Design of polyethylene glycol-polyethylenimine nanocomplexes as non-viral carriers: mir-150 delivery to chronic myeloid leukemia cells†

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Running Title: PEG-PEI Nanocomplexes for miRNA Delivery

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Abstract

MicroRNAs (miRNAs) are acknowledged as indispensable regulators relevant in many biological processes, and they have been pioneered as therapeutic targets for curing disease. miRNAs are single-stranded, small (19-22 nt) regulatory non-coding RNAs whose deregulation of expression triggers human cancers, including leukemias, mainly through dysregulation of expression of leukemia genes. miRNAs can function as tumor suppressors (suppressing malignant potential) or oncogenes (activating malignant potential) like actors of complex diseases.

To address the issue of overcoming instability and low transfection efficiency in vitro, the polyethylene glycol-polyethyleneimine (PEG-PEI) nanoparticle was used as non-viral vector carrier for miR-150 transfection, which is downregulated in chronic myeloid leukemia.

PEG-PEI [PEG\(_{550}^{(550)}\cdot g\cdot PEI_{1800}^{(1800)}\)]/miRNA nanocomplexes were synthesized and characterized by particle size distribution, polydispersity index and zeta potential, surface charge, their cytotoxicity, and transfection efficiency. Interaction with human leukemia cells (K-562 and KU812) and control cells NCI-BL2347 with them has been investigated. The transfection efficiency of PEG-PEI/miRNA at N/P 26 rose 6.7 fold above the control by qRT-PCR. The size of homogenous nanocomplexes (PBI<0.5) was 160.8±11 nm.

The data indicate that PEG-PEI may be an encouraging non-viral carrier for altering miRNA expression in the treatment of chronic myeloid leukemia, with many advantages such as relatively high miRNA transfection efficiency and low cytotoxicity.

Keywords: Leukemia; MicroRNA; Nanoparticles; Polyethylenimine; Poly(ethylene glycol)
Introduction

Gene delivery system has attracted much attention in recent years. However, a safe and efficient gene delivery that can be achieved by either viral or non-viral methods remains a crucial barrier to successful gene therapy (Choi et al., 2001). The widespread use of the viral system is affected by the limited size of genetic material delivered, severe safety risks, and a lack of targeting interaction to certain cells. Therefore, non-viral gene delivery has become a promising alternative since the vectors could be synthesized of high quality and purity degree, with less immunogenic response than viral vectors (Ghiamkazemi et al., 2010). Non-viral gene vectors, including liposomes and cationic polymers, have received much attention because of the ease of preparation, lack of immunogenicity, and ability to be modified for potential targeted delivery (Chen et al., 2011). Encapsulation of nucleic acid in biodegradable polymer offers potentially a way to protect DNA or RNA from degradation and control their release. The vector used should have sufficiently small size (<300nm) to efficiently enter target cells. If the particle size is >300 nm, the vector cannot enter cells, and if the particle size would be very small, aggregation in the blood may occur (Ghiamkazemi et al., 2010).

Various non-viral vectors have been described for delivery of nucleic acids. Among the cationic polymers, polyethylenimine (PEI) is an effective transfection reagent due to easy synthesis and multiple modifications for non-viral gene delivery. PEI concentrates negatively charge DNA via electrostatic interaction and form polyethylenimine/DNA nanoparticles. Many factors, such as the molecular weight, degree of grafting, ionic strength of the solution, zeta potential, particle size, cationic charge density, molecular structure, sequence and conformational flexibility, influence the transfection efficiency and cytotoxicity of these non-viral vector systems. PEI polymers with
different molecular weights and degrees of branching, as well as several modifications of the PEI backbone, remain under intense investigations for potential in vivo use. They enter nuclei through several steps, including cellular uptake, escaping from nucleases, endosomal release, and DNA decondensation inside nuclei (Howard, 2009). The efficiency of PEI depends on its structure and molecular weight, the amine/phosphate (N/P) ratio of the complexes, and the quantity of complexed nucleic acids (David et al., 2010; Endres et al., 2012). The small complexes formed with PEI (polyplexes) are stable enough to transport genetic material into cells (Breunig et al., 2008). High-molecular-weight PEI is regarded as one of the most effective non-viral cationic vectors for gene transfection.

