State Key Laboratory of Bioactive Substances and Functions of Natural Medicines[1], Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing; Yanbian University Hospital[2], Yanji; Department of Pathology[3], Beijing Institute of Basic Medical Sciences, Beijing, China

Preparation and characterization of polymeric nanoparticles for siRNA delivery to down-regulate the expressions of exogenous and endogenous target genes

WEI HUANG[1], MING LV[1], ZHONG-GAO GAO[1,2], MING-JI JIN[1], YUAN-JI XU[3], XIAO-DAN YU[3], ZHE-HU JIN[2], XUE-ZHE YIN[2]

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Zhong-gao Gao, State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China
zggao@imm.ac.cn

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Gene silencing induced by RNA interference using small interfering RNA (siRNA) provides a promising therapeutic approach for cancers. However, the lack of siRNA delivery vector has limited the development of siRNA therapy. The purpose of this study was to use the novel copolymer $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$ to prepare siRNA-loaded nanoparticles for siRNA delivery. The results suggested that $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$ could load siRNA to form nanoparticles with particle size less than 200 nm in a narrow distribution. Moreover, a certain density of positive charge existed onto the surfaces of nanoparticles. MTT assay results demonstrated that $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$/siRNA nanoparticles showed very low cytotoxicity. The gene silencing efficiency of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$/siRNA nanoparticles was investigated through luciferase reporter gene assays. The expression of exogenous luciferase gene was significantly down-regulated at a range of N/P ratio from 50 to 125, and was maximally inhibited at the N/P ratio of 125 with 54% and 59% reduction in MCF-7 and HepG2 cells, respectively. In the 4T1-luc cell line expressing luciferase stably, the silencing of endogenous luciferase gene also has a similar overall profile with maximal 54% reduction of luciferase expression. These results suggested that $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$/siRNA nanoparticles could serve as a kind of highly efficient siRNA delivery system for down-regulating the expression of exogenous and endogenous target genes.

1. Introduction

Small interfering RNA (siRNA) are a kind of double-stranded RNA molecules with a length of about 20–25 bp, which can induce RNA interference (RNAi) reponse to down-regulate the expression of target genes such as disease-related genes. Hence, siRNA provides a promising new kind of therapeutics for serious diseases such as various cancers (Bumcrot et al. 2006). The siRNA itself cannot actively enter cells, and are also easy to be degraded by nuclease. So, vectors are required to protect and deliver siRNA into cells. Although viral vectors are highly efficient, their applications are limited by immunogenicity, potential pathogenicity and difficult preparation (Couto and High 2010; Elsabahy et al. 2011). On the contrary, non-viral vectors attract more and more attention due to their advantages including good biocompatibility, no immunogenicity, no pathogenicity and easy preparation (Kim and Kim 2009; Takahashi et al. 2009). Currently, the study of non-viral vectors became a very hot topic in the field of siRNA delivery (Shegokar et al. 2011).

Recently, it has been demonstrated that the introduction of hydrophobic moieties could enhance tissue permeability and cellular affinity of gene delivery vectors (Batrakova et al. 2001; Wang et al. 2002). Due to good biocompatibility and biodegradability, polycaprolactone (PCL) is often used as hydrophobic moiety to construct drug delivery vectors (Kim et al. 2001). In addition, PEGylation is the most common modification strategy in the development of nanoscale drug delivery systems (nano-DDS) (Hoffman 2008). PEGylation not only improves the nano-DDS stability (Morris et al. 2007), but also extends its blood circulation time *in vivo* to increase drug utilization (Gref et al. 1994), specially performing the enhanced permeability and retention (EPR) effects for cancer therapy (Wang et al. 2008). Moreover, PEGylation also increases the safety of nano-DDS due to good biocompatibility of PEG.

