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A charge-switched nano-sized polymeric carrier for protein delivery

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ABSTRACT

A novel synthetic nanocomplex was constructed from glycol chitosan (GCS) grafted with 2,3-dimethylmaleic anhydride (DMA) (denoted as 'GCD' hereafter) and lysozyme (isoelectric point = 10.9) as a model protein. This is a core-shell supramolecular assemble formed through electrostatic interactions between anionic GCD and cationic lysozyme at a pH 7.4. The pH-sensitivity of the nanocomplexes originates from the dissociation of DMA block from GCD at a slightly acidic pH (*i.e.*, pH 6.8), resulting in an increased electrostatic repulsion between cationic GCS and cationic lysozyme. This pH-induced charge switching of GCD provides a mechanism for triggered protein drug release from the nanocomplexes triggered by the small change in pH (pH 7.4–6.8).

1. Introduction

Proteins are, in effect, the main actuator of the most basic functions for cell life (Zhang, 2002). The specific biological function of proteins for a specific cell is one of the fundamental concepts for protein-based therapeutics (Mueller et al., 2009). This has inspired synthetic designs of various vehicles that can carry proteins to a purposed disease site (Walker et al., 1997; Maham et al., 2009; Lee et al., 2003, 2005, 2007a, 2008a; Oh et al., 2007, 2009; Oh and Lee, 2008; Chae et al., 2008). Although liposomes (Walker et al., 1997; Maham et al., 2009), polyionic complex (Lee et al., 2007c, 2009), microspheres (Lee et al., 2007b), and PEGylation (Chae et al., 2008) have increased the half-life of proteins and enhanced protein stability in the body, the lack of disease selectivity for pathologic tissues still remains unresolved. Therefore, recent efforts in the protein-carrier design have focused on providing advanced functionality to increase disease selectivity (Lee et al., 2008a,b; Lee and Youn, 2008; Schlossbauer et al., 2009).

Acidosis in specific cells is usually regarded as a phenotype of diseases such as neuromuscular diseases (*e.g.*, myasthenia gravis, amyotrophic lateral sclerosis, and muscular dystrophy) (Cesaroni et al., 2009), chronic obstructive pulmonary disease (COPD) (Chabot et al., 2009), rheumatoid arthritis (Paleolog and Fava, 1998), and solid tumors (Leeper et al., 1994; Engin et al., 1995; Ferrari, 2005). For example, the extracellular pH (pH_e) of clinical solid tumors ranges from 7.2 to 6.5 (Leeper et al., 1994; Engin et al., 1995; Volk et al., 1993) and rheumatic joints display a pH of approximately 6.0 (Paleolog and Fava, 1998), which is caused by anaerobic respiration and subsequent glycolysis (Leeper et al., 1994; Tannock and Rotin, 1989). Thus, the development of a novel protein-carrier that responds to the acidosis may be a favorable strategy to improve therapeutic efficacy of labile protein drugs.

Recently, Kataoka group developed a protein nanocarrier with charge-conversion polymer (*e.g.*, poly(L-aspartic acid) grafted with citraconic acid (CA)) (Lee et al., 2007c, 2009). The polyionic complex micelles (constructed with a charge-conversion polymer and a counter ionic protein at a pH 7.4) responded to endosomal acidic pH (*e.g.*, pH 5.5) and electrostatic repulsed (or released) protein resulting from a charge conversion of polymer from negative to positive. It is important to note that this system targeted realistic endosomal acidic pH.

The aim of this study is to develop a novel synthetic nanovehicle for delivering proteins into solid tumors or rheumatic joints with acidosis (particularly, pH 7.2–6.5). We preferentially investigated the pH-responsive protein-releasing behavior of a novel synthetic nanovehicle.

2. Materials and experimental design

2.1. Materials

Glycol chitosan (GCS, M_w = 250 kDa; degree of deacetylation = 82.7%), 2,3-dimethylmaleic anhydride (DMA), dimethylsulfoxide (DMSO), dimethylformamide (DMF), triethylamine (TEA), acetonitrile, trifluoroacetic acid, sodium azide, pyridine, fluorescamine, lysozyme (chicken egg white), and *Micrococcus lysodeikticus* (ATCC 4698 were purchased from Sigma–Aldrich (St.

