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RESEARCH PAPER

Microcosmic Mechanism of Dication for Inhibiting Acylation of Acidic Peptide

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ABSTRACT

Purpose For long-effective peptide formulation based on poly(d, l-lactic-co-glycolic acid) (PLGA) microspheres, acylation often leads to peptide instability during its release and reduced drug efficacy. Among the reported solving strategies, adding dication such as Ca^{2+} and Mn^{2+} in the formulation was the most convenient method for inhibiting basic peptide acylation. However, the strategies for the acidic peptide still remain unexplored, possibly due to the peptide's changeable charge state in acid environment within degraded PLGA microspheres. Moreover, the previous studies mainly focusing on the macroscopical adsorption of peptide to PLGA cannot demonstrate the inhibition mechanism.

Methods Acylation inhibition for acidic peptide (exenatide) by dications (Ca^{2+}, Mn^{2+} and Zn^{2+}) was studied for the first time, and Quartz Crystal Microbalance with Dissipation (QCM-D) was innovatively employed to analyze microcosmic mechanism of the inhibition.

Results These dications played different roles in acylation inhibition of acidic peptide. The effects of dications on acylation outside or inside PLGA microspheres indicated that Ca^{2+} did not work, Mn^{2+} played a weak role, and Zn^{2+} possessed the greatest inhibition.

Conclusions Zn^{2+} was the most effective dication for the acylation inhibition because of the complex formation and its steric-hindrance effect, which was a new function for this dication.

KEY WORDS acidic peptide · acylation · exenatide · PLGA · QCM-D

ABBREVIATIONS

Ca^{2+}	Calcium ion
DMSO	Dimethyl sulfoxid
GA	Glycolic acid
HAc	Acetic acid buffer
HPLC-MS	HPLC mass spectrometry
ICP-OS	Inductively coupled plasma optical emission spectrometry
LA	Lactic acid
Mn^{2+}	Manganese ion
M_w	Molecular weight
PEG	Polyethylene glycol
pI	Isoelectric point
PLGA	Poly(d,l-lactic-co-glycolic acid)
PVA	Polyvinyl alcohol
QCM-D	Quartz Crystal Microbalance with Dissipation
RP-HPLC	Reversed phase high performance liquid chromatography
SDS	Natrium dodecyl sulphate
SEC-HPLC	Steric exclusion chromatography
SPG	Shirasu Porous Glass
T2DM	Type 2 diabetes mellitus

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Zn^{2+}	Zinc ion
ΔD	Dissipation shift
Δf	Frequency shift

INTRODUCTION

Poly(d,l-lactic-co-glycolic acid) (PLGA) as a form of microsphere has been widely investigated to encapsulate protein/peptide drugs (1,2). However, more and more researches have reported the acylation between PLGA and the encapsulated protein/peptide drugs (3,4), which often leads to drug instability and bio-activity loss. The reaction easily occurs between the peptide/protein with labile N-terminus or lysine and PLGA. Lots of peptides/proteins possess these primary amine groups, so the possibility of the harmful reaction with the polymer is increased.

Many efforts have been devoted to overcome this issue. For example, PEGylation was very likely to shield the peptide from its binding or active site. Adjusting the pH inside microspheres might decrease the reaction velocity (5). Among the reported inhibition methods, adding dication in the formulation, such as Ca^{2+} and Mn^{2+}, attracted more attentions due to the easy feasibility (4). By these dications, the stabilities of octreotide (Oct), salmon calcitonin (sCT) and human parathyroid hormone (hPTH) were highly preserved (6). However, all these investigated peptides possessed high isoelectric point (pI) (pI for Oct, sCT and hPTH were 8.3, 8.0 and 10.4, respectively), and the strategy for preventing acidic peptide from acylation has not been reported. The possible reason is that pH of the microclimate inside PLGA microsphere is often acidic owing to accumulation of degraded polymer (7,8). Acidic peptide may be in positive, neutral or negative charge state at different time, which is more complicated than basic peptide in low pH.

Moreover, the previous study for inhibiting acylation was investigated by the indirect measurement of the concentration of non-adsorptive peptide in supernatant to explore macroscopical adsorption of the peptide to PLGA (4). This method was not only inaccurate, but could not indicate how the dications played their roles in the reaction. Therefore, the acylation inhibition mechanism of the dications has not been well explained. To minor the reaction microcosmically and illustrate the mechanism in nature, Quartz Crystal Microbalance with Dissipation (QCM-D) was employed in this study, which was widely used to monitor biological events, such as peptide adsorption, interactions between proteins (9).

In our study, exenatide, a therapy for type 2 diabetes mellitus (T2DM), possessing a low pI 4.96 was used as a model acidic peptide. It has three potential acylation sites with labile primary amines (2 lysine residues at positions 12 and 27 with pKa 10.1 and 1 N-terminus with pKa 7.8) (10). Three kinds of dications, Ca^{2+}, Mn^{2+} and Zn^{2+}, were added in the system to investigate their effects on the acylation. Then, QCM-D was employed to analyze the reaction mechanism of different dications on the acylation between peptide and PLGA. Finally, the acylation inhibition in PLGA microspheres was studied.

MATERIALS AND METHODS

Materials

PLGA with mol ratio of D,L-lactide/glycolide 75/25 (M_w 13 kDa) was purchased from Lakeshore Biomaterials (Birmingham, AL, USA). Exenatide was provided by Hybio Pharmaceutical Co., Ltd. (Shenzhen, China). Acetonitrile and trifluoroacetic acid (both in HPLC grade) were purchased from Dikma Co., Ltd. (Lake Forest, USA). All other reagents were analytical grade.