However, PEI has high cytotoxicity that limits its clinical application (Huang et al., 2010). Therefore, covalent linkage with non-ionic and hydrophilic polymers, such as polyethylene glycol (PEG), has been used to minimize toxicity. A moderate prolongation of the half-life of the polyplexes in vivo after intravenous administration was also observed due to reduced macrophage clearance (Ozcan et al., 2010). Hydrophobic PEG modification of PEI may create a more non-ionic surface of polyplexes which could possibly enhance transfection efficacy due to better interaction with the cell membrane, and therefore improve endocytotic uptake; they have proved successful for PEI-based nucleic acid delivery (Beyerle et al., 2011). PEG has also improved the solubility of polyethylene glycol-grafted polyethylenimine (PEG-PEI) complexes, minimized aggregation, and reduced non-specific interactions with proteins and phagocytic removal in physiological fluids (Choi et al., 2001; Bergstrand et al., 2009; Chen et al., 2011; Bege et al., 2011). The hydrophilic PEG shell may reduce protein adsorption while improving stability, possibly leading to increased blood circulation times in vivo (Howard, 2009).

miRNAs that play an important role in the regulation of gene expression are single-stranded non-coding RNA molecules of 18-25 nucleotide length. Deregulation of miRNA expression is important
in pathogenesis of many genetic and multifunctional disorders, and might provide a promising strategy to treat cancer by targeting the specific proteins involved in the mechanism of proliferation, invasion, anti-apoptosis, drug resistance, and metastasis (Chen et al., 2010). miRNAs might have more than one target, and a gene that codes a single protein might be targeted by more than one miRNA. This situation raises the possibility that over 1/3rd of human genes might be regulated by miRNAs. From this perspective, post-transcriptional regulation becomes very relevant (Sales et al., 2010).

Many miRNAs are downregulated in cancer, some of which inhibit proto-oncogenes translation in normal cells. They are therefore acknowledged as tumor suppressor miRNAs in cancer (Waldman et al., 2009). miR-15a and miR-16-1 inhibit tumorogenesis by targeting BCL-2 oncogene (Cimmino et al., 2005).

Downregulation of miR-150 expression at diagnosis in blast crisis and most of hematological relapses (Machová Poláková et al., 2011). miR-150 functions as a tumor suppressor and its downregulation induces phosphoinositide-3-kinase (PI3K), Protein Kinase B (AKT) pathway activation, pioneering telomerase activation and immortalization of cancer cells (Watanabe et al., 2011).

Based on reports that miR-150 downregulation is associated with chronic myeloid leukemia (Agirre et al., 2008; Machová Poláková et al., 2011), we have tried to transfect mir-150 by PEG-PEI-based nanoparticles into leukemia cells. Different types of PEI with varying molecular weights were used for gene delivery. PEI-based gene transfection technology induces cytotoxicity depending on its molecular weight and concentration. A major focus was to reduce cytotoxicity by using PEG-modified PEI polymer while keeping the high yield of miRNA transfer.
Having synthesized and characterized a PEG-PEI copolymer, 9 different nanoparticle formulations were prepared with it and miRNA was encapsulated in them. This was followed by characterization and stability studies. To measure miR-150 condensation, gel retardation was used.

**Materials and methods**

**Materials and reagents**

Branched polyethylenimine (PEI) (MW: 1800 Da) and PEG monomethyl ether (mPEG) (MW: 550 Da) were purchased from Alfa-Aesar (Ward Hill, MA, USA). Hexamethylene diisocyanate (HMDI) was provided from Fluka (Switzerland). Chloroform, dichloromethane, diethyl ether and light petrol were supported from Merck KGaA (Darmstadt, Germany). All other chemicals were of analytical grade.

