Available online at www.sciencedirect.com
SCIENCE DIRECT
Polymer 46 (2005) 8107–8112
polymer
www.elsevier.com/locate/polymer

# Complex formation between plasmid DNA and self-aggregates of deoxycholic acid-modified chitosan

Kuen Yong Lee[a,*], Ick Chan Kwon[b], Won Ho Jo[c], Seo Young Jeong[b,*,1]

[a]Department of Bioengineering, Hanyang University, Seoul 133-791, South Korea
[b]Biomedical Research Center, Korea Institute of Science and Technology, Seoul 136-791, South Korea
[c]School of Materials Science and Engineering, Seoul National University, Seoul 151-744, South Korea

Received 17 February 2005; received in revised form 1 June 2005; accepted 21 June 2005
Available online 27 July 2005

**Abstract**

The use of non-viral delivery systems has numerous advantages in the area of gene therapy as this approach could circumvent the safety issues that may occur when using viral vectors. Hydrophobically modified chitosan containing five deoxycholic acid groups per 100 anhydroglucose units was synthesized to prepare self-aggregated nanoparticles in aqueous media and utilized to form complexes with plasmid DNA. Formation and various characteristics of DNA/chitosan self-aggregate complexes were investigated by electrophoresis, zeta potential measurement, and dynamic light scattering method. It was hypothesized that complex formation between DNA and chitosan self-aggregates can be strongly dependent on the charge ratio, pH, and incubation time. Migration of DNA on an agarose gel was completely retarded over a charge ratio (+/−) of 4/1 at pH 7.2, and the mean diameter of the complexes was determined to be 350 nm with unimodal size distribution (+/− =4/1; pH 7.2; incubation time, 30 min). The complex formation behavior was also regulated by pH of the media. The incubation time was unlikely to be the critical factor affecting the complex formation behavior due to the instant electrostatic interactions between negatively charged DNA and positively charged chitosan self-aggregates. This approach to controlling the characteristics of DNA/self-aggregate complexes could be critical in the design and tailoring of novel gene delivery systems using non-viral vectors.


*Keywords:* Chitosan self-aggregate; DNA; Complex

## 1. Introduction

Gene therapy is a recent and exciting approach designed to introduce genetic materials into appropriate cells, to alleviate symptoms and/or to prevent the occurrence of particular diseases [1,2]. In this approach, genes are transferred into cells, typically mediated by either viral or non-viral vectors. Several viral vectors such as retroviruses and adenoviruses have been extensively employed in this approach [3]. However, their safety in many clinical uses has been an issue due to their toxicity, immunogenicity, and inflammatory responses in the body [4,5]. Regardless of their low transfection efficiency, non-viral vectors have been extensively investigated as an alternative delivery vehicle of genes. Critical design parameters of a new non-viral gene delivery system include the stability, binding capability to DNA, transfection efficiency, and low immunogenic and biohazardous activity [6,7].

Non-viral delivery systems include direct DNA injection [8], microencapsulation [9], cationic liposomes [10–14], and cationic polymers [15–19]. Liposomes are one of the most frequently used delivery systems to introduce DNA into cells [20,21]. In particular, cationic liposomes have shown potential as a gene delivery vehicle. They can reduce the net negative surface charge of DNA, resulting in the reduction of electrostatic repulsion at the surface of cell membranes, which can facilitate DNA transfer across rather impermeable cell membranes [10–14]. However, their in vivo applications are limited to local delivery due to their low stability and rapid degradation in the body [22].

An alternative approach to the development of non-viral vectors is using soluble cationic polymers designed to form

* Corresponding authors. Tel.: +82 2 2220 0482; fax: +82 2 2298 4101.
*E-mail addresses:* leeky@hanyang.ac.kr (K.Y. Lee), syjeong@khu.ac.kr (S.Y. Jeong).
[1] Present address: Department of Pharmaceutics, Kyung Hee University, Seoul 130–701, South Korea

0032-3861/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.polymer.2005.06.082

complexes with DNA. Simple mixing of DNA with poly-L-lysine or DEAE-dextran results in the formation of polyelectrolyte complexes that can transfer DNA into cells. These systems still show low transfection efficiency, cytotoxicity, and limited efficacy for systemic administration due to the rapid clearance in the body following intravenous injection [16,23]. Therefore, new types of polymers such as poly(ethylene glycol)-*b*-poly-L-lysine, poly-*N*-(2-hydroxypropyl) methylacrylamide-*b*-poly(trimethylammonioethyl methacrylate chloride), poly(ethylene imine), and cationic cascade polymers have been synthesized and their efficacy has been tested as novel gene delivery carriers [24–28].

Chitosan is the second most abundant natural polymer and has many useful applications in the area of biomedical engineering, including gene delivery, due to its cationic character at physiological conditions. Chitosan shows limited solubility at neutral pH, and forms polyelectrolyte complexes with polyanions, often resulting in precipitation. Deoxycholic acid is the main component of bile acids, which are biologically the most detergent-like molecules in the body and can self-associate in water and form micelles. It was hypothesized that chitosan modified with deoxycholic acid can also form stable colloidal nanoparticles at physiological conditions which could be useful for the delivery of DNA. We have previously reported that deoxycholic acid-modified chitosan can form self-aggregated nanoparticles in a physiological buffer solution, and their various physicochemical characteristics have been investigated by using a fluorometer and light scattering method [29,30]. We have also reported preliminary data on potential applications of the chitosan self-aggregates as a gene delivery carrier [31]. In this study we hypothesized that the complex formation behavior between chitosan self-aggregates and plasmid DNA can be controlled by the charge ratio, pH, and incubation time. Various physicochemical characteristics of DNA/chitosan self-aggregate complexes were investigated by gel electrophoresis, dynamic light scattering, and zeta potential measurements.

## 2. Experimental section

### 2.1. Materials

Biomedical grade chitosan ($M_v = 7 \times 10^4$, degree of deacetylation = 80%) was supplied from Samchully Pharm. Co. (Seoul, Korea). Deoxycholic acid (>99% purity) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma (St Louis, MO). DNA molecular weight marker II (λDNA/Hind III digested) was purchased from Boehringer Mannheim GmbH (Germany).

### 2.2. Preparation of chitosan self-aggregates

Chitosan was hydrophobically modified with deoxycholic acid in the presence of EDC as previously reported [29]. Chitosan was dissolved in 1% acetic acid solution and the same volume of methanol was added to the solution. Deoxycholic acid (0.34 mol/mol chitosan) in methanol was then added to the 1% chitosan solution, followed by the drop-wise addition of EDC (0.18 mol/mol deoxycholic acid) while stirring at room temperature. Phase separation did not occur during the reaction. After 24 h, the reaction mixture was poured into the methanol/ammonia solution (7/3, v/v). The precipitates were filtered off, washed thoroughly with distilled water, methanol, and ether, and then followed by vacuum drying at room temperature. The degree of substitution, defined as the number of deoxycholic acid groups per 100 anhydroglucose units of chitosan, was determined from the C/N ratio obtained by elemental analysis. The deoxycholic acid-modified chitosan was then suspended in phosphate buffered saline (PBS) solution (pH 7.2) at 37 °C for 48 h, followed by ultrasonication (30 W, three times for 2 min) using a pulse function (pulse on, 5.0 s; pulse off, 1.0 s).

### 2.3. Plasmid

The plasmid pCMV-CAT encoding chloramphenicol acetyltransferase (5.8 kb) was grown in *E. coli*, extracted by the alkali lysis technique, and purified by using a QIAGEN® Plasmid Midi kit. The purity of the plasmid consisting of supercoiled and open circular forms was checked by electrophoresis on a 1.0% agarose gel, and the concentration of DNA was determined by measuring the ratio of the UV absorbance at 260–280 nm.

### 2.4. Complex formation between chitosan self-aggregates and plasmid DNA

Chitosan self-aggregate/DNA complexes were prepared at various charge ratios, pH, and incubation times by the addition of a DNA solution (1 mg/mL) to a solution of chitosan self-aggregates (20 mg/mL) at room temperature. Complex formation was confirmed by electrophoresis on a 1.0% agarose gel with tris-acetate (TAE) running buffer at 100 V for 30 min. UV transillumination of the gel was employed with ethidium bromide to visualize DNA. Zeta potential of DNA/self-aggregate complexes was measured using Malvern Zetasizer 4 (Malvern Instruments, UK).

### 2.5. Measurements

Dynamic light scattering (DLS) experiments were carried out with an argon ion laser system (Lexel Laser Model 95) tuned at 488 nm. The scattering angle ($\theta$) was varied from 30 to 135°. The intensity autocorrelation was measured at a scattering angle of 90° with a Brookhaven

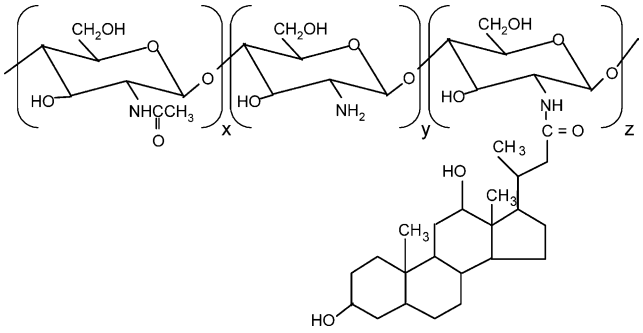


Fig. 1. Chemical structure of deoxycholic acid-modified chitosan ($x=20$, $y=75$, and $z=5$ mol%).

BI-9000AT digital autocorrelator at $25\pm0.1$ °C. When the difference between the measured and the calculated baselines was less than 0.1%, the correlation function was accepted. A non-linear regularized inverse Laplace transformation technique (CONTIN) was used to obtain the distribution of decay function [32]. Mean diameter ($d$) was evaluated by the Stokes–Einstein equation. Steady-state fluorescence spectra were recorded on an ISS K2 multifrequency phase and modulation fluorometer (ISS, Champaign, IL). Samples were excited using a 300 W Xenon arc lamp (ILC Technology, Sunnyvale, CA). For the measurement of the emission intensity of pyrene, the slit openings for excitation and emission were set at 1.0 and 0.5 mm, respectively. The excitation wavelength ($\lambda_{ex}$) was 336 nm, and the spectra were accumulated with an integration time of 5 s/1 nm and used to determine the critical aggregation concentration (CAC) of chitosan self-aggregates.

## 3. Results and discussion

### 3.1. Characteristics of chitosan self-aggregates

Deoxycholic acid was covalently coupled to an amino group of chitosan using water-soluble carbodiimide (Fig. 1). The degree of substitution of deoxycholic acid was determined to be 5 per 100 anhydroglucose units of chitosan. Deoxycholic acid-modified chitosan formed self-aggregates in aqueous media with a mean diameter of less than 200 nm, which can be controlled by the degree of substitution of hydrophobic groups to the chitosan backbone and pH and ionic strength of the media [29]. Fluorescence spectroscopy provided a method to determine the critical aggregation concentration (CAC), which was $1.3\times10^{-2}$ mg/mL for the chitosan self-aggregates used in this study, which was lower than the critical micelle concentration (CMC) of typical surfactants (e.g. 2.3 mg/mL for sodium dodecyl sulfate in water and 1.0 mg/mL for deoxycholic acid in water). The lower CAC value of the deoxycholic acid-modified chitosan as compared with those of low molecular weight surfactants may indicate that a small amount of the chitosan derivative can form self-aggregates and maintain their stability in dilute conditions. The increased amount of hydrophobic groups attached to chitosan further reduced the CAC values [29].

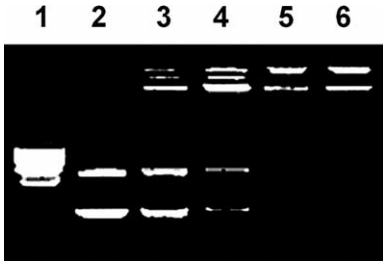


Fig. 2. Migration of DNA/self-aggregate complexes on an agarose gel. pCMV-CAT was mixed with an increasing amount of self-aggregates ([self-aggregates]=2 mg/mL; incubation time, 30 min; pH 7.2). Lane 1, DNA molecular weight marker II; lane 2, DNA only; lane 3, charge ratio (+/−)=1/1; lane 4, 2/1; lane 5, 4/1; lane 6, 8/1.

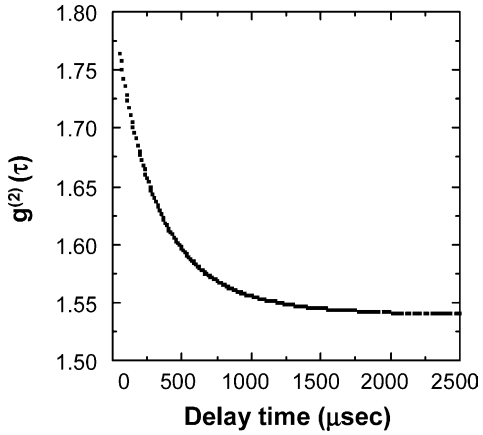


Fig. 3. Intensity autocorrelation function ($g^{(2)}(\tau)$) of DNA/self-aggregate complexes in PBS solution (pH 7.2; [self-aggregate]=2.0 mg/mL; charge ratio (+/−)=4/1; $\theta=90°$; $T=25$ °C).

### 3.2. Complex formation between chitosan self-aggregates and plasmid DNA

We first confirmed the complex formation between

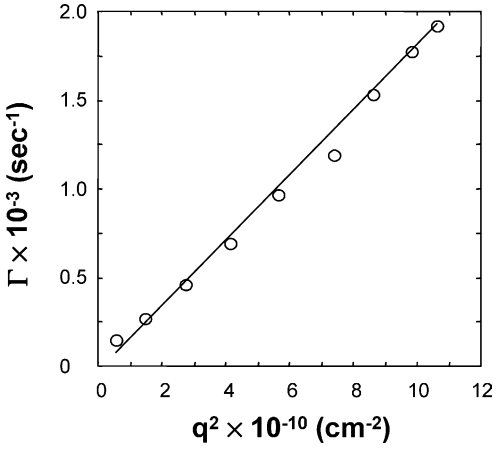


Fig. 4. $\Gamma$ vs. $q^2$ for the diffusive mode of DNA/self-aggregates in PBS solution (pH 7.2; [self-aggregate]=2.0 mg/mL; $T=25$ °C).

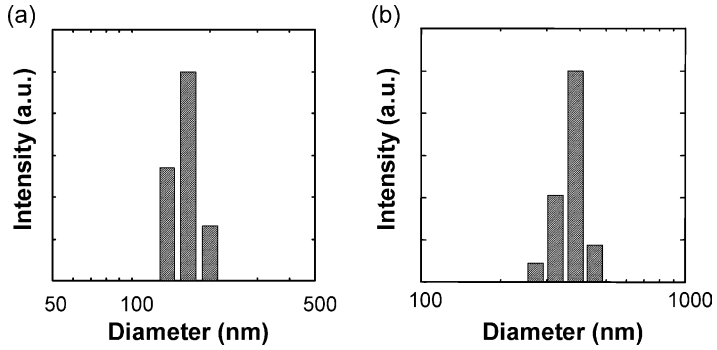


Fig. 5. The change of size distribution (a) before and (b) after complex formation between chitosan self-aggregates and DNA ([self-aggregate] = 2 mg/mL; charge ratio (+/−) = 4/1; pH 7.2).

plasmid DNA and chitosan self-aggregates by electrophoresis on an agarose gel. When DNA is mixed with self-aggregates, electrostatic interactions mainly drive the formation of complexes. The migration of DNA on the agarose gel was retarded because of the charge neutralization and/or an increase in the molecular size of the complexes (Fig. 2). The charge ratio (+/−) was defined as a ratio between the number of amino groups in the chitosan self-aggregates and the number of phosphate groups in DNA. When the charge ratio was greater than 4/1, migration of DNA was completely retarded.

We next measured the size of the complexes by the dynamic light scattering method. A typical intensity autocorrelation function ($g^{(2)}(\tau)$) of DNA/self-aggregate complexes in PBS solution (pH 7.2) at $\theta = 90°$ and $T = 25$ °C is shown in Fig. 3. Under the assumption of the polydisperse system, CONTIN algorithm was adopted to calculate the mean diameter and size distribution of the complexes. A plot of $\Gamma$ vs. $q^2$ for DNA/self-aggregate complexes in PBS solution (pH 7.2) is shown in Fig. 4. The $q$ is a scattering vector [$=(4\pi n/\lambda_0)\sin(\theta/2)$], where $n$ is the refractive index of the scattering medium and $\lambda_0$ is the wavelength of the incident light in a vacuum. Since a linear relationship is obtained, the measured $\Gamma$ can be predominantly attributed to a diffusive mode. The DNA/self-aggregate complexes in PBS solution (pH 7.2) had the mean diameter of 350 nm with unimodal size distribution, which was greater than that of chitosan self-aggregates only ($d = 160$ nm). Interestingly no significant change in the size distribution of the complex particles was observed (Fig. 5).

We next investigated changes of the mean diameter and zeta potential of the complexes at various charge ratios. The mean diameter of the complexes was slightly increased with an increase of the charge ratio until 4/1 and then suddenly increased over 4/1 (Fig. 6(a)). This is likely due to the intermolecular cross-linking between DNA strands by self-aggregates, a phenomenon typically observed with either high DNA concentrations or an excess amount of polycations [12]. Zeta potential is a function of the surface charge that develops when any material is placed in a liquid and is a good index of the magnitude of the electrostatic properties of colloidal particles. An initial negative value of the zeta potential was observed at a low charge ratio of the complexes (Fig. 6(b)). The negative value of zeta potential at the charge ratio of 1/1 could be due to the low effective charge density of amino groups in chitosan at the physiological condition, and the access of DNA to positive charges on chitosan being sterically hindered by the inherent rigidity of the chitosan backbone. Complexes between self-aggregates and DNA showed an increasing zeta potential in parallel with increasing charge ratios. It was considered that a positive zeta potential value could benefit the complexes

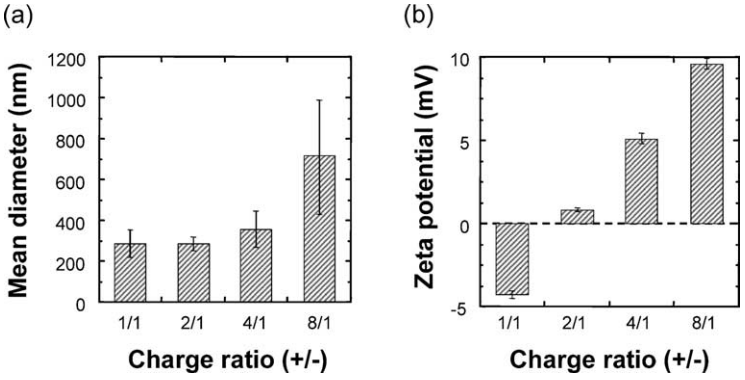


Fig. 6. Effect of charge ratio (+/−) on (a) size and (b) zeta potential of self-aggregates/DNA complexes ([self-aggregates] = 2 mg/mL; incubation time, 30 min; pH 7.2; $n = 3$; mean ± SD).

as a gene delivery system because the cell membrane has a net negative zeta potential value.

### 3.3. Effect of pH on complex formation

The effect of the pH of the media on the complex formation was next investigated. Complexes were formed in PBS solutions at different pH but the charge ratio (+/−) was kept constant at 4/1. Since chitosan has a p$K_b$ value of 7.7 [33], the amino groups in chitosan have an almost unionized form above pH 8.0. The free DNA dissociated from the complex was observed on an agarose gel above pH 8.0 (lanes 6 and 7 in Fig. 7). DNA bands disappeared at low pH (lanes 3 and 4). Two reasons could cause this phenomenon. One is the formation of more compact complexes due to the high charge density of chitosan at low pH that disrupts the intercalation of ethidium bromide. The other is the degradation of DNA by contaminants, such as nucleases in the self-aggregates. To verify whether the DNA bands disappeared by the degradation of DNA, DNA was extracted from the complexes by treatment with phenol/chloroform [34]. An intact form of supercoiled DNA was isolated from the complexes, indicating a compact complex formation at low pH (data not shown).

### 3.4. Effect of incubation time on complex formation

Complex formation behavior between DNA and chitosan self-aggregates was further studied by varying the incubation time (Fig. 8). DNA/self-aggregate complexes were formed within a few minutes, and the release of DNA was not observed during the incubation time periods. Since the dominating driving force for the complex formation is electrostatic interactions, the complexes may form rapidly. Degradation of DNA in the presence of self-aggregates was not observed during 4 h of incubation. Extracted DNA still formed the supercoiled structure, indicating no damage occurred to DNA during the incubation time period used in this experiment (data not shown). The mean diameter of DNA/self-aggregate complexes was also measured by using the dynamic light scattering method as a function of incubation time (Fig. 9). The mean diameter was kept nearly constant (350 nm) over the incubation time used in this experiment, even in the presence of serum. The complexes remained stable after 48 h of incubation (data not shown).

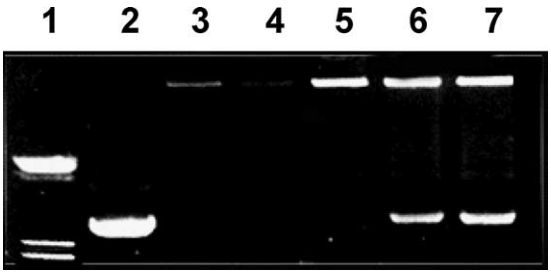


Fig. 7. Effect of pH on the formation of DNA/self-aggregate complexes at a fixed charge ratio (+/−) of 4/1 and constant incubation time of 30 min ([self-aggregates]=2 mg/mL). Lane 1, DNA molecular weight marker II; lane 2, DNA only; lane 3, pH 5.5; lane 4, pH 6.5; lane 5, pH 7.2; lane 6, pH 8.0; lane 7, pH 9.5.

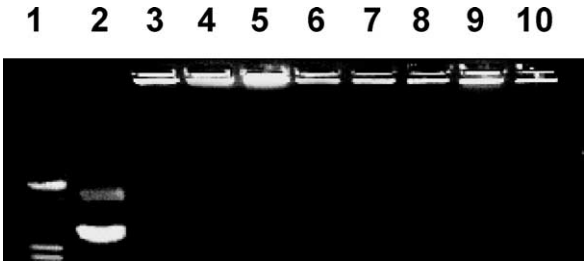


Fig. 8. Effect of incubation time on the formation of DNA/self-aggregate complexes at a fixed charge ratio (+/−) of 4/1 and pH 7.2 ([self-aggregates]=2 mg/mL). Lane 1, DNA molecular weight marker II; lane 2, DNA only; lane 3, 1 min; lane 4, 5 min; lane 5, 10 min; lane 6, 30 min; lane 7, 1 h; lane 8, 2 h; lane 9, 3 h; lane 10, 4 h.

## 4. Conclusions

Colloidally stable self-aggregates in a physiological buffer solution can be obtained by hydrophobic modification of chitosan with deoxycholic acid. These self-aggregates can form complexes when mixed with plasmid DNA through electrostatic interactions. The characteristics of DNA/chitosan self-aggregate complexes varied depending on the charge ratio and pH of the media. However, formation and stability of the complexes was not significantly influenced by incubation time. This approach to controlling the physicochemical properties of DNA/chitosan self-aggregate complexes could be critical in designing novel non-viral gene delivery systems using water-soluble

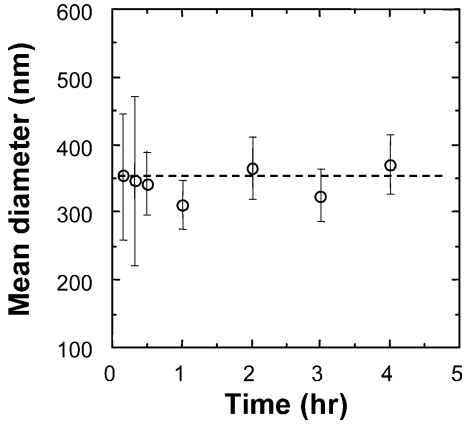


Fig. 9. Changes in the mean diameter of DNA/self-aggregates complexes over incubation time in the presence of 10% serum in PBS solution ([self-aggregates]=2 mg/mL; charge ratio (+/−)=4/1; pH 7.2; $n$=3; mean ± SD).

polymers, which can regulate the transfection efficiency of DNA delivered into mammalian cells.

## References


[1] Brady SI, Crystal RG. Ann NY Acad Sci 1994;716:90.
[2] Tomlinson E, Rolland AP. J Controlled Release 1996;39:357.
[3] Templeton NS, Lasic DD. Gene therapy: Therapeutic mechanisms and strategies. New York: Marcel Dekker; 2000. p. 1–130.
[4] Thomas M, Klibanov AM. Appl Microbiol Biotechnol 2003;62:27.
[5] Schmidt-Wolf GD, Schmidt-Wolf IGH. Trends Mol Med 2003;9:67.
[6] Maulik S, Patel SD. Molecular biotechnology—therapeutic applications and strategies. New York: Wiley; 1997.
[7] De Smedt SC, Demeester J, Hennink WE. Pharm Res 2000;17:113.
[8] Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani G, et al. Science 1990;247:1465.
[9] Chang PL, Shen N, Westcott AJ. Hum Gene Ther 1993;4:433.
[10] Felgner PL, Ringold GM. Nature 1989;337:387.
[11] Gershon H, Ghirlando R, Guttman SB, Minsky A. Biochemistry 1993; 32:7143.
[12] Reimer DL, Zhang YP, Kong S, Wheeler JJ, Graham RW, Bally MB. Biochemistry 1995;34:12877.
[13] Gao XA, Huang L. Gene Ther 1995;2:710.
[14] Hofland HEJ, Shephard L, Sullivan SM. Proc Natl Acad Sci USA 1996;93:7305.
[15] Laemmli UK. Proc Natl Acad Sci USA 1975;72:4288.
[16] Kawai S, Nishizawa M. Mol Cell Biol 1984;4:1172.
[17] Kabanov AV, Astafyeva IV, Chikindas ML, Rosenblat GF, Kiselev V I, Severin ES, et al. Biopolymers 1991;31:1437.
[18] Boussif O, Lezoualc'h F, Zanata MA, Mergny MD, Scherman D, Demeneix B, et al. Proc Natl Acad Sci USA 1995;92:7297.
[19] Kim JS, Maruyama A, Akaike T, Kim SW. J Controlled Release 1997; 47:51.
[20] Fraley RP, Subramani S, Berg P, Papahad-jopoulos D. J Biol Chem 1980;255:10431.
[21] Nicolau C, Cudd A. Crit Rev Ther Drug Carrier Syst 1989;6:239.
[22] Lew D, Oarker SE, Latimer T, Kuwaharararum-Dell A, Doh SG, Yang ZY, et al. Hum Gene Ther 1995;6:553.
[23] Ishkawa Y, Homcy CJ. Nucleic Acids Res 1992;20:4367.
[24] Wolfert MA, Schacht EH, Toncheva V, Ulbrich K, Nazarova O, Seymour LW. Hum Gene Ther 1996;7:2123.
[25] Haensler J, Szoka FC. Bioconjugate Chem 1993;4:372.
[26] K.-Latallo DA, Bielinska JR. Proc Natl Acad Sci USA 1996;93:4897.
[27] Roberts JC, Bhalgat MK, Zera RT. J Biomed Mater Res 1996;30:53.
[28] Wang DA, Narang AS, Kotb M, Gaber AO, Miller DD, Kim SW, et al. Biomacromolecules 2002;3:1197.
[29] Lee KY, Jo WH, Kwon IC, Kim YH, Jeong SY. Macromolecules 1998;31:378.
[30] Lee KY, Jo WH, Kwon IC, Kim YH, Jeong SY. Langmuir 1998;14: 2329.
[31] Lee KY, Jo WH, Kwon IC, Kim YH, Jeong SY. J Controlled Release 1998;51:213.
[32] Provencher SW. Comput Phys Commun 1982;27:213.
[33] Muzzarelli RAA. Chitin. New York: Pergamon; 1977.
[34] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.