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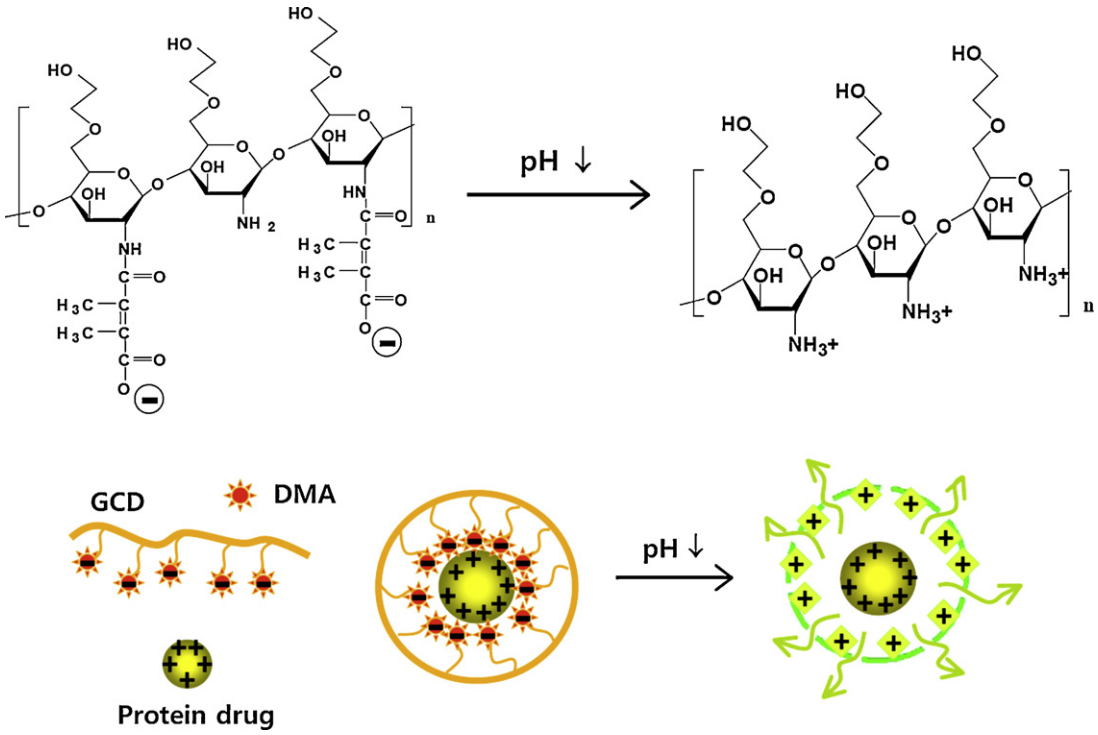


Fig. 1. Schematic presentation depicting the charge-switched mechanism of the GCD/Protein nanocomplex.

Louis, MO, USA). The BCA-protein assay kit was obtained from Pierce (Milwaukee, USA).