In our previous study, we have synthesized a kind of copolymer $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$ as non-viral vector for gene delivery (Huang et al. 2011). This kind of copolymer has a novel structural composition with mPEG segments (molecular weight of 5 kDa), PCL segments (molecular weight of 1.2 kDa), PEI segments (molecular weight of 10 kDa) and 1.4 graft density of $mPEG_{5k}\text{-}PCL_{1.2k}$ segments. Taking molecular structures into account, $mPEG_{5k}\text{-}PCL_{1.2k}$ segments introduced at the primary amine sites of PEI_{10k} segments can provide biocompatibility for $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$ molecules. Moreover, the hydrophilic segments $mPEG_{5k}$ incorporated at the end of $PCL_{1.2k}$ segments can easily carry out the PEGylation of drug delivery systems based on $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$ copolymers. Importantly, PEI_{10k} segments have a certain den-

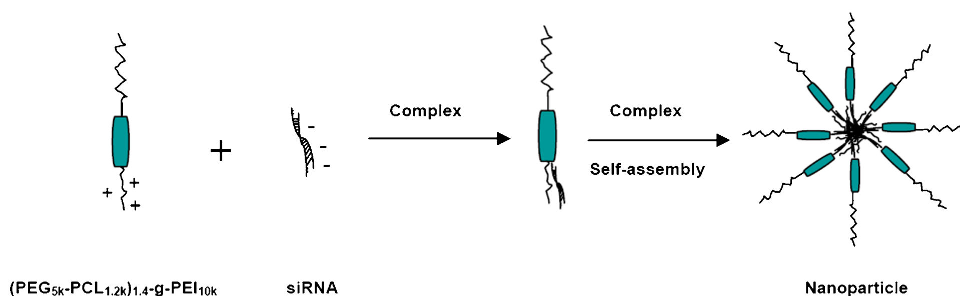

Fig. 1: Schematic representation of $(PEG_{5K}\text{-}PCL_{1.2K})_{1.4}$-g-$PEI_{10K}$/siRNA nanoparticles formation

sity of positive charge, and can complex the negatively charged nucleic acid molecules by electrostatic interaction. In fact, we have used $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ to delivery green fluorescent protein (GFP) gene into mammalian cells to get the high expression of GFP (Huang et al. 2011). However, the potential of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ as siRNA delivery vector has still not been explored in detail so far. Therefore, in this study, we used the novel copolymers $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ to prepare siRNA-loaded nanoparticles for siRNA delivery. The nanoparticles were used to deliver siRNA into cells to down-regulate the expression of exogenous and endogenous target genes. The physicochemical properties, cytotoxicity and gene silencing efficiency of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles were investigated in this study.

2. Investigations, results and discussion

2.1. Preparation and characterization of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles

Due to the protonation of primary amine groups in PEI_{10k} segments, a certain density of positive charge exists onto the surface of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ molecules. Consequently, $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ can complex the negative charged siRNA molecules through electrostatic interaction. On the other hand, $PCL_{1.2k}$ segments contribute to the property of hydrophobicity, which leads to the amphiphilicity of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ molecules. From the above two aspects, it was inferred that $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ could load siRNA to form nanoparticles (Fig. 1). In the actual preparation, we have prepared $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles at various N/P ratios. Moreover, the particle size and zeta potential of nanoparticles were also well characterized, respectively. The results showed that $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ could load siRNA to form nanoparticles with particle sizes less than 200 nm in a narrow range (Fig. 2), which just also confirmed our above inference. With the increase in N/P ratio, the particle size of nanoparticles showed a decreasing trend. Additionally, $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles had a positive zeta potential less than 20 mV (Fig. 3), which suggested that a certain density of positive charge existed on the surface of nanoparticles. The zeta potential of nanoparticles gradually increased with the increase in N/P ratio (Fig. 3), which may result from the gradual enhancement of the proportion of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ in the nanoparticles suspension.

