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Gene-carried chitosan-linked-PEI induced high gene transfection efficiency with low toxicity and significant tumor-suppressive activity

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ABSTRACT

PEI and chitosan are considered to be promising non-viral gene delivery vectors. To improve the transfection efficiency of chitosan, we linked chitosan with polyethylenimine (PEI, Mw = 1.8 kDa) by 1,1′-carbonyldiimidazole to form a complex. The composition, particle size, as well as the zeta potential of this chitosan-linked-PEI (CP) complex were measured. And the DNA binding ability, cytotoxicity, and gene transfection efficiency of CP complex were also investigated in cancer cells. In HepG2, A549 and HeLa cells, CP complex exhibited lower cytotoxicity as compared with PEI25KDa (Mw = 25 kDa), a positive control proved to be an efficient gene transfection polymer. Likewise, it showed good transfection efficiency in these cancer cell lines. Specifically, the long-term transfection efficiency of CP was higher than PEI25KDa as demonstrated by the *in vitro* cancer cell model. The confocal laser scanning microscopy data showed the time for CP to enter the nucleus was 4 h, which was longer than that of PEI25KDa but shorter than that of chitosan. Furthermore, CP complexes were used as a gene carrier to deliver the CCL22 gene into H22 cells. When these gene-altered cells were inoculated in mice, the tumor growth rate was significantly decreased, indicating the CP copolymer was a promising vector for the therapeutic gene delivery.

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1. Introduction

Gene therapy can be defined as the transfer of a therapeutic gene of interest into the targeted cells or organs with the consequent expression of the transgene. In the past several years, the gene therapy has received significant attention due to its potential application to replace dysfunctional genes for the treatment of acquired diseases (Huang, 1999; Marxhall et al., 1999). The success of gene transfection is largely dependent on the development of vectors that could efficiently deliver a gene to cells with minimum toxicity. In this regard, two kinds of vectors, viral vectors and non-viral vectors have been developed. Viral vectors have been proved to be the most efficient carrier in gene delivery, but its clinical application has been limited due to safety issues. Therefore, non-viral vectors, such as chitosans and PEI, have been increasingly proposed as safer alternatives to viral vectors for their potential advantages such as the ease of synthesis, cell/tissue targeting properties, low immune response, as well as the unrestricted plasmid size (Leong et al., 1998).

Chitosans, a family of linear binary polysaccharides, comprised of beta (Huang, 1999; Koping-Hoggard et al., 2004; Leong et al., 1998; Marxhall et al., 1999) linked 2-amino-2-deoxy-β-D-glucose (GlcN; D-unit) and the N-acetylated analogue (GlcNAc; A-unit), have been proposed as biocompatible alternative cationic polymers that are suitable for non-viral gene delivery (Koping-Hoggard et al., 2004). However, this system has a significant limitation owing to its low transfection efficiency (Kim et al., 2005). One of the primary causes of poor gene delivery efficiency is the insufficient release of chitosans from endosomes into the cytoplasm (Kim et al., 2005). Another non-viral vector, PEI, is regarded to be the most effective cationic polymer for gene delivery (Densmore et al., 2000). Its high proton-buffering capacity results in rapid osmolysis of the endosomes, and the PEI–DNA complexes escape into the cytosol and are subsequently transported into the nucleus (Kichler et al., 2005). However, PEI is also associated with dose-dependent toxicity, especially at high molecular weight, and its long-term safety has been a main concern because it is non-biodegradable. It has therefore not yet been used in human studies (Fischer et al., 1999; Kunath et al., 2003). In our previous studies (Zhao et al., 2008, 2009), we obtained

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a chitosan/DNA/PEI complex simply using incubation method. By adjusting the N/P ratio, defined as the ratio of moles of the amine groups of copolymer to moles of phosphates of DNA, the complex showed high transfection efficiency and low toxicity. However, some problems still exist. For example, we could not remove the slight PEI/DNA complex in our previous experiments. The PEI which had been used in the incubation was 25 kDa. At this molecular weight, it is not biodegradable and may cause safety issues. To resolve these problems, a new complex, chitosan-linked-PEI (CP), was synthesized in the present study by using the low molecule weight of PEI (Mw = 1200 Da). It was reported that linking the low molecular weight PEI with the high molecular one using degradable linkers would form new PEIs with suitable molecular weights. It is desired that the toxicity will be greatly reduced for those new PEIs as it could be degraded into low molecular weight PEI with much less toxicity after delivering DNA into cells. Furthermore, those new PEIs could have good transfection efficiency for gene delivery (Ahn et al., 2002; Forrest et al., 2003; Gosselin et al., 2001; Petersen et al., 2002). However, during the reactions of the multifunctional low molecular weight PEIs with the degradable linkers, gelation might occur easily (Flory, 1953). Even though soluble products could be obtained through carefully adjusting the reaction conditions, it was difficult to control the structures, molecular weights, and constitutions of the products. Also, the reproducibility was poor. So we proposed to link chitosan with low molecule weight PEI. And the PEI length could be well controlled by adjusting the feed molar ratio of aziridine to amine of chitosan.

Several groups have reported the pilot research in this area. Park et al. (2001, 2000) prepared modified galactosylated chitosan (GC)/DNA complexes, and these systems could efficiently transfect liver cells, express asialoglycoprotein receptors, which specifically recognize the galactose ligands on chitosan. However, the transfection efficiency of these complexes was still low. Also, Kim et al. (2005) and Park et al. (2003) reported that chitosan coupled to urocanic acid (UA), which bears an imidazole ring, could play a crucial role in endosomal rupture through a proton-sponge mechanism. This group also demonstrated that when PEI (Mw = 10 kDa) was combined with a water-soluble chitosan (WSC)/DNA complex, the transfection efficiency was enhanced *via* the proton-sponge effect, while cell survival was not markedly decreased. Another group (Wong et al., 2006) designed a chitosan-graft-PEI (CHI-g-PEI) copolymer which has shown good results by using an imine reaction between peroxidate oxidized chitosan and low molecular weight PEI.