Incubation of Medium with PLGA and Exenatide

Firstly, $CaCl_2$, $MnCl_2$ and $ZnCl_2$ (50 mM) in acetic acid buffer (HAc) with pH 4.5 and no ion added HAc were prepared. Exenatide (1 mM) was dissolved in the above solutions and mixed with DMSO or DMSO containing PLGA (0.5%*w*/*v*) at volume ratio 1:9. Then, eight groups were obtained: exenatide with no ion, exenatide with Ca^{2+}, exenatide with Mn^{2+}, exenatide with Zn^{2+}; exenatide+PLGA with no ion, exenatide+PLGA with Ca^{2+}, exenatide+PLGA with Mn^{2+}, exenatide+PLGA with Zn^{2+}. Finally, all the groups were incubated in 37°C, and sampled at day 0.5, 1, 2, 3, 4 and 7.

Determining Stability of Exenatide

The stability of exenatide was determined by a reversed phase HPLC (RP-HPLC) system at room temperature with a C18 (250 mm × 4.6 mm × 5 μm, Syncronis, Thermo) chromatographic column as previously reported (8,11). The chromatography was performed with a linear gradient elution from 35 to 44% acetonitrile in ultrapure water containing 0.1% trifluoroacetic acid for 21 min.

The SEC-HPLC was performed with a Column Superdex 75 10/300 GL (GE, Sweden). The mobile phase was 0.05 M phosphate buffer and 0.15 M NaCl at flow rate 0.4 mL/min.

QCM-D Measurement

QCM-D technique was widely used to measure the real-time macromolecule adsorption in liquid-phase research applications (9,12). In this study, exenatide adsorption on the PLGA film immersed in flowing phase was measured by this technique. The general QCM-D principle is to apply an AC

voltage in the megahertz range across an AT-cut piezoelectric quartz crystal and to record the resonance frequency of the crystal. The sensor crystals were coated by PLGA with a spin coater at a speed of 8000 rpm for 40 s. The QCM-D experiments were firstly conducted at 37°C using exenatide solution containing no ion, Ca^{2+}, Mn^{2+} or Zn^{2+} at flow rate 50 μL/min. When the adsorption of exenatide reached saturation, degassed buffer was injected into the chamber to desorb exenatide at the same flow rate until balance was achieved. To investigate the interaction between exenatide and PLGA, the buffer with 1% SDS was further injected into the chamber to elute the peptide adsorbed by hydrophobic interaction.

Preparation and Characterization of PLGA Microspheres

Four kinds of exenatide-loaded PLGA microspheres with or without dications were prepared by SPG premix membrane emulsification combined with $W_1/O/W_2$ double emulsion-solvent evaporation method. First, $CaCl_2$, $MnCl_2$ or $ZnCl_2$ (30 mg) was dissolved in 1 mL aqueous solution (W_1) containing exenatide (30 mg). The W_1 was emulsified with 8 mL organic solvent (methylene dichloride, O) containing PLGA (10%, *w/v*) by ultrasonication (S-450D Digital Sonifier, Branson, USA) on 120 W for 60 s in ice bath to form W_1/O. Next, the W_1/O was mixed with external aqueous phase (W_2) containing PVA (2%, *w/v*) and NaCl (3%, *w/v*) to form coarse $W_1/O/W_2$ emulsions. Then they were poured into premix reservoir and extruded through the SPG membrane (50.2 μm) by N_2 to achieve uniform-sized droplets. After that, they were solidified at room temperature for 5 h. Finally, the microspheres were collected by centrifugation, washed with distilled water for five times and obtained after freeze-drying.

The particle size and size distribution was measured by Mastersizer 2000 (Malvern, UK). The size distribution was referred as Span value and calculated as follows:

$$\text{Span} = \frac{D_{v,90\%} - D_{v,10\%}}{D_{v,50\%}}$$

where $D_{v,90\%}$, $D_{v,50\%}$ and $D_{v,10\%}$ are volume size diameters at 90, 50 and 10% of the cumulative volume, respectively. The smaller Span value indicates the narrower size distribution.

Encapsulation Efficiency of Exenatide and Dications

To determine the encapsulation efficiency of exenatide, 5 mg of microspheres were dissolved in 150 μL acetonitrile, and then 850 μL 0.01 M HCl was added to dissolve exenatide. Next, the concentration of exenatide in mixture was determined as described above. The loading efficiency and encapsulation efficiency of the microspheres were calculated by the following equations:

$$\text{Loading efficiency}\,(\%,\ w/w) = \frac{\text{Mass of drug in microspheres}}{\text{Mass of microspheres}} \times 100\,\%$$

$$\text{Encapsulation efficiency}(\%,\ w/w) = \frac{\text{Loading efficiency}}{\text{Theoretical loading efficiency}} \times 100\%$$

To determine the encapsulation efficiency of dications, 2 mg of microspheres were degraded in HCl (pH 2). The concentrations of the dications were determined by ICP-OES (Thermo iCAP 6300).

RESULTS AND DISCUSSION

The medium composed of DMSO and HAc was a good system to study the stability of exenatide *in vitro* (11), because PLGA and exenatide were both dissolved in the form of molecule, making them contact each other with high frequency. Moreover, it was also very accessible to determine the stability of exenatide by RP-HPLC.