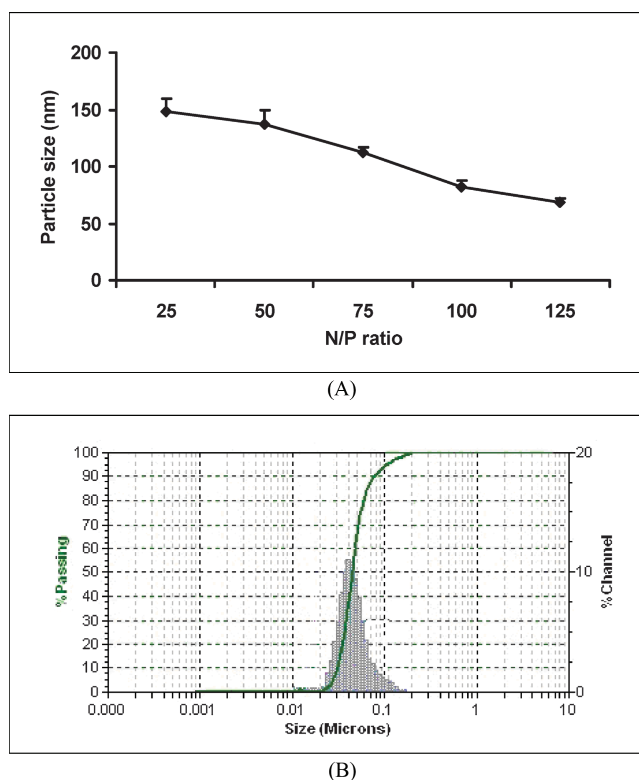

Fig. 2: Particle size and distribution of $(PEG_{5K}\text{-}PCL_{1.2K})_{1.4}$-g-$PEI_{10K}$/siRNA nanoparticles. (A) Particle size of nanoparticles at various N/P ratios. (B) Particle size distribution of nanoparticles at the N/P ratio of 125

2.2. Cytotoxicity of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles

It has been demonstrated that the transmembrane of charged nanoparticles can be carried out through the means of membrane perforation (Hong et al. 2006). Unfortunately, the cavities produced by membrane perforation can induce extravasation of intracellular fluid, which resulted in the cytotoxicity of cell membrane structural damage (Leroueil et al. 2008; Mecke et al. 2004). So, the positively charged nanoparticles show an inevitable toxicity effect on cell growth. In this study, MTT assays were performed to evaluate the effect of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA at various N/P ratios on cell viability. The results show that cell viabilities in various groups all reached more than 88% in MCF-7 cells and 93%

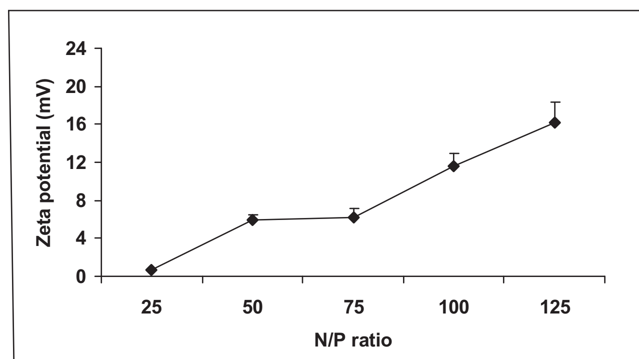

Fig. 3: Zeta potential of $(PEG_{5K}\text{-}PCL_{1.2K})_{1.4}$-g-$PEI_{10K}$/siRNA nanoparticles at various N/P ratios