In this study, we designed and developed a novel CP (CHI-l-PEI) copolymer by an imine reaction between chitosan and low molecular weight PEI to reduce cytotoxicity of the complex and enhance its transfection efficiency. The physicochemical properties, cytotoxicity, transfection efficiency, as well as the long-term gene expression of this CP/DNA complex were investigated *in vitro*. The therapeutic gene CCL22 was used in the *ex vivo* study and its anti-tumor effects were evaluated in the murine model. It was first report that a therapeutic gene was delivered by the novel non-viral vectors and notable anti-tumor effects were observed in the murine model.

2. Materials and methods

2.1. Materials

Chitosan (Mw = 100 kDa) was purchased from Yuhuan Biochemistry Co., Ltd. (Yuhuan, China). Polyethylenimine (PEI, Mw = 25 kDa, 1200 Da) was purchased from Aldrich Chemical Co., Ltd. (Milwaukee, WI, USA). 1,1′-Carbonyldiimidazole (CDI) was from Pierce (Rockford, IL, USA). Luciferase activity assay kits and the protein assay kit were obtained from Biyuntian Biologic Co., Ltd. (Nanjing, China). Dulbecco's modified eagle's medium (DMEM) and trypsin were obtained from Gibco BRL (Gaithersberg, MD, USA). Fetal bovine serum (FBS) was purchased from Sijiqing Biologic Co., Ltd. (Hangzhou, China). Lipofectmine2000 was purchased from Invitrogen. All other chemical were analytical grade.

Plasmids DNA (pGL3 and pEGFP-N1) were kindly provided by the Institute of Infectious Diseases, Zhejiang University (Hangzhou, China). The plasmids DNA were amplified using *Escherichia coli* DH5α and purified using AxyPrep Maxi Plasmid Kit (Axygene Biotechnology Limited, Hangzhou, China). The purity of the plasmids 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 UV absorbance at 260 and 280 nm. DNA from protamine was used for the measurement of particle sizes.

2.2. Cell line

Human cervical adenocarcinoma cell line (HeLa) cells, A549 human lung carcinoma cells, HepG2 (human hepatoblastoma cells) cells and H22 (peripheral hepatoma cells) cells were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in DMEM medium containing 10% FBS at 37 °C and humidified 5% CO_2.

2.3. Synthesis of copolymer

0.5 g chitosan powder (100 kDa) was dissolved in 20 ml 0.5% HAc solution with a pH of 7.0 and the mixture was stirred over night. CDI was then added at a molar ratio of CDI: amine of chitosan = 2:1, followed by stirring for 1 h. The PEI (1200 Da) was added dropwise into the solution under stirring at a molar ratio of PEI:amine of 2:1. The polymerization was performed at room temperature over night. The resulting crude product was purified by dialysis in water for 48 h. Finally, the light white powder was yielded by lyophilizing.

2.4. Characterization of copolymer

The composition of the prepared CP copolymer was estimated by measuring ^{1}H nuclear magnetic resonance (^{1}H NMR) (Avance^{TM} 600, Bruker, Germany)

2.5. Cell viability assays

The CP solution and PEI solution were filtering through 0.22 μm aseptic filter membranes before there were incubated with cells. For cell viability assay, HeLa, HepG2 and A549 cells were seeded into 96-well plates at a density of 5×10^3 cells/well, After 24 h, different concentrations of CP and PEI were added with 100 μl fresh serum-free DMEM medium to replace the culture medium. And cells were incubated at 37 °C in a humidified 5% CO_2-containing atmosphere for 6 h. Then it was replaced by fresh DMEM medium with 10% fetal bovine serum. After being incubated for 42 h, 20 μl of MTT solution (5 mg/ml) was added to each well and the absorbance was measured according to the manufacture's instruction. The cell viability was calculated as following:

$$\text{cell viability } (\%) = \frac{\text{OD595 (sample)}}{\text{OD595 (control)}} \times 100$$

where OD595 (sample) represents a measurement from the group treated with vectors and OD595 (control) represents the group treated with PBS buffer only. All the experiments were carried out in triplicate to ascertain the reproducibility.

2.6. Preparation of CP/DNA complex

All chitosan-l-PEI/DNA complexes were freshly prepared before use. The charge ratio (N/P) of the chitosan-l-PEI/DNA complexes are defined as the molar relation of amine groups in the cationic molecule, which represent the positive charges, to phosphate groups in the DNA, which represent the negative charges. For calculation of N/P ratios, 330 Da was used as an average mass per charge for DNA. Complexes were prepared by adding copolymer solution to equal volumes of calf thymus DNA (Sigma) (for size and zeta potential measurements), to pGL3-control solution (for luciferase assay), or to pEGFP-N2 (for *in vitro* GFP transfection) with gentle vortexing and incubation at room temperature for 30 min.

2.7. Gel retardation assay

Different amounts of CP (charge ratio from 2 to 10) were combined to DNA (0.5 μg of pGL3) and the effect of CP on condensation of DNA was investigated by electrophoresis on a 1% agarose gel with Tris-acetate (TAE) running buffer at 100 V for 30 min. DNA was visualized with ethidium bromide (0.2 μg/ml).

2.8. Measurement of particle sizes and zeta potential

The particle sizes and surface charge (represented by the surface zeta potential) of the synthesized CP/DNA complexes with different charge ratios were measured using a laser diffraction spectrometry (Malvern Zetasizer 3000HS, Malvern, UK). The volume of the samples was 1 ml containing a final DNA concentration of 50 μg/ml.