A tetrazolim salt WST-1[2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] was obtained from Roche Applied Science (Mannheim, Germany). Cell culture medium and fetal bovine serum (FBS) were purchased from Biological Industries (Kibbutz Beit-Haemek, Israel) and also Sigma-Aldrich (St Louis, MO, USA). Low melting agarose was supplied from Amresco (Solon, OH USA) and Tris-borate-EDTA (TBE) buffer was purchased from Biorad (Marnes-la-Coquette, France). Human leukemia cell lines (human myeloid leukemia cell lines (K562, KU812) and human control cell line NCI-BL2347 were obtained from ATCC (USA). Fluorescently labeled siRNA (siGLO Red Transfection Indicator) and Negative Control siGLO RISC-free control were purchased from Dharmacon, Thermo Scientific, USA). miRNeasy Kit was obtained from Qiagen. miRCURY LNA™
Universal RT microRNA system kit, hsa-miR-150-5p LNA PCR primers, Universal cDNA synthesis and SYBR Green were provided from Exiqon.

**Cell Culture**

Human chronic myeloid leukemia model cell lines (K562, KU812) and control cells NCI-BL2347 were cultured and maintained in RPMI-1640 medium (Gibco), Iscove's Modified Dulbecco's Medium (Biological Industries), containing 2 mM L-glutamine supplemented with 10% inactivated fetal bovine serum and 1% penicillin/streptomycin in a standard cell culture incubator at 37°C, under a humidified 95% air and 5% CO₂ atmosphere. Before each experiment, cells were split at 5x10⁵ cells/ml in the RPMI 1640 medium and cell suspensions was aliquoted into flasks for subsequent treatments.

**Synthesis and characterization of PEG-PEI copolymers**

PEI-PEG copolymers were synthesized in a 2-step procedure for coupling reaction between PEG and PEI (Petersen et al., 2002). In the first step, mPEG was activated by 4g being dissolved in CH₂Cl₂ in a flask fitted with a reflux condenser and an oil bubbler. HMDI was added and the mixture heated under reflux for 8 h before the polymer was precipitated with petrol. After purification, residues of solvents were removed at reduced pressure and a viscous liquid was obtained. The availability of a single hydroxy group reactive toward amino groups of PEI was measured spectroscopically [Nuclear Magnetic Resonance Spectroscopy (NMR), Fourier Transformed Infrared Spectroscopy (FTIR)]. In the second step, 0.8 g PEI and 0.77 g activated PEG were dissolved in 100 mL of CHCl₃ and CH₂Cl₂, respectively. The PEI solution was added drop wise into the PEG solution and the mixture heated (60°C) under reflux for 24 h. The copolymer was precipitated in diethyl ether and dried in vacuo.
The physicochemical characterization of the synthesized polymers were done spectrometrically using Nuclear Magnetic Resonance Spectroscopy, Fourier Transformed Infrared Spectroscopy and Differential Scanning Calorimetry (DSC). 1H NMR spectra were recorded in CDCl₃ (Aldrich) using a Bruker Advance 400 apparatus. Integration of the signals in 1H NMR spectra for -CH₂-CH₂-O- and for -CH₂-CH₂-ND- yielded the composition of the copolymers. Indices in the nomenclature of the copolymers were calculated from this integration and from the MW provided by the suppliers. FTIR spectroscopy was conducted on a Perkin Elmer Spectrum 100 spectrometer. Thermal characteristics of copolymers were carried out using a Perkin Elmer, DSC 8000. Measurements were made in nitrogen at a heating and cooling rate of 10⁰C/min (temperature range -100 to 120⁰C). All the determinations were done in triplicate.

**Preparation of miRNA-copolymer nanocomplexes**

PEG-PEI copolymers were dissolved to at different concentrations (1-100 μg/mL), according to various N/P ratios. The PEG-PEI copolymer was added to 10 mM HEPES buffer and mixed continuously until solubilized. This copolymer and a commercially available tumor suppressor miRNA (miR-150) were mixed at the ratio of 100:1 μg/ml:nM (PEG-PEI:miRNA) and incubated at room temperature for 15 min to form the nanocomplex structure.