Identification of Acylated Derivatives

After incubation of the system containing exenatide and PLGA for 4 days at 37°C, the medium was detected by HPLC-MS. As shown in Fig. 1, compared with the control (day 0), the acylated derivatives were presented after exenatide peak (4184.4 Da): single glycolic unit (4242.6 Da, Exenatide-GA), one lactic and one glycolic unit (4313.7 Da, Exenatide-LA-GA) and double lactic units (4328.4 Da, Exenatide-2LA).

Although the pH of this system was 4.5, which was close to the exenatide pI (4.96) making the peptide charge neutral, the acylation still occurred. It was because the pKa of N-terminus was 7.8, whereas that of Lys residue was higher. Therefore,

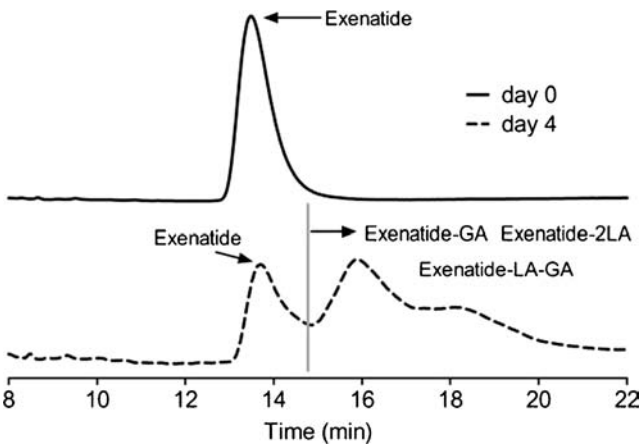

Fig. 1 Identification of acylated derivatives at Day 0 (*solid line*) and Day 4 (*dashed line*) after incubation at 37°C.

these groups were reactive independent of the charge state (13). Meanwhile, PLGA with free-acid end group was nucleophilically attacked by amino groups of exenatide forming acylated derivatives (14).

Inhibition of Acylation by Different Dications

As we stated above, it has been widely studied that dications such as Ca^{2+} and Mn^{2+} could inhibit acylation. Moreover, Zn^{2+} was also chosen due to its unique function as a complex-former with peptide or protein (15,16). All the dications at concentration of 50 mM were co-incubated with exenatide and PLGA in the medium at 37°C.

After incubation for 4 days (Fig. 2a), it seemed that there was no difference between Ca^{2+} and the control group (no ion addition), both presenting significant acylated derivatives, whereas Mn^{2+} showed less. Contrarily, the stability of exenatide co-incubated with Zn^{2+} was highly preserved with no acylated products.

The molar absorptivity coefficient under Beer's law was the same for the parent peptide and its derivatives, so the acylated derivatives were quantified as follows: acylated exenatide (%)=impurity areas after exenatide peak/ sum of all peak areas×100% (11). As shown in Fig. 2b, it was clearly illustrated that Ca^{2+} did not have inhibition effect on the acylation as the control. On the other hand, Mn^{2+} partly restrained the acylation within first 4 days, but the effect was weakened since then. However, there were almost no acylated derivatives when Zn^{2+} was added. In order to ensure the stability of exenatide during incubation, the groups without PLGA were also analyzed. As illustrated in Fig. 2c, all the chromatograms were identical, indicating the stability of exenatide in all the groups was not influenced. It was also concluded that the factor influencing the stability of exenatide was the presence of PLGA.

Therefore, combined with the above results, Zn^{2+} could inhibit acylation, Mn^{2+} had week effect, and Ca^{2+} did not work. The roles that Ca^{2+} and Mn^{2+} played in acidic peptide were different from those in basic peptide, because these two dications strongly inhibited acylation of basic peptide. To further demonstrate this mechanism, QCM-D was used to microcosmically analyze the influence of the dications on the acylation.