2.9. GFP transfection

HeLa cells were seeded into the 24-well plate at a density of 1×10^5 cells/well, cultured for 18 h before transfection. CP/DNA complex was prepared by mixing 1 μg of EGFP plasmid with appropriate amounts of mass ratios from 2:1 to 10:1 and incubated for 30 min at room temperature. The Lipofectmine2000 was used as the positive control. The final volumes were adjusted to 500 μl by serum-free DMEM medium. All the solutions were filtered through a 0.22 μm aseptic filter before mixing. The vector/DNA complex were added to the 24-well plate and incubated for 6 h at 37 °C under 5% CO_2 atmosphere. Then the DMEM was replaced with the fresh DMEM with 10% serum. After 48 h, fluorescence signal was observed by the fluorescence-inverted microscope.

2.10. Assay of luciferase activity

To evaluate the transfection efficiency, CP/DNA complexes (1 μg pGL3 plasmid with mass ratio = 10:1) were prepared to transfect in HeLa cells, A549 cells and HepG2 cells. PEI/DNA complex (N/P = 10, 1 μg DNA) was also prepared using the similar methods. The transfect procedure was the same as mentioned in Section

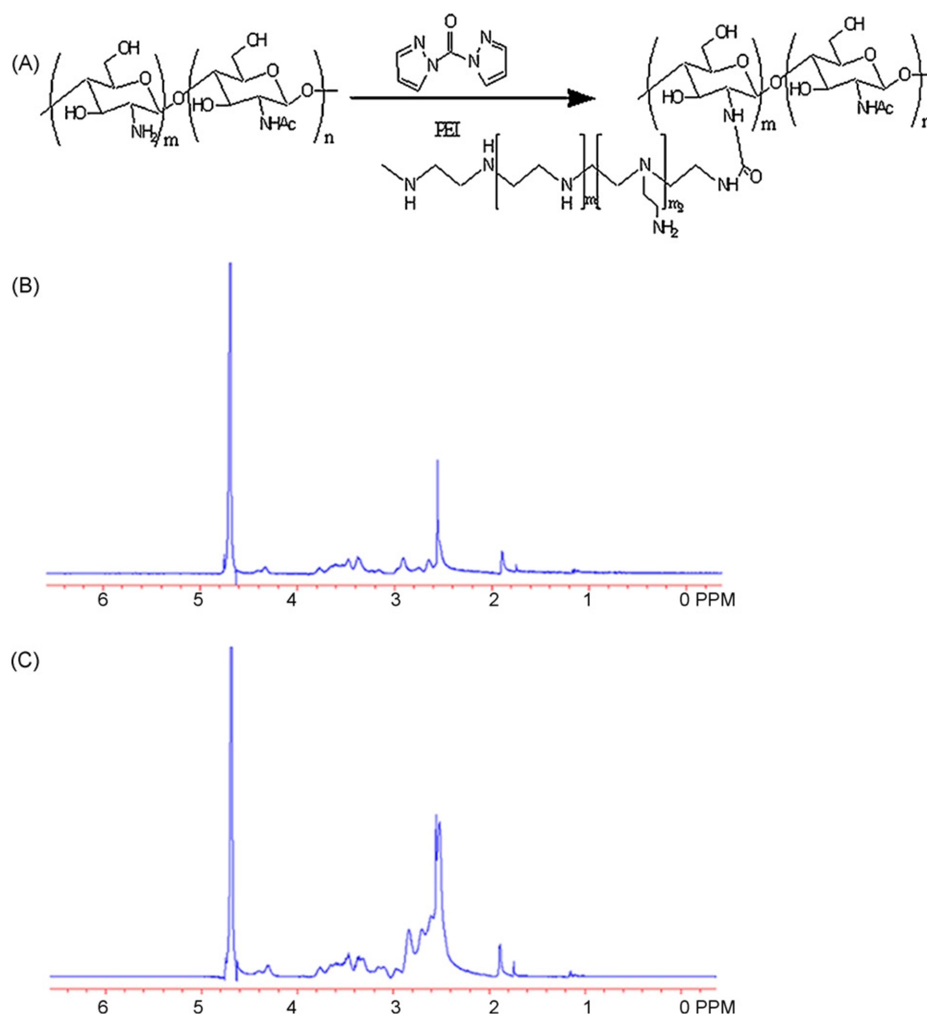


Fig. 1. (A) Reaction scheme for synthesis of chitosan-linked-PEI. (B) ^{1}H NMR spectra of water-soluble oligo-chitosan. (C) ^{1}H NMR spectra of chitosan-linked-PEI.