**Characterization of miRNA-copolymer nanocomplexes; zeta potential and size measurements**

Characterization of the nanoparticle formulations involved parameters such as particle size distribution, polydispersity index, zeta potential and morphological properties. Particle size distribution and zeta potential measurements were made by the laser diffraction method (Nanosizer Coulter N4 Plus, Margency, France), using 6 replicates in each case. Polydispersity index was used to check homogeneity. The zeta potential values were measured in specific cuvettes by using Zetasizer 4-Malvern at a 45⁰ angle and 25⁰C. Wirth regard to nanoparticle
stability, these measurements were made 6 consecutive times per sample. Nanocomplexes of T1, T3 and T9 copolymers with miR-150 were prepared, and coded F1, F2 and F3, respectively. The data represent the average ± standard deviations.

**Gel retardation assay**

Gel electrophoresis was used to measure miRNA condensation. PEG-PEI (T1,T3,T9)/miRNA complexes prepared at different N/P ratios were loaded onto 4% agarose (low melting point) gel and run with 1x Tris/Borate/EDTA (TBE) buffer at 55 V for 2 h. The miRNA band was visualized using safeview under a ultraviolet (UV) imaging system.

**Cell Proliferation Assay-WST-1**

Human chronic myeloid leukemia cell lines (K562, KU812) and control cells NCI-BL2347 were seeded in 96-well plates at 10,000 cells/well. After 24 h they were treated with the different N/P ratios of formulations at 100, 10 and 1 μg/ml.

siGLO RISC-Free Control was used for cytotoxicity analysis of PEG-PEI (T1,T3,T9)/nucleic acid complexes. Each concentration was analyzed in triplicate. Transfection was performed with 1 nM siGLO RISC-Free Control per well to form the PEG-PEI/siGLO complexes. After additional incubation for 48 h, the medium was replaced with 100 μL serum-free medium, and 20 μl WST-1 solution (5 mg/mL) were added per well. WST-1 test is based on the activity of mitochondrial lactate dehydrogenases (LDH) of vital cells that convert WST-1 (water soluble tetrazolium) to a water soluble formazan. The absorbance of each well was assayed by means of microplate reader at 450 nm with reference of 620 nm. Cell viability was calculated as % ratio between the absorbance of each sample and the absorbance of complete growth medium.
**Transfection efficiency analysis**

Chronic leukemia and control cells were seeded into 6-well plates at 1X10^6 cells per well before transfection. PEGPEI+ siGLO Red Transfection Indicator and PEGPEI/mir-150 complexes were prepared, DY-547-labeled (Rhodamine filter) siGLO and copolymer solution at different concentrations were added to 1 mL HEPES. The PEG-PEI/miRNA and siGLO complexes were gently mixed and incubated for 10–15 min at room temperature. The original cell culture media was replaced with 1 mL fresh and complete culture media per well. The complexes were added in 3 µl and the mixture rocked gently for 4 h at 37°C, after which the transfection media was exchanged with fresh complete RPMI. The cells were observed under the fluorescence microscope (Olympus, Japan), and images were recorded.

**Confirmation of Transfection Efficiency of mir-150 by Flow Cytometer**

To measure the percentage of transfected cells in total cells and also transfection efficiency confirmation 48 h after transfection, cells were washed and resuspended in 500 µL PBS before being analyzed by BD Accuri C6 Flow Cytometry (FL1-A channel), with the data being analyzed using CFlow Software (Accuri Cytometers, Inc.).