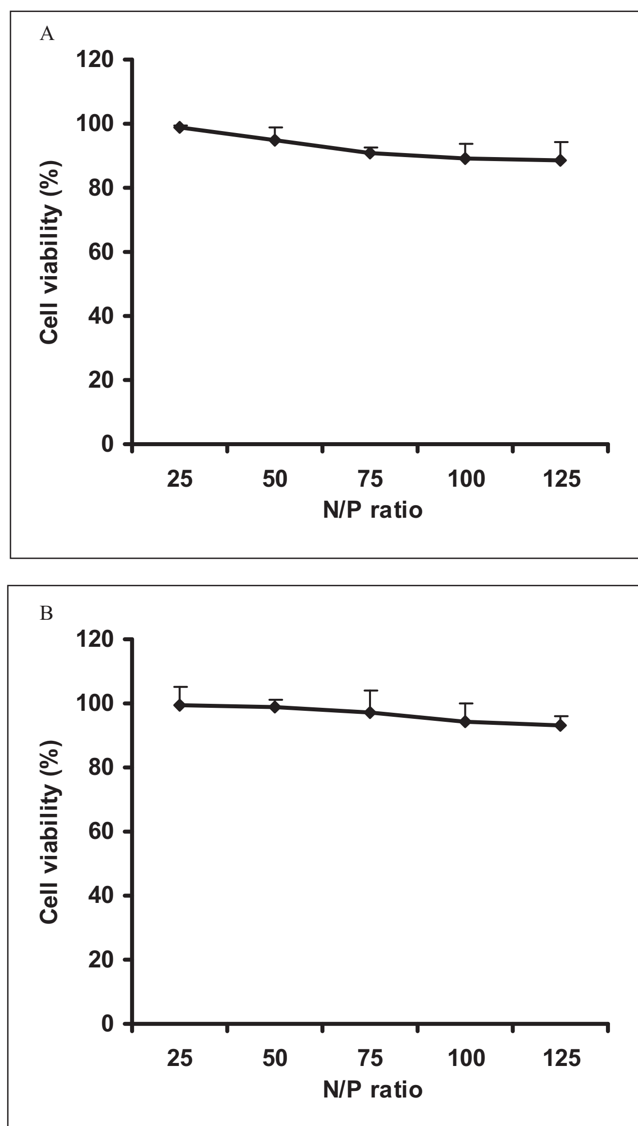

Fig. 4: Effect of $(PEG_{5K}\text{-}PCL_{1.2K})_{1.4}$-g-$PEI_{10K}$/siRNA nanoparticles at various N/P ratios on the viability of MCF-7 (A) and HepG2 (B) cells

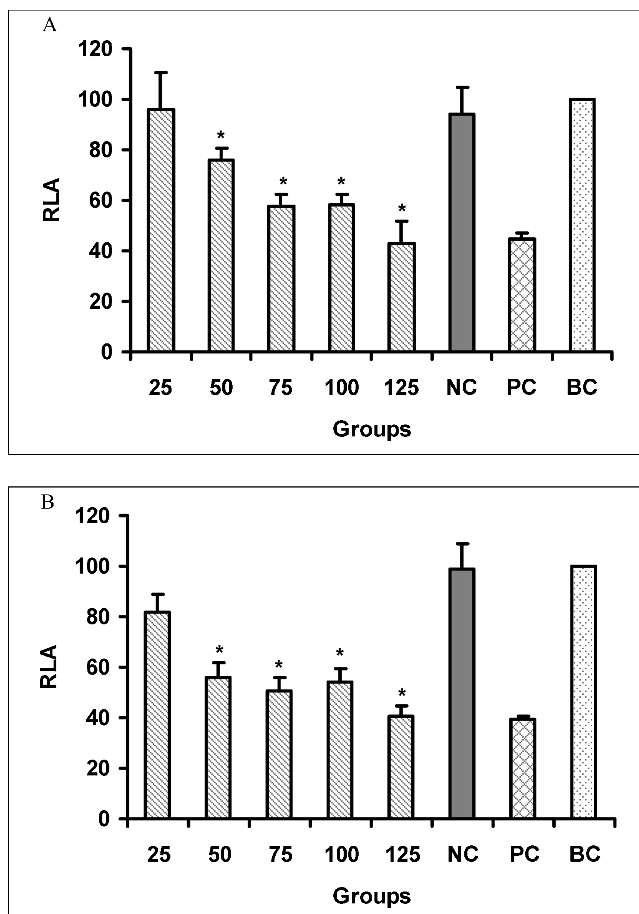

Fig. 5: Expression down-regulation of exogenous firefly luciferase gene in MCF-7 (A) and HepG2 (B) cells by $(PEG_{5K}\text{-}PCL_{1.2K})_{1.4}$-g-$PEI_{10K}$/siRNA nanoparticles at various N/P ratios. The numbers in the groups also respectively represent the N/P ratio of nanoparticles in each group. PC: positive control (Lipofectamine 2000); NC: negative control (Naked siRNA); BC: blank control (Rnase-free water). RLA: relative luciferase activity. The reported data represent fold induction relative to the activity of blank control (expressed as 100%). The experiment was performed in triplicate

in HepG2 cells (Fig. 4), which suggested that the cytotoxicity of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles was very low. As discussed above, this kind of low cytotoxicity may result from the low density of positive charges on the surface of nanoparticles. In addition, although the zeta potential of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles gradually enhanced with N/P ratio increasing in the range of 25 to 125, the cell viability showed a very slow decline with the increasion of N/P ratio (Fig. 4). It is suggested that the N/P ratio in the range of 25 to 125 had very little influence on the cytotoxicity of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles.