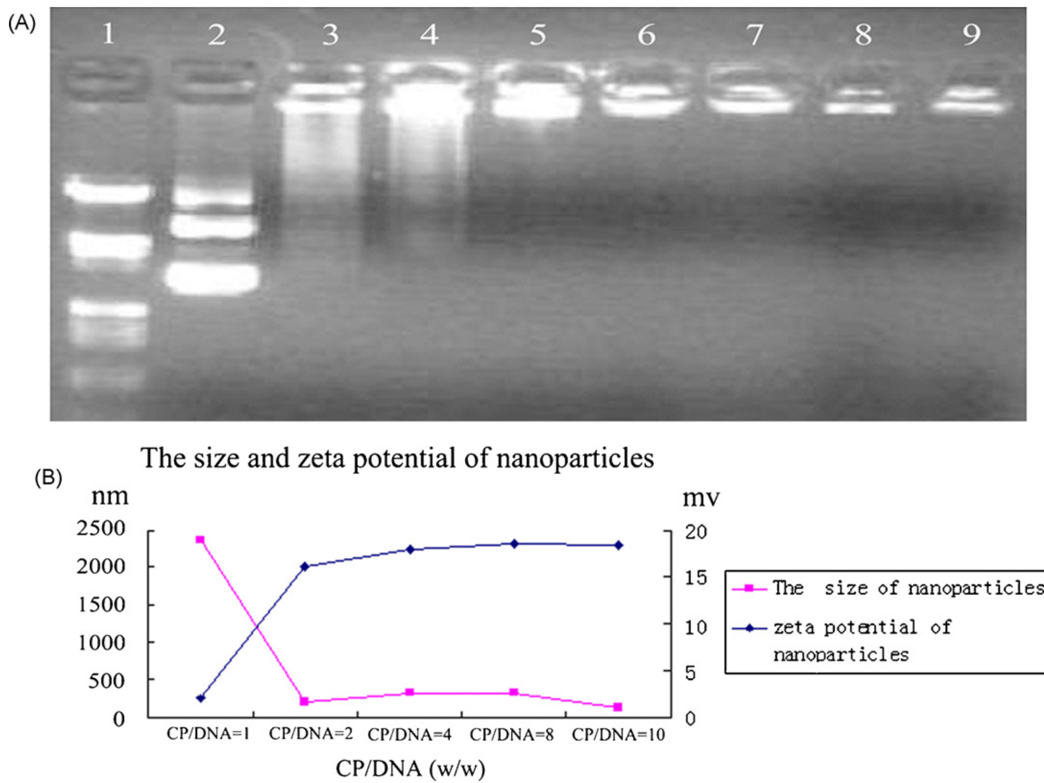


Fig. 2. (A) Gel retardation assay of CP/DNA (w/w) nanoparticles. Lane 1: marker; 2: naked DNA; 3: (w/w) = 0.5; 4: (w/w) = 1; 5: (w/w) = 2; 6: (w/w) = 3; 7: (w/w) = 4; (B) particle sizes and surface charges of copolymer/DNA complexes at various (w/w) ratios.

2.9. After 24 and 96 h, the luciferase assay was carried out according to the manufacture's instruction (Promega, USA). Light units (LUs) due to luciferase activity were measured with chemiluminometer (Autolumat LB953, EG&G Derthold, Germany). All the experiments were carried out in triplicate to ascertain the reproducibility.

2.11. Confocal laser scanning microscopy

The HepG2 cells were plated on glass coverslips in 24-well plates at 5×10^4 cells/well. The cells were incubated for 18 h. Then the media were replaced with serum-free media, containing polymer/DNA-FITC (1 μg) complex at (w/w) ratio of 10. After

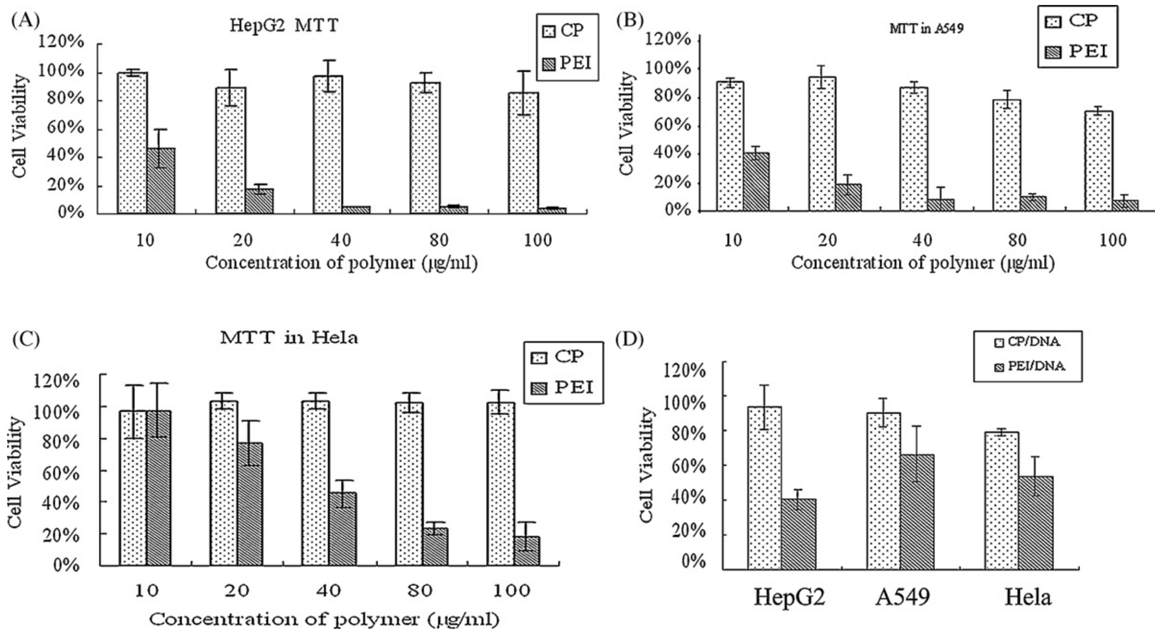


Fig. 3. Cytotoxicity of CP and PEI at various concentrations in different cell lines. (A) HepG2, (B) A549 and (C) HeLa. (D) Cytotoxicity of complex in three cell lines, CP/DNA (mass ratio) = 10, PEI/DNA (N/P) = 10 (n = 3, error bars represent standard deviation). Data are expressed as mean ± standard deviations.

incubation for 0.5, 2, 4, and 6 h, cells were washed in PBS, and fixed with 4% paraform. The nucleus was stained with PI for 30 min in room temperature and washed for three times. The coverslips were mounted on microslides. Cells were observed with a confocal laser scanning microscope (MRC-1024, Bio-Rad, UK).

2.12. Tumor regression for ex vivo experiment

Five weeks old ICR mice were inoculated intradermally with 1.5×10^6 H22 cells that had been transfected with CP/CCL22 (mass ratio 10:1), PEI/CCL22 (N/P = 4), and chitosan/CCL22 (N/P = 4) for 4 h. The dose of CCL22 is 20 μg per mouse. All cells are in serum-free DMEM before injection. Tumor volume was calculated by measuring the length and width of the tumor three times a week. Mice weights were also measured. Each group included at least 6 mice. Meanwhile the MTT of H22 cells were also conducted to deplete the false positive.