**Real-Time qRT-PCR Analysis: Nanoparticle Mediated miR-150 Transfection**

After transfection for 48 h, RNA was extracted using miRNeasy Kit (Qiagen, Germany). qRT-PCR was carried out with a miRCURY LNA Universal RT microRNA system kit (Exiqon). Twenty µl of resulting cDNA were subjected to PCR reactions using specific hsa-miR-150-5p LNA PCR primers and SYBR Green. A negative control lacking cDNA was used to detect possible contamination, and a Universal cDNA synthesis kit was used for reverse transcription. Twenty ng RNA and RNA “spike-in” for expression normalization were used. Inhibitors and degradation were kept under control.
**Statistical analysis**

Possible variations of the particle size distribution and polydispersity index studies under the concept of stability tests have been compared with the one-way ANOVA test. Post-hoc comparison of the groups involved the Tukey test (significance taken as p<0.05). Data are expressed as mean ± standard deviation (SD). Statistical analyses used SPSS 15.0 software.

**Results and discussion**

**PEG - PEI Copolymer Synthesis**

Copolymers with different molecular structures were synthesized by using mPEG and PEI polymers having different molecular weights. T₉ and T₃ were white powders, but the T₁ copolymer was yellowish in color. The amounts of the copolymers and synthesis efficiencies are given in Table 1. Copolymer synthesis was completed with high efficiency.

The molecular structures of the resulting copolymers were evaluated using NMR, FTIR spectroscopies. ¹H NMR (in CDCl₃) and FTIR confirmed the structure of the expected PEI-PEG copolymer [(−CH₂CH₂O−) 3.65 ppm, (−CH₂CH₂NH−) 3.08 ppm and N-H amine 3270, 1541, 1465 cm⁻¹].

Thermal analysis verified the successful formation of the copolymers.

PEI derivatives and synthesized copolymers were characterized by Infrared Spectroscopy, Nuclear Magnetic Resonance Spectroscopy and Differential Scanning Calorimetry. The synthesized copolymer, with respect to the spectrum of the PEI polymer, showed that N-H stretching at 3,270 cm⁻¹, C-H 2,900 cm⁻¹, C=O 1,650 cm⁻¹, N-H 1,541 cm⁻¹, CH₂ pulse at 1,359 cm⁻¹ and C-O-C
stretching’s at 1,359, 1,270, 1,145 cm\(^{-1}\), which are specific points for the polymer. These polymers were used for the preparation of nanocomplexes.

The \(^1\)H-NMR spectra of T\(_1\), T\(_3\) and T\(_9\) had characteristic peaks belonging to the PEG chain (-CH\(_2\)CH\(_2\)O-) and PEI (-CH\(_2\)CH\(_2\)NH-) polymer (3.6-3.8 and 2.9-3.1, respectively), which is the evidence that the synthesis was successfully completed. The melting point increased with the increasing molecular weight and results were in good accordance with the studies on PEG-PEI, as shown in Figure 1 (-55, -40 and +90\(^\circ\)C).

Beyerle et al. (2011) investigated the structure–function relationship in relation to cytotoxicity with free PEGylated PEI copolymer varying in PEG molecular weight and chain length of PEI (25 kDa). Greater PEG content and decreased chain length correlated with lower cytotoxicity in murine alveolar epithelial-like type II cells and alveolar macrophages evaluated by LDH release. Lipid mediator detection of 8-isoprostanes (8-IP) and prostaglandin E2 (PGE2) in cell supernatants as indicators of oxidation stress response and lipid peroxidation were decreased with shorter PEG chain length. The authors proposed PEG shielding of the PEI charge gave improved performance. Masking of the surface charge to reduce interactions, therefore, is a successful strategy to prevent membrane disruption and consequent cytotoxicity (Petersen et al., 2002).

Investigation of the proinflammatory potential of the 2 different polymers and their modifications showed that unmodified PEI (25 kDa and 8.3 kDa) causes only mild inflammation despite its strong toxicity (Davis, 2009; Bramsen et al., 2010). As explained above, PEGylated PEI, however, had remarkable signs of inflammation despite its reduced cytotoxic effects. In response to the PEG modified PEIPEG copolymers, high levels of IgM in BALF were detected after polyplex instillation (Beyerle et al., 2011).
Nanoparticle Preparation and Characterization Studies

The particle size distribution (PSD), polydispersity index (PDI) and zeta potential (ζ) results of the nanoparticle miRNA complexes are summarized in Table 2. Nanoparticles were found to be within the acceptable range as 160.8-252.6 nm. These were obtained after 3 measurements and the results are expressed with their standard deviations.