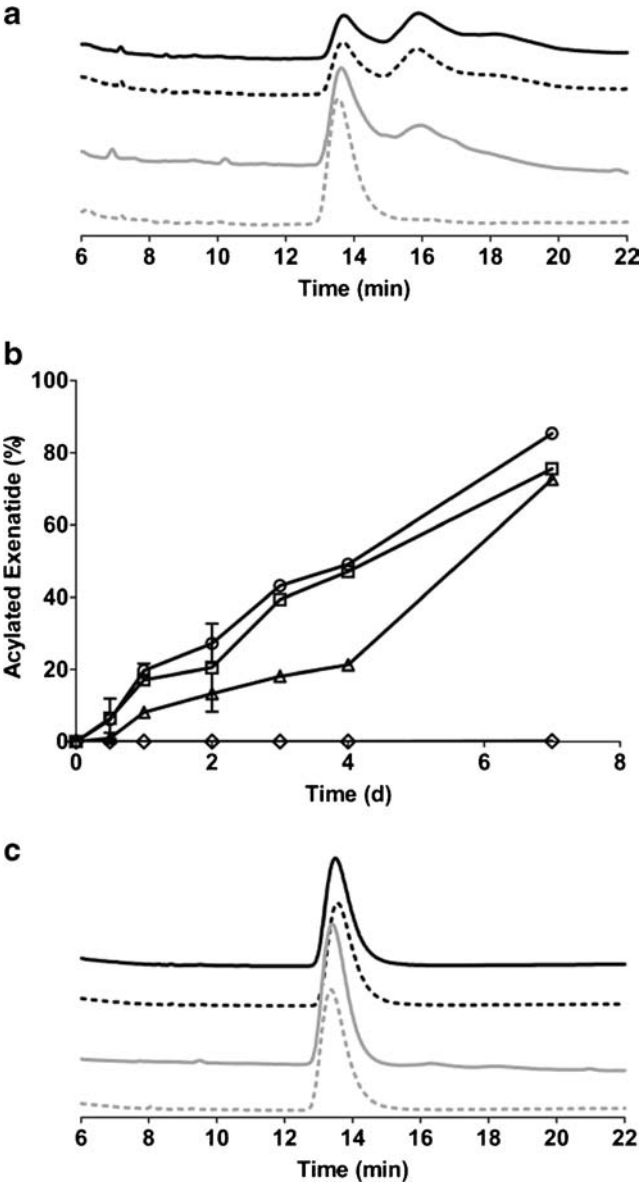

Fig. 2 (**a**) RP-HPLC chromatogram of medium containing exenatide and PLGA co-incubated with no dication addition (control, *black solid line*), Ca^{2+} (*black dashed line*), Mn^{2+} (*gray solid line*) and Zn^{2+} (*gray dashed line*) at Day 4; (**b**) quantification of acylated exenatide vs. time (mean ± SD, $n=3$), no dication addition (*white circle*), Ca^{2+} (*white square*), Mn^{2+} (*white triangle*) and Zn^{2+} (*white diamond*); (**c**) RP-HPLC chromatogram of medium containing exenatide co-incubated with no dication addition (control, *black solid line*), Ca^{2+} (*black dashed line*), Mn^{2+} (*gray solid line*) and Zn^{2+} (*gray dashed line*) without PLGA at Day 4.

Microcosmic Investigation on Acylation by QCM-D

It has been widely accepted that the first step for acylation is caused by adsorption of peptide to PLGA via electrostatic interaction and hydrophobic interaction, and then primary amine groups in peptide nucleophilically attacks to PLGA (13,17). In previous studies, the detection of adsorption was often performed by suspending PLGA powder with peptide in aqueous phase and indirectly measuring the concentration of peptide in supernatant (4), which could not reflect the adsorption mechanism accurately. QCM-D, however, is widely used to monitor biological events, such as peptide adsorption on material surface. It can monitor two parameters: frequency (f) and dissipation (D). During a given adsorption process, Δf is effective mass loading caused by the adsorbates and the solvent entrapped, whereas ΔD is a qualitative assessment of flexibility/viscoelasticity of the structural variations in thin films (18,19).

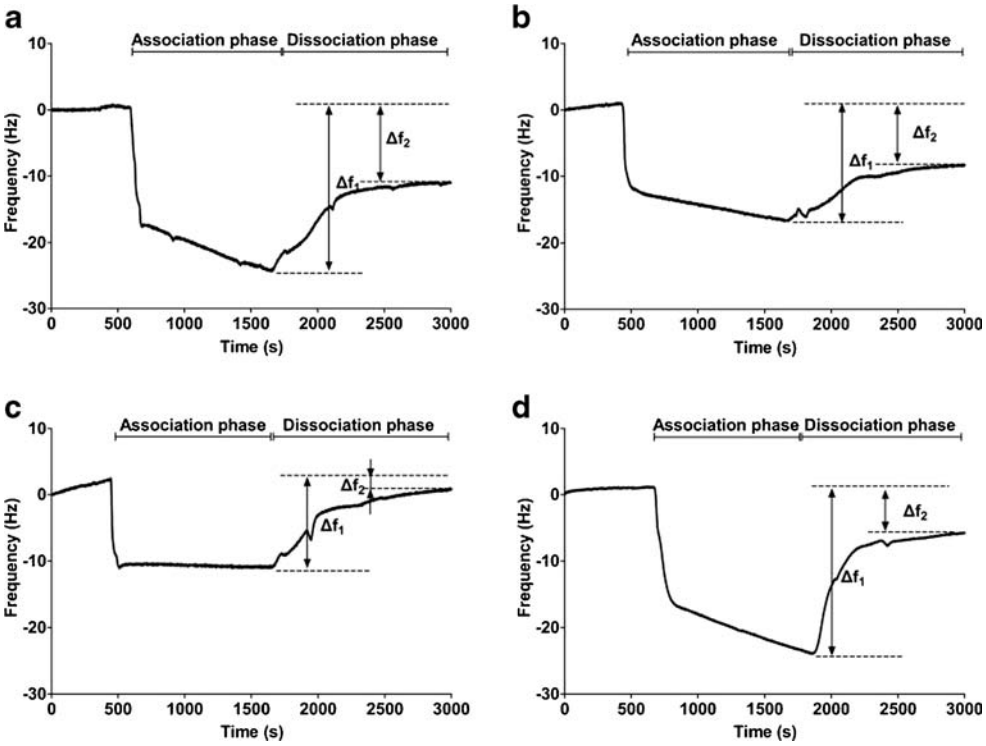

Fig. 3 Diagram of frequency vs. time for exposure of PLGA film to exenatide solution (4 mg/mL) co-incubated with (**a**) no ion (control), (**b**) Ca^{2+} (50 mM), (**c**) Mn^{2+} (50 mM) and (**d**) Zn^{2+} (50 mM) at association phase, followed to HAc buffer at dissociation phase. Δf_1 represents the changes in frequency before and after association phase, Δf_2 represents the changes in frequency before association phase and after dissociation phase.