2.3. *Expression down-regulation of exogenous target gene by $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles*

Reporter genes are usually used as silencing targets to investigate the potential of siRNA delivery vectors because their expressed products are very easy to detect (Veldhoen et al. 2006; Xu et al. 2010). However, these reporter genes such as luciferase gene and GFP gene are exogenous relative to mammalian cells. In the experiments of gene silencing *in vitro*, mammalian cells are firstly required to be transfected with a reporter gene. Then, the interesting vector was used to deliver the siRNA into cells to silence the expression of reporter genes. Finally, the delivery potential of siRNA vector was evaluated according to the silencing efficiency of reporter gene expression (Katas and Alpar 2006). In this study, we selected the exogenous GL3 firefly luciferase reporter gene as a silencing target due to the high detection sensitivity of expressed luciferase (Wang et al. 2007). The siRNA delivery capability of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ was characterized using the siRNA targeting to silence the expression of GL3 luciferase gene.

In the actual transfection experiments, MCF-7 and HepG2 cells were firstly co-transfected with pGL3-Control and pGL 4.75 plasmids. Here, the plasmid pGL3_Control carries the GL3 firefly luciferase reporter gene serving as the silencing target. The plasmid pGL4.75 can express *Rennila* luciferase, and was used as internal control to correct the transfection efficiency (Chen et al. 2003). Subsequently, $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles at various N/P ratios were used to transfect cells to down-regulate the expression of exogenous GL3 luciferase gene. The results of dual-luciferase reporter gene assays in MCF-7 and HepG2 cells are shown in Fig. 5. As we can see, there were no remarkable differences of luciferase activity between negative and blank controls. It is suggested that the naked siRNA could not down-regulate the expression of the target gene, which also exactly proved that siRNA needed a vector to be delivered into cells to exert its gene silencing effect. In the positive control group, compared to negative control, the expression of luciferase gene was

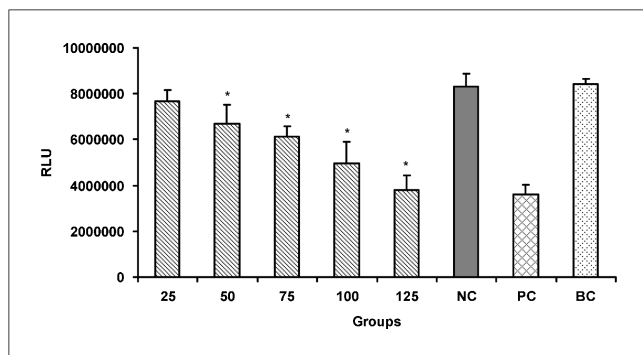

Fig. 6: Down-regulation of endogenous firefly luciferase gene expression in 4T1-luc cells by $(PEG_{5K}\text{-}PCL_{1.2K})_{1.4}$-g-$PEI_{10K}$/siRNA nanoparticles at various N/P ratios. The experiment was performed in triplicate. The numbers in the groups also respectively represent the N/P ratio of nanoparticles in each group. PC: positive control (Lipofectamine 2000); NC: negative control (Naked siRNA); BC: blank control (Rnase-free water). RLU: relative light unit

significantly inhibited by Lipofectamine2000/siRNA complexes with 52% and 60% reduction in MCF-7 and HepG2 cells, respectively. It proved that the positive control and designed siRNA targeting GL3 luciferase gene were both effective. Most important of all, the luciferase gene expression could be significantly down-regulated by $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles at a range of N/P ratio from 50 to 125 ($^{*}P < 0.05$). At the N/P ratio of 125, the luciferase gene expression was silenced with 54% and 59% reduction in MCF-7 and HepG2 cells respectively, and the silencing efficiencies reached the silencing level of positive control. Therefore, it was concluded that $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ could serve as siRNA delivery vector to efficiently down-regulate the expression of exogenous target gene.