Formulations prepared with copolymers that had different physicochemical properties showed different particle size distribution, polydispersity index, zeta potential and miRNA transfection rates. Particles with mean size <300 nm were suitable for non-viral gene delivery (Ghiamkazemi et al., 2010); parallel results were found by Kim et al. (2005) Wu et al. (2006) and Glodde et al. (2006) stated that nanoparticle size is increased in parallel with the increase in polymer molecular weight in formulation. Depending on a mean particle size <200 nm, F1 formulation (T1 and miR-150) was considered suitable.

The zeta potential is the total ionic strength of a colloidal system, which is important in testing stability. Particles tend to aggregate in a higher ratio as the zeta potential comes closer to zero (Couvreur and Vauthier, 2006). When compared with F1 and F2 formulations, the high zeta potential value of F3 may be explained by the high positive charge of PEI polymer (25kDa). In accordance to this finding, Liu et al. (2011) and Zheng et al. (2012) found that zeta potential values of the formulations were prepared with high MW PEI had high values because of the positive charge on the surface.

Naked siRNA has poor intracellular uptake, limited blood stability, and nonspecific immune stimulation. To correct these problems, modified siRNA, cationic carriers and ligand-targeting
have been used in this type of vector delivery. PEGylation is also used to improve stability, solubility, and bioavailability. Compared with F1 and F2, longer mPEG chain length decreased the zeta-potential of polyplexes, which was attributed to the increased steric hindrance associated with the longer PEG blocks. Electrostatic interactions between polycationic polymers and nucleic acid, largely responsible for the stability of the polyplexes, are strongly influenced by the ionic strength of the surroundings. F3 was most stable, as confirmed by its higher zeta potential.

**Gel retardation assay**

N/P values (the ratio of amino group in PEI with respect to phosphate group in miRNA) of PEG-PEI (T1, T3, T9) / miR-150 complex mixtures at the ratios of 100:1, 10:1 and 1:1 (µg/ml:nM) were determined as 26, 2.6 and 0.26 for T1; 24, 2.4 and 0.24 for T3; and 20, 2.0 and 0.20 for T9. By gel electrophoresis, the T1 miR-150 complex with a N/P ratio of 26 was retarded, indicating the miRNA was complexed with the nanoparticle. Even though very slight, T3 also showed some retardation [N/P 24, (Figure 2)].

The negative charges of phosphate groups of nucleic acids could be neutralized by PEG-positive charges of PEI copolymer and the mobility of nucleic acids could be retarded in gel (Wu et al., 2010). In nNanocomplexes having different N/P ratios (0.26-26) were analyzed, at a N/P ratio of 26 (F1) miRNA was completely neutralized. Wu et al. (2010) used naked siRNA as the control group, which showed weakened bands were weakened similar with the increase in N/P ratio (2.5-30). They also found that at N/P ratio of 10, retarded band was brighter, and at N/P ratios ≥10, PEG-PEI and siRNA formed complexes. Mao et al. (2006) showed that there was no significant difference with respect to retardation among the formulations [PEI(25k)-g-PEG(2k)10, PEI(25k)-g-PEG(5k)5 and PEI(25k)-g-PEG(20k)1], and complete retardation was achieved at N/P ratio of 3.
Transfection efficiency

Transfection efficiency of mir-150 via nanoparticle (1 nM:100 µg/ml) and siGLO Red Transfection Indicator in a leukemia model cell line was determined by fluorescence microscope (Figure 3). Nanoparticle-mediated transfection of mir-150 was determined as effective by fluorescence microscopic imaging.

