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Nanoscaled buffering zone of charged (PLGA)_n-*b*-bPEI micelles in acidic microclimate for potential protein delivery application

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Abstract

Poly(lactide-*co*-glycolide) (PLGA) has most often been employed for the controlled release of protein formulations because of its safety profile with non-toxic degradation products. Nevertheless, such formulations have been plagued by a local acidic microenvironment and protein-polymer interactions, which result in chemical and physical denaturation of loaded proteins and often unfavorable release profiles. This study investigated the pH change of inner PLGA microsphere (MS) using charged (PLGA)_n-*b*-branched polyethyleneimine (bPEI) micelles. The designed micelles can be transformed into either micelle or reverse micelle (RM) depending on the solvent and RM can form microspheres. In addition, (PLGA)_n-*b*-bPEI can be modified into (PLGA)_n-*b*-(carboxylated bPEI) via carboxylation of the primary amines. Cationic micelle (CM) or anionic micelle (AM) were complexed with counter-charged proteins leading to nanosized particles (approximately 100 nm). In the micelle/protein complexes, the micelles mostly maintained their proton buffering capacity, and consequently, prevented or delayed the typical decrease in pH caused by degradation of PLGA in aqueous solution. Reconstitutable micelle/protein complexes allowed for increased and fine-tuned protein loading (~20 wt% when using CM1 (CM prepared from PLGA_{36kDa}-*b*-bPEI_{25kDa})/insulin complexes) in PLGA MS. In CM2 (CM prepared from (PLGA_{36kDa})₂-*b*-bPEI_{25kDa})/insulin (4 of weight ratio (WR) of micelle to protein; WR4)-loaded PLGA MS, CM2 strongly prevented the micellar nanoenvironmental pH (pH 6.6 within 5 days and then approximately pH 8.5) to be acidified in PLGA MS for 9 weeks, unlike CM2-free PLGA MS. In conclusion, our findings propose that the proton buffering capacity and protein loading in PLGA MS can be tuned by controlling the complexation ratios of micelles and proteins, polymeric architectures of (PLGA)_n-*b*-bPEI copolymers and WR of micelle/protein complexes and PLGA (or RM).

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Keywords

Charged Micelle; Microclimate pH; Microsphere; Nanoenvironmental pH; Poly(lactide-*co*-glycolide); Protein Delivery

1. Introduction

Poly(lactide-*co*-glycolide) (PLGA) microsphere (MS) has been favored as an injectable protein depot because of its degradability, biocompatibility and the repeated approval of many biomedical and pharmaceutical PLGA products by the FDA [1, 2]. However, proteins incorporated in PLGA MS were often exposed to unfavorable microenvironments with hydrophobicity and acidity [1]. The interactions of proteins with the hydrophobic surfaces of PLGA and other protein molecules mostly caused physical denaturation (*e.g.*, deformation and aggregation) [1]. Additionally, the acidity ($\sim$ pH 1.5–4) that results from degrading PLGA MS under physiological conditions [3–6] is responsible for poor release kinetics (*e.g.*, a triphasic release pattern and incomplete release) and some physical but mostly chemical denaturation (*e.g.*, deamidation, chain cleavage, and acylation) due to acid-catalyzed mechanisms [1, 7, 8].

To protect proteins in PLGA MS, conventional approaches include adding excipients. Surfactants (*e.g.*, poly(ethylene glycol), polysorbate, glycol chitosan, and poloxamer) and saccharides (*e.g.*, mannitol, trehalose, sucrose, and cyclodextrin) have been added to minimize protein-protein and protein-PLGA interactions [9–16]. For acidity-caused issues, basic divalent salts have been extensively used to dampen the acidity [17–19]. Schwendeman and his colleagues added a poorly soluble base (*e.g.*, magnesium hydroxide) into PLGA MS. This modification improved the release and stability of encapsulated proteins [17, 18]. However, too strong of a base (*e.g.*, calcium hydroxide) induced the formation of covalent protein aggregates as a result of alkali-induced thiol-disulfide exchange [19]. Such excipient approaches have helped protein protection to a certain degree in specific aspects, but the protection has been limited.

Charged polymers have been used to shield proteins having counter-charges and to form micron-sized polymer/protein aggregates [20–22] or nanocomplexes [22–24]. For example, ionic complexation of insulin with poly(methacrylic acid) or chondroitin sulfate at pH 3 showed enhanced insulin encapsulation in PLGA MS and improved insulin stability at the w/o interface [22, 23]. However, the polymers used in these approaches protected proteins from hydrophobicity but not from acidity, because there is no proton buffering capacity in weak acidic pH-neutral pH ranges.

Our previous studies applied poly(L-histidine) (polyHis) and oligo(vinylsulfadimethoxine) (OVSDM) as polyelectrolytes due to their proton buffering capacity in acidic pH ranges [25, 26]. Their block copolymers with poly(ethylene glycol) (PEG) had a pK_b (or pK_a) value around pH 7.0 [25, 27] and were complexed with their counter-charged proteins having low pIs (*e.g.*, insulin (pI 5.4) and bovine serum albumin (pI 4.9)) or high pI (*e.g.*, lysozyme (pI 10.9)) [28–31]. The polyelectrolyte/protein complexes significantly improved protein stability [28–31] and protein release kinetics [30, 31]. In addition, the strategy acidified the microclimate pH in PLGA MS [31]. However, the systems still demanded stronger binding properties with proteins, larger buffering capacities within the degrading PLGA environment and stability at neutral pHs.