3. Results and discussion

3.1. Synthesis and characterization of copolymer

PEI-l-chitosan was synthesized by reaction between chitosan and PEI with the presence of CDI (Fig. 1). In the reaction, CDI played an important role as a linking agent. The amino group of chitosan was firstly reacted with one imidazolyl of CDI, and then another imidazolyl of CDI linked with PEI's amino group. The molar ratio of CDI to the amine of chitosan was kept to be 2:1.The feed molar ratio of PEI to amine of chitosan was 5:1. Composition of synthesized copolymer was analyzed by ^{1}H NMR. As shown in Fig. 1, the proton peaks area appeared at 3.0–2.5 ppm were increased after synthesis, which indicated that PEI was grafted to the chitosan chain. It could be calculated from the proton peak area that every two chitosan monomers grafted one PEI monomer to form the complex. After PEI was grafted, CHI-l-PEI was completely water-soluble at physiological pH conditions due to the hydrophilic property of PEI, although chitosan itself is only soluble in acidic conditions.

3.2. Characterization of chitosan-linked-PEI/DNA complexes

The formation of the chitosan-l-PEI/DNA (CP/DNA) complexes was confirmed using agarose gel electrophoresis and their particle size and zeta potential were evaluated. Fig. 2(A) demonstrates that the migration of DNA was completely retarded when the CP/DNA ratio (w/w) of complex was around four, indicating DNA bound tightly and completely with CP so there is little free DNA to escape. Generally, small particle size and positive surface charge would lead to higher internalization rates of complexes. As shown in Fig. 2(B), the particle size was determined to be 2500 nm when the CP:DNA (w/w) ratio was 1. However, at a ratio higher than 2, the sizes of the complexes were less than 250 nm. It could be explained by the facts that the combining of polymer complexes with DNA is through ionic interactions (Kubota et al., 2000). Thus, at high CP to DNA ratios, there are net electrostatic repulsive forces to prevent aggregation among complexes. Fig. 2(B) showed the zeta potential assay results of the CP/DNA complexes with increased (w/w) ratios. It was shown that the zeta potential was negative when the ratio of CP:DNA (w/w) complex was 1, where the complex could not form

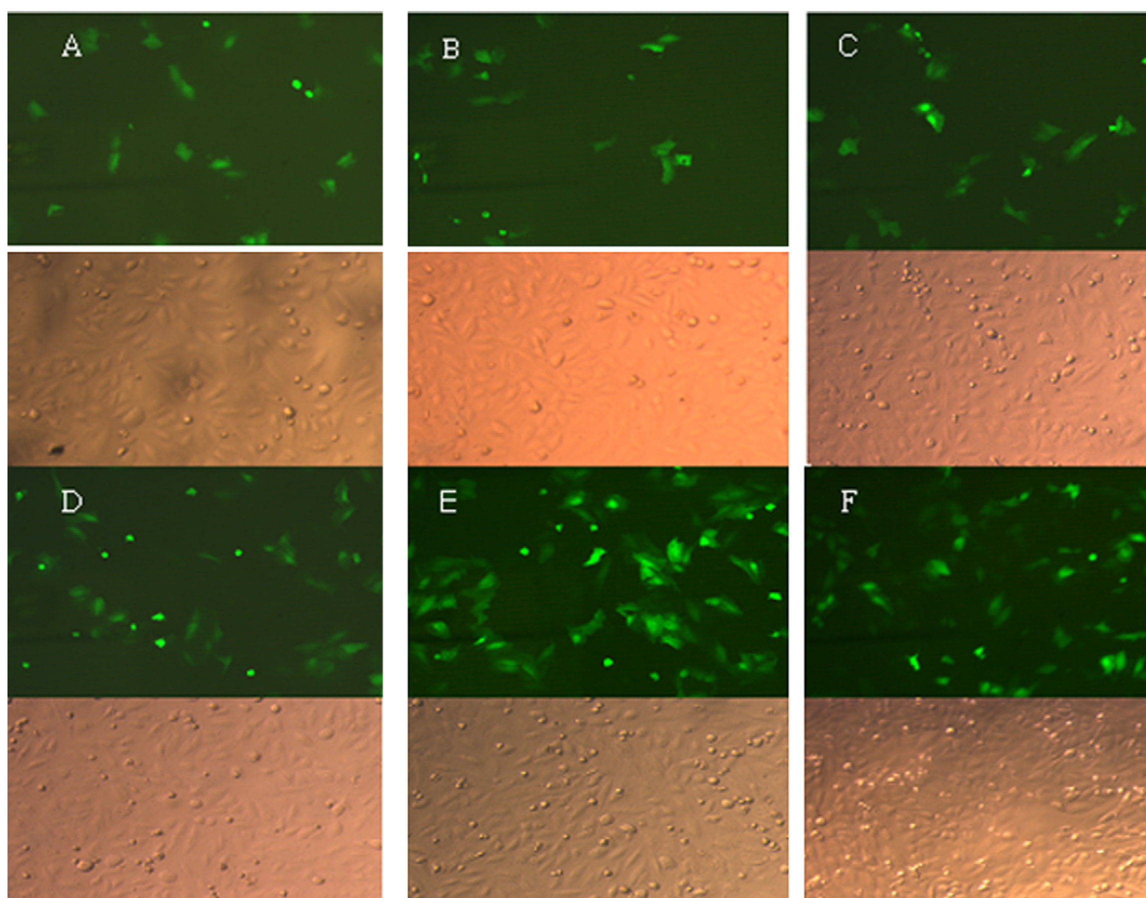


Fig. 4. Transfection efficiency of CP/DNA (EGFP-control) complex at various (w/w) ratios in HeLa cells after 48 h. (A) (w/w) = 1, (B) (w/w) = 2, (C) (w/w) = 4, (D) (w/w) = 5, (E) (w/w) = 10 and (F) Lipofectamine2000.

completely. With the increasing of CP:DNA (w/w) ratio, the zeta potential of the complex rapidly increased to positive values. These results, together with the results obtained from particles size assay and gel retardation experiments, suggested that the CP/DNA could form positive potential nanoparticles when the CP:DNA (w/w) was up to 4.