2.2. Synthesis of GCD

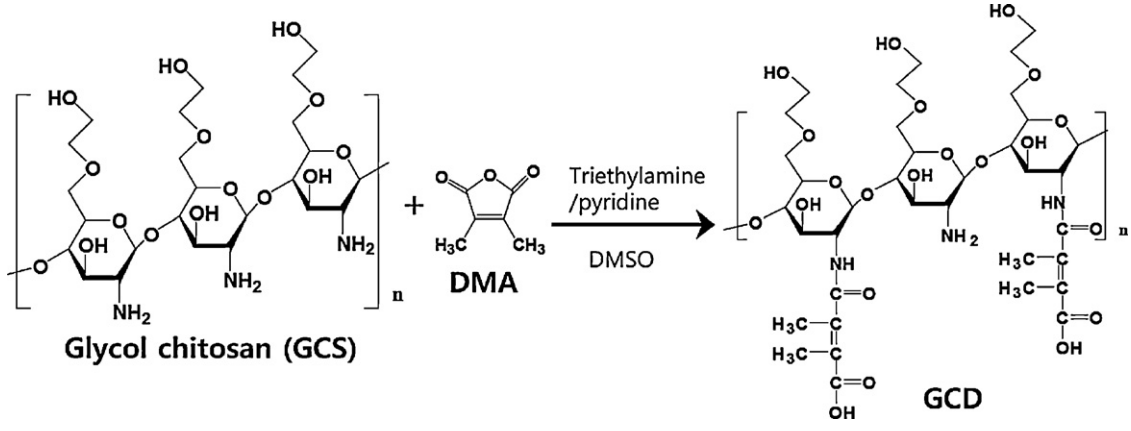
DMA (150 mg) was grafted to GCS (100 mg) in DMSO (5 ml) with TEA (0.5 ml) and pyridine (0.1 ml) at room temperature for 7 days. The resulting solution was dialyzed against pH 9.0 borate buffer (1 mM) for 3 days, followed by the lyophilization. The degree of substitution (DS, defined as the number of DMA blocks per 1 primary amine of GCS) was estimated from the ^{1}H NMR (DMSO-d_6 with TMS) peaks using the integration ratio of the peaks from δ 1.88 (–C**H**$_3$, DMA block) and δ 2.73 (–C**H**–, repeating sugar unite of GCS).

2.3. Preparation and characterization of nanocomplex

GCD and lysozyme with different weight ratios (GCD:lysozyme = 1–10:1) were dissolved in a HCl (or NaOH)–$Na_2B_4O_7$ buffer solution (pH 8.0, 1 mM) solution at room temperature. The resulting solution was vigorously mixing at 14,000 rpm for 30 s and then was mixed with phosphate buffer saline (PBS) solution (ionic strength = 0.15, pH 7.4–5.0).

Average particle size was measured by PCS, which was conducted 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°. The zeta potential of the nanocomplex solution (GCD 0.5 mg/ml, lysozyme 0.1 mg/ml) at different pHs (pH 7.4–5.0) was also measured using a Zetasizer 3000. In addition, the nanocomplex solution was exposed to different pH conditions (pH 7.4–5.0) at 37 °C for 24 h before measuring the particle size and the zeta potential.

The morphology of the nanocomplex (GCD 0.5 mg/ml, lysozyme 0.1 mg/ml) with pH (pH 7.4 and 6.8, ionic strength = 0.15) was confirmed by FE-SEM (Hitachi s-4800, Japan). The FE-SEM sample was prepared by casting a dilute nanocomplex solution (lysozyme 0.1 mg/ml) with pH 7.4 or pH 6.8 solutions on a slide glass, which was then dried *in vacuo*.



Scheme 1. Synthesis of GCD.

The complexation efficiency of lysozyme to GCD was measured using a BCA-protein assay kit. Briefly, the nanocomplex solution was exposed to 0.1N HCl for 4 h after dialyzing the nanocomplex solution for 1 day at pH 7.4 (ionic strength = 0.15) (in order to remove noncomplexed lysozyme). The obtained solution was analyzed using a BCA-protein assay kit. The complexation efficiency of lysozyme was approximately 80 ± 2%.

2.4. Release of DMA from the nanocomplex

GCD (1 mg) was dissolved in deionized water (1 ml). 100 μl of the resulting solution was mixed with 1 ml of PBS pH 6.8 (ionic strength = 0.15) and pH 7.4 (ionic strength = 0.15). This solution was incubated at 37 °C for 0–24 h and then mixed with 20 μl of fluorescamine (2.5 mg/ml) in DMF at room temperature for 30 min. The fluorescent intensity of each solution was measured using a Spectrofluorometer (Shimadzu RF-5301PC) at excitation wavelength 365 nm and at emission wavelength 465 nm. The positive control was estimated from the fluorescent intensity of GCD treated with 0.1 M HCl for 24 h and the negative control was deionized water.

