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The regulation of DNA adsorption and release through chitosan multilayers

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ABSTRACT

To sustain transgene expression, chitosan was studied to immobilize DNA using layer-by-layer assembly to form polyelectrolyte multilayers (PEMs). Higher DNA concentrations and longer deposition periods demonstrated more DNA adsorptions to PEMs. By adjusting pH and the molecular weight of chitosan, PEM structures were manipulated. Chitosan molecules adsorption to PEMs increased when they were at pH 6 because of their low protonation. Furthermore, the configuration of chitosan favored a coiled-form when the pH was high, as the intramolecular repulsion decreased. Therefore, interdiffusion of polyelectrolytes in PEMs was promoted to increase DNA adsorption, especially for chitosan with high molecular weight. For the release experiments, because PEMs fabricated by lower pH chitosan owned less chitosan molecules, DNA release was enhanced. However, this phenomenon did not happen to chitosan with high molecular weight, which should be due to the entanglement between polymer chains. This comprehensive approach should be beneficial to substrate-mediated gene delivery applications.
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1. Introduction

Chitosan, the linear and typically 20% acetylated (1,4) linked 2-amino-deoxy-β-D-glucan, is isolated from marine chitin (Muzzarelli, 2012; Muzzarelli et al., 2012). Owing to its cationicity, chitosan lends itself to incorporate polyelectrolytes *via* layer-by-layer (LbL) technique (Bertrand, Jonas, Laschewsky, & Legras, 2000; Peyratout & Dahne, 2004). Through alternating stepwise coating of polyanions and chitosan, polyelectrolyte multilayers (PEMs) are deposited on biomaterial surfaces. These electrostatic self-assembly multilayers can be utilized, for example, to immobilize poly (styrene sulfonate) and silk fibroin on substrate to improve osteoblast adhesion (Cai, Hu, Jandt, & Wang, 2007). Anionic protein has been prepared as PEMs with chitosan/silicate for sustained protein delivery applications (Li et al., 2012). Chitosan has been used to tether DNA onto material surfaces in LbLs for biosensor applications (Liu & Hu, 2007) and for gene delivery (Mansouri et al., 2006; Roy, Mao, Huang, & Leong, 1999; Wang, Li, Liu, & Wang, 2010). Chitosan has no cytotoxicity as a point of difference from poly-L-lysine and polyethyleneimine (Han, Mahato, Sung, & Kim, 2000) and it protects plasmid DNA against degradation by DNase (Bao & Song, 2009). Chitosan is a good biomaterial for DNA protection and an appropriate carrier that facilitate gene transfer.

Although these examples suggest that gene delivery using chitosan in LbL assemblies is feasible, research concerning the control of DNA adsorption and release from PEMs is scarce. Nondestructive erosion is the main mechanism of DNA release from PEMs. Certain parameters such as pH (Wang, Sun, & Ji, 2011), ionic strength (Ren, Wang, Ji, Lin, & Shen, 2005), or redox potential gradients (Sato & Anzai, 2006), alter the interactions between DNA and polycations. For clinical requirements, changes in these factors must be suitable for applications in physiological environments, and the release rate should be appropriate. For example, the ionic strength and molecular weight of chitosan have been reported to control DNA adsorption during LbL assembly (Pedano et al., 2004). Therefore, we hypothesized that the composition of chitosan/DNA PEMs can be regulated by the assembly process, which may not only determine the adsorption but also the release of DNA. The influences of different parameters, such as the deposition periods,

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concentrations of DNA solutions, pH and molecular weight of chitosan, were thoroughly evaluated in this study.

2. Experimental

2.1. Materials

Chitosans with molecular weights of 5, 190–310, and 310–370 kDa were purchased from Sigma–Aldrich. Chitosan with a molecular weight of 15 kDa was purchased from Polyscience (Polyscience, PA, USA) and chitosan with a molecular weight of 10 kDa was purchased from Charming & Beauty Co. (Taiwan).

2.2. Plasmid DNA preparation

Plasmid DNA, pEGFP-C3 (Clontech, Mountain View, CA, USA), encoding green fluorescent protein (GFP), was used as a reporter gene for the *in vitro* experiments to determine delivery efficiencies. It was amplified in *Escherichia coli* DH5α. The amplified plasmid DNA was isolated by sodium hydroxide and then precipitated by polyethylene glycol (PEG) purification. Finally, restriction enzyme mapping, polymerase chain reaction (PCR) detection, and DNA sequencing were performed to determine the quality of purified plasmid DNA.

2.3. Preparation of chitosan and DNA solutions

Chitosans with different molecular weights were dissolved in 0.35 M sodium acetate buffer with final concentrations of 1 mg/ml. The final pH was adjusted by 2 N HCl. Plasmid DNA solutions were prepared in 20 mM Tris buffer containing 0.5 M NaCl and 1 mM EDTA at pH = 7. All solutions were filtered through 0.22 μm membranes before the LbL experiments.

2.4. Preparation of chitosan films

Chitosan was dissolved in 0.35 M acetic acid for a final concentration of 0.5 wt.%, which was added to 96-well multiplates at 50 μl per well. The plates were incubated at 37 °C overnight to evaporate the acetic acid solvent. Each well was thoroughly washed with ddH_2O before LbL experiments.

2.5. Fabrication of chitosan/DNA multilayered films

Chitosan films were successively dipped in chitosan and DNA solutions with volumes of 50 μl for 20 min of adsorption in each cycle and subsequently rinsed three times with ddH_2O between steps. These cycles were repeated until the desired bilayer number was achieved.

2.6. Fourier transform infrared (FTIR) spectroscopy

Infrared spectra of the chitosan film and chitosan/DNA multilayers were obtained using FTIR (FT/IR 410, Jasco, USA). The IR spectrum of the DNA was obtained using the KBr-pellet method (1 wt.% DNA in KBr).

2.7. In situ cell transfection

A human embryonic kidney cell line (HEK-293T) was used to evaluate gene transfer efficiency that cells were directly seeded on the chitosan/DNA multilayers with culture medium (DMEM with 10 vol.% FBS). In addition, nanoparticles of DNA complexed with chitosan were used as a control group. One hundred microliters of DMEM with 0.5 μg of DNA and 1.25 μg of chitosan (100 μl/well) was added to HEK-293T cells seeded in the chitosan-coated 96-well multiplates one day before transfection (Sato, Ishii, & Okahata, 2001). The expression of pEGFP-C3 was examined by fluorescent microscopy (Eclipse Ti-U, Nikon, Japan).