3.3. Cytotoxicity of CP copolymer

Cytotoxicity was one of the main concerns for the copolymer, particularly for *in vivo* delivery. Fig. 3(A–C) showed the results of *in vitro* cytotoxicity of the copolymer analyzed by MTT assay in three cell lines (HepG2, A549 and HeLa cells). As shown in Fig. 3, the cytotoxicity of CP copolymer was much lower than that of PEI (25 kDa). The cytotoxicity of PEI is probably caused by polymer aggregation on cell surfaces, impairing important membrane functions. The high positive charge of polymer can cause cell membrane damage. The examined zeta potential of PEI/DNA is nearly 40 (Zhao et al., 2009), which is much higher than CP/DNA complex. Also, the cationic polymers may interfere with critical intracellular processes of cells. Particularly, the primary amine was reported to disrupt PKC function through disturbance of protein kinase activity(Farhood et al., 1992; Zhang et al., 2005). It has been reported that high molecular weight PEI is significantly more toxic than low molecular weight PEI (Fischer et al., 1999; Kunath et al., 2003). Therefore we choose low molecular weight PEI (Mw = 1200 Da) and chitosan, both of which are degradable, to synthesis co-polymers. The biocompatibility and long-term safety of CP could be improved significantly as compared with PEI (25 kDa). These results are in agreement with the findings by Jiang et al. (2007). Moreover, CP/DNA complexes were also evaluated for the cytotoxicity. No cytotoxicity was detected in three types of cells treated with CP/DNA complex. In contrast, the PEI/DNA-treated group was shown to have a high cytotoxicity (Fig. 3(D)).

3.4. Transfection efficiency of CP/DNA complex

To optimize the transfection efficiency of CP by carefully controlling the mass ratio of CP and DNA (w/w), we transfer complex into HeLa cells and the Lipofectamine2000 was used as the positive control. The mass ratio varied from 2:1 to 10:1. Plasmid pEGFP-N1 encoding green fluorescence protein (GFP) was used to examine the GFP expression in cells. Fig. 4 showed the transfection efficiency of the copolymer increased with the increasing of mass ratios. When the mass ratio reaches 5, the transfection efficiency of the complex is similar with that of the Lipofectamine2000. When it reaches 10, the transfection efficiency is greater than that of Lipofectamine2000. With the increasing amount of CP, the content of grafted PEI (1.2 kDa) helped complex to escape from endosome more easily, so that it will not cause cytotoxicity to the cells. Other than the results obtained after 48 h, we also get a result after 7 days. Fluorescence signal could be observed when transfections were mediated by CP after 7 days. However, at the same time, the GFP expression could not be detected when the transfection was mediated by Lipofectamine2000 (data not shown).

To further investigate the transfection efficiency of CP copolymer, we also performed an *in vitro* luciferase activity assay to investigate the transfection efficiency of CP copolymer in three cell lines (HeLa, HepG2 and A549). The highest transfection efficiency was observed in the HepG2 cell line (Fig. 5). Furthermore, we evaluated the long-term transfection efficiency of copolymer and PEI by measuring the luciferase activity at 24 and 96 h. It was found that the transfection efficiency of the copolymer increased or at least maintained at certain level with the increasing of time, whereas the transfection efficiency of PEI25KDa was decreased. It is known that a key cellular barrier impeding the transfection efficiency of non-viral gene vectors is the inefficient release of endosomally trapped DNA into the cell cytosol. It was generally believed that PEI could escape from endosome through the proton-sponge mechanism and thus facilitate gene entry into the nucleus (Kichler et al., 2005). And the proton-sponge ability of the polymer may be caused by the high amine content in PEI, which also results in high cytotoxicity of the polymer, so that the transfection of DNA delivered by PEI could not last for long-term. The PEI-l-chitosan is synthesized by linking the low molecular weight of PEI with chitosan. The total amount of amine content in complex is similar with PEI25KDa, which allows the complex to escape the endosome easily due to a higher buffering capacity. And moreover, the release rate of DNA from the complex could be decreased by retardant effect of the chitosan. Thus the gene expression could be lasted for long time. Additionally, the copolymer may have a weaker condensation capability than PEI25KDa, which would result in an easy release of DNA from the complexes after escape from the endosome, so that it could increase the transfection efficiency of DNA.

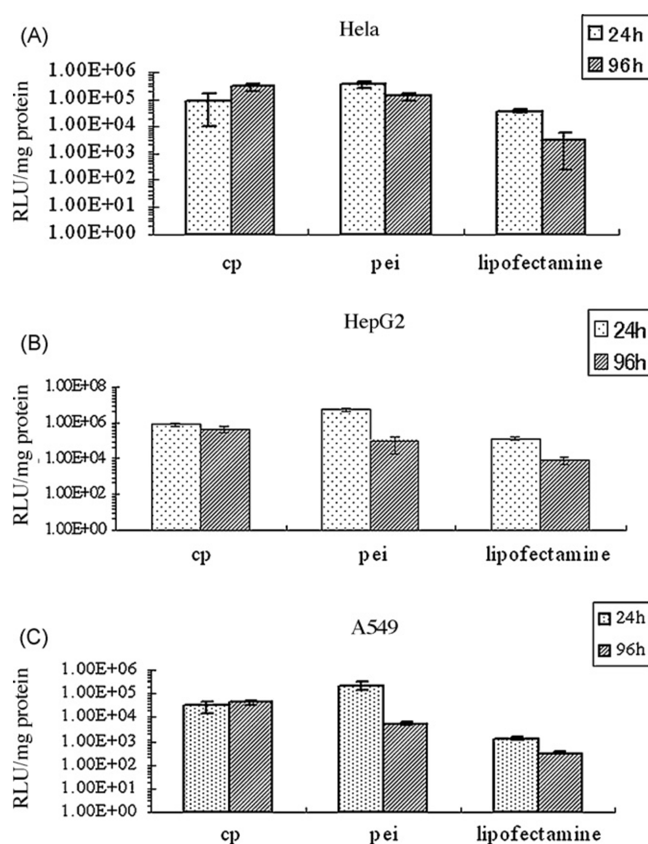


Fig. 5. Transfection efficiency of CP/DNA (pGL3-control) complex at various (w/w) ratios in various cell lines. (A) HeLa, (B) HepG2 and (C) A549 ($n = 3$, error bars represent standard deviation). Data are expressed as mean ± standard deviations.