As shown in Fig. 3a, after association phase, a high Δf_1 (24.8 Hz) was in the no ion addition group, suggesting that amounts of peptide adsorbed on the PLGA film. Figure 3b and c shows that when Ca^{2+} and Mn^{2+} were added, their Δf_1 both presented smaller values (17.5 and 13.1 Hz, respectively), indicative of less adsorption. Therefore, it was deduced that Ca^{2+} and Mn^{2+} could partly impede the adsorption of peptide on PLGA. On the other hand, it was clearly shown that when Zn^{2+} was added, the Δf_1 was close to the control (24.7 Hz, Fig. 3d). Thus, it seemed that Zn^{2+} might not reduce the adsorption of peptide on PLGA.

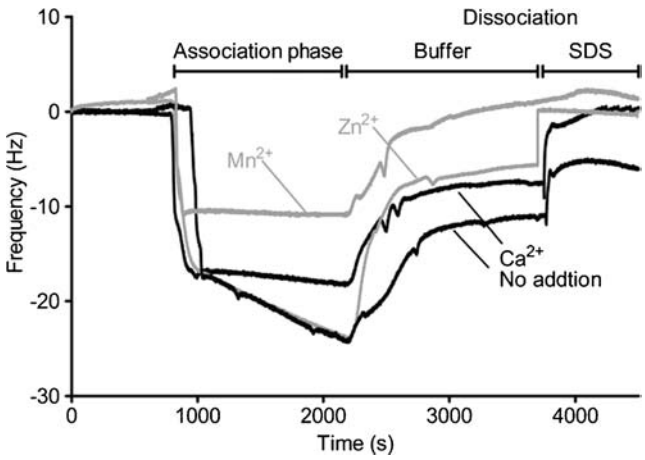

Fig. 4 Diagram of frequency vs. time for exposure of PLGA film to exenatide solution (4 mg/mL) co-incubated with no ion (control), Ca^{2+} (50 mM), Mn^{2+} (50 mM) and Zn^{2+} (50 mM) at association phase, then at dissociation phase exposed to HAc buffer and HAc buffer with 1% SDS.

During the dissociation phase, the flowing phase was replaced by buffer without peptide and ion. After dissociation, Δf_2 of the no ion addition, Ca^{2+}, Mn^{2+} and Zn^{2+} group were 11.7, 9.4, 1.23 and 6.9 Hz, respectively (Fig. 3). The higher value means more peptide absorbed tightly on the PLGA film.

In no ion addition group, the highest adsorption occurred during the association phase, and nearly 50% ($\Delta f_2/\Delta f_1$) of the peptide adsorbed on PLGA by nonspecific interaction after dissociation. The high adsorption before and after dissociation phase, reflected by Δf_1 and Δf_2, both increased the contact probability between peptide and PLGA, making acylation readily occur. The Δf_2 in Ca^{2+} group was higher than the other two ion groups and lower than the control. It was demonstrated that although Ca^{2+} co-incubated with exenatide in the medium partly inhibited the adsorption, more peptide

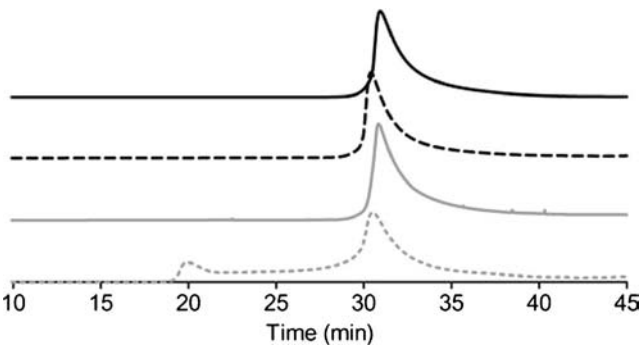

Fig. 5 SEC-HPLC of exenatide co-incubated with no dication addition (*black solid line*), Ca^{2+} (*black dashed line*), Mn^{2+} (*gray solid line*) and Zn^{2+} (*gray dashed line*).

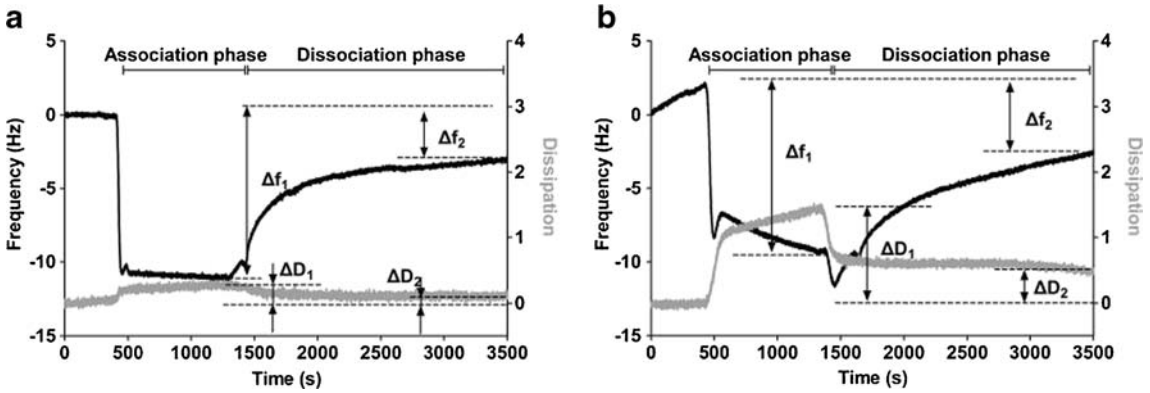

Fig. 6 Diagram of frequency (*black line*) and dissipation (*gray line*) vs. time for exposure of PLGA film to exenatide solution (0.1 mg/mL) co-incubated with (**a**) no ion (control), (**b**) Zn^{2+} (50 mM) at association phase, followed to HAc buffer at dissociation phase. Δf_1 and ΔD_1 represent the changes in frequency and dissipation before and after association phase, Δf_2 and ΔD_2 represent the changes in frequency and dissipation before association phase and after dissociation phase.

nonspecifically adsorbed on PLGA ($\Delta f_2/\Delta f_1$, 53.7%), which in turn provided the precondition for acylation. Therefore, Ca^{2+} had no effect on the inhibition. As for Mn^{2+}, the smallest Δf_2 suggested that very few peptide molecules tightly adsorbed on PLGA, so the acylation could be partly restrained. On the other hand, Zn^{2+} had a low Δf_2, and only 27.9% ($\Delta f_2/\Delta f_1$) of the peptide nonspecifically adsorbed on PLGA after dissociation. However, it was still not a good interpretation for the acylation inhibition temporarily.

To investigate which interaction between peptide and PLGA played the important role, 1% SDS was used to further dissociate the peptide that still adsorbed on the PLGA by hydrophobic interaction (20). As shown in Fig. 4, after SDS elution, only the no ion addition group still showed large shift, indicative of peptide adsorption by electrostatic interaction on the PLGA. Almost no peptide left in the other groups. Therefore, it was demonstrated that the peptide adsorbed on the PLGA both by hydrophobic and electrostatic adsorption. When Ca^{2+}, Mn^{2+} and Zn^{2+} were used, electrostatic interaction was absolutely shielded, so only hydrophobic interaction played the part in adsorption.

In conclusion, Ca^{2+} could not inhibit acylation because of its high adsorption by hydrophobic effect, and Mn^{2+} could partly inhibit the reaction due to less adsorption. Even so, the reversible adsorption during association phase still increased the possibility for nucleophilic attack followed by acylation reaction.

To further demonstrate the inhibition mechanism of Zn^{2+}, the medium with different dications were detected by SEC-HPLC. As illustrated in Fig. 5, there was another peak before the exenatide peak when Zn^{2+} was used, indicative of aggregations (complex formation), which was in line with previous

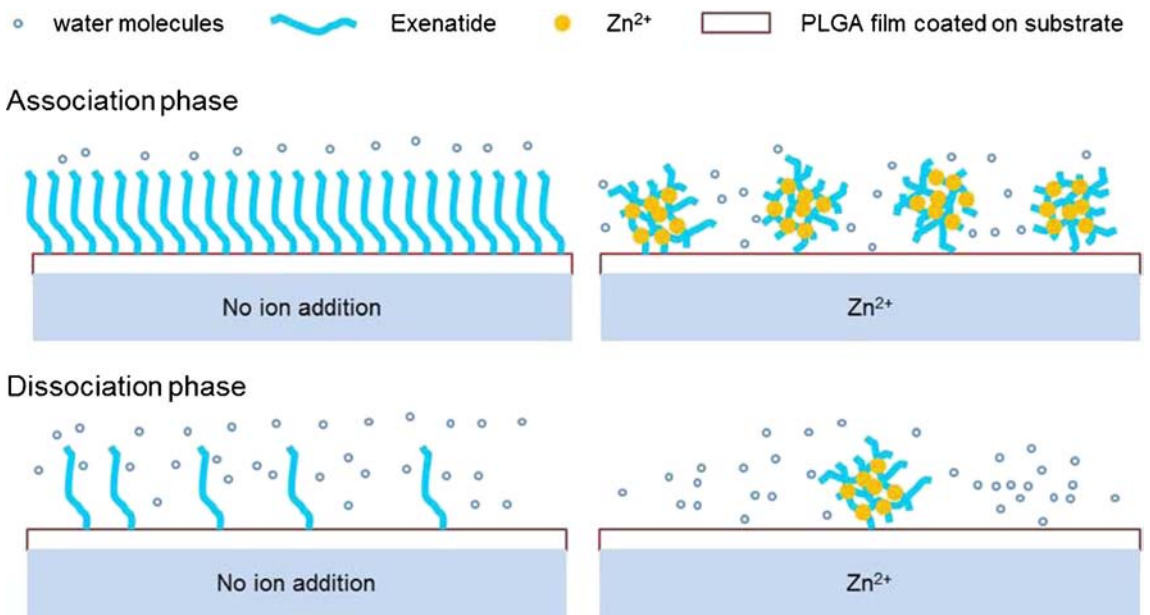

Fig. 7 Schematic diagram for mechanism of acylation inhibition by Zn^{2+}.

Table I Characteristics of PLGA Microspheres Prepared by Different Dications

Dication	Size/ μm	Span	Encapsulation efficiency of exenatide/%	Encapsulation efficiency of dication/%
None	20.41	0.676	90.34 ± 5.10	–
Ca^{2+}	21.67	0.656	95.69 ± 1.13	55.7 ± 4.24
Mn^{2+}	22.09	0.645	92.31 ± 2.65	66.0 ± 3.37
Zn^{2+}	22.12	0.677	94.68 ± 2.54	64.7 ± 7.93

study (21,22). The complex was easily formed due to combination of Zn^{2+} and the N-terminal region of exenatide (Histidine) (23). Thus, based on this result, it was deduced that the inhibition of acylation might be associated with the complex formation.

