHs destabilized the pores of the PMs (Figure 1). In particular, the FE-SEM image of the H50-PM sample at pH 6.0 showed highly enlarged pores. Figure 5 shows the BET plot $\{1/[V(P/P_0\text{-}1)]$ *vs.* $P/P_0\}$ of the PMs measured using a high performance surface area and porosity analysis instrument (BELSORP-max).[24] The BET specific surface area (calculated using BELSORP-max software based on BET Theory)[24] of the H0-PM sample at pH 7.4 was 2.0 m^2/g; this value is not significantly different with that of the HO-PM sample at pH 6.0 (Figure 5(a)). However, the BET specific surface areas of H20-PM at pH 7.4 and pH 6.0 were 5.1 and 11.4 m^2/g, respectively (Figure 5(b)). The BET specific surface areas of H50-PM at pH 7.4 and pH 6.0 were 10.6 and 127.2 m^2/g, respectively (Figure 5(c)). The average pore diameters (calculated using BELSORP-max software based on BET Theory)[24] of H0-PM, H20-PM, and H50-PM at pH 7.4 were 11, 12, and 27 nm, respectively. However, the average pore diameters of H0-PM, H20-PM, and H50-PM at pH 6.0 were 13, 23, and 120 nm, respectively. These data indicate that the incorporation of HA-DEAP increases the surface areas and average pore diame-

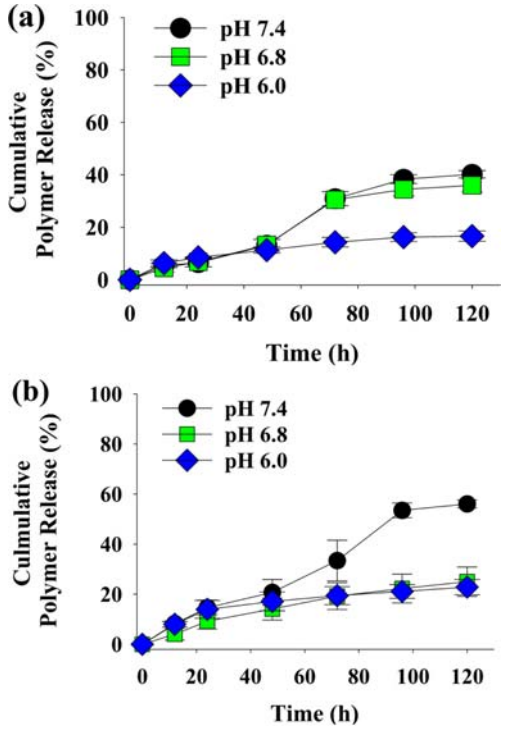

Figure 6. pH-Dependent cumulative HA-DEAP release profiles for (a) H20-PM and (b) H50-PM at pH 7.4-6.0 for 120 h of incubation (n=3).

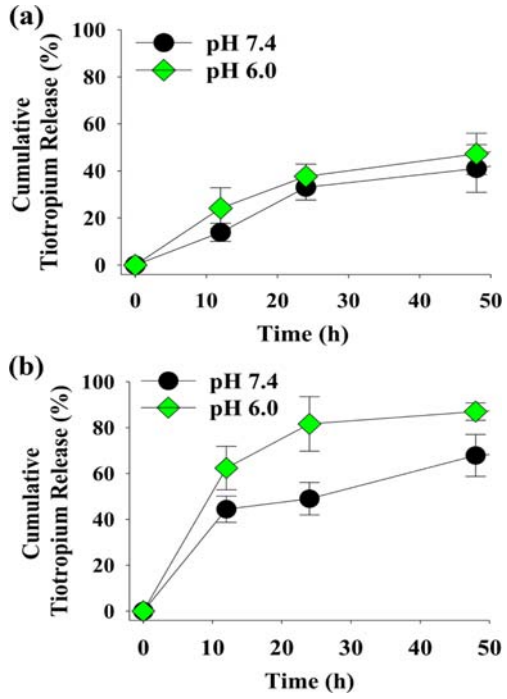

Figure 7. pH-Dependent cumulative tiotropium release profiles from (a) H20-PM and (b) H50-PM at pH 7.4 and 6.0 for 50 h of incubation (n=3).

ters of the PMs in acidic environments.

pH-Activated Drug Release. The drug release profiles also indicate the HA-DEAP incorporation accelerates drug release in acidic environments. First, we monitored the HA-DEAP release profiles as a function of pH. Figure 6 shows that as the pH of the solution decreased from 7.4 to 6.0, the cumulative HA-DEAP release decreased to less than 20 wt%, probably due to the aggregation of the HA-DEAP moieties in the PMs at acidic pHs (pH 6.8 or 6.0). Next, we monitored the tiotropium release profile for the H20-PM (Figure 7(a)) and H50-PM samples (Figure 7(b)). Interestingly, the cumulative amount of tiotropium released from H50-PM after 48 h incubation depends on the pH of solution: 84 and 64 wt% tiotropium was released at pH 6.0 and pH 7.4, respectively. However, the cumulative amount of tiotropium release from H20-PM after 48 h incubation was not significantly different as a function of pH: 46 and 40 wt% tiotropium was released at pH 6.0 and pH 7.4, respectively. These drug release profiles indicate that an optimum amount (*e.g.*, H50-PM) of HA-DEAP can stimulate the accelerated release of encapsulated tiotropium under conditions relevant in acute or chronic respiratory acidosis.

Conclusions

In this study, we fabricated porous PLGA microparticles bearing pH-sensitive HA-DEAP moieties. These microparticles released drugs in acidic environments. This system could potentially be used as an inhalation-based platform for the treatment of acidic pulmonary diseases. Of course, further *in vitro/in vivo* studies involving therapeutic activity and toxicity are necessary.

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