2.5. Protein release test

Protein release from the nanocomplex (GCD 0.5 mg/ml, lysozyme 0.1 mg/ml) in PBS (ionic strength = 0.15, pH 7.4, 0.01 wt.% sodium azide) was measured using a BCA-protein assay kit. Briefly, the dialysis membrane tube (Spectra/Por MWCO 15K) containing a nanocomplex solution (1 ml) was immersed in a vial containing 10 ml of PBS solution adjusted to different pHs (pH 7.4, 6.8, 6.0). The release test of lysozyme from the nanocomplex was performed under mechanical shaking (120 rpm) at 37 °C. A small volume of the release medium was removed at each time point and analyzed with a microplate reader at 562 nm.

2.6. HPLC analysis

HPLC (Agilent 1100 series, USA) was used to confirm the degradation of lysozyme released from nanocomplexes. The mobile phase consisted of acetonitrile, distilled water, and trifluoroacetic acid (50:50:0.1, vol%). The flow rate was 1 ml/min and the injection volume was 10 μl. Lysozyme was detected at 220 nm.

2.7. In vitro bioactivity test

The *M. lysodeikticus* cell (ATCC 4698) solution (0.24 mg/ml, PBS pH 7.4 or pH 6.8 ionic strength = 0.15) was incubated with or without nanocomplexes (GCD 0.5 mg/ml, lysozyme 0.1 mg/ml). Photographic image of *M. lysodeikticus* cells suspension was obtained after 0–120 min incubation. Conventional red light was applied to the opposite side of the cell suspension before photographing.

3. Results and discussion

Fig. 1 shows the change in structure of a core charge-switched nanocomplex (180 nm size) consisting of a glycol chitosan derivative and protein at changing pH. Biodegradable glycol chitosan (GCS, M_w = 250 kDa, degree of deacetylation = 82.7%) was grafted with DMA (Scheme 1), which was used to construct the nanocomplex with a model protein, lysozyme (isoelectric point = 10.9). The degree of substitution (DS, defined as the number of DMA blocks per 1 primary amine of GCS) was 0.98, as determined by ^{1}H NMR. The different weight ratio of GCS grafted with DMA and lysozyme yielded nanocomplexes that were 180–300 nm in diameter (see Supporting Figure 1).