2.8. UV–vis spectrometry

The progressive buildup of DNA/chitosan multilayers on chitosan films was monitored using a UV–vis spectrometer (Epoch, Biotek, Winooski, VT, USA). Measurements were taken after each deposition step (Lang & Lin, 1999). The amount of deposited DNA was determined by the absorbance at 260 nm which is the maximal absorption peak of nucleic base chromophores while chitosan does not exhibit any absorbance.

2.9. Quartz crystal microbalance (QCM) analysis

Piezoelectric quartz crystals consisting a 9 MHz AT cut quartz slab with a layer of gold electrode on each side were obtained from ANT Technology (Taipei, Taiwan). The frequency variation was recorded by the Affinity Detection system (ANT, Taipei, Taiwan). The area of the quartz was 0.091 cm^2, and the oscillatory frequency change (ΔF) corresponds to a mass change of 0.391 ng.

Quartz chips were pre-treated with sulfuric acid and hydrogen peroxide for cleaning and then were thoroughly rinsed with ddH_2O and then air-dried. Chitosan solutions (0.5 wt.%) were coated with a volume of 50 μl on the QCM chip surfaces and then were incubated at 37 °C overnight to evaporate the acetic acid solvent. The chips were thoroughly rinsed with ddH_2O before measurement.

2.10. Atomic force microscopy

The morphologies of sample surfaces were illustrated by atomic force microscopy (AFM, SPA400 Seiko). Image capture was performed in wet PEMs in the tapping mode with a scan range of 10 μM × 10 μM.

2.11. Contact angle assessment

The water contact angles of chitosan/DNA multilayers were determined by the Drop Shape Analysis system (DSA10, Kruss GmbH, Hamburg, Germany). Four measurements per substrate were taken and the data were demonstrated as average with standard deviation.

3. Results and discussion

3.1. Characterization of layer-by-layer assembly of DNA/chitosan multilayered films

To stably maintain negative-charged nucleic acids on biomaterial surfaces, chitosan was used as cationic polymer for multilayer deposition. Infrared spectroscopy was used to confirm the functional groups of deposited multilayer molecules. Both chitosan and DNA illustrated their specific peaks in IR spectra (Fig. 1). Chitosan exhibited two characteristic absorption peaks at 1560 cm^{-1} and 1153 cm^{-1}, which were attributed to its amine groups and saccharide structures, respectively (Boonsongrit, Mueller, & Mitrevej, 2008). The peak adsorption of un-deacetylated amide groups was at 1654 cm^{-1} (Osman & Arof, 2003). The peaks of the DNA spectrum occurred at 1221 cm^{-1} and 1060 cm^{-1} and were ascribed to its antisymmetric and symmetric stretching of phosphate ester groups, respectively (Malins et al., 2005). Guanine residues exhibited stretching vibration of carboxyl and scissoring vibration of amine groups occurred at 1692 cm^{-1} (Malins et al., 2005). The spectrum of the DNA/chitosan multilayers fabricated by LbL assembly

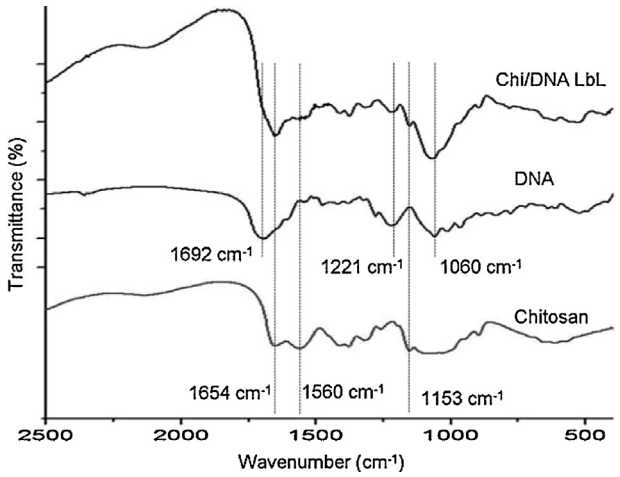

Fig. 1. The FT-IR spectra of chitosan (bottom), DNA (middle), and the chitosan/DNA build-up PEMs (top).

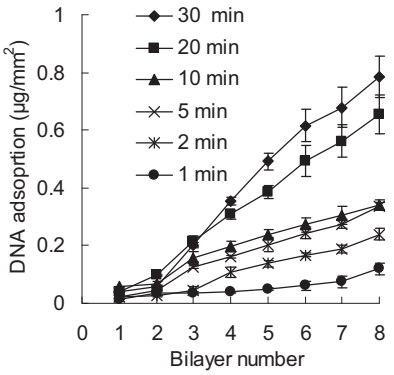

Fig. 3. The effect of deposition duration on DNA adsorption. The substrates were immersed in DNA and chitosan solutions in different durations each step from 1 to 30 min and the adsorbed of DNA was analyzed using UV spectrometry (chitosan: MW = 190–310 kDa, pH = 5) (n = 3).

on the chitosan film demonstrated characteristic spectral peaks of both DNA and chitosan absorption, suggesting that electrostatic interaction successfully immobilized DNA and chitosan on the substrate surfaces.

The transformed abilities of the DNA/chitosan multilayers were also evaluated. HEK 293T cells were cultured on chitosan/DNA multilayered films to demonstrate that DNA release was accessible. Ten DNA/chitosan bilayers were deposited and HEK 293T cells were directly seeded on the surface. GFP expression could be observed at day 3, was highest at day 5, and was still detectable at day 10, although expression was reduced (Fig. 2). In contrast, conventional administration using chitosan/DNA complexed nanoparticles illustrated the highest GFP expression at day 3. Green fluorescence decayed quickly and was undetectable at day 10. These results demonstrated that LbL assembly can immobilize DNA for *in situ* cell transfection, which can not only increase, but also elongate transgene expression.

The late expression of chitosan-mediated transfection has also been studied by Koping-Hoggard's group. They compared the intracellular trafficking of DNA carried by PEI or chitosan (Koping-Hoggard et al., 2001). For PEI/DNA delivery, the internalized endosomes ruptured at 24 h because PEI has wide range of buffer capacity which elicited the proton sponge effect to break the endosome. In contrast, chitosan/DNA complexes stayed in complete endosomes until 72 h post-transfection. Afterward, the degraded chitosan molecules increased osmolarity to promote DNA release from endosome. Therefore, the onset of eGFP expression in our study was at day 3.