3.5. Cellular internalization of CP/DNA complex

Cellular internalization of CP/DNA complex was observed under confocal laser scanning microscopy. Fig. 6 shows the time-dependent changes of CP/DNA complex internalization. It was observed that the complexes were internalized in 0.5 h and they were distributed mostly into the endochylema. The complexes entered into the nucleus in 4 h and more DNA could be detected in nucleus after 6 h. In contrast, PEI25KDa could escape from endosome and enter the nucleus more quickly and the DNA carried by the chitosan is difficult to enter the nucleus (Feng et al., 2006;

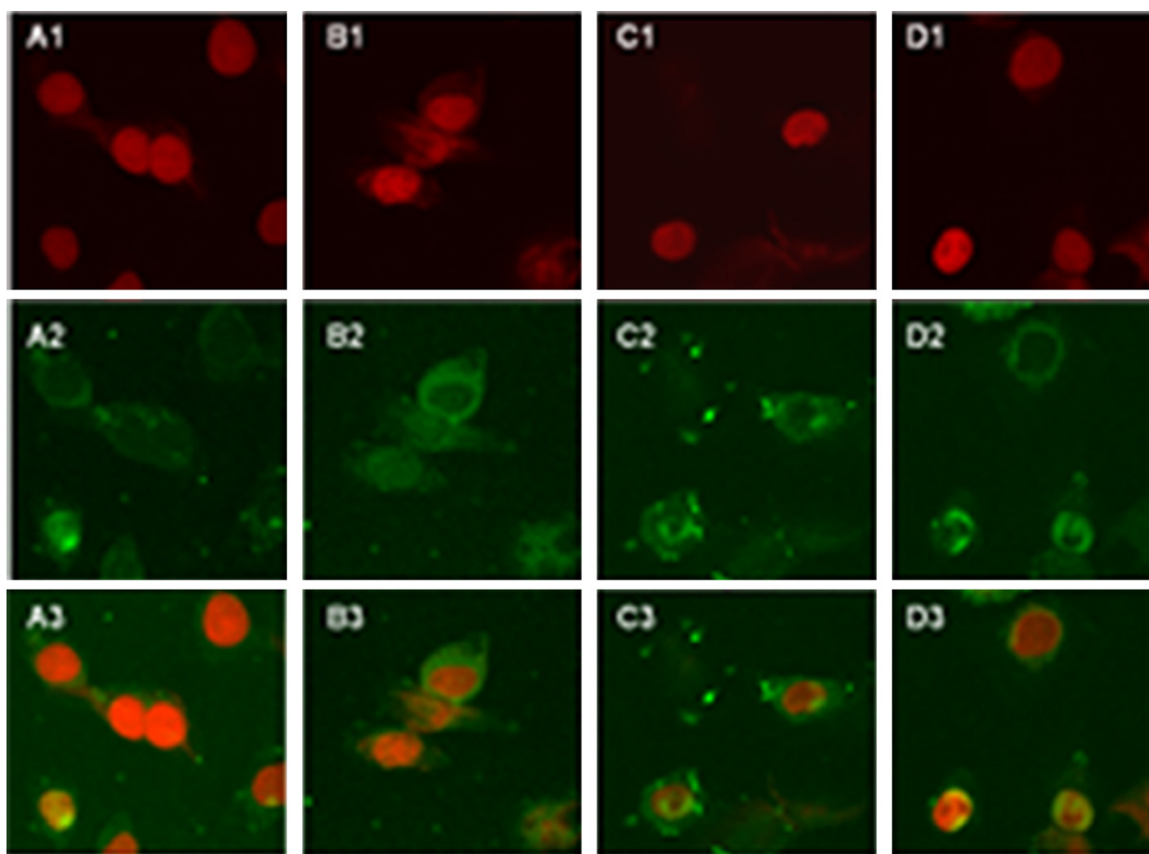
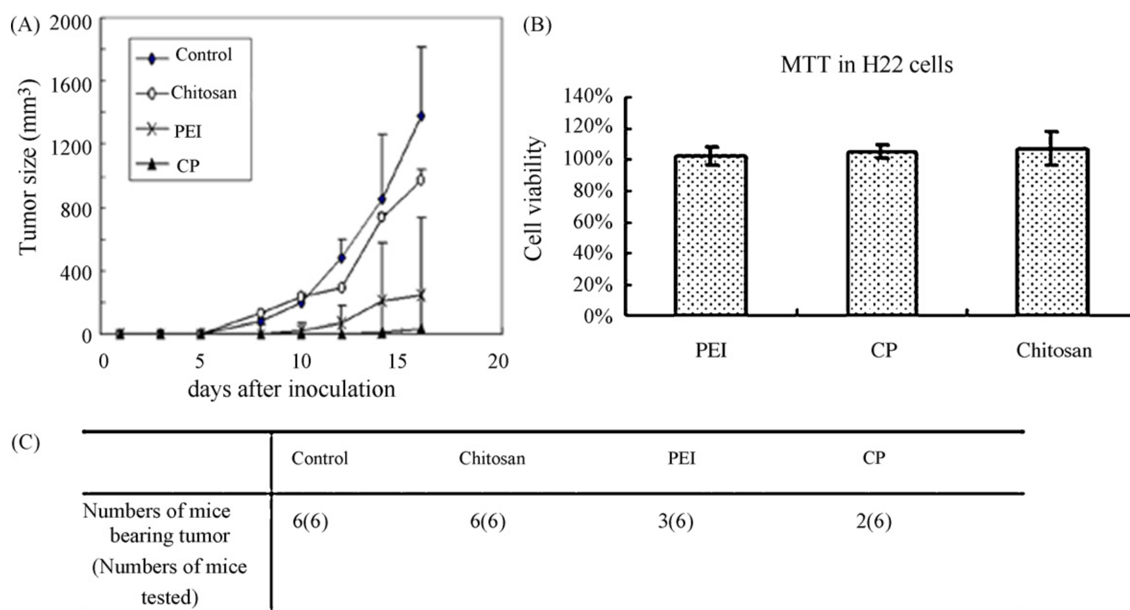