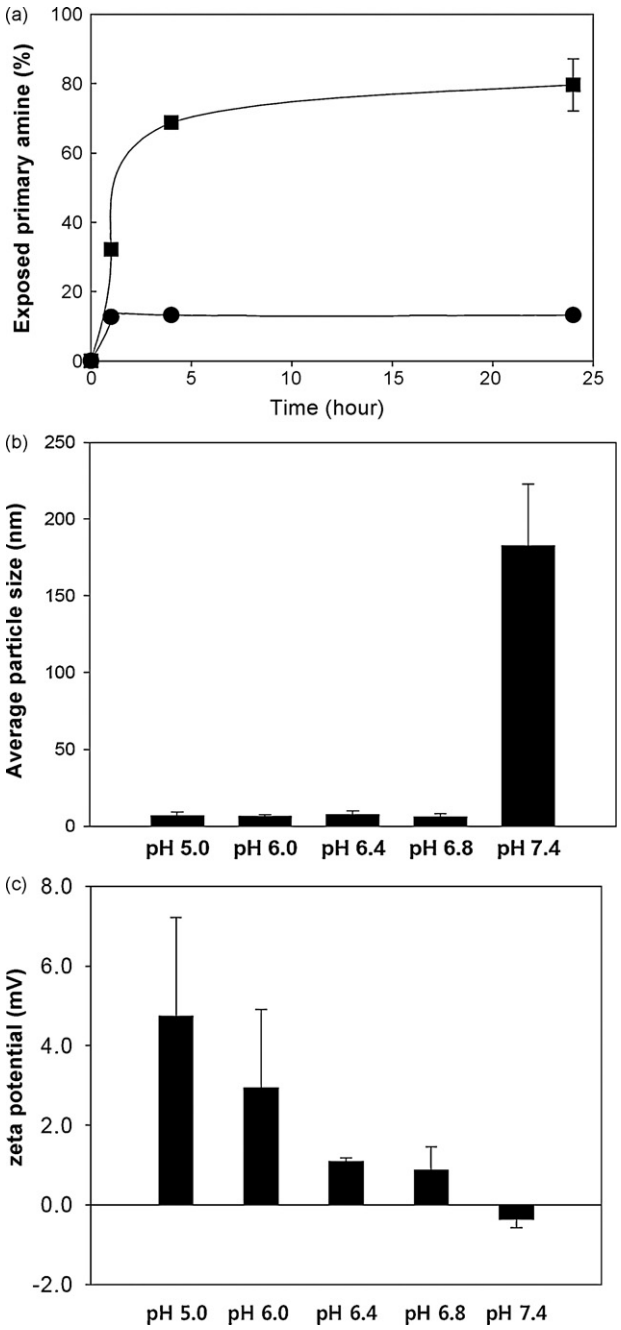


Fig. 2. (a) Exposed primary amine (%) on GCD (0.1 mg/ml) as a function of pH: pH 7.4 (●) and pH 6.8 (■). (b) Change in particle size of the GCD/lysozyme nanocomplex (GCD 0.5 mg/ml, lysozyme 0.1 mg/ml) at different pH values. (c) Zeta potential change of the GCD/lysozyme nanocomplex (GCD 0.5 mg/ml, lysozyme 0.1 mg/ml).

The construct was designed such that DMA-conjugated primary amine would be free primary amine at a slightly acidic pH (Oh et al., 2009). This dissociation mechanism of DMA may be due to the hydrolysis of an amide bond by a slightly acidic aqueous solution (Lee et al., 2007c; Oh et al., 2009). This would then result in a large extent of protein release from the nanocontainer, which may be caused by an increased electrostatic repulsion between GCS and lysozyme (Fig. 1).

To evaluate this proposed mechanism, the release rate of DMA from the nanocomplexes was calculated by measuring the primary amine concentration using a fluorescamine method at a wavelength of 465 nm. As shown in Fig. 2a, the exposed primary amine (%) of GCD increased sharply at pH 6.8 (*e.g.*, tumor pH_e). Approx-

imately 70% of DMA-conjugated primary amine was changed to free primary amine in the pH 6.8 solution (ionic strength = 0.15) within 4 h, while 87% of DMA-conjugated primary amine remained intact in the pH 7.4 solution (ionic strength = 0.15). This result is comparable with that obtained when citraconic acid-conjugated primary amine dissociates citraconic acid only at pH 5.5 (similar to endosomal/lysosomal pH) (Lee et al., 2009).

Interestingly, the magnitude of particle size changes of the nanocomplex with GCD was enormous at each pH; 180 nm in diameter at pH 7.4 and 6 nm at pH 6.8 (Fig. 2b). Based on these results, we hypothesized that the nanocomplexes are relatively stable at pH 7.4, but are decomplexed at pH 6.8.

Moreover, as the pH of solution decreased from pH 7.4 to 5.0, the zeta potential of nanocomplex increased from to −0.4 mV to +4.6 mV (Fig. 2c). This indicates that DMA-conjugated primary amines degraded back into cationic primary amines, which led to an increase in non-shielded free amine of the lysozyme residues.

Images obtained from FE-SEM reveal that nanocomplexes were finally decomposed at pH 6.8 (Fig. 3). Overall, these data suggest that our nanovehicles complex with proteins at normal pH and decomplex at a slight acidic pH condition. Thus, these novel nanovehicles hold great promise to selectively deliver proteins to various classes of cells undergoing acidosis.

In addition, we expect that a high DMA concentration of GCD will increase the stability of the nanocomplex during blood circulation due to the strong electrostatic interaction between the high molecular weight of GCD and lysozyme. Indeed, when the PBS concentration in the nanocomplex solution was increased up to 300 mM, the nanostructure of the GCD/lysozyme nanocomplexes was still persevered and particle size turn over was still observed at increasing pH (see Supporting Figure 2).