2.4. Expression down-regulation of endogenous target gene by $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles

In RNAi therapy, siRNA are delivered into cells to down-regulate the expression of endogenous target genes located in genomic DNA (Kenny et al. 2011; Mao et al. 2011). In order to simulate the actual situation of endogenous target genes, the reporter gene can be integrated into the cell genome DNA to become a kind of endogenous gene through cell cloning technique (Iijima et al. 2007). So, the reporter gene labeled cell line can be used to evaluate the gene silencing potential of a siRNA delivery vector against endogenous target genes. In the 4T1-luc cell line, the GL3 firefly luciferase reporter gene has been randomly integrated into the genome DNA to become an endogenous gene. The 4T1-luc cell line can stably express the GL3 firefly luciferase. Here, we used this cell line to investigate the gene silencing potential of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles against endogenous GL3 firefly luciferase gene. In cell transfection, $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles at various N/P ratios were used to transfect 4T1-luc cells to down-regulate the expression of endogenous GL3 luciferase gene. The results of luciferase reporter gene assays showed that the silencing efficiency of luciferase gene expression in 4T1-luc cells was similar to those in MCF-7 and HepG2 cells (Fig. 6). The naked siRNA could not inhibit the endogenous luciferase gene expression. Compared to naked siRNA, the positive control Lipofectamine2000/siRNA complexes showed 56% silencing efficiency of luciferase expression. Similarly, the expression of endogenous luciferase gene was significantly down-regulated at a range of N/P ratio from 50 to 125 in nanoparticles groups ($^{*}P < 0.05$). Also, the luciferase gene expression was maximally silenced at the N/P ratio of 125 with 54% reduction, which reached the same silencing level as positive control. Accordingly, it was also demonstrated that $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ could deliver siRNA into cells to efficiently down-regulate the expression of endogenous target genes. On the other hand, $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles showed nearly the same silencing efficiency among three cell lines including MCF-7, HepG2 and 4T1-luc. It was suggested that $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles had no remarkable cell-type dependence, which became an advantage of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ as siRNA delivery vector.

In summary, our results suggested that the novel copolymers $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ were able to load siRNA to form nanoparticles. These nanoparticles can deliver siRNA into cells to effectively down-regulate the expression of exogenous and endogenous target genes without cell-type dependence. Additionally, $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles showed very low cytotoxicity. Therefore, $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles could serve as a promising and highly efficient siRNA delivey system. In the future study, the transfection mechanism of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ will be explored for better understanding of its siRNA delivery potential. Moreover, further studies on siRNA therapy will be performed using $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles, which eventually leads to a kind of siRNA delivery vector for clinical application.

3. Experimental

3.1. Materials

Copolymers $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ (Mw 19.5 kDa) were synthesized according to our previous work (Huang et al. 2011). QIAprep Spin Miniprep Kit was purchased from Qiagen company; Lipofectamine 2000 transfection reagent was a product of Invitrogen company; Cell culture medium and fetal bovine serum were provided by Hyclone company; Dual-Luciferase Reporter Assay System Kit, pGL3_Control and pGL4.75 reporter plasmids was obtained from Promega company; Other chemicals were analytical grade.

3.2. Plasmid extraction and siRNA synthesis

The plasmid pGL3_Control and pGL4.75 were respectively transformed into DH5α *E. coli* competent cells, and spread onto LB agar plates containing 100 μg/ml Ampicillin. After inverted culture overnight, the single colonies were picked up into LB culture medium to culture for 16 h. After collection of *E. coli* by centrifugation, plasmids were extracted using QIAprep Spin Miniprep Kit. The purity and concentration of extracted plasmids were assayed by agrose gel electrophoresis and UV spectrophotometry using a BioPhotometer (Eppendorf, Germany).

The siRNA with the sequence of 5'-CUUACGCUGAGUACUUCGATT-3' was synthesized by Dharmacon company. The siRNA sequence was designed to target the GL3 luciferase gene (Acc. No.U47296, GenBank), corresponding to the coding region 153–173 relative to the first nucleotide of the start codon.

3.3. Preparation and characterization of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles

The $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$ copolymers were dissolved in double distilled water at various concentrations, and then filtered through 0.22 μm membranes for sterilization. The siRNA was diluted with Rnase-free water at a specific concentration. Then, equal volumes of copolymers and siRNA solution were mixed and vortexed for 30 s to generate the $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}$-g-$PEI_{10k}$/siRNA nanoparticles suspension at various N/P ratios (the ratios of moles of the primary amine groups of cationic polymers to those of the phosphate ones of siRNA). The nanoparticle suspensions were diluted with double distilled water to measure particle size and zeta potential using a Nicomp380/ZLS analyzer (Particle Sizing Systems, USA).