3.2. The effect of deposition duration on DNA adsorption

DNA has a characteristic UV absorption peak at 260 nm; however, chitosan does not have any absorbance in this region, and therefore the amounts of DNA maintained on substrate surfaces in this study can be measured (Fig. 1s). First, the effect of the assembly time was examined. Different deposition periods of DNA and chitosan solutions on chitosan films were performed from 1 to 30 min (Fig. 3). Longer deposition times resulted in more DNA coated on the surfaces, suggesting that the equilibrium was not achieved if the deposition time was too short. Because incubation longer than 20 min did not show significant enhancement of DNA deposition ($p > 0.05$ for all bilayer number), 20 min was applied for both chitosan and DNA deposition in the following experiments.

3.3. The effect of DNA concentration on its deposition

To determine the relation between DNA concentrations and their deposition, different concentrations of DNA solution ranging from 0.1 to 1 g/L were used in LbL assemblies. The deposition of DNA increased with the number of bilayers linearly in all concentrations, and higher concentrations of DNA resulted in more DNA maintained on the substrates (Fig. 4a). Furthermore, increased

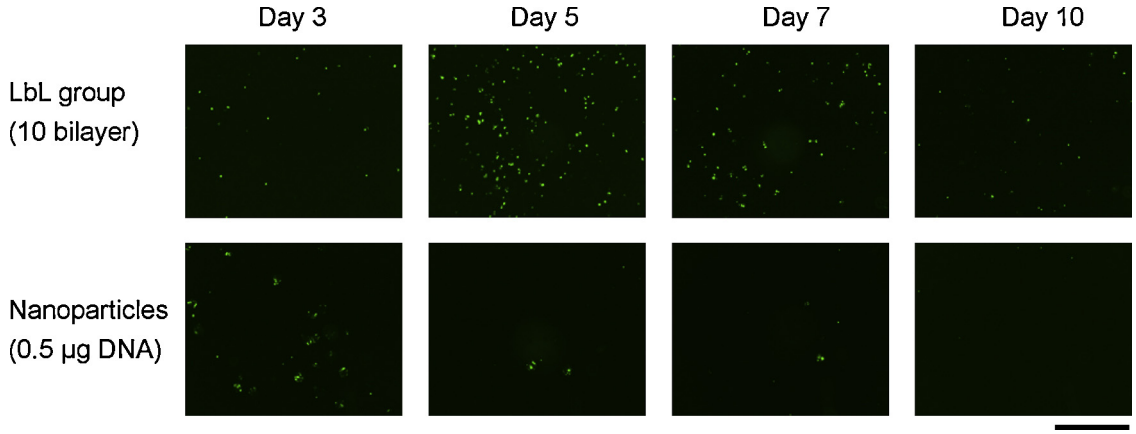

Fig. 2. The *in situ* transfection on LbL surfaces. HEK-293T cells were directly cultured on surfaces with 10 bilayers of DNA/chitosan deposition for *in situ* transfection. Chitosan and DNA complexed as nanoparticles were used as the control group to transfect HEK-293T cells which were seeded on chitosan-coated 96-well multiplates one day before transfection (chitosan: MW = 190–310 kDa, pH = 5) (scale bar = 1 mm).

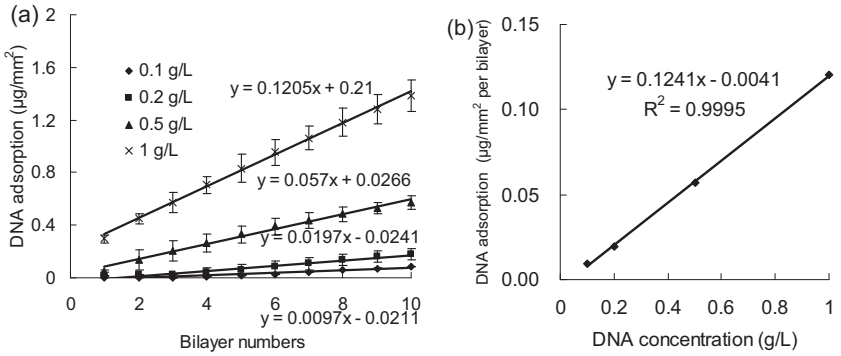

Fig. 4. The effect of DNA concentrations on their adsorption. (a) Different concentrations of DNA solutions from 0.1 g/L to 1 g/L were used for LbL assembly. The adsorption curves were linearly regressed by the least-squares method and the slopes of regression indicated the adsorptive DNA per bilayer. (b) The amount of DNA adsorption per bilayer increased with the concentrations of DNA solutions linearly (chitosan: MW = 190–310 kDa, pH = 5) (n = 3).

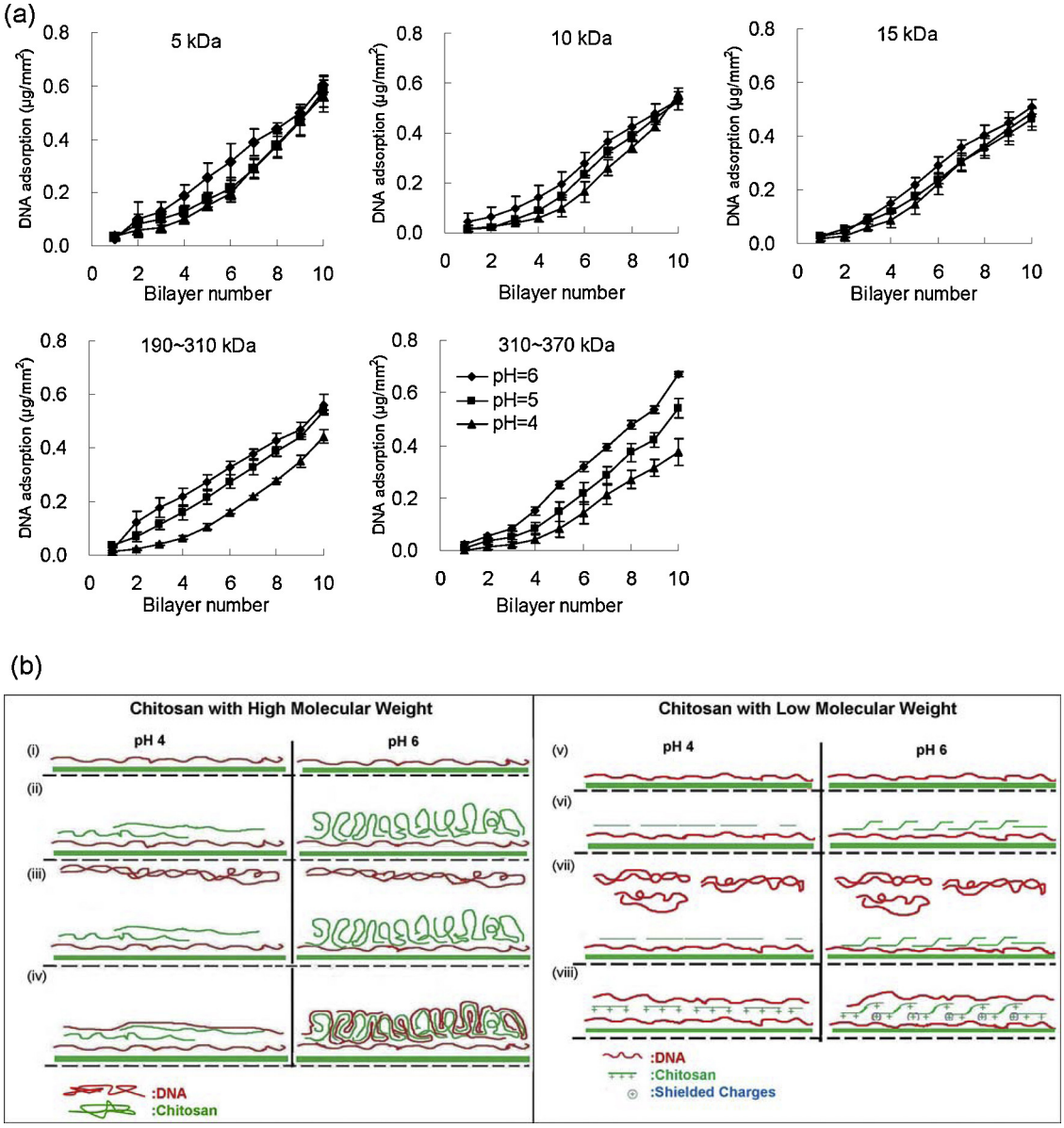

Fig. 5. The adsorption of DNA using chitosan solutions with different molecular weights at different pH values. (a) Chitosan with molecular weights of 5, 10, 15, 190–310, and 310–370 kDa at different pH values—pH 4 (triangle), pH 5 (square), and pH 6 (diamond)—were used to deposit with DNA. The adsorption of DNA was analyzed using UV spectrometry at each bilayer (n = 3). (b) The scheme of possible pH effect mechanisms of LbL assembly using chitosan with high or low molecular weights.

amounts of DNA deposited per bilayer linearly corresponded to the concentrations of DNA solutions, suggesting that the total amount of DNA on material surfaces can be simply controlled by the number of bilayers and DNA concentration (Fig. 4b). DNA solution with a concentration of 0.5 g/L was used in the following studies.

3.4. The effects of molecular weight and pH values of chitosan solutions on DNA deposition

The conditions of LbL assembly to control polyelectolyte deposition on material surfaces for drug delivery applications have been broadly studied. For PEMs fabricated with chitosan, Kujawa et al. have tried to immobilize chitosan and hyaluronan. Their results demonstrated that the adsorption of polyelectrolytes varies with the molecular weight of chitosan (Kujawa, Moraille, Sanchez, Badia, & Winnik, 2005). In addition, chitosan and polyglutamic acid solutions at different pH values have been used in LbL assembly (Song et al., 2009). Their results demonstrate that charge densities of weak polyelectrolytes differ according to the pH values of the polymer solutions, which highly affects the composition of PEMs. These studies suggest that molecular weights and pH values of polymer solutions may critically determine the conformation of polymer molecules and charge densities.

Different molecular weights of chitosan were dissolved in a sodium acetate buffer. To maintain the positive charges available on chitosan molecules, which have a pK_a of 6.5, the pH values of chitosan solutions were adjusted to 4, 5, and 6, separately (Fig. 5a). Different pH values did not cause significant differences in low molecular weight chitosan groups (5, 10, and 15 kDa). All groups maintained DNA deposition of approximately 0.5–0.6 μg/mm^2 during the assembly of 10 bilayers. In contrast, higher pH values caused greater DNA deposition in high molecular weight chitosan groups (190–310 and 310–370 kDa) in which the amounts of DNA after 10 bilayer depositions were pH 4 < pH 5 < pH 6.

The differences of adsorption behaviors between low- and high-molecular-weight chitosans may be due to changes in molecular conformations caused by varying pH. Chitosan molecules with high molecular weight (190–310 and 310–370 kDa) have relatively long backbones, which may result in random-coiled structures (Song et al., 2009). Equal surfaces of chitosan were coated to attract the same amount of DNA at the first layer (Fig. 5b-i). However, when the first layer of chitosan was deposited, pH values of the chitosan solution affected the charge density of the chitosan molecules (Fig. 5b-ii). Because the pK_a of chitosan is 6.5, the degree of protonation of chitosan was low at a high pH condition (*i.e.* pH 6), which made chitosan molecules more coiled. The coiled conformation may induce steric hindrance during adsorption due to shielding of some amines, thus requiring more adsorbed chitosan molecules to balance the negative charges on the surfaces (Fig. 5b-iii). Therefore, when the second layer of DNA was deposited, the extra positive charges from hidden amines would cause DNA to diffuse inward, *i.e.* interdiffusion (Fig. 5b-iv). This interdiffusion phenomena is due to unbalanced charges inside of the PEM, which may cause chitosan or DNA molecules to interpenetrate with each other (Zacharia, Modestino, & Hammond, 2007). In contrast, the degree of protonation of chitosan should be high at low pH conditions. The positive groups would repel each other and cause polymer chains to extend in linear conformations. Under these conditions, chitosan adsorption decreased (Fig. 5b-ii). Additionally, because these linear-form chitosan molecules were relatively rigid compared to coiled chitosan molecules at pH 6, the deposited chitosan layers were more compact causing a reduction in the interdiffusion of polyelectrolytes, and thus the layer-by-layer structure was maintained (Fig. 5b-iv). Because more chitosan molecules were deposited and interdiffusion of DNA was enhanced at high pH conditions, the amounts of DNA adsorption per layer were pH 4 < pH 5 < pH 6.

On the other hand, the polymeric chains of low-molecular-weight chitosan (5, 10, and 15 kDa) are small. Their conformations

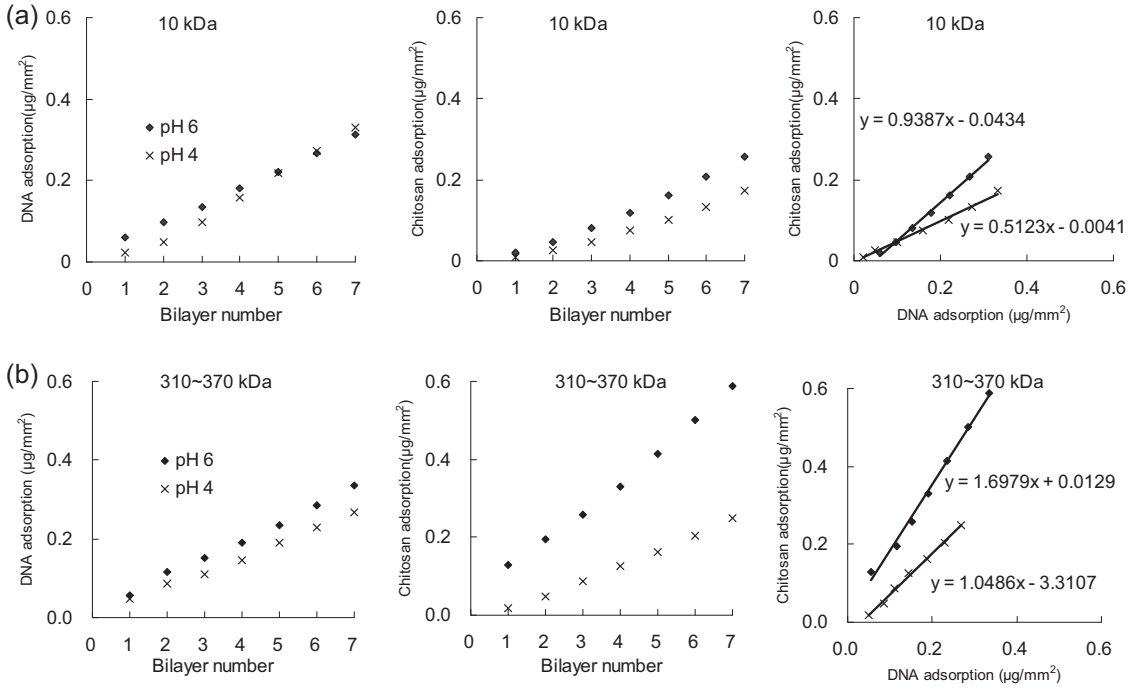

Fig. 6. QCM analysis of chitosan solutions at different pH values. Chitosan with molecular weight of (a) 10 kDa and (b) 310–370 kDa were assembled with DNA (0.5 g/L). Mass changes on the substrate were quantified by QCM. The adsorption of DNA and chitosan using chitosan solutions with pH 6 (diamond) and pH 4 (cross) were monitored continuously with different bilayer numbers. The correlation between DNA and chitosan deposition in different bilayers were also analyzed to determine the adsorption of chitosan per unit mass of DNA.

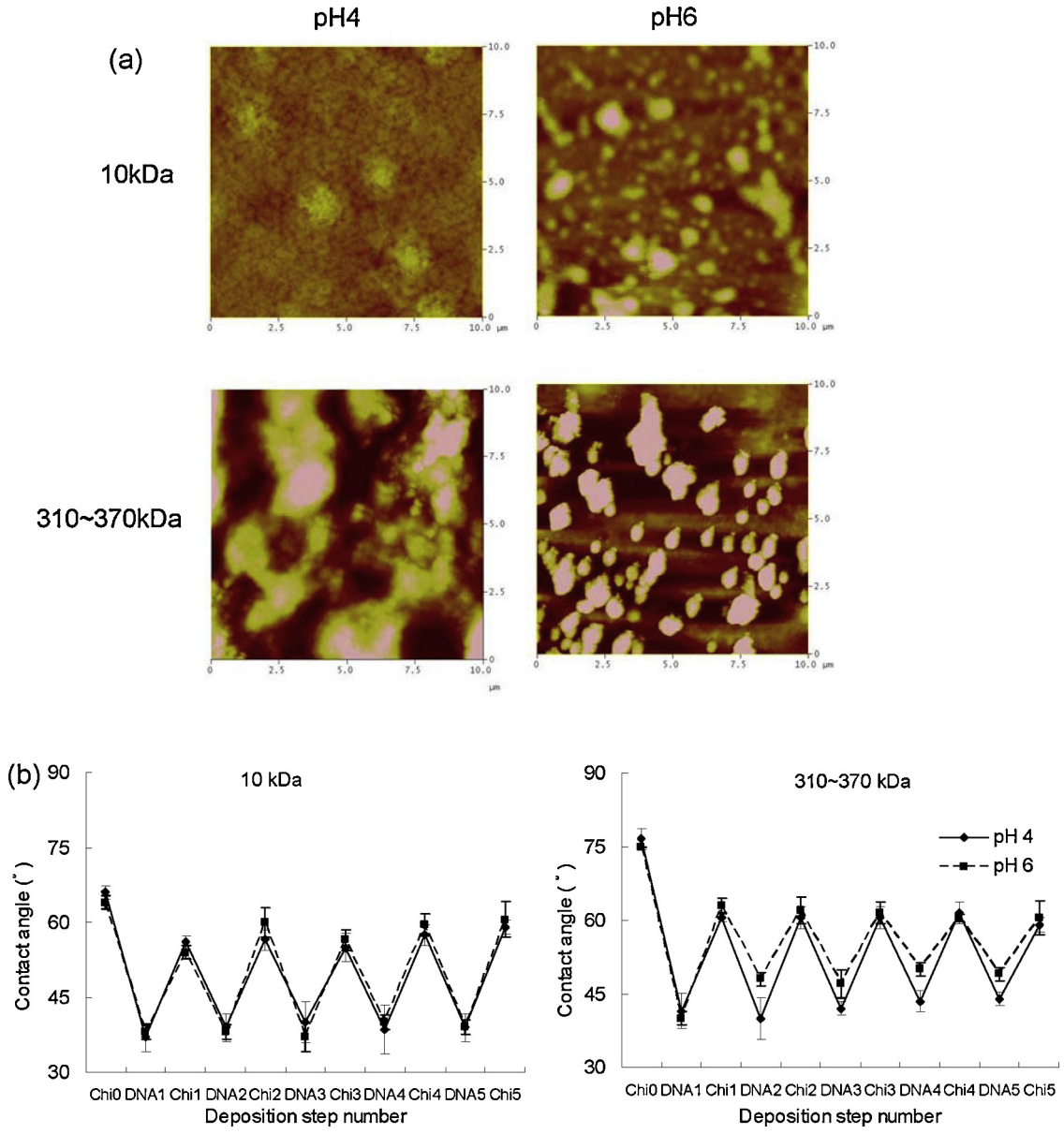

Fig. 7. Surface properties of LbL surfaces. (a) Atomic force microscopy (AFM) was applied to determine the surface morphologies of PEMs assembled by DNA and chitosans with molecular weights of 10 kDa and 310–370 kDa at pH 4 and pH 6. (b) Hydrophobicities of these film were also analyzed by water contact angle analysis ($n = 4$).

are relatively linear compared to high-molecular-weight chitosans, which reduces steric hindrance and electrostatic attractions between chitosan molecules and DNA. Like high-molecular-weight groups, low-molecular-weight chitosan solutions at different pH values exhibited different levels of protonation. Higher pH induced more chitosan adsorption (Fig. 5b-vi). The repelling force of amines in chitosan molecules may be reduced at high pH conditions; however, the conformation change was limited due to short chains. Compared to high-molecular-weight groups, with which interdiffusion may occur in loose polyelectrolyte distribution, low-molecular-weight chitosans were compacted so that their adsorptions were close to sheet-form depositions (Bieker & Schönhoff, 2010). Although some amines might be shielded at high pH conditions, because the project area was constant, the positive charges per area should be constant due to their 2D depositions (Fig. 5b-vii). Therefore, the amounts of DNA adsorption per layer were independent of the pH of chitosan solutions for low-molecular-weight groups (Fig. 5b-viii).

3.5. The pH effect on the composition of polyelectrolyte multilayers

To prove our hypothesis that DNA was stably maintained in multilayers by lower amounts of chitosan deposition at low pH, a QCM experiment was performed to determine the mass changes of DNA and chitosan deposition during LbL assembly. Chitosans with molecular weights of 10 and 310–370 kDa and with pH of 4 and 6 were examined. For the 10 kDa group, the total adsorptions of DNA and chitosan were increased with the steps continuously, and the amount of deposited DNA and chitosan in different bilayers were analyzed, respectively (Fig. 6a). As the result of the UV spectrometry, there were no significant differences of total DNA adsorption between pH 4 and pH 6. However, the deposition of chitosan at pH 4 was less than that at pH 6, and the differences increased with bilayer numbers. The correlation of DNA and chitosan deposition demonstrated that 1 $\mu g/mm^2$ of DNA deposition corresponded to 0.51 $\mu g/mm^2$ of chitosan deposition at pH 4, whereas DNA

deposition was almost equal to that of chitosan at pH 6. For the 310–370 kDa group, the adsorption of chitosan at pH 6 was also higher than that at pH 4 (Fig. 6b). The adsorption of DNA at pH 6 was slightly higher than that at pH 4, which was consistent with our UV spectrum results (Fig. 5a). The deposition ratio of chitosan to DNA at pH 4 and pH6 were 1.049 and 1.698, respectively, and these trends were similar to that of the 10 kDa group. These results suggest that chitosan molecules may equip more positive charges in an acidic environment, which resulted in less chitosan deposition.

Atomic force microscopy was also applied to illustrate the surface of deposited PEMs (Fig. 7a). Chitosan with molecular weight of 10 kDa at pH 4 displayed an extremely smooth surface after 7 bilayer depositions, and the smoothness slightly decreased at pH 6. In contrast, chitosan with molecular weight of 310–370 kDa exhibited bumpy surfaces. Large globular conformation was found at pH 4 and pH 6 conditions, which resulted in numerous sharp spikes on the surface. The *z* ranges (maximum height) of PEMs assembled by DNA and chitosans with molecular weight of 10 kDa at pH 4 and pH 6 as well as 310–370 kDa at pH 4 and pH 6 were 89, 150, 294, and 467 nm, respectively. The maximum height increased with pH values for both the 10 kDa and 310–370 kDa groups, suggesting that higher pH recruited more chitosan on the surfaces which enhanced surface roughness.

Contact angle analysis was used to investigate the change of surface properties with deposition processes (Fig. 7b). For the 10 kDa group, there were almost identical surface properties for both pH conditions: the chitosan ending films showed contact

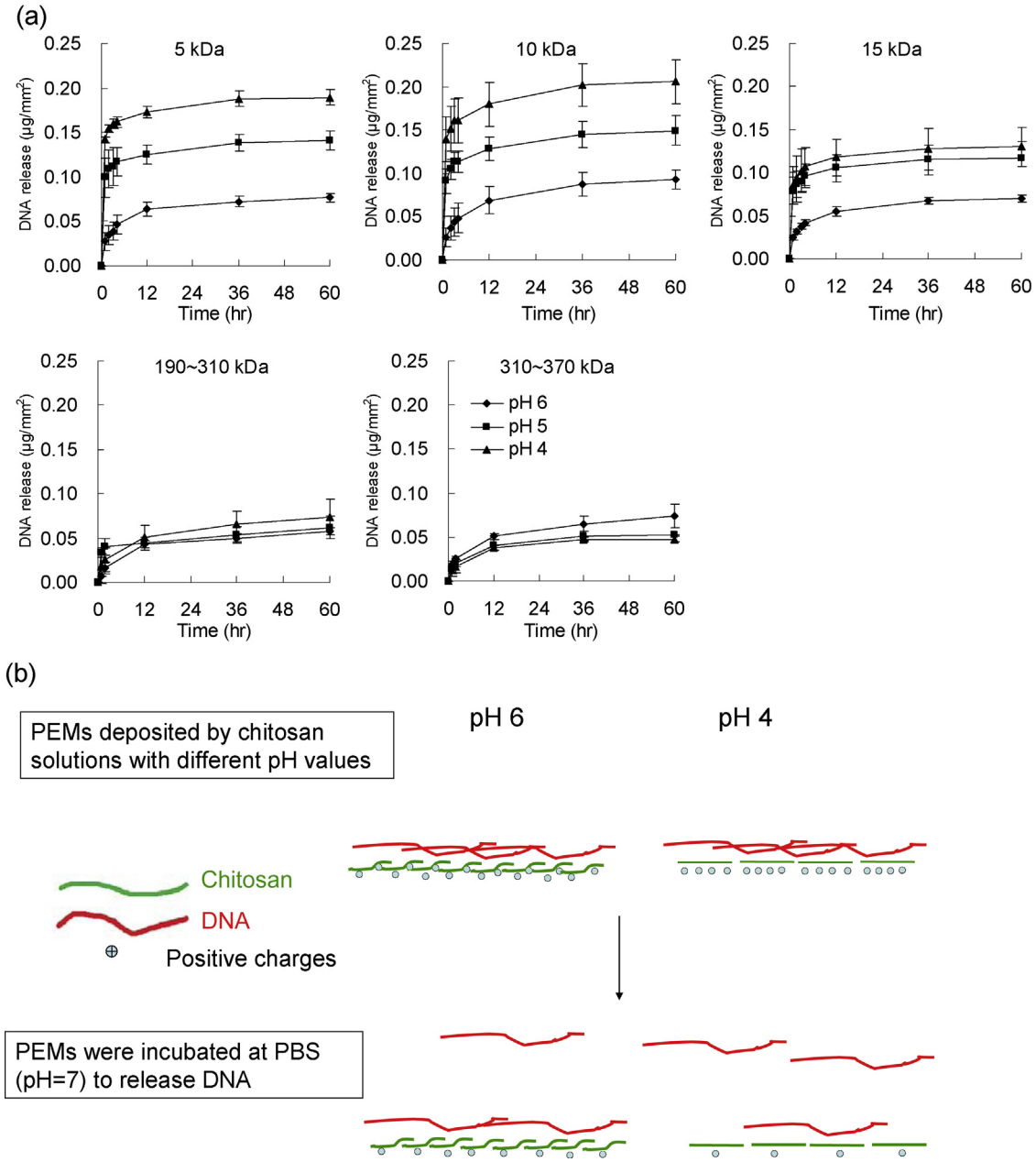

Fig. 8. The release of DNA from PEMs using chitosan solutions with different molecular weights at different pH values. (a) Chitosan with molecular weights of 5, 10, 15, 190–310, and 310–370 kDa at different pH values—pH 4 (triangle), pH 5 (square), and pH 6 (diamond)—were used to deposit with DNA for 10 bilayers. These PEMs were immersed in PBS at 37 °C and the release of DNA was sampled at different time points and analyzed using UV spectrometry ($n = 3$). (b) The scheme of possible mechanisms of DNA release from PEMs fabricated by chitosan solutions with different pH values.

angles around 60°, in contrast to 40° for the DNA ending films. The hydrophobicity of the surface alternatively changed with the upmost layer, indicating that the deposited chitosan and DNA should be assembled on material surfaces in a layered manner. For the 310–370 kDa group, the contact angles of the chitosan and DNA ending films were similar to those of the 10 kDa group when chitosan solutions at pH 4 were used for deposition. However, the deposition of chitosan solutions at pH 6 demonstrated a different wettability: contact angles of DNA ending films were raised to 50°, although the hydrophobicity of chitosan ending films were similar to those at pH 4. These results supported our hypothesis that high pH conditions made chitosan with high molecular weight take coiled forms. This would unbalance amines and may attract DNA diffusion into the chitosan layer, which should cause partial chitosan exposure on the surface to increase contact angles.

Similar results had been demonstrated by Bieker and Schönhoff's group: they found that the charge densities of polyelectrolytes changed with pH conditions, which further influenced PEM adsorption (Bieker & Schönhoff, 2010). In addition, Song et al. have fabricated chitosan/polyglutamic acid PEMs by LbL assembly, and their results demonstrated that the chitosan adsorption per layer increases with increasing pH, which exactly match our results (Song et al., 2009). These results all indicated that when the pH is closer to the pKa of the polyelectrolytes, weaker interaction between polyelectolytes results in more adsorption of polyelectrolyte for charge balance. Strong interdiffusion of polymers within the film thus occurs, leading to an exponential increase in film thickness with layer number.

3.6. *The effects of molecular weight and pH of chitosan solutions on DNA release*

Because pH may affect charges in weak polyelectrolytes, the structure of multilayers should be sensitive to the pH value during LbL assembly. Our DNA adsorption results demonstrated that the molecular weight and the pH of chitosan solutions may determine the compositions of PEMs. Next, these films would be investigated to determine if different compositions of PEMs regulate DNA release behaviors.

After DNA deposition on substrates by chitosan with different molecular weights and pH values, these multilayers were incubated in PBS at 37 °C to simulate their release in an *in vitro* environment (Fig. 8a). For molecular weights of 5 kDa and 10 kDa, DNA deposited by chitosan solutions with lower pH demonstrated higher release abilities: the release of pH 4, pH 5, and pH 6 groups were 0.18, 0.12, and 0.06 $\mu g/mm^2$, respectively. This effect should be due to chitosan molecules having more positive charges at lower pH, and thus the total number of chitosan depositions was less than that of the high pH group (Fig. 8b). When these multilayers were immersed in PBS, all of the groups were at the same pH condition (pH = 7). As the pKa of chitosan is 6.5, the neutral environment reduced positive charges of chitosan molecules and thus DNA cannot be stably maintained within the multilayers. Due to less chitosan molecules were deposited in low pH groups, the binding force of chtiosan was susceptible that more DNA molecules were released with time than that of high pH groups. However, this pH effect was reduced when the molecular weight of chitosan increased. For chitosan with molecular weight of 15 kDa, DNA release at pH 4 reduced to the same level of pH 5, around 0.12 $\mu g/mm^2$, whereas the DNA release at pH 6 was as low as that of the 5 kDa and 10 kDa groups. When chitosan molecular weight rose to 190–310 kDa and 310–370 kDa, the release behaviors were independent of pH conditions. These results should be due to greater entanglement between DNA and chitosans of higher molecular weight, which physically bound deposited DNA and chitosan within multilayers even when the positive charges of chitosan molecules were reduced.

In our transfection experiment, gene expression began at day 3 and the highest expression of cells on PEMs was at day 5 (Fig. 2). The release experiment demonstrated that the DNA delivery at this LbL system can be divided into 2 stages. The burst release happened at the first 6 h, but DNA was still gradually delivered until 60 h. The sustained delivery at the second stage should be the reason that the transgene expression on PEM was elongated.

4. Conclusions

In this study, we demonstrated that chitosan can immobilize DNA using a LbL method. The amounts of DNA adsorption are linearly correlated to the bilayer number and the concentrations of DNA solutions. Chitosan solutions with higher pH induce more DNA deposition during LbL assembly for high-molecular-weight chitosans because of their complicated conformation. Due to the reduction of chitosan charge densities with rising pH, the adsorption of chitosan increases to enhance interdiffusion of DNA and increases DNA amounts in PEMs. In contrast, the adsorption of DNA is independent to the pH conditions for chitosan with low molecular weight because these chitosan molecules are short and the deposition is likely in a sheet format. Due to less chitosan deposition using lower pH chitosan solutions, more DNA is released at neutral conditions because of weaker electrostatic forces. However, because interdiffusion of DNA in chitosan occurs in high-molecular-weight groups, entangled polymer chains may still physically tether DNA, and thus DNA release is independent of the pH of chitosan solutions during PEMs fabrication. These results provide a comprehensive reference for DNA/chitosan multilayers deposition using LbL assembly and should be useful in applications of *in situ* gene delivery.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.08.088.

References

Bao, J. B., & Song, C. X. (2009). Chitosan chip and application to evaluate DNA loading on the surface of the metal. *Biomedical Materials*, *4*(1), 011002.

Bertrand, P., Jonas, A., Laschewsky, A., & Legras, R. (2000). Ultrathin polymer coatings by complexation of polyelectrolytes at interfaces: Suitable materials, structure and properties. *Macromolecular Rapid Communications*, *21*(7), 319–348.

Bieker, P., & Schönhoff, M. (2010). Linear and exponential growth regimes of multilayers of weak polyelectrolytes in dependence on pH. *Macromolecules*, *43*(11), 5052–5059.

Boonsongrit, Y., Mueller, B. W., & Mitrevej, A. (2008). Characterization of drug–chitosan interaction by ^{1}H NMR, FTIR and isothermal titration calorimetry. *European Journal of Pharmaceutics and Biopharmaceutics*, *69*(1), 388–395.

Cai, K., Hu, Y., Jandt, K. D., & Wang, Y. (2007). Surface modification of titanium thin film with chitosan via electrostatic self-assembly technique and its influence on osteoblast growth behavior. *Journal of Materials Science: Materials in Medicine*, *19*(2), 499–506.

Han, S., Mahato, R. I., Sung, Y. K., & Kim, S. W. (2000). Development of biomaterials for gene therapy. *Molecular Therapy*, *2*(4), 302–317.

Koping-Hoggard, M., Tubulekas, I., Guan, H., Edwards, K., Nilsson, M., Varum, K. M., et al. (2001). Chitosan as a nonviral gene delivery system. Structure–property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. *Gene Therapy*, *8*(14), 1108–1121.

Kujawa, P., Moraille, P., Sanchez, J., Badia, A., & Winnik, F. M. (2005). Effect of molecular weight on the exponential growth and morphology of hyaluronan/chitosan multilayers: A surface plasmon resonance spectroscopy and atomic force

microscopy investigation. *Journal of the American Chemical Society*, *127*(25), 9224–9234.
Lang, J., & Lin, M. H. (1999). Layer-by-layer assembly of DNA films and their interactions with dyes. *Journal of Physical Chemistry B*, *103*(51), 11393–11397.
Li, W., Xu, R. F., Zheng, L. Q., Du, J., Zhu, Y. L., Huang, R., et al. (2012). LBL structured chitosan-layered silicate intercalated composites based fibrous mats for protein delivery. *Carbohydrate Polymers*, *90*(4), 1656–1663.
Liu, Y., & Hu, N. F. (2007). Loading/release behavior of (chitosan/DNA)(n) layer-by-layer films toward negatively charged anthraquinone and its application in electrochemical detection of natural DNA damage. *Biosensors & Bioelectronics*, *23*(5), 661–667.
Malins, D. C., Gilman, N. K., Green, V. M., Wheeler, T. M., Barker, E. A., & Anderson, K. M. (2005). A cancer DNA phenotype in healthy prostates, conserved in tumors and adjacent normal cells, implies a relationship to carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(52), 19093.
Mansouri, S., Cuie, Y., Winnik, F., Shi, Q., Lavigne, P., Benderdour, M., et al. (2006). Characterization of folate–chitosan–DNA nanoparticles for gene therapy. *Biomaterials*, *27*(9), 2060–2065.
Muzzarelli, R. A. A. (2012). Nanochitins and nanochitosans, paving the way to eco-friendly and energy-saving exploitation of marine resources. *Polymer Science: A Comprehensive Reference*, 153–164.
Muzzarelli, R. A. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M., & Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydrate Polymers*, *87*(2), 995–1012.
Osman, Z., & Arof, A. K. (2003). FTIR studies of chitosan acetate based polymer electrolytes. *Electrochimica Acta*, *48*(8), 993–999.
Pedano, M. L., Martel, L., Desbrieres, J., Defrancq, E., Dumy, P., Coche-Guerente, L., et al. (2004). Layer-by-layer deposition of chitosan derivatives and DNA on gold surfaces for the development of biorecognition layers. *Analytical Letters*, *37*(11), 2235–2250.
Peyratout, C. S., & Dahne, L. (2004). Tailor-made polyelectrolyte microcapsules: From multilayers to smart containers. *Angewandte Chemie-International Edition*, *43*(29), 3762–3783.
Ren, K. F., Wang, Y. X., Ji, J., Lin, Q. K., & Shen, J. C. (2005). Construction and deconstruction of PLL/DNA multilayered films for DNA delivery: Effect of ionic strength. *Colloids and Surfaces B-Biointerfaces*, *46*(2), 63–69.
Roy, K., Mao, H. Q., Huang, S. K., & Leong, K. W. (1999). Oral gene delivery with chitosan–DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nature Medicine*, *5*(4), 387–391.
Sato, H., & Anzai, J. (2006). Preparation of layer-by-layer thin films composed of DNA and ferrocene-bearing poly(amine)s and their redox properties. *Biomacromolecules*, *7*(6), 2072–2076.
Sato, T., Ishii, T., & Okahata, Y. (2001). In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. *Biomaterials*, *22*(15), 2075–2080.
Song, Z. J., Yin, J. B., Luo, K., Zheng, Y. Z., Yang, Y., Li, Q., et al. (2009). Layer-by-layer buildup of poly(L-glutamic acid)/chitosan film for biologically active coating. *Macromolecular Bioscience*, *9*(3), 268–278.
Wang, D., Li, H., Liu, B., & Wang, X. (Eds.). (2010). *Chitosans: New vectors for gene therapy*. Hauppauge, NY: Nova Science Pub Inc.
Wang, X. F., Sun, J. K., & Ji, J. A. (2011). pH modulated layer-by-layer assembly as a new approach to tunable formulating of DNA within multilayer coating. *Reactive & Functional Polymers*, *71*(3), 254–260.
Zacharia, N. S., Modestino, M., & Hammond, P. T. (2007). Factors influencing the interdiffusion of weak polycations in multilayers. *Macromolecules*, *40*(26), 9523–9528.