The following procedures were used to verify that proteins were released from the nanocomplexes at lower pH value. After vigorously shaking the GCD and lysozyme, the solution was transferred to a dialysis membrane tube (Spectra/Por MWCO 15K) immersed in a vial containing 10 ml of PBS solution at different pHs (pH 7.4, 6.8, 6.0). So the solution also contained unloaded free lysozyme. The lysozyme release test was performed under mechanical shaking (120 rpm) at 37 °C. Fig. 4a shows that approximately 30% of the lysozyme was released from the nanocomplex solution within 2 h at pH 7.4. Considering the complexation efficiency of lysozyme was approximately 80% (data not shown), it appears that only 10% of the lysozyme was released from the nanocomplexes at pH 7.4. However, over 90% of the lysozyme was rapidly released from the nanocomplex solution within 8 h at pH 6.0 and 70% was released within 24 h at pH 6.8. HPLC analysis was also performed to confirm that the lysozyme was not degraded after release from the nanocomplexes (Fig. 4b).

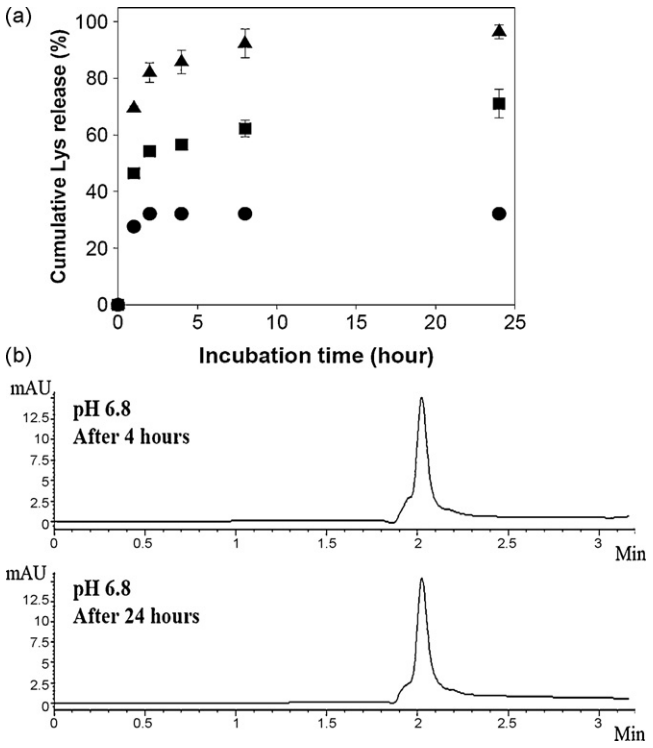


Fig. 4. (a) Cumulate release behavior of lysozyme from the GCD/lysozyme nanocomplex (GCD 0.5 mg/ml, lysozyme 0.1 mg/ml) over 24 h. The pH of the solution was adjusted to pH 7.4 (●), pH 6.8 (■), and pH 6.0 (▲). Each data point represents an average with standard deviation ($n = 3$). (b) HPLC assay of lysozyme released from GCD/lysozyme nanocomplex after 4 and 24 h.

Fig. 5 shows impressive contrast due to the bioactivity of lysozyme for the various cases. The *M. lysodeikticus* cell suspension (Lee et al., 2007b) was used to observe full lysozyme activity after the disintegration of the nanocomplexes at a slight acidic pH. After 30 min of incubation, lysozyme was rapidly released from nanocomplex at pH 6.8 accelerating the catalytic activity of the *M. lysodeikticus* cell suspension, resulting in increased transmit-