In order to indicate the effect of Zn^{2+} on the adsorption more obviously, at the fixed Zn^{2+} concentration (50 mM), peptide concentration was reduced (0.1 mg/mL) to make the dication excess. As shown in Fig. 6, higher obvious dissipation shift (ΔD_1) was presented in Zn^{2+} group after association phase. Combined with the above result, the possible reason was that one of the exenatide molecules from the complex anchored to the PLGA surface, and the trapped interlayer water within the complex moved with shear oscillation of the crystal, resulting in higher ΔD_1. Therefore, the adsorbed molecules ranged loosely on the PLGA film (Fig. 7). The exenatide molecules without ion closely packed on the film with less water trapped interlayer, which was considered as a rigid substance, so the ΔD_1 was lower. However, Δf_1 was almost identical (11.5 Hz vs. 11.3 Hz for control and Zn^{2+} group), suggesting the same quality of exenatide molecules deposited or absorbed on the PLGA film. This also indirectly supported the conjecture of complex formation between Zn^{2+} and exenatide. After dissociation, Zn^{2+} group showed slightly high Δf_2 compared with control (4.4 Hz vs. 3.2 Hz), but the number of binding exenatide molecule on the PLGA would be less because of the complex formation (Fig. 7). Although Zn^{2+} could not inhibit the hydrophobic adsorption of exenatide on PLGA greatly, the steric hindrance of the formed complex did restrain the nucleophilic attack of primary amine groups to PLGA, meanwhile it also blocked the adsorption site for the free exenatide to bind PLGA. Thus, the acylation could be greatly inhibited when Zn^{2+} was added in the system.

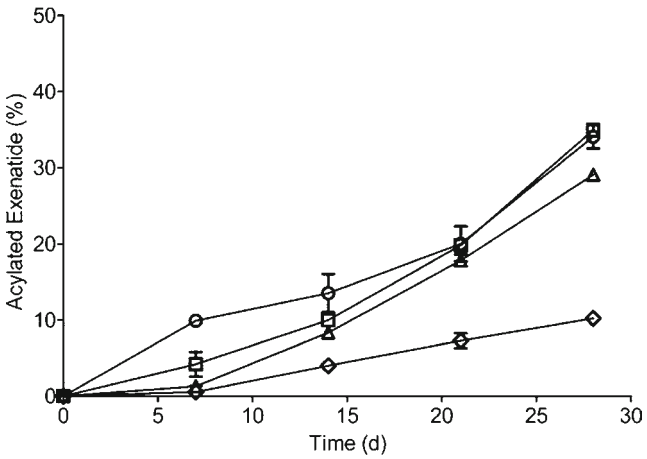

Fig. 8 Quantification of acylated exenatide in PLGA microspheres vs. time (mean ± SD, $n = 3$), no dication addition (*white circle*), Ca^{2+} (*white square*), Mn^{2+} (white triangle) and Zn^{2+} (*white diamond*).

Acylation Inhibition in Microspheres

To further determine whether the acylation inhibition still took effect in microspheres, exenatide-loaded PLGA microspheres were prepared by SPG membrane emulsification. Based on this technique, uniform-sized PLGA microspheres could be prepared, ensuring reproducibility and accuracy of the experiment.

As shown in Table 1, after addition of different dications in the formulation, all the microspheres with size of 20 μm possessed high encapsulation efficiency of peptide, and the encapsulation efficiencies of dication were also at the same level.

Exenatide was extracted from the microspheres after incubation. As shown in Fig. 8, in the early phase (0~7 days), Mn^{2+} and Zn^{2+} could greatly inhibit acylation, and Ca^{2+} showed weak inhibition. With incubation further proceeding, the inhibition effect of Ca^{2+} and Mn^{2+} both gradually got weaker, because the dications were also released companied with exenatide. At late phase (14~28 days), especially for Ca^{2+}, there was no difference on the ratio of formed acylated derivatives compared with the no addition group. Mn^{2+} still presented a weaker effect, and Zn^{2+} could significantly inhibit the acylation. Therefore, it was concluded that Zn^{2+} possessed high acylation inhibition effect for exenatide in PLGA microspheres.

CONCLUSION

Different dications were investigated to analyze their effects on the acylation of acidic peptide with PLGA. It was found that Ca^{2+} had no influence on the inhibition of acylation, which was opposite with previous work for basic peptide. Mn^{2+} had weak inhibition, but Zn^{2+} had the strongest effect. Combined with the analysis by QCM-D, the nature of the inhibition mechanism was demonstrated. Ca^{2+} did prevent the peptide from adsorption during the association phase but with more hydrophobic adsorbed peptide after dissociation. Mn^{2+} presented the same trend in the association phase but with very few adsorbed peptide after dissociation, suggesting most of the adsorption was reversible. While Zn^{2+} could form complex with exenatide and shield more binding sites, leading to less

acylated derivatives. Moreover, Zn^{2+} also took strong effect on acylation inhibition in exenatide-loaded PLGA microspheres. These promising results provide a guidance for acylation inhibition of other acidic peptides.

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REFERENCES

1. Qi F, Wu J, Fan QZ, He F, Tian GF, Yang TY, *et al.* Preparation of uniform-sized exenatide-loaded PLGA microspheres as long-effective release system with high encapsulation efficiency and biostability. Colloids Surf B. 2013;112:492–8.
2. Qi F, Wu J, Yang T, Ma G, Su Z. Mechanistic studies for monodisperse exenatide-loaded PLGA microspheres prepared by different methods based on SPG membrane emulsification. Acta Biomater. 2014;10(10):4247–56.
3. Lucke A, Fustella E, Tessmar J, Gazzaniga A, Gopferich A. The effect of poly(ethylene glycol)-poly(D, L-lactic acid) diblock copolymers on peptide acylation. J Control Release. 2002;80(1–3):157–68.
4. Schwendeman SP, Sophocleous AM, Zhang Y. A new class of inhibitors of peptide sorption and acylation in PLGA. J Control Release. 2009;137(3–4):179–84.
5. Lucke A, Kiermaier J, Gopferich A. Peptide acylation by poly(alpha-hydroxy esters). Pharm Res. 2002;19(2):175–81.
6. Zhang Y, Schwendeman SP. Minimizing acylation of peptides in PLGA microspheres. J Control Release. 2012;162(1):119–26.
7. Liu YJ, Schwendeman SP. Mapping microclimate pH distribution inside protein-encapsulated PLGA microspheres using confocal laser scanning microscopy. Mol Pharm. 2012;9(5):1342–50.
8. Qi F, Wu J, Hao D, Yang T, Ren Y, Ma G, *et al.* Comparative studies on the influences of primary emulsion preparation on properties of uniform-sized exenatide-loaded PLGA microspheres. Pharm Res. 2014;31(6):1566–74.
9. Freichels H, Pourcelle V, Auzély-Velty R, Marchand-Brynaert J, Jérôme C. Synthesis of poly(lactide-co-glycolide-co-ε-caprolactone)-graft-mannosylated poly(ethylene oxide) copolymers by combination of "clip" and "click" chemistries. Biomacromolecules. 2012;13(3):760–8.
10. Kim JY, Lee H, Oh KS, Kweon S, Jeon OC, Byun Y, *et al.* Multilayer nanoparticles for sustained delivery of exenatide to treat type 2 diabetes mellitus. Biomaterials. 2013;34(33):8444–9.
11. Liang R, Zhang R, Li X, Wang A, Chen D, Sun K, *et al.* Stability of exenatide in poly(d, l-lactide-co-glycolide) solutions: a simplified investigation on the peptide degradation by the polymer. Eur J Pharm Sci. 2013;50(3–4):502–10.
12. Yates EA, Terry CJ, Rees C, Rudd TR, Duchesne L, Skidmore MA, *et al.* Protein-GAG interactions: new surface-based techniques, spectroscopies and nanotechnology probes. Biochem Soc Trans. 2006;34:427–30.
13. Na DH, DeLuca PP. PEGylation of octreotide: I. Separation of positional isomers and stability against acylation by poly(D, L-lactide-co-glycolide). Pharm Res. 2005;22(5):736–42.
14. Murty SB, Goodman J, Thanoo BC, DeLuca PP. Identification of chemically modified peptide from poly(D, L-lactide-co-glycolide) microspheres under in vitro release conditions. AAPS PharmSciTech. 2003;4:E50.
15. Yin DF, Lu Y, Zhang H, Zhang GQ, Zou H, Sun DX, *et al.* Preparation of glucagon-like peptide-1 loaded PLGA microspheres: characterizations, release studies and bioactivities in vitro/in vivo. Chem Pharm Bull. 2008;56(2):156–61.
16. Kwak HH, Shim WS, Choi MK, Son MK, Kim YJ, Yang HC, *et al.* Development of a sustained-release recombinant human growth hormone formulation. J Control Release. 2009;137(2):160–5.
17. Na DH, Lee JE, Jang SW, Lee KC. Formation of acylated growth hormone-releasing peptide-6 by poly(lactide-co-glycolide) and its biological activity. AAPS PharmSciTech. 2007;8(2). Article 43.
18. Limson J, Odunuga OO, Green H, Hook F, Blatch GL. The use of a quartz crystal microbalance with dissipation for the measurement of protein-protein interactions: a qualitative and quantitative analysis of the interactions between molecular chaperones. S Afr J Sci. 2004;100(11–12):678–82.
19. Chen HB, Su XD, Neoh KG, Choe WS. QCM-D analysis of binding mechanism of phage particles displaying a constrained heptapeptide with specific affinity to SiO2 and TiO2. Anal Chem. 2006;78(14):4872–9.
20. van de Weert M, Hennink WE, Jiskoot W. Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm Res. 2000;17(10):1159–67.
21. Yu L, Li K, Liu XJ, Chen C, Bao YC, Ci TY, *et al.* In vitro and in vivo evaluation of a once-weekly formulation of an antidiabetic peptide drug exenatide in an injectable thermogel. J Pharm Sci. 2013;102(11):4140–9.
22. Li K, Yu L, Liu XJ, Chen C, Chen QH, Ding JD. A long-acting formulation of a polypeptide drug exenatide in treatment of diabetes using an injectable block copolymer hydrogel. Biomaterials. 2013;34(11):2834–42.
23. Qian F, Ni NN, Burton LS, Wang YF, Desikan S, Hussain M, *et al.* Sustained release subcutaneous delivery of BMS-686117, a GLP-1 receptor peptide agonist, via a zinc adduct. Int J Pharm. 2009;374(1–2):46–52.