To find polyelectrolytes that have a strong buffering capacity and ionic interactions with proteins, this study selected PLGA-*b*-branched polyethyleneimine (bPEI) copolymers recently designed for gene delivery [32]. Typically, bPEI shows a broad-range (pH 3–10)

proton-buffering region [32–34] due to the coexistence of primary, secondary and tertiary amines. At pH 7–7.5, approximately 20% of the amines in bPEI are ionized, and a one-unit adjustment in pH, within the range of pH 3–7, causes a 10% total change in the protonation of the amines [33]. Based on these facts, bPEI is ideal for preventing the acidification in PLGA MS and complexing with anionic proteins. In addition, the primary amines of bPEI were modified to carboxylic acids to interact with cationic proteins. Protein loading contents and bPEI's buffering powers were modulated by controlling feed ratios of PLGA and PLGA-*b*-bPEI during the construction of PLGA-based MS. Thus, this study assessed whether charged micelles interact with counter-charged proteins and maintain the proton-buffering capacity in the micelle/proteins and whether the micelle/protein complexes prevent PLGA acidification.

2. Materials and methods

2.1. Materials

PLGA_{36kDa} (Resomer®503H; approximate MW 36 kDa) and PLGA_{48kDa} (Resomer®504H; approximate MW 48 kDa) having a 1:1 (mole/mole) ratio of lactide:glycolide and a carboxylic group at one end were purchased from Boehringer Ingelheim Pharm GmbH & Co. KG (Germany). Human recombinant insulin, IgG, lysozyme, bPEI_{25kDa} (M_w 25 kDa; M_n 10 kDa), triethylamine (TEA), 4-dimethylaminopyridine (DMAP), *N,N'*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), succinic anhydride (SA), fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RITC), Ca²⁺-free and Mg²⁺-free Dulbecco's phosphate buffered saline (DPBS), 4-(2-hydroxy-ethyl)-1-piperazine (HEPES), poly(vinyl alcohol) (PVA), sodium hydroxide (NaOH), methylene chloride, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), d₂-methylene chloride (CD₂Cl₂), deuterium oxide (D₂O) and sodium deuteroxide (NaOD) were purchased from Sigma-Aldrich Co. (St. Louis, MO).

2.2. Synthesis and characterization of (PLGA)_n-*b*-bPEI_{25kDa} copolymers and their derivatives

As previously described [32], (PLGA)_n-*b*-bPEI_{25kDa} copolymers ($n=1, 2$) such as PLGA_{36kDa}-*b*-bPEI_{25kDa}, (PLGA_{36kDa})₂-*b*-bPEI_{25kDa}, and (PLGA_{48kDa})₂-*b*-bPEI_{25kDa} were synthesized by conventional condensation between the carboxylic acid group of PLGA and the amine groups of bPEI_{25kDa} as shown in Fig. 1(a). In detail, PLGA (50 μ mol or 100 μ mol for diblock or triblock copolymers, respectively) and bPEI_{25kDa} (50 μ mol) were dissolved separately in DMSO (100 mL) and stirred independently for 4 hr. Then, the two polymer solutions were mixed together with DCC (0.5 or 1 mmol for diblock or triblock copolymers, respectively), NHS (1 equivalent of DCC), and TEA (0.1 mL). Then, polymerization was carried out at room temperature (RT) for 48 hr with constant stirring. Approximately 100 mL of deionized water (DIW) was added to the reaction mixture and stirred for 2 hr; then, the polymer solution was transferred to a dialysis tube (MWCO 15 kDa) and dialyzed against DIW for 2 d to remove any unreacted bPEI_{25kDa}. Unreacted PLGA was removed by filtration at a later stage following micelle formation. The dialysis solution was lyophilized to obtain the resulting block copolymers. Their yields generally were above 90%. The chemical structure was confirmed by ¹H-NMR spectroscopy. As reported in our previous paper [32], the block ratios of PLGA to bPEI_{25kDa} were calculated from ¹H-NMR spectra after complete degradation of PLGA blocks by 1 N NaOH.

To introduce carboxylic acids into (PLGA_{36kDa})₂-*b*-bPEI_{25kDa}, the block copolymer (10 μ mol) was first dissolved in DMF (50 mL). Excess SA (5 equivalents of primary amines of bPEI_{25kDa}) and excess DMAP (1 equivalent of SA) were used for converting all primary amines into all carboxylic groups (Fig. 1(a)). Unreacted SA was removed by dialyzing the

block copolymer against DIW for 2 d using a MWCO 15 kDa dialysis membrane. The dialysis solution was lyophilized to obtain the resulting block copolymer, (PLGA_{36kDa})₂-*b*-(Carboxylated bPEI_{25kDa}). Its yield was almost 100%.