Fig. 6. Intracellular distribution of FITC-plasmid/CP complexes was observed with a confocal fluorescence microscope in HepG2 cells. (A) 0.5 h Post-incubation; (B) 2 h post-incubation; (C) 4 h post-incubation; (D) 6 h post-incubation. The upper panel shows the nucleus (red) and the middle panel shows the FITC-plasmid (green). The lower panel shows the over-laid of 1 and 2.

Hashimoto et al., 2006). It is supposed that the ability and the time to escape from the endosome and enter nucleus of vectors are very important for both the transfection efficiency and the cytotoxicity. The CP complex can escape from endosomes at a proper rate which increased the transfection efficiency while the cytotoxicity was not increased.

3.6. Tumor-suppressive activity induced by therapeutic gene-carried CP

The efficiency of the CP copolymer in delivering the DNA is demonstrated using the transfection system by combining the copolymer and a therapeutical gene, chemokine CCL22, and



	Control	Chitosan	PEI	CP
Numbers of mice bearing tumor (Numbers of mice tested)	6(6)	6(6)	3(6)	2(6)

Fig. 7. (A) Growth of H22 tumor cells in ICR mice. H22 cells were transfected with CCL22 chemokine gene by CP, PEI, chitosan *in vitro* for 4 h.Then the cells were gathered and were intradermally injected into the flank of mice at 1×10^6 cells. Tumor volume was calculated after measuring the length and width of the tumor at indicated periods of time, and data are expressed as the mean ± SE of results obtained from six mice. (B) The cells survival before inject into mice (c)% of mice bearing tumor.

assessed by its abilities in suppressing the growth of tumor cells. Chemokines consist of a superfamily of small secreted proteins that attract their target cells by interacting with G protein-coupled receptors expressed on these cells (Gao et al., 2003). Researchers have paid attention to chemotactic activity of chemokines for immune cells, and have expected that they may be able to play a pivotal role in cancer treatment, because the basis and premise of immunotherapy is the accumulation of immune cells in tumor tissues. More than 40 chemokines have been identified so far, but only a few have been demonstrated to be candidates for cancer therapy as sole agents or with adjuvant (Gao et al., 2003; Okada et al., 2004; Yoshie et al., 2001). Macrophage-derived chemokine/CCL22 are chemotactic for memory $CD4^{+}$ T cells via CCR4 (Campbell et al., 1999). In our previous study, the anti-tumor effect of CCL22-encoding adenovirus vector has also been demonstrated (Gao et al., 2004). It was supposed that tumor cells, genetically altered to secrete chemokines by an efficient gene transfer system *in vitro*, would generate the anti-tumor immune response in the murine models. The *in vivo* interaction of T cells with the tumor cells should induce anti-tumor immunity, resulting in suppression of tumor growth. The gene transfection and expression were achieved by incubating H22 cells with the copolymer *in vitro*. The tumor sizes of mice were greatly reduced after injection of the genetically altered cells (Fig. 7). CP showed similar transfection efficiency as PEI. In all of the six mice examined, only two and three mice bared tumors in CP group and PEI group respectively. This is a little different from the *in vitro* results where PEI showed the highest transfection efficiency in 24-h incubation. However, the increased gene expression was observed in the group treated by CP with the increasing of the cultivation time. And the fluorescence signal induced by CP was higher than that of PEI25KDa after 96 h. We supposed that DNA encapsulated in the CP nanoparticle may result in the sustained release of protein and prolonged the duration of gene expression due to the retardant effect of chitosan, therefore induced the stronger tumor-suppressive activity. On the other hand, the immune process is a long-term procedure and it takes time for the tumor to reach a certain volume. In the previous experiments, we have already demonstrated that CP could express gene which lasted for at least 7 days while the gene transfection efficiency of PEI was decreased dramatically at the same time. At the initial stage of tumor growth, the immune response would be more important to determine whether the tumor would grow or not. It was demonstrated that when the tumor cells were transfected with PEI/DNA complex at the N/P ratio of 4:1 and inoculated into the mice, the mice survived. Thus, the PEI/DNA complex with an N/P ratio of 4:1 was proved to be a safe complex as compared with that with the N/P ratio of 10:1, which could cause a high cytotoxicity *in vitro*, while the transfection efficiency was a little lower.

4. Conclusions

In this study, a novel gene carrier, CP copolymer, was successfully synthesized and investigated for its physicochemical properties, cytotoxicity and gene transfection efficacy. The results showed that CP could form a complex with DNA and showed compatible physicochemical properties for a gene delivery system. This copolymer had low cytotoxicity and high gene transfection efficiency in HepG2, A549 and HeLa cells when compared with PEI25KDa in the long-term transfection efficiency. Particularly, when CCL22 gene-altered H22 cells were inoculated into mice, the tumor growth rate was significant inhibited. Therefore, CHI-l-PEI could be a promising vector for the therapeutic gene delivery.

Acknowledgements

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