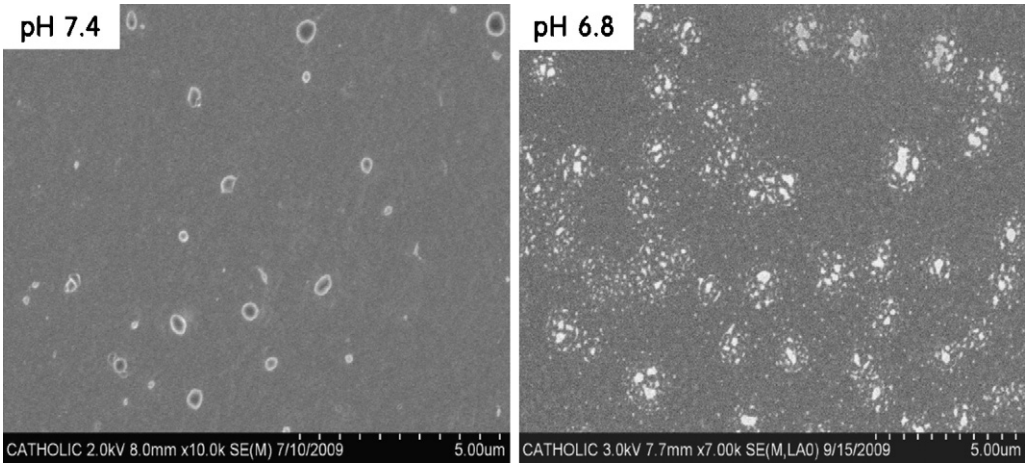


Fig. 3. FE-SEM images of the GCD/lysozyme nanocomplex (GCD 0.5 mg/ml, lysozyme 0.1 mg/ml) at pH 7.4 and pH 6.8. Each data point represents an average with standard deviation ($n = 3$).

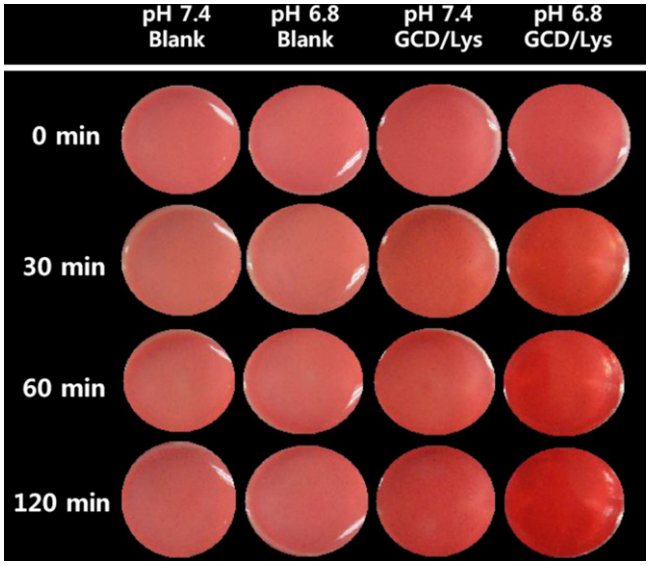


Fig. 5. Photographs of *Micrococcus lysodeikticus* cell suspension (0.24 mg/ml) when red light was emitted from the opposite side. The cell suspensions with GCD/lysozyme nanocomplex (GCD 0.5 mg/ml, lysozyme 0.1 mg/ml) or without nanocomplex (blank) were incubated at pH 7.4 or pH 6.8 for 0–120 min at 37 °C. The pH of the solution was adjusted to pH 7.4 or pH 6.8.

tance of red light emitted from the opposite side. This is comparable with that at pH 7.4 that red light was highly scattered by intact cell suspension.

4. Conclusion

Our core charge-switching nanocomplexes displayed an advanced protein-releasing mechanism when the pH was dropped from pH 7.4 to 6.8. This system was capable of distinguishing minute differences in pH, which may be very beneficial for selectively delivering shielded high molecular weight proteins into target site, as a 'Trojan horse'. Furthermore, this system can be used as a novel cellular imaging probe to detect cells undergoing acidosis. To confirm the potential use of this nanocomplexed vehicle, further *in vitro* evaluations and *in vivo* investigations are needed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2010.03.028.

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