Cytotoxicity Studies

Cytotoxic effects of nanoparticles at 100, 10 and 1 µg/ml in the leukemia model cell lines (K562, KU812) and in the control cell model (NCI-BL2347) were examined with HEPES given as control. Cytotoxicity reached 12, 10, and 9% in K562 cell line transfected with 100 µg/ml T1 at 1, 2 and 3 days, respectively. Cytotoxicity was detected 11, 13, and 17% in K562 cells+T1+siGLO at these same times. In KU812 cells transfected with 100 µg/ml T1, 10, 12 and 14% cytotoxicity was found in this same time-dependent manner. For KU812+T1+siGLO, the corresponding figures were 14, 12 and 16%. And for NCI-BL2347 cells transfected with 100 µg/ml T1, they were 17, 15 and 16. Cytotoxicity was correspondingly 16, 16 and 17% in NCI-BL2347 cells transfected with 100 µg/ml T1+ siGLO. No significant cytotoxicity was determined in all cell lines PEG-PEI/siGLO RISC-free control nanocomplexes (Figure 4). Thus PEG-PEI can be used as a valuable non-viral miRNA delivery carrier according to its high transfection efficiency and low cytotoxicity in leukemic and control cell lines. A lower toxicity with low Mw PEI (~10 kDa) compared to high Mw (~800 kDa) in L929 cells had been reported by Ballarín-González and Howard (2012). In particular, branched PEI (25 kDa, the gold standard) is highly effective in transfection efficiency owing to its high buffering capacity (due to the presence of secondary and tertiary amines). In contrast, linear polyethylenimine (25 kDa) is relatively nontoxic, but is less water soluble and gives poor transfection efficiency (Goyal et al., 2012).
Validation of Nanoparticle Mediated miR-150 Transfection

Validation of Nanoparticle Mediated miR-150 Transfection (100:1 μg/ml:nM) in chronic myeloid leukemia cell models and control cells employed qRT-PCR 48 h after transfection. Expression analysis was calculated with $C_T$ and Log2 transformation was performed. miR-150 expression was upregulated in K562, KU812 and NCI-BL2347 cell lines 6.68, 6.50, 1.48 fold, respectively (Figure 5) compared with the untransfected control group.

Confirmation of Transfection Efficiency of mir-150 via Flow Cytometer

The percentage of transfected cells in total cells and also transfection efficiency confirmation were assessed by flow cytometry. Transfection efficiency of mir-150 by nanocomplex PEG-PEI in cell lines showed that it was: K562 cell line 78.2±2.2, KU812 76.9±3.0, NCI-BL2347 77.5±4.0 relative to untransfected cell lines (Figure 6).

The precise function of PEGylation may depend on many factors including the PEG $M_w$, PEI $M_w$, stoichiometry of PEG grafting, type of nucleotide, and environment. Although PEG-PEI copolymers have been primarily used for plasmid delivery, there is strong evidence that PEGylated PEI can be adapted to function as an effective carrier for cellular delivery of small oligonucleotides. Several reports have characterized the properties and transfection capacity of PEGylated PEI25K complexed with oligonucleotides (Kichler et al., 2001; Fiandaca and Bankiewicz, 2010). Overall, they provide valuable insight into the influence of the PEG chain length, extent of PEG grafting, and method of PEGylation on the polyplex structural properties, nuclease protection, complement activation, serum stability, transfection efficiency, and in vivo distribution. To a lesser extent, similar types of studies using much smaller molecular weight PEI2K have also described the physiochemical properties and transfection potential of PEG-PEI-AO polyplexes (Bertschinger et al., 2006; Swami et al., 2009). In addition, improvements in the transfection efficiency of PEG-PEI-
oligonucleotide polyplexes have been attained by covalent attachment of cell-targeting ligands to PEG (Bertschinger et al., 2006; Liu et al., 2010).

Conclusion

Nanoparticles prepared with low molecular weight of PEG-PEI may be a promising non-viral carrier for altering miRNA expression in the treatment of chronic myeloid leukemia with many advