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Synthesis and evaluation of chitosan-graft-polyethylenimine as a gene vector

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The objective of this study was to prepare a series of chitosan-graft-polyethylenimine (chitosan-g-PEI) copolymers as gene carriers with high transfection efficiency and low cytotoxicity. Chitosan-g-PEIs with different molecular weights and segments were successfully synthesized by both oxidation and imine reactions and then characterized by ^1H NMR, IR, UV and DSC. All types of Chitosan-g-PEIs prepared were found to interact efficiently with plasmid DNA (pIRES2-EGFP-p53) on DNA retardation assays. The Chitosan-g-PEI/DNA complex had a diameter of approximately 200 nm and a surface potential of $\zeta = +10.0$ mV when the N/P ratio was 15/1. Optimal transfection efficiency of the chitosan-g-PEI/DNA complex was observed at N/P = 45/1 on HepG2 cells, with significantly lower toxicity compared with the gold standard PEI 25 kd. Moreover, the results showed that the toxicity increased with increasing molecular weight of the PEI segment in chitosan-g-PEI. Based on these results, chitosan-g-PEI with different chitosan and PEI segments could be used for gene expression on different levels, and some of them may appear as potential candidates for gene delivery systems.

1. Introduction

The treatment of disease at a genetic level has advanced greatly in the past two decades, and an engineered virus was the first carrier for gene delivery (Cavazzana-Calvo et al. 2000; Kay et al. 2000; Hacein-Bey-Abina et al. 2002). However, safety issues have limited the use of viruses as gene vectors, motivating researchers to seek other safer, less pathogenic and immunogenic gene delivery alternatives, including lipid-based vectors, chemically modified viruses, inorganic materials, and cationic polymer-based gene delivery systems (Duncan et al. 1979; Boeckle and Wagner 2006; Wong et al. 2007).

Among various cationic polymers, polyethylenimine (PEI) is still considered to be the gold standard, as, compared to others, it facilitates effective DNA binding and protection, combined with a high endosomolytic competence, which all contribute to the superior transfection efficiency of the corresponding non-viral vectors. However, the clinical development of PEI has been sluggish due to its acute toxicity and lack of biodegradability. Therefore, various systems have been developed to decrease the significant toxicity of PEI *in vitro* and *in vivo*, such as using low molecular weight PEI, grafting PEG to the PEI backbone (von Harpe et al. 2000; Lungwitz et al. 2005), preparing Pluronic-PEI copolymers and poly(*N*-(2-hydroxypropyl) methacrylamide)[pHPMA]-derivatives, and so on. All these studies aimed to explore strategies that could be used to improve the biological properties of PEI polymers (El-Aneel 2004). Chitosan (Cs) is a natural cationic polysaccharide that shown potential as non-viral vector for gene delivery. However, the weak specificity and low transfection efficiency of chitosan

must be overcome for it to be used in clinical trials. Therefore, chemical modification of chitosan by hydrophilic, hydrophobic, pH-sensitive, thermosensitive and cell-specific ligand groups has been studied extensively, as the functional groups on the chitosan backbone allow the simple coupling of extracellular and intracellular targeting ligands (Dang and Leong 2006). The primary cause of the weak gene expression of chitosan is the inefficient release from the endosome into the cytoplasm; thus the proton loading capability of chitosan in the endosome must be enhanced, which is just the advantage given by PEI. In view of above disadvantages, chitosan-g-PEI has been investigated as a gene carrier to reduce the cytotoxicity of PEI and improve the transfection efficiency of chitosan (Wong et al. 2006). For example, chitosan 100 kd and PEI 1800 have been grafted as a novel gene vector and showed high transfection efficiency with low cytotoxicity (Jiang et al. 2007).

Compared with the previous study, in this study, a series of chitosan-graft-polyethylenimine (chitosan-g-PEI) copolymers with chitosans with relatively low molecular weights (20 kd, 10 kd, 5 kd) and different molecular weights of PEI (25 kd, 10 kd, 1800 d) was prepared, with the aim of investigating the relationship between the structure of chitosan-g-PEI and its transfection efficiency and cytotoxicity. A new method was used in the oxidation reaction to control the oxidation ratio of chitosan directly by UV spectrophotometry. Moreover, the effects of various synthesis reaction parameters were investigated, including reaction time, reaction temperature and ratio between chitosan and periodate. We then investigated the DNA condensation, protection capability, cytotoxicity and transfection efficiency of different types of chitosan-g-PEI.

2. Investigations, results and discussion

2.1. Synthesis of copolymers

The approach described in this paper represents an easy and efficient method to obtain fairly stable chitosan-g-PEI with chitosan (5 kd, 10 kd, 20 kd) and PEI (1800 d, 10 kd, 25 kd) as gene vectors. The results demonstrated that chitosan and PEI could be grafted successfully by oxidation and imine reactions and the novel copolymers exhibited properties of both chitosan and PEI, such as solubility and pH values. Chitosan possesses low water-solubility which limits its use in gene delivery system, given that both transfection and investigation of characteristics need an aqueous medium (pH = 7.4). By grafting PEI to chitosan, the resulting chitosan-g-PEI was completely soluble in both pure water and PBS (pH = 7.4), while at the same time cationic characteristics were conferred which could increase the ability of chitosan to interact with pDNA and improve its ability to adhere to cell membrane.

Compared with the prior study, the oxidation ratio of chitosan was calculated by the periodate consumption method and ^1H NMR spectroscopy, respectively. Although oxidation values from the periodate consumption method were slightly lower than from ^1H NMR spectra, the trend of the oxidation ratio of the chitosan was correctly reflected by this practical and simple new method. This means that the oxidation ratio could be obtained directly during the synthesis process, and the extent of the oxidation reaction could be controlled as required.

In order to optimize the oxidation reaction process, some factors were investigated including the reaction time, reaction temperature and molar ratio of chitosan and periodate. As the results show, oxidation ratios of 33.4%, 33.5% and 33.8%, respectively were obtained when the reaction time was 3 h, 6 h and 48 h. The oxidation ratio was 33.4%, 33.6% and 34.2% at reaction temperatures of 0 °C, 4 °C and 25 °C, respectively. No significant differences were observed when reaction time and reaction temperature were changed ($p > 0.05$). The oxidation ratio of chitosan was determined every 10 min for 3 h in order to further investigate the oxidation rate. It was found that the oxidation ratio was almost constant after reacting for 30 min. This means that the oxidation reaction is complete in a short time and there is no need to maintain the reaction for a long time. Compared with the 48 h reaction time used in a prior study by Hu-Lin Jiang et al. (2007), choosing 6 h as the reaction time in this study allows copolymers to be obtained more effectively and saves time. Therefore, a reaction time of 6 h and a reaction temperature of 4 °C were chosen to ensure successful oxidation of chitosan with the least side reactions. The degree of oxidation of chitosan was 30.2%, 42.1% and 53.4% when the molar ratios of chitosan and periodate were 1:0.4, 1:0.5 and 1:0.6, respectively. This result demonstrated that the oxidation ratio was determined by the molar ratios of chitosan and periodate, since one mole of periodate ion cleaves one mole of the carbon-carbon bond of chitosan to dialdehyde.

Therefore, different oxidation ratios of chitosan could be obtained by using different molar ratios of chitosan and periodate, so that chitosan-g-PEI with a specified PEI grafting ratio could be synthesized. The chitosan-g-PEI copolymers prepared in this study are listed in the Table, including different PEI graft ratios, and molecular weights of the chitosan segments and PEI segments. Thus the relationship between the Chitosan-g-PEI structure and the *in vitro* properties of the complexes could be assessed.

2.2. Characterization of chitosan-g-PEI

The ^1H NMR spectra of chitosan, chitosan oxidation product and chitosan-graft-PEI (Sample 8) are shown in Fig. 1.

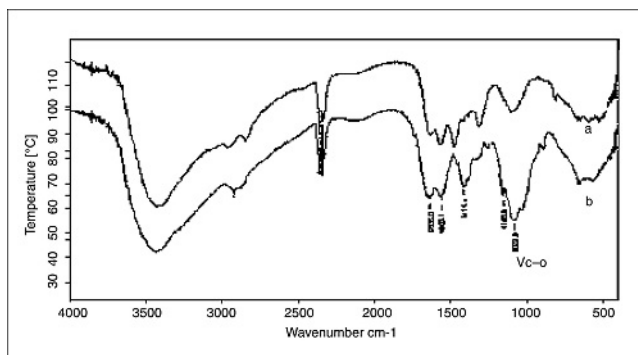


Fig. 1: FT-IR spectrum of chitosan-g-PEI (a) and chitosan (b)

Chitosan (^1H NMR): $\delta = 4.87$ (1-H, -CH); $\delta = 3.18$ (2-H, -CH); $\delta = 3.7\sim 3.9$ (3~6-H, -CH); $\delta = 2.07$ (-COCH₃, -CH₃). Chitosan-g-PEI (^1H -NMR): $\delta = 2.3\sim 3.4$ (4H, -CH₂-CH₂-). After the oxidation reaction was completed, the 2-carbon proton peak of chitosan decreased ($\delta 3.18$, 2-H), in accordance with the work of Jiang et al. (2007), due to cleavage of the carbon-carbon bond by periodate ion, on addition of periodate to the vicinal diols, 1,2-dioxygenated groups and 1,2-aminoalcohols were also oxidatively cleaved by periodate, forming a dialdehyde (Jiang et al. 2007). The oxidation ratio of chitosan was found to be 40.2% which was determined from integral values of the 2-carbon proton peak of chitosan. The PEI graft ratio was 27.2% which was determined from the repeating unit [-CH₂CH₂-] of PEI. The oxidation ratio of chitosan and the PEI graft ratio of the other chitosan-g-PEI copolymers prepared were also determined by ^1H NMR (data not listed).

IR spectra of chitosan and chitosan-g-PEI (Sample 8) are displayed in Fig. 2. Some differences are apparent in a pair of peaks near wave numbers 1100 cm⁻¹ and 1460 cm⁻¹ associated with the absorption of C-O and C-H, respectively. The chitosan-g-PEI peak was weaker than that of chitosan at 1100 cm⁻¹ which may be attributed to the reduction of hydroxide. Also, the chitosan-g-PEI peak at 1460 cm⁻¹ had a stronger absorption than that of chitosan. This phenomenon could be used as evidence that PEI had been grafted on to the chitosan backbone.

Equilibrium phase transition temperatures of chitosan, PEI, and chitosan-g-PEI were measured using differential scanning calorimetry (DSC). It was found that the thermal characteristics of chitosan and PEI were all changed after the reaction (spectrum not shown).

The electrolytic nature of different aqueous polymer solutions were also investigated in this study. The pH values of aqueous solutions of chitosan and the chitosan oxidation product were 5.0 and 4.5, respectively. After grafting PEI to the chitosan oxidation product, the pH value of chitosan-g-PEI (Sample 8) aqueous solution was 7.4 which was similar to that of PEI aqueous solution. This might be attributed to the large number of amine groups in the PEI structure. Introducing more amine groups to chitosan could provide more protons and improve the ability of chitosan to adhere to cell membranes. Consistent results were observed with all types of chitosan-g-PEI (1-9). According to UV results, the UV absorption of chitosan and its oxidation product (Sample 8) at 300 nm was 0.23 and 0.58, respectively, which is attributable to the aldehyde group of the chitosan oxidation product. Also, the UV absorption peak of chitosan-g-PEI at 300 nm disappeared due to the C = N between chitosan and PEI replacing the aldehyde group.

2.3. Preparation of chitosan-g-PEI/DNA complexes

The condensation capability of chitosan-g-PEI (Sample 7) with DNA was evaluated using gel electrophoresis. Figure 3

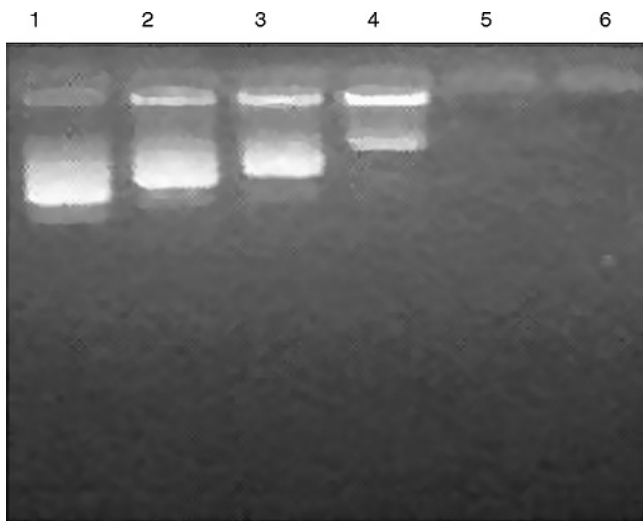


Fig. 2: The electrophoretogram of chitosan-g-PEI/DNA complex Left-1:DNA; left 2-6:Chitosan-g-PEI/DNA with N/P = 1/4, 1/2, 1, 2, 4

Table: Chitosan-g-PEI with different structural characteristics prepared in this study

Sample	PEI graft ratio	Chitosan MW/Da	PEI MW/Da	N/P ratio of DNA totally retarded
1	30%	20,000	1800	3
2	40%	20,000	1800	3
3	50%	20,000	1800	3
4	50%	20,000	1800	4
5	50%	10,000	1800	1
6	50%	5,000	1800	3
7	50%	20,000	1800	2
8	50%	20,000	10,000	4
9	50%	20,000	25,000	2

demonstrates that the migration of DNA was completely retarded when the N/P ratio of chitosan-g-PEI and DNA was $\geq 2/1$. Moreover, the condensation capability of chitosan-g-PEI (1-9) was different as shown by the results in the Table. The condensation capability of chitosan-g-PEI improved with increasing PEI graft ratio. DNA mobility was already fully retarded at N/P = 1 for Sample 5, suggesting that chitosan-g-PEI with 10 kd chitosan had the strongest DNA binding capability. Further, DNA was totally retarded at N/P = 4/1 when the molecular

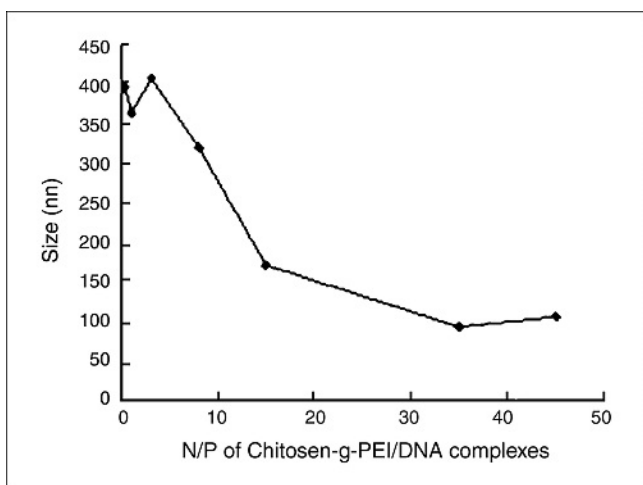


Fig. 3: Average size of complexes with various N/P of chitosan-g-PEI/DNA

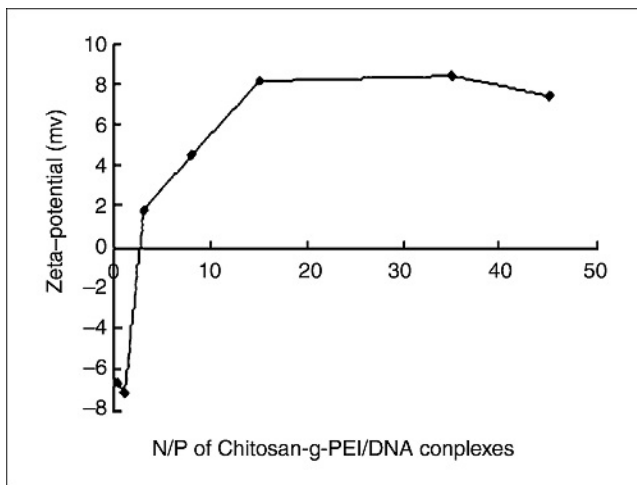


Fig. 4: Average zeta potential of complexes with various N/P

weight of the PEI segment was 10 kd, while DNA was totally retarded at N/P = 2/1 for Sample 7 (PEI 1800 d) and Sample 9 (PEI 25 kd).

To examine the interaction of chitosan-g-PEI with plasmid DNA, zeta potential and particle size were also measured at different N/P ratios. As shown in Fig. 4, the diameters of chitosan-g-PEI (Sample 8)/pDNA complexes at various N/P ratios were in the nano-size range and the polydispersity index was lower than 0.2. The particle size of the complex was largest at N/P = 3, which is the saturation point between chitosan-g-PEI and DNA, the interaction being weakest at this point. With additional chitosan-g-PEI, particle size sharply decreased, finally settling at 200 nm (N/P > 15), which is suitable for *in vivo* delivery. Meanwhile, the zeta potential of the complex became positive at N/P = 3 (Fig. 5), and reached +10 mV at N/P = 15, which was similar to the zeta potential of PEI.

The particle size of chitosan-g-PEI/DNA in physiological media is especially significant for cellular uptake and consequently for transfection efficiency, as DNA is a large molecule and its compaction is essential for cell internalization. As we know, it would

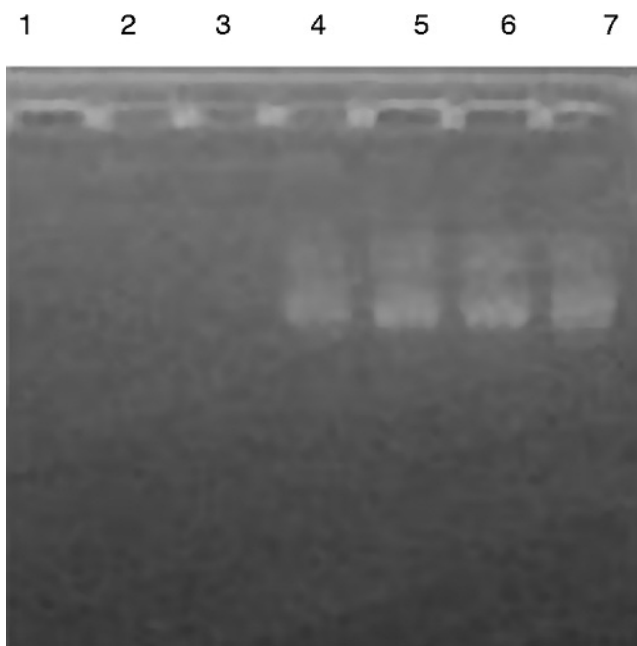


Fig. 5: The effect of heparin with different concentration disaggregation on complexes (sample 8) Left: 1-6: Concentration ($\mu\text{g/mL}$) of heparin are 0, 20, 30, 50, 100, 150; 7: Naked DNA

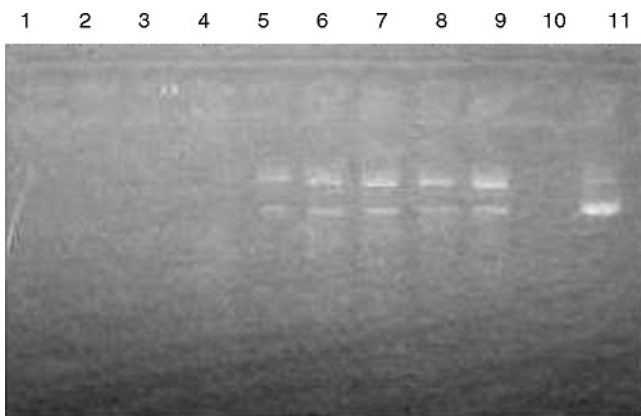


Fig. 6: Effect of compounds (sample 8) with different N/P ratio on degradation by DNase I 1–9: compound + DNase I (N/P ratio was 1/4, 1/2, 1, 2, 4, 5, 6, 8, 10) 10: Naked DNA + DNase I; 11: Naked DNA

be easier for small particles to enter cells via an endocytosis pathway than larger ones, but large particles are likely to adhere to the cell membrane, which could improve the transport process efficiently. However, attention should be paid to the fact that large particles have more potential to induce toxicity compared with small particles. Therefore both transfection efficiency and toxicity should be considered seriously. Zeta potential of the chitosan-g-PEI/DNA complex is another factor that should be investigated in gene delivery systems. The positive charge on the surface of the complex could facilitate the ability to adhere to the negatively charged cell membrane, and thus could induce and increase cellular uptake (van der Aa et al. 2007). The cationic charge of chitosan-g-PEI/DNA also determines the interaction between the complex and negative biological components in cells. Moreover, the condensation process could be understood clearly from the change of zeta potential value of the DNA surface as addition of chitosan-g-PEI.

2.4. Effects of heparin on stability of chitosan-g-PEI/DNA complexes

The effects of heparin on the stability of Chitosan-g-PEI/DNA complex were investigated according to the method of Bertschinger et al. (2006). Different amounts of polyanionic heparin were added to assess the release of DNA from chitosan-g-PEI/DNA complex (Sample 8) at pH 7.4 (Fig. 6). Some of the DNA was released initially when the concentration of heparin reached 50 $\mu\text{g}/\text{mL}$, and the degree of DNA release increased with additional amounts of heparin. The fluorescence intensity was almost equal to that of naked DNA at the final heparin concentration of 100 $\mu\text{g}/\text{mL}$, which could be interpreted as complete release of DNA from the complex. This result agreed with the conclusion shown previously that heparin efficiently disrupted chitosan-g-PEI/DNA particles (Moret et al. 2001).

To further analyze the effect of heparin on the stability of the chitosan-g-PEI/DNA complex, different amounts of heparin were added to each chitosan-g-PEI (1–9)/DNA complex at N/P = 4/1. A linear relationship between the heparin concentration and the amount of DNA released was observed for all the chitosan-g-PEIs. Furthermore, the results showed that the degree of release of DNA from the chitosan-g-PEI (1–9)/DNA complex was different after adding 50 $\mu\text{g}/\text{mL}$ heparin. Incomplete release of DNA by the action of heparin was observed for chitosan-g-PEI(5)/DNA and chitosan-g-PEI(9)/DNA, the fluorescence intensity being approximately 50% and 75%, respectively compared with naked DNA. With other chitosan-g-PEI (1, 2, 3, 4, 6, 7, 8)/DNA complexes, DNA was released completely at the same heparin concentration. Compared with the results from

gel retardation studies, it was found that the degree of release of DNA from the complex was consistent with the condensation capability of the chitosan-g-PEI. This means that chitosan-g-PEI with a strong condensation capability with DNA is not easily disaggregated by heparin.

The interaction between heparin-like compounds and cationic polymers may have a negative effect on chitosan-g-PEI-mediated gene transfer *in vivo*. For example, these polyanions are present in blood and may interact with chitosan-g-PEI following the injection of chitosan-g-PEI/DNA particles (Dash et al. 1999). On the other hand, it has been proved that the release of plasmid DNA from the complex plays an important role in successful gene transfection. When the chitosan-g-PEI/DNA complex is released from endosome to cytoplasm, some negative component, like heparin, is needed to disaggregate the complex and permit DNA to enter the nucleus to carry out gene expression. Therefore, suitable gene vectors should provide effective protection to DNA and allow DNA release as required.

2.5. Stability of the complexes to DNase I

In order to evaluate the protection given by chitosan-g-PEI to DNA, naked DNA and chitosan-g-PEI (sample 8)/DNA complex were both incubated with DNase I (Fig. 7). The results showed that naked DNA was quickly and completely degraded by DNase I; whereas DNA released from chitosan-g-PEI/DNA had the same fluorescence intensity compared to untreated DNA after addition of heparin when the N/P ratio > 4. That is to say, DNA was completely protected from DNase I degradation when the N/P ratio > 4 (Sample 8) and the plasmid DNA would be more resistant to nuclease degradation in the presence of chitosan-g-PEI. Moreover, the dependence of the enhanced particle stability on increasing N/P ratio was observed after addition of DNase I to a series of chitosan-g-PEI (1–9)/DNA complexes. All of the chitosan-g-PEI (1–9) complexes have the ability to protect DNA from DNase I when N/P > 4.

2.6. Transfection efficiency

The dependent relationship between transgene efficiency and N/P ratio was also demonstrated in this study. Transfection experiments using chitosan-g-PEI (Sample 8) on HepG2 cells at different N/P ratios suggested that the optimal N/P ratio was 45/1 as illustrated in Fig. 8. Chitosan-g-PEI/DNA complexes with low N/P ratios from 3/1 to 20/1 showed no transfection activity. The transfection efficiency was improved when the N/P ratio was above 25/1. Also, the green fluorescence intensity of gene expression increased as the N/P ratio increased from 25 to 45, but the transfection efficiency decreased sharply when the N/P increased to 50, which is attributed to the higher cytotoxicity of the complex with an excess of polymers. However, the naked DNA had no transfection activity at all. The reason why the optimal transfection efficiency was obtained at much higher N/P ratios than those needed to retard DNA in this study was that the excess transfection agent, which was not associated with DNA, also played a role in the delivery process (Kichler et al. 2000). It has been estimated that each fifth or sixth amino nitrogen of PEI is protonated at physiological pH (Suh et al. 1994) and only those positively charged amino groups will interact ionically with the negatively charged DNA. The increased positive net charge of the corresponding complex could improve cell interaction and enhance cellular and nuclear uptake and retention (Oh et al. 2002). Previous work has shown that adding such large amounts of cationic polymers may result in increased cytotoxicity. The reason why the chitosan-g-PEI showed low cytotoxicity in this study was the introduction of degradable chitosan and the use

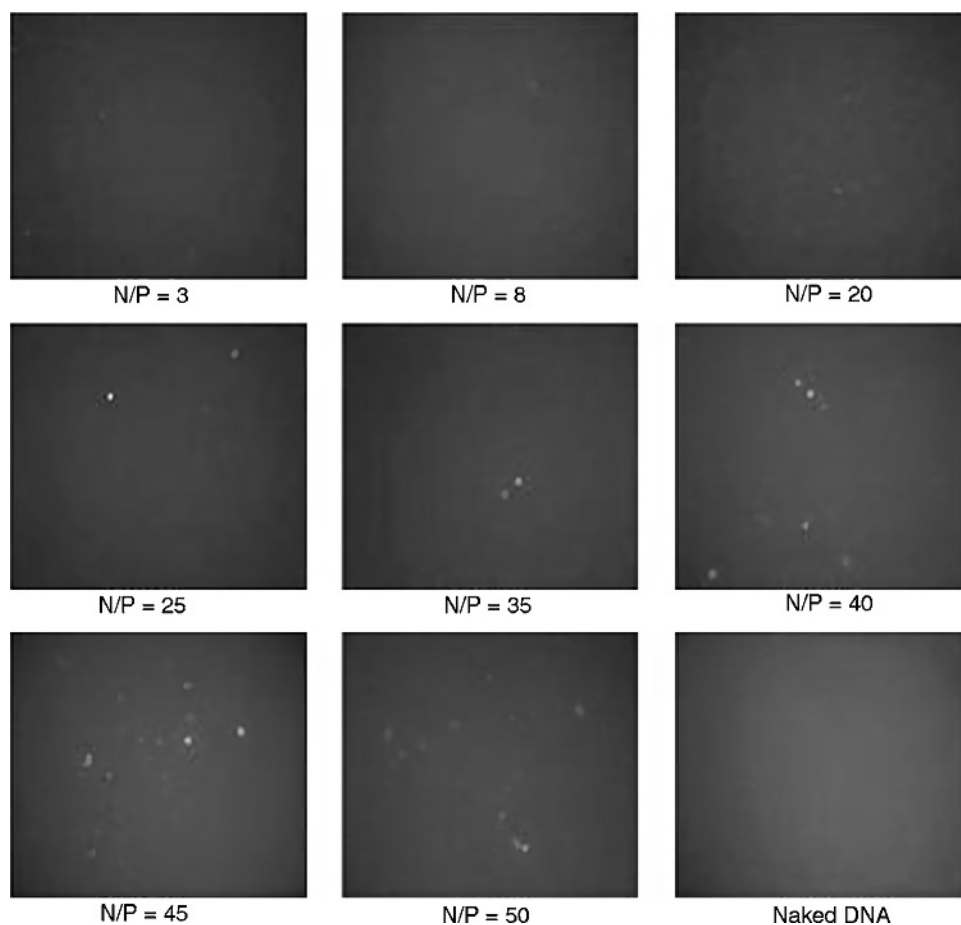


Fig. 7: Fluorescent microcopy photos of HepG 2 cells, 24 h after transfection

of low molecular weight PEI instead of high molecular weight PEI (Xiong et al. 2007).

The results with the series of chitosan-g-PEI(1–9) highlighted that at a PEI graft ratio of 0.5, chitosan 10 kd and chitosan 20 kd with PEI 10 kd and PEI 1800 d showed better transfection efficiency (data not listed). Excess PEI in chitosan-g-PEI was found to cause high cytotoxicity, leading to low cell viability and low transfection efficiency, while insufficient PEI in the chitosan-g-PEI would not provide enough condensation and protection to DNA. In terms of the PEI segment, chitosan-g-PEI with 25 kd PEI showed low gene expression for the high cytotoxicity of high molecular weight PEI. And chitosan-g-PEI with 10 kd and 1800 d PEI could provide high transfection efficiency while

having low cell toxicity. As regards the molecular weights of the chitosan segments, it has been demonstrated that relatively low molecular weight chitosan could provide high gene expression and present low risk *in vivo* (Germershaus et al. 2007).

2.7. Toxicity

Cytotoxicity is an important issue in gene carrier evaluation. The cytotoxicity of chitosan-g-PEI (1–9) complexes and their corresponding polyplexes were tested by MTT assay. As shown in Fig. 9, the viability of HepG 2 cells decreased sharply with increasing molecular weight of the PEI segment. The average cell viability of chitosan-g-PEI at a concentration of 50 $\mu\text{g}/\text{mL}$ was less than 10% when the molecular weight of PEI segments was 25 kd. Other copolymers showed lower cytotoxicity (50% to 80% cell viability) at different concentrations (50 $\mu\text{g}/\text{mL}$, 100 $\mu\text{g}/\text{mL}$ and 150 $\mu\text{g}/\text{mL}$). It has been demonstrated that low molecular weight PEI causes less damage to cell membranes compared to high molecular weight PEI even when grafted to chitosan. Therefore low molecular weights PEI should be used in chitosan-g-PEI to guarantee the safety of the vectors, such as the 1800 d and 10 kd PEI in this study.

In other experiments, chitosan-g-PEI and PEI 1800 d appeared to be less toxic to cells than PEI 25 kd. The average cell viability with chitosan-g-PEI and PEI 1800 d was over 80% at a concentration of 50 $\mu\text{g}/\text{mL}$, while for PEI 25 kd it was less than 10% at the same concentration.

Any transfection method tends to induce toxicity since a large amount of the complex is needed to ensure that enough of the complex enters the cell nucleus and implements gene expression, and such an amount of cationic polymers certainly induces toxicity. Non-virus vectors will also induce an intense and non-specific immune response even it is much lower than that

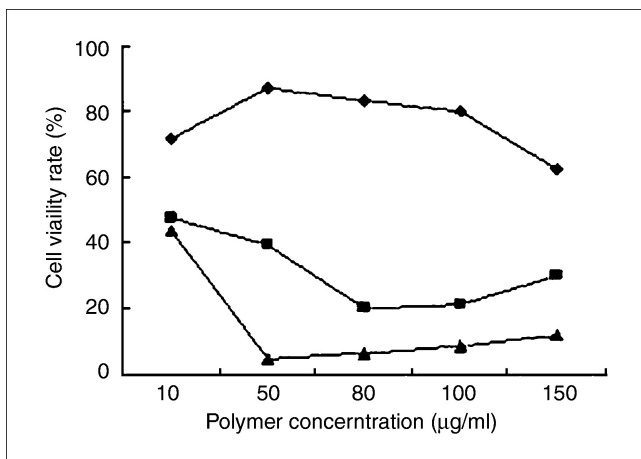


Fig. 8: Cytotoxicity of chitosan-g-PEI with different MW of PEI (◆-1800 d; ■-10 kd; ▲-25 kd)

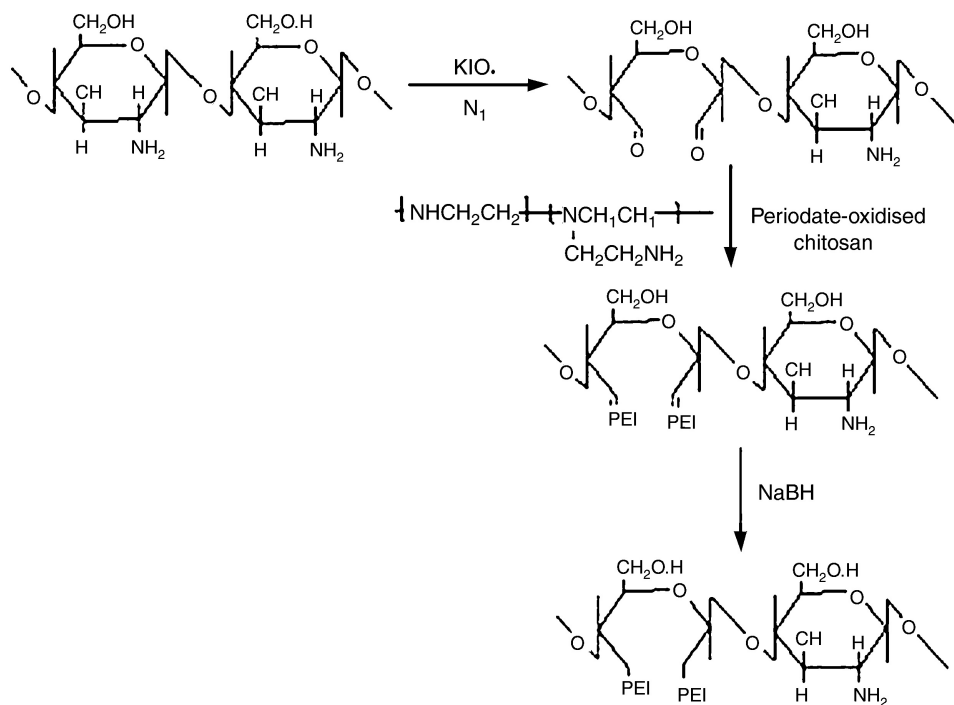


Fig. 9: Reaction scheme for synthesis of chitosan-g-PEI

induced by the virus vectors. The toxicity of cationic polymers depends mainly on two factors. Firstly, the pro-inflammatory cytokine which possesses toxicity and limits gene expression is induced by cationic polymers after administration. Secondly, the interaction between cationic polymers and the cell membrane leads to cell aggregation and membrane damage. Since the chitosan derivatives are relatively non-toxic compared with PEI, the strategy combining low molecular weight PEI with chitosan gave low cytotoxicity and same transfection efficiency compared with PEI 25 kd.

Based on these studies, it is suggested that chitosan-g-PEIs with different chitosan and PEI segments would implement gene expression at different levels. Compared with a prior study by Hu-Lin Jiang (Jiang et al. 2007) not only was chitosan-g-PEI with 100 kd chitosan and PEI 1800 d suitable for gene delivery systems, but also PEI 10 kd, chitosan 10 kd, and chitosan 20 kd have the potential to be safe and efficient gene carriers as demonstrated in this study. Of all types of chitosan-g-PEI, the chitosan-g-PEI sample with 10 kd chitosan and PEI 1800 d was superior to the others. Given that there are many different types of PEI and chitosan which could be used as gene carriers, such as linear PEI, branch PEI, and chitosan derivatives, there must be an optimum combination of PEI and chitosan. More comprehensive studies with these vectors are currently being conducted to improve their transfection efficiency further and reduce their cytotoxicity.

3. Experimental

3.1. Materials

Branched PEI (1.8 kd and 10 kd) was purchased from Nippon Shokubai (Japan), and branched PEI (25 kd) was obtained from Sigma-Aldrich (UK). Chitosan (5000, 10,000 and 20,000 Da) was obtained from ZheJiang Jinke (China). Potassium periodate and sodium borohydride were purchased from Sinopharm Chemical Reagent Co (China). 96-well plates were obtained from Costar (Corning, USA). Agarose was from Biorline (UK). MTT, ethanol and bovine serum albumin were purchased from Sigma (UK).

3.2. Synthesis of copolymers

Two steps were involved in the synthesis of chitosan-graft-PEI. In the first step, periodate-oxidized chitosan was prepared (Jiang et al. 2007). First, the

specified amounts of both chitosan and potassium periodate were each dissolved in sodium acetate buffer (pH 4.5). A degassing process was applied to each of the two solutions using N_2 prior to mixing, and the temperature was then adjusted as necessary. The reaction between chitosan and potassium periodate to produce periodate-oxidized chitosan was controlled by the addition of ethylene glycol (10% v/v) to end the reaction after the desired time and also to eliminate excess periodate. The resultant periodate-oxidized chitosan solution was then dialysed against 0.2 M NaCl (adjusted to pH 4.5) (Spectra/Por[®] membrane: MWCO = 3500) for 24 h to convert the chitosan to the hydrochloride salt. The same dialysis procedure was then repeated but this time against deionised water adjusted to pH 4.5 (Spectra/Por[®] membrane: MWCO = 3500) for 24 h. Finally periodate-oxidized chitosan was obtained by freeze drying after the dialysis process was complete.

In previous studies, the degree of oxidation of chitosan was determined by 1H NMR after the freeze dried oxidized chitosan had been obtained. Although this method is accurate and clear, it is neither convenient nor direct. A new method was used to analyse the consumption of periodate using ultraviolet spectrophotometry in order to control the oxidation process more efficiently. First, the standard curve of periodate was established. The concentration of periodate in the reaction solution was then determined to calculate the consumption of periodate. Based on the amount of periodate consumed, the oxidation ratio of chitosan was obtained. Therefore the degree of oxidation could be controlled during the process.

In the second step, PEI (of different molecular weights) was reacted with the periodate-oxidized chitosan (different oxidation ratios), the reaction being catalyzed by magnetic stirring for 2 days at 4 °C. Subsequently, the solution was treated with sodium borohydride (2 g $NaBH_4/g$ chitosan). After completion of the reaction, the mixture was dialysed using a Spectra/Pors membrane (MWCO 12–14 K) against NaCl (0.2 M, pH 4.5) for 24 h and against deionized water (pH 4.5) at 4 °C for 24 h to remove unreacted PEI. After the dialysis process was complete, the copolymer was lyophilized. The reaction scheme is shown in Fig. 10.

3.3. Characterization of copolymer

Synthesized copolymer was characterized by Nuclear Magnetic Resonance (1H NMR) (AVACE AV-500, Bruker), Infrared Spectra (FTIR Nicolet Impact 410), UV (ultraviolet spectrophotometry) and Netzsch DSC 204.

3.4. Preparation and characterization of chitosan-g-PEI/DNA complex

Chitosan-g-PEI was dissolved in PBS buffer (pH 7.4) to make the concentration 0.1 mg/mL. The pDNA was quantified by UV absorbance at 260 nm (UVIpro GAS7300, Uvitrc, UK). To form complexes at different N/P ratios (nitrogen to phosphate), 0.1 mg/mL of pDNA in TE buffer (Tris-HCl, EDTA) was pipetted into different amounts of the copolymer solutions, and mixed by pipetting up and down. Then the solutions were vortexed at

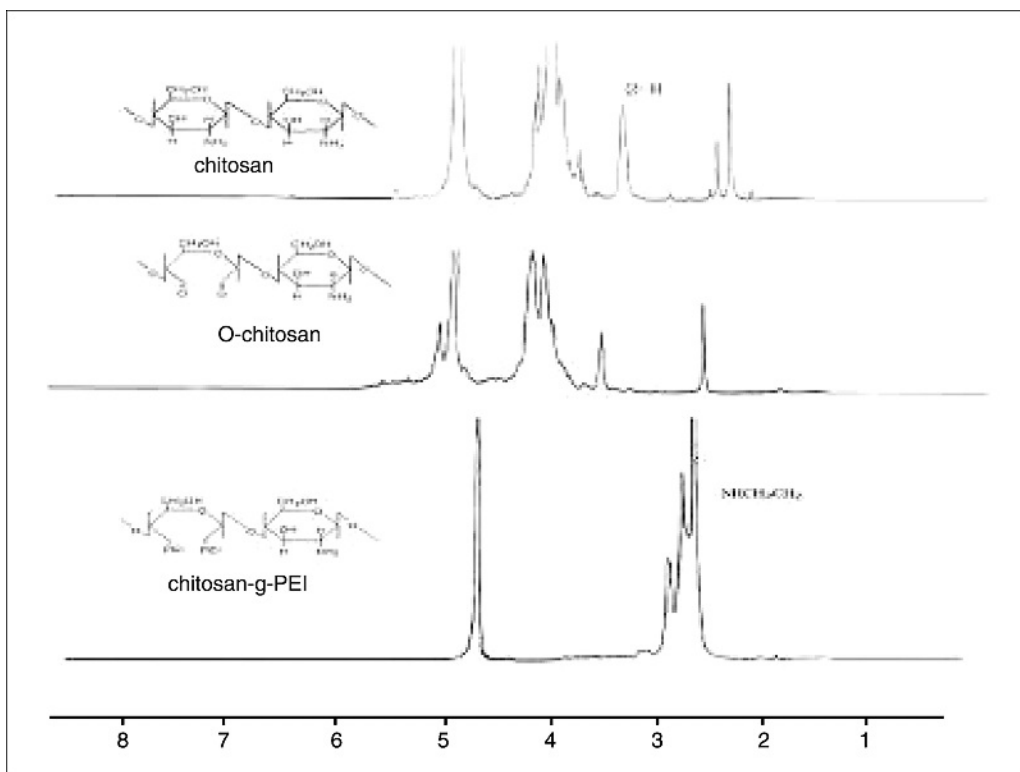


Fig. 10: ^1H NMR spectra of chitosan, O-chitosan and chitosan-g-PEI

low speed for 30 s to avoid pDNA shearing, and allowed to form chitosan-g-PEI/DNA complexes for 30 min at room temperature before use. The complexes formed were characterized by their capability to retard pDNA migration through 0.6% agarose gel containing 5 $\mu\text{L}/100\text{ mL}$ Goldview. 15 μL of the complexes in different N/P ratios were electrophoresed at 90 mV.

3.5. Chitosan-g-PEI/DNA particle stability assay

In order to assess the release of DNA from complexes in the presence of negative components, a complex formed with chitosan-g-PEI and p53 at a ratio of 4/1 (N/P) was treated with various amounts of heparin (0, 10 $\mu\text{g}/\text{mL}$, 20 $\mu\text{g}/\text{mL}$, 30 $\mu\text{g}/\text{mL}$, 50 $\mu\text{g}/\text{mL}$, 80 $\mu\text{g}/\text{mL}$, 100 $\mu\text{g}/\text{mL}$ and 150 $\mu\text{g}/\text{mL}$). All the samples were incubated with constant mixing at room temperature and then centrifuged in a microcentrifuge for 1 min. The release of DNA was determined by gel electrophoresis. The release of DNA from complexes with different types of chitosan-g-PEI was also tested at a heparin concentration of 50 $\mu\text{g}/\text{mL}$ according to the method described above.

To investigate the protection given by chitosan-g-PEI to DNA in the presence of DNase, DNA and different types of chitosan-g-PEI/DNA complex were incubated with DNase I using the same method as described above.

3.6. Transfection of HepG 2 cells

HepG-2 (human hepatoblastoma cells) cells were maintained in DMEM (Dulbecco's Modified Eagle Media) supplemented with 10% (v/v) FBS (Fetal Bovine Serum), penicillin (100 units/mL) and streptomycin (100 units/mL) at 37 °C in the presence of 5% CO_2 , then the cells were sub-cultured regularly using 0.02% EDTA.

Cells were seeded at 1×10^5 cells/well in a 24-well plate and grown overnight in DMEM supplemented with 10% FCS (fetal calf serum) and penicillin/streptomycin (100 units/mL, units/mL) at 37 °C in the presence of 5% CO_2 . Four hours prior to transfection, the medium was removed and replaced with DMEM alone, and the plates returned to the incubator. Following this, in the absence of serum, 200 μL of complex-containing medium were added slowly to each well in quadruplicate to give 2 $\mu\text{g}/\text{well}$ of pDNA and the plates were then incubated for 6 h. Complexes were removed and replaced with cell culture medium. Before the medium was removed the plates were incubated for 48 h to allow protein expression.

3.7. Cytotoxicity studies

The experiment was carried out in 96-well plates and 1×10^4 cells per well were seeded. Various concentrations of copolymers, ranging from

10 to 150 $\mu\text{g}/\text{mL}$, were prepared in the PBS medium. To each well 200 microlitres of the sample solution were added and subsequently incubated with the cells for 48 h at 37 °C in the presence of 5% CO_2 . The medium was then removed and replaced with 20 μL of MTT solution and after further incubation for 4 h, 100 μL DMSO were added. The absorbance values were determined at 540 nm using a micro plate reader. The cell viability (%) was calculated as the absorbance ratio of samples compared with the untreated control (100% cell viability).

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