In addition, one (PLGA_{36kDa})₂-*b*-bPEI_{25kDa} polymeric molecule was labeled with one RITC and one FITC (as a feed) in DMSO. In brief, (PLGA_{36kDa})₂-*b*-bPEI_{25kDa} (2 μmol), RITC (2 μmol), and FITC (2 μmol) were dissolved in DMSO (10 mL) and reacted for 1 d at RT under a dark condition. Although the isothiocyanate groups of FITC and RITC were almost completely reacted with the primary amines of the block copolymer, dialysis of the block copolymer against DIW for 2 d using a MWCO 15 kDa dialysis membrane was performed to remove any possible unreacted fluorescent dyes. The dialysis solution was lyophilized to obtain the resulting block copolymer, (PLGA_{36kDa})₂-*b*-RITC&FITC-bPEI_{25kDa}.

2.3. Preparation and characterization of charged micelles and micelle/protein complexes

Charged micelles of block copolymers were prepared using standard dialysis techniques as previously described [32]. The micelles were lyophilized and then reconstituted by adding a HEPES solution (25 mM, pH 7.4) prior to use. Reconstituted micelles were mixed with proteins in HEPES buffer (25 mM, pH 7.4 for IgG and lysozyme or pH 8.5 for insulin) and incubated at RT for 30 min to form “fresh” micelle/protein complexes. The complexes were lyophilized and stored at -20°C. “Reconstituted” micelle/protein complexes were prepared by adding DIW to the lyophilized complexes. As characterized in Table 1, cationic micelles (CM) prepared from PLGA_{36kDa}-*b*-bPEI_{25kDa}, (PLGA_{36kDa})₂-*b*-bPEI_{25kDa}, and (PLGA_{48kDa})₂-*b*-bPEI_{25kDa} were called CM1, CM2, and CM3, respectively. Anionic micelles (AM) prepared from (PLGA_{36kDa})₂-*b*-(carboxylated bPEI_{25kDa}) were named as AM2. Protein-micelle complexes were characterized by the weight ratio (WR) of micelles to proteins (i.e. WR2 equals a micelle to protein weight ratio of 2:1).

The particle size and surface charge of the micelles and the micelle/protein complexes dispersed in HEPES solution (25 mM, pH 7.4) were measured at RT using a Zetasizer 3000HSA (Malvern Instruments Inc., Worcestershire, UK) with a fixed wavelength of 677 nm and a constant angle of 90°.

The proton buffering capacities of micelle/protein complexes were measured via traditional acid-base titration methods [26]. CM/insulin and AM2/lysozyme complexes (in DIW) prepared at WR2 (for CM1), WR4 (for CM2 and AM2), and WR6 (for CM3) were lyophilized and reconstituted in 10 mM NaCl (adjusted at pH 8.5) prior to use. The micelle/protein solution (1 mg/mL of protein; 2 mL) and the micelles solutions (2 mg/mL for CM1, 4 mg/mL for CM2 and AM2, and 6 mg/mL for CM3) were titrated with 0.1 N HCl at RT, and their pH changes were monitored. Their proton buffering capacities were compared at a pH range of 7.4-4 to determine the behavior of the complexes during acidification of PLGA MS. The buffering capacity was calculated as follows:

$$\text{Buffering Capacity} = \frac{\Delta V_{\text{HCl}} \times C_{\text{HCl}}}{m_{\text{bPEI}_{25\text{kDa}}}}$$

Here, ΔV_{HCl} is the volume of 0.1 N HCl, C_{HCl} is the concentration of HCl solution (0.1 N), and $m_{\text{bPEI}_{25\text{kDa}}}$ is the moles of amines in bPEI_{25kDa} or carboxylated bPEI_{25kDa} in the micelles. The estimated proton buffering capacities of the micelle/protein complexes were compared with those of the micelles and proteins.

2.4. Preparation and characterization of insulin- and micelle/insulin-loaded PLGA MS

PLGA-based MS was manufactured by a conventional double emulsion ($W_1/O/W_2$) method [1]. As an oil phase (O), PLGA_{36kDa} or (PLGA_{36kDa})₂-*b*-bPEI_{25kDa} (0.1 g) was dissolved or dispersed in methylene chloride (0.5 mL), respectively. Separately, protein drugs or micelle/protein complexes were dissolved or reconstituted, respectively in aqueous solution (25 μ L or 100 μ L, respectively; W_1). The two different phased solutions (O and W_1) were vortexed for 1 min, and then, the W_1/O emulsion in 2% PVA aqueous solution (50 mL; W_2) was re-emulsified with a homogenizer at 3000 rpm for 10 min. To evaporate the methylene chloride, the MS-containing solution was gently stirred for at least 4 hr at RT. The MS was rinsed four times with DIW to remove the PVA and then lyophilized.

Micelle/protein complexes for loading in MS were prepared from their lyophilized complexes. The lyophilized micelle/protein complexes (6 mg of protein) were reconstituted in DIW (6 mL). Then, the micelle/protein complexes were centrifuged at 3000 rpm for 5 min to reduce the volume of W_1 (0.1 mL). After the supernatant was discarded, about 83% of the micelle/protein complexes in DIW were retrieved. The concentrated micelle/protein complex (5 mg of protein) was used for MS preparation (100 mg of MS).