3.4. Cell culture

Human breast cancer cell line MCF-7 and human hepatocellular carcinoma cell line HepG2 were provided by the Cell Resource Center in Institute of Basic Medical Sciences in Peking Union Medical College & Chinese Academy of Medical Sciences. The luciferase-labeled mouse mammary

tumor cell line 4T1-luc was provided by the Department of Pathology in Beijing Institute of Basic Medical Sciences. In a cell incubator at 37 °C and 5% CO_2, these cell lines were respectively cultured with the culture mediums containing 10% fetal bovine serum including DMEM medium for MCF-7, MEM/NEAA medium for HepG2 and RPMI1640 medium for 4T1-luc. Sub-culture was carried out when cells grew to about 80% confluence. Cells in logarithmic growth phase were used to perform the following cell experiments.

3.5. MTT assay

MCF-7 and HepG2 cells in logarithmic growth phase were seeded into a 96-well plate at a density of 5000/well and 8000/well in a total volume of 100 μL. Blanks were prepared by adding 100 μL medium alone. After 24-hour cell culture, 20 μL of $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$/siRNA nanoparticles at various N/P ratios were added in wells in various sample groups. Cells in wells without addition of nanoparticle samples were used as control. Five plicate wells were included in each group. After 24-hour cell culture, 20 μL of MTT solution was added into each well, and incubated at 37 °C for 4 h. Then, 150 μL DMSO was added into each well, and the plate was gently shaken for 10 min. Finally, the optical density (OD) was measured at 560 nm using a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, USA). The cell viability was calculated according to the following formula.

$$\text{Cell viability}(\%) = [(OD_{\text{nanoparticles}} - OD_{\text{blank}})/(OD_{\text{control}} - OD_{\text{blank}})] \times 100\%$$

3.6. Expression down-regulation of exogenous target gene

MCF-7 and HepG2 cells in the logarithmic growth phase were seeded into a 24-well plate at a density of 6×10^4 cells/well. After 24-hour cell culture, cells in each well were co-transfected with 400 ng pGL3_Control reporter plasmid and 1 ng pGL4.75 reference plasmid using Lipofectamine 2000 transfection reagent. After 4-hour transfection, the medium in each well was replaced with fresh culture medium. Subsequently, $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$/siRNA nanoparticles at various N/P ratios were added into wells in test groups. Rnase-free water and naked siRNA were used as blank and negative controls, respectively. In the positive control group, siRNA were transfected into cells using Lipofectamine 2000 reagent. In the above groups, three plicate wells were included in each group, and the amount of siRNA added into each well was 4 pmol. After another 36-hour cell culture, dual-luciferase reporter gene assay was performed to measure the activities of firefly luciferase and *Renilla* luciferase in each well using TriStar LB941 luminometer (Berthold, Germany) and Dual-Luciferase Reporter Assay System Kit. Finally, the activity of firefly luciferase was normalized to that of *Renilla* luciferase. The experiment was performed in triplicate.

3.7. Expression down-regulation of endogenous target gene

4T1-luc cells in logarithmic growth phase were seeded into a 24-well plate at a density of 5×10^4 cells/well. After 24-hour cell culture, the medium in each well was replaced with fresh culture medium. Subsequently, $(mPEG_{5k}\text{-}PCL_{1.2k})_{1.4}\text{-g-}PEI_{10k}$/siRNA nanoparticles at various N/P ratios were added into wells in test groups. Rnase-free water and naked siRNA were used as blank and negative controls, respectively. In the positive control group, siRNA were transfected into cells using Lipofectamine 2000 reagent. The amount of siRNA added into each well was 4 pmol. After another 36-hour cell culture, luciferase reporter gene assay was performed to measure the activity of firefly luciferase in each well. The experiment was performed in triplicate.

3.8. Statistical analysis

All experiments were performed in triplicate. Quantitative data represented the mean ± standard deviation (SD). Statistical analysis was performed using SPSS v 13.0 software. Differences between groups were evaluated with Student's *t* test. $P < 0.05$ was considered statistically significant.

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