A modified two-step extraction method [35] was used to measure protein (*i.e.*, insulin) content in protein or micelle/protein-loaded MS. MS (10 mg) dissolved in acetonitrile (1 mL) was vortexed and then centrifuged at 13200 rpm for 10 min. After discarding the supernatant, 1 N NaOH (1 mL) was added to the precipitate. Then, the solution was incubated for 12 hr and was centrifuged at 13200 rpm for 10 min. The supernatant was neutralized with 1 N HCl. The protein concentration was measured with the BCATM protein assay. The Protein Loading Efficiency (%) and Protein Loading Content (wt%) were calculated using the follow equations:

$$\text{Protein Loading Efficiency (\%)} = \frac{m_{\text{protein,actual}}}{m_{\text{protein,theoretical}}} \times 100$$

$$\text{Protein Loading Content (wt\%)} = \frac{m_{\text{protein,actual}}}{m_{\text{MS,actual}}} \times 100$$

Here, m_x is the mass of x.

The morphology and size of the MS were evaluated with a scanning electronic microscope (SEM; Hitachi S-3000N). MS was mounted on carbon tape that was gold-coated by a sputter, and the samples were monitored at 3 kV. To observe the cross-sectional morphology of the MS, they were dispersed into a 20% agarose solution. After cooling, the MS-loaded gel was thin-cut and lyophilized. MS in the lyophilized film was monitored by SEM.

2.5. Prediction of micellar nanoenvironmental pHs in micelle solution and micelle/insulin-loaded PLGA MS

CM2, containing both FITC and RITC, was dispersed in DPBS at different pHs, and their fluorescent intensity ratios were monitored at 495 nm (Ex)/515 nm (Em) for FITC and 540 nm (Ex)/610 nm (Em) for RITC. In general, the maximal emission wavelength of RITC is 580 nm, but the RITC fluorescence at 580 nm showed very weak pH-induced change [36]. Thus, the emission of RITC fluorescence was monitored at 610 nm because of negligible pH effect, resulting in improved ratiometric pH sensitivity with pH-sensitive FITC. To predict local nanoenvironmental pHs around CM2, different pH solutions with both FITC and RITC were prepared.

To monitor micellar nanoenvironmental pH in micelle/insulin-loaded PLGA_{36kDa} MS, CM2/insulin (WR 4) was loaded into PLGA_{36kDa} MS. The MS (10 mg) was dispersed in

DPBS (10 mL; pH 7.4) and incubated at 37°C. The solution pH and fluorescent intensity of FITC and RITC were measured with a pH meter and a microplate reader, respectively. To compare CM2/insulin (WR4)-loaded PLGA_{36kDa} MS, the solution pH of PLGA_{36kDa} MS was monitored.

3. Results and Discussion

3.1. Characteristics of (PLGA)_n-b-bPEI_{25kDa} copolymers and their micelles

As shown in Fig. 1(a), (PLGA)_n-b-bPEI_{25kDa} copolymers were synthesized by a conjugation reaction between monocarboxylated PLGA (PLGA_{36kDa} or PLGA_{48kDa}) and bPEI_{25kDa}. The chemical structure was confirmed by ¹H-NMR spectroscopy [32]. The synthesized PLGA_{36kDa}-b-bPEI_{25kDa}, (PLGA_{36kDa})₂-b-bPEI_{25kDa}, and (PLGA_{48kDa})₂-b-bPEI_{25kDa} had 0.85, 1.93, and 2.38 PLGA blocks per bPEI_{25kDa}, respectively, and their bPEI_{25kDa} weight percentages were 24.6 wt%, 12.6 wt%, and 8.1 wt%, respectively (Table 1).

In aqueous solution, amphiphilicity of the block copolymers was used to generate the micellar structures via typical dialysis. The formed cationic micelles (CM) were structured with a hydrophobic PLGA core and a hydrophilic bPEI_{25kDa} shell (Fig. 1(b)) and structurally confirmed by their ¹H-NMR spectra in D₂O (Fig. 2). As previously reported, their ¹H-NMR spectra in a 1 N NaOD aqueous solution showed the characterized peaks of both the bPEI_{25kDa} and PLGA blocks [32]. However, when the (PLGA)_n-b-bPEI_{25kDa} copolymers were reconstituted in D₂O, their ¹H-NMR spectra predominantly showed peaks specific to the bPEI_{25kDa} block but none from the PLGA block because the solution NMR detects only soluble molecules (Fig. 2). The confirmed CM structure should be beneficial for forming ionic complexes with molecules or macromolecules with an anionic nature.

To evaluate the charged nature of CMs in aqueous solution, their surface charges were monitored. As shown in Fig. 3, the cationic character of the micelles was confirmed by a 15–35 mV surface charge. Additionally, their sizes were 60–130 nm in a diameter. CMs were lyophilized to avoid different batch-to-batch sizes and surface charges of fresh micelles and were reconstituted prior to use. No significant difference in size and surface charge of fresh CM and reconstituted CM was seen, as confirmed in our previous report [32]. However, CMs interact only with anionic proteins (AP) to form CM/AP complexes. To extend their applications to cationic proteins (CP), one candidate CM made of (PLGA_{36kDa})₂-b-bPEI_{25kDa} (CM2) was modified into an anionic micelles (AM2) via carboxylation of CM2's primary amines (Fig. 1(a)). The charge conversion caused the positive surface charge (approximately 35 mV) of CM2 to change to an overall negative surface charge (approximately –20 mV) for AM2, as shown in Fig 3.

In addition, it was investigated whether the block copolymers can generate reverse micellar structures, which should form a lipophilic PLGA shell and lipophobic bPEI_{25kDa} core in lipophilic solvents such as methylene chloride. As shown in Fig. 2, when the block copolymers were reconstituted in CD₂Cl₂, PLGA blocks were significantly detected but bPEI_{25kDa} blocks disappeared, unlike the ¹H-NMR spectra in D₂O. These results indicate that the block copolymers can form reversed micelles (RM) in organic solvents. When adding PLGA-b-bPEI block copolymers into methylene chloride, the block copolymer formed RM. As with PLGA, only RM generated MS via a W/O/W method.

3.2. Characteristics of micelle/protein complexes

As a negatively charged model protein, insulin with pI 5.4 and approximate weight of 6 kDa was selected due to its availability and therapeutic impact on diabetic patients. In HEPES solution (25 mM, pH 7.4), CM/insulin complexes were prepared by changing WRs of CM per insulin. As shown in Fig. 4(a) and 4(b), higher WRs formed smaller CM/insulin

complexes. The number-based size distribution of CM/insulin complexes clearly showed that micron-sized CM/insulin aggregates were changed into nanosized CM/insulin complexes with increasing CM doses per insulin (Fig. 4(b)). CMs, having a higher bPEI_{25kDa} content, demanded lower WRs to construct smaller CM/protein complexes due to different contents of bPEI_{25kDa} as protein-anchoring shells in CMs. CM/insulin complexes prepared with WR 2 for CM1, WR 4 for CM2 and WR>10 for CM3 showed similar particle sizes to each individual CM. In addition, most CM/insulin complexes showed slightly less or similar surface charges compared with cationic surface nature of each individual CM (Fig. 4(a)). However, when low WRs of CM per insulin were applied, the surface charges of CM/insulin complexes approached a neutral value (*e.g.*, WR 1 of CM2/insulin complexes) and then would become more negative ($WR \ll 1$). Like the CM cases [32], CM/insulin complexes also showed no significant size and surface charge difference between fresh and reconstituted complexes (Fig. 4(b) and 4(c)). The results suggest WR-dependent configurations of CM/insulin complexes, as depicted in Fig. 4(d). On exposing a few CMs to a large amount of anionic insulin ($WR \ll 1$), the insulin may anchor randomly inside and at the surface of micelles, resulting in a dynamic state of mixed surface charges. This, in turn, causes the formation of larger particles by electrostatic interactions between the insulin-loaded micelles. However, at $WR \gg 1$ (large amounts of CM with little insulin), the similar size and surface charge of the CM/insulin complexes and the CMs indicate that most of the insulin is buried in the bPEI_{25kDa} shell forest rather than being exposed to the shell surface of CM.

The extended applications of CM were examined by complexing them with larger sized proteins. A CM candidate, the reconstituted CM2 was electrostatically complexed with IgG (~150 kDa). Like CM2/insulin complexes, CM2/IgG complexes were micron-sized aggregates at WR 3, but nanoparticles approximately 150 nm in size with WR 6 (Fig. 5 and Fig. S1). Surface charges of CM2/IgG complexes (Fig. 5) showed similar trends to those of the CM2/insulin complexes (Fig. 4(a)). In addition, AM2 (a carboxylated CM2) can interact with a positively charged protein (*e.g.*, lysozyme 14.7 kDa, pI 10.9). The resulting AM2/lysozyme complexes were around 100 nm in diameter and had negative surface charges at WR 2 (Fig. 5 and Fig. S2). These findings may indicate that charged micelles can load counter-charged proteins at least 150 kDa in size without producing a significant change in size compared to the micelle.

When the CM formed complexes with anionic proteins (AP) at high WRs, their surface charges showed minimal changes compared to that of CMs. However, unlike CM/AP complexes, AM2/lysozyme complexes at high WR (*e.g.*, WR 5) showed a significant change in their surface charge compared with that of AM2. The results are unclear, and different chemical functional groups (*e.g.*, amines and carboxylic acid) of charged micelles may be relevant to the findings. Most primary amines of the bPEI_{25kDa} shell in CMs could be exposed on the shell surface. However, some succinic acid derivatives of the carboxylated bPEI_{25kDa} shell in AM2 could be folded or hidden by weak electrostatic attractions with secondary amines located at the inner area of the carboxylated bPEI_{25kDa} shell, resulting in a smaller surface charge of AM2 than expected. On adding CP (*e.g.*, lysozymes), these folded/hidden succinic acid derivatives could be exposed to the micellar surface to complex lysozymes due to exchange process of electrostatic interaction between secondary amines of carboxylated bPEI_{25kDa} shell and lysozymes with succinic acid derivatives.

In PLGA-based MS, micellar nanoprotection of proteins from local acidity would be induced by proton buffering capacity of the charged micelles. The buffering function of CM and AM could result from their protonable secondary and tertiary amines and was monitored by acid titration. Additionally, for AM, the carboxylic acids derived from primary amines can buffer pH acidification. To compare proton buffering capacities among CMs, CMs and

CM/insulin complexes containing similar amounts of bPEI_{25kDa} based on the wt% of bPEI_{25kDa} in the block copolymers (Table 1) were studied. CM's proton buffering capacities produced in pH ranges from pH 7.4 to pH 5.1 were 12.0% for CM1 (22.85 μmol of amines in 2 mL), 12.0% for CM2 (23.40 μmol of amines in 2 mL) and 12.2% for CM3 (22.57 μmol of amines in 2 mL) compared with that of bPEI_{25kDa} (17.2%), as previously reported (Fig. 6) [32]. After complexation with insulin, the proton buffering capacity of CM was monitored for changes. When the pH of insulin (2 mg in 2 mL of 10 mM NaCl) was dropped from pH 7.4 to pH 5.1 by titration with 0.1 N HCl, the insulin was consumed, ΔV_{HCl} (12 μL). If the complexation with insulin does not cause damage after proton buffering of CM, the CM/insulin complexes need 12 μL (0.1 N HCl) more than CM. However, as shown in Fig. 6 and Table S1, CM/insulin complexes consumed 4 μL more for CM1/insulin (WR 2), 6.5 μL more for CM2/insulin (WR 4) and 7 μL more for CM3/insulin (WR 6) compared to their counterpart CMs. The reduced consumption of 0.1 N HCl may indicate that CMs' proton buffering was slightly damaged upon insulin complexation.

In addition, the pH range 7.4-4.0 was investigated to evaluate which amines in bPEI_{25kDa} of CM contribute to its complexation with insulin. As shown in Table S1, ΔV_{HCl} (pH 7.4-7.0) and ΔV_{HCl} (pH 7.0-6.0) of the CM/insulin complexes were lower than the sum of the ΔV_{HCl} for CM and insulin in each pH range. However, in the ranges of pH 6.0-5.0 and pH 5.0-4.0, the sum of CM's ΔV_{HCl} and insulin's ΔV_{HCl} was almost the same as the ΔV_{HCl} of the CM/insulin complexes. These results suggest that the amines, which can buffer pH in the range of pH 7.4-6.0 are used to complex with insulin. Thus, the findings indicate that the electrostatic complexation between CM and insulin is caused by CMs' primary amines and in part, by their secondary amines.

Unlike CM2, AM2 has secondary amines, tertiary amines and carboxylic acids because only the primary amines of bPEI_{25kDa} in CM2 are carboxylated with succinic anhydride. The estimated amount of secondary and tertiary amines (14.81 μmol) and carboxylic acids (6.65 μmol) in AM2 (8 mg in 2 mL) is based on the amine distribution in bPEI_{25kDa} (1°:2°:3°=31%:39%:30%) [37]. Because of the coexistence of amines and carboxylic acids, the proton buffering capacity could not be calculated based only on amines. Thus, the proton buffering capacity of AM2 was estimated in acidic pH ranges compared with that of CM2. As shown in Table S1, ΔV_{HCl} (pH 7.4-7.0) of AM2 was lower than that of CM2 due to the reduced number of secondary amines (9.13 μmol for CM2 and 8.37 μmol for AM2) in 8 mg of micelles. However, although secondary and tertiary amines of AM2 were reduced compared to CM2, ΔV_{HCl} (pH 7.0-6.0), ΔV_{HCl} (pH 6.0-5.0) and ΔV_{HCl} (pH 5.0-4.0) of AM2 were higher than those of CM2. Especially, ΔV_{HCl} (pH 6.0-5.0) of AM2 was almost 3-fold higher than that of CM2. These results might be caused by carboxylic groups derived from succinic anhydride in AM2 because succinic acid has two pK_a 5.57 and 4.19. Upon adding 0.1 N HCl solution, ionized carboxylic groups could capture protons for their protonation. Especially, it is noted that ΔV_{HCl} (pH 6.0-5.0) of AM2/lysozyme was lower than the sum of ΔV_{HCl} (pH 6.0-5.0) for AM and lysozyme. This result might indicate that the complexation between AM2 and lysozyme partially consumed the carboxylic acids of AM2.

3.3. Micelle/protein complexes-loaded PLGA-based microspheres

As model systems, insulin and CM2/insulin complexes (WR 4) were introduced into PLGA_{36kDa} MS. As shown in Fig. S3, insulin-loaded PLGA_{36kDa} MS and CM2/insulin (WR 4)-loaded PLGA_{36kDa} MS were approximately 20–30 μm and 30–40 μm in diameter, respectively. When targeting an insulin content of 5 wt% in the MS, the actual insulin content was 4.68 ± 0.13 wt% (mean $\pm$ standard deviation; $n=3$) for the insulin-loaded PLGA_{36kDa} MS and 4.5 ± 0.21 wt% ($n=3$) for the CM2/insulin (WR 4)-loaded PLGA_{36kDa} MS, and their insulin-loading efficiencies were 93.6 ± 0.7 % and 90 ± 1.0 %, respectively.

The results are quite reasonable and in excellent yield when compared to other polyelectrolyte/protein complex-loaded PLGA MS (60–95% for protein-loading efficiency and 2–4 wt% for protein content in MS) [22, 24, 30, 31].

A MS loaded with 5 wt% protein would not be sufficient for long-term sustained protein delivery after a single injection. This issue proposes that the protein content in PLGA MS should be tuned or be as high as possible. However, protein loading is frequently limited by volume ratios of W_1 phase (for soluble insulin) and O phase, because high W_1/O volume ratios generally cause hollow or porous PLGA MS. That is, to maximize protein loading in MS, W_1 volume should be as small as possible when preparing polyelectrolyte/protein complexes. Under these conditions, large polyelectrolyte/protein complexes may form due to the highly concentrated solutions of polyelectrolyte and proteins. Unlike other polyelectrolyte/protein complexes, our micelle/protein complexes were reconstitutible without significant changes in their size and surface charge. This unique character allowed for the use of a minimal W_1 volume.

In our micelle/protein complexes, their WRs should be as low as possible or the bPEI_{25kDa} contents in the micelles should be as high as possible in order to maximize protein loading in the PLGA MS. To pursue this aim, CM1 was selected as a candidate micelle. Without an optimization process, CM1/insulin complexes (WR 1.5, total 50 mg) in an aqueous solution were mixed with PLGA_{36kDa} (35 mg) in methylene chloride to form CM1/insulin (WR 1.5)-loaded PLGA_{36kDa} MS. This trial (23.5 wt% of target insulin content in the MS) produced actual insulin content of 19.6 wt%. These results might suggest that protein-loading contents in MS could be tuned by controlling complexation ratios of micelles and proteins, polymeric architectures of (PLGA)_n-*b*-bPEI_{25kDa} copolymers and weight ratios of micelle/protein complexes and PLGA (or RM).

3.4. Monitoring nanoenvironmental pH

To investigate whether the corona (bPEI or carboxylated bPEI) of the designed micelles has a separate pH environment from the solution pH, CM2 was selected as a representative micelle. CM2 was dual-labeled with pH-sensitive FITC and pH-insensitive RITC because dual labeling with fluorophores having different pH sensitivities has been applied for monitoring the inner pHs of micron-sized materials (*e.g.*, intracellular pHs in cells) [38–41]. First, pH monitoring was investigated with fluorophores in aqueous solution and on micelles and then with fluorophore-labeled CM2 in PLGA MS.

First, FITC/RITC intensity ratios (F/R IR) of the fluorophore solution were measured as the pH was decreased. The fluorophore solution showed a sharp drop in the F/R IR in the pH range of 7.4–6.0 and then a slow change in the ratios below pH 6.0 (Fig. 7). However, the fluorophore-labeled CM2 in aqueous solution showed a somewhat higher F/R IR than the fluorophore solution at the same solution pH (Fig. 7). This result indicated that the pHs near CM2 were higher than the solution pH. Specifically, when CM2 was exposed to a solution at pH 4.0, the micellar nanoenvironmental pH of CM2 was approximately 6.0 (Fig. 7). These findings suggest that the bPEI corona has a proton buffering capacity with an isolated pH nanoenvironment that is segregated from the solution (or bulk) pH environment.

Maintenance activity of CM2's nanoenvironmental pH was evaluated by exposing CM2 to an initial pH of 7.4 at 37°C (Fig. 8). The solution pHs were sharply decreased to pH 6.2–6.3 within 10 days and then the pH value was maintained at 6.2–6.3 for 9 weeks. In aqueous solution, slow degradation occurs when PLGA is used as the micellar core. This PLGA degradation could cause decreased solution pHs over time [6, 17, 31]. For CM2, its F/R IR dropped from 1.6 to approximately 1.2 within 8 hr. This change means that CM2's nanoenvironmental pH was decreased from pH 7.4 to pH 7.0. The F/R IR was constant for

approximately 2 weeks and then decreased slightly to 0.9–1.0 (close to pH ~6.9) from weeks 3 to 9. This result might propose that CM2's strong buffering capacity maintains CM2's nanoenvironmental pHs and solution pHs.

To understand whether micelles complexed with proteins preserve their own nanoenvironmental pHs in PLGA MS, CM2/insulin complexes (WR 4) were loaded into PLGA_{36kDa} MS. The pH buffering of CM2/insulin (WR 4)-loaded PLGA_{36kDa} MS (1 mg/mL) was compared with that of PLGA_{36kDa} MS (1 mg/mL). When incubating PLGA_{36kDa} MS at 37°C, the solution pH continuously dropped to approximately 6.7 after 2 weeks and 4.4 after 9 weeks (Fig. 9). Its pH decrease was caused by PLGA degradation [6, 17, 31].

In the case of CM2/Insulin (WR 4)-loaded PLGA_{36kDa} MS, its solution pH slowly decreased to ~pH 6.8 within 2 weeks and held constant at pH 6.8 for 9 weeks (Fig. 9). This solution pH change came from the proton buffering activity of CM2 because CM2 can delay the acidification and degradation of PLGA MS. CM2's pH buffering seems to be superior to 3% MgCO₃-containing PLGA MS because, although they have similar MS sizes, it showed a continuous drop in solution pH over time and had an approximate solution pH of 6.4 for 5 weeks [5]. For inner pH buffering of PLGA MS having CM2/Insulin (WR 4), its F/R IR continuously dropped to 0.6 (close to pH ~6.6) within 5 days and then gradually recovered to ~1.1 (close to pH 6.9) after 2 weeks. Then, the F/R IR reached a constant value (estimated to around pH 8–8.5) for 9 weeks. This result may indicate that bPEI corona of CM2 can buffer the inner pH environment of PLGA MS because the PLGA microclimate pHs generally reached a pH of ~3–4 within 1 day and was maintained below pH 5 for 2 months [4]. In addition, CM2-induced pH buffering looks to be more powerful than MgCO₃-loaded PLGA MS [5]. However, the increasing nanoenvironmental pH in PLGA MS may be linked to insulin release from CM2/insulin complexes in PLGA_{36kDa} MS, resulting in the exposure of free primary amines having pK_a 9.5–11 [33].

Our findings support that our approach of incorporating proteins into protonable micelles in 'PLGA MS' creates a separate nano-environment that protects proteins from the microclimate of the degrading PLGA microspheres. The buffering power of the micelles prevented acidification in the micelle corona and maintained a pH within a range. This feature suggests a strong potential for detailed investigation of acid-catalyzed chemical reactions and denaturations, such as acylation, chain scission and deamidation. This approach also predicts the minimal intra- and inter-molecular interactions of insulin. The proposed system is unique and scalable for translational study. The micelles will be extended to long-term pDNA or siRNA delivery systems because nucleic acids can be degraded in strongly acidic environments. In addition, the micelles will provide extra room to load a second agent in its core, if necessary, which could suppress inflammatory reactions, foreign body reaction and fibrosis that are observed near PLGA MS implantation sites.

4. Conclusion

The goal of this study was to create an isolated buffering zone in PLGA MS using protonable and charged (PLGA)_n-*b*-bPEI micelles and simultaneously tune protein-loading contents using the charged micelles. Using the designed micelles, their counter-charged proteins were complexed, and the micelle/protein complexes were reconstitutable in the viewpoints of their size and surface charge. After complexing with proteins, the micelles strongly maintained their proton buffering capacities. When using the micelles with the highest bPEI_{25kDa} content, reconstitutable micelle/protein complexes reached approximately 20 wt% protein in PLGA MS. Finally, the micelle/protein complex-containing PLGA MS was able to buffer acidification of the inner pH in PLGA MS. The findings could propose

that the charged (PLGA)_n-*b*-bPEI micelles endow pH buffering for protein protection and have potential for high protein loading in PLGA MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
 (a) Synthetic scheme of (PLGA)_n-bPEI block copolymers and their carboxylated copolymers and (b) schematic representation of the structures of the block copolymers, micelles, micelle/protein complexes, and micelle/protein-loaded PLGA microspheres.

Fig. 2. ^1H -NMR spectra and micellar structures of $(\text{PLGA})_n$ - b - $\text{bPEI}_{25\text{kDa}}$ block copolymers in D_2O and CD_2Cl_2 .

Fig. 3. Size and surface charge of charged micelles derived from $\pm$ (PLGA)_n-*b*-bPEI_{25kDa} block copolymers.

Fig. 4. (a) CM- and WR-dependent hydrodynamic (Z_{avg}) particle sizes and surface charges of the CM/insulin complexes ($n=3$, Mean $\pm$ SD) and (b) number-based particle sizes ($n=3$, Mean $\pm$ SD), (c) surface charges of fresh and reconstituted CM2/insulin complexes ($n=3$, Mean $\pm$ SD), and (d) WR-dependent estimated configuration of CM/insulin complexes.

Fig. 5. Hydrodynamic particle sizes and surface charges of the CM2/IgG complexes and the AM2/Lysozyme complexes. (n=3, Mean $\pm$ SD)

Fig. 6. Acid titration curves of micelles (CM or AM) and their protein complexes (CM/insulin or AM/lysozyme). Protein concentration was 1 mg/mL. (n=3, Mean±SD)

Fig. 7.
Solution pH-dependent fluorophore intensity ratios of fluorophore solutions (FITC and RITC) and fluorophore-labeled CM2

Fig. 8. Time-dependent fluorophore intensity ratios, solution pH and estimated micellar pH of fluorophore-labeled CM2 incubated in DPBS (initial pH 7.4) at 37°C. (n=3, Mean±SD)

Fig. 9. Time-dependent fluorophore intensity ratios, solution pH and estimated nanoenvironmental pH of CM2/insulin (WR 4)-loaded PLGA_{36kDa} MS incubated in DPBS (initial pH 7.4) at 37°C. (n=3, Mean±SD)

Table 1

Characteristics of (PLGA)_n-*b*-bPEI₂₅kDa copolymers

	Code Name	Type of block copolymer	Feed block ratio of PLGA per bPEI ₂₅ kDa	Actual block ratio of PLGA per bPEI ₂₅ kDa ^a	M _n of PLGA- <i>b</i> -bPEI ₂₅ kDa copolymers	Weight percentage of bPEI ₂₅ kDa in PLGA- <i>b</i> -bPEI ₂₅ kDa copolymers ^b
PLGA ₃₆ kDa- <i>b</i> -bPEI ₂₅ kDa	CM1	Diblock	1	0.85	40.6 kDa	24.6 %
(PLGA ₃₆ kDa) ₂ - <i>b</i> -bPEI ₂₅ kDa	CM2	Triblock	2	1.93	79.5 kDa	12.6 %
(PLGA ₄₈ kDa) ₂ - <i>b</i> -bPEI ₂₅ kDa	CM3	Triblock	2	2.38	124.2 kDa	8.1 %

^a calculated by ¹H-NMR;

^b calculated based on M_n (10 kDa) of bPEI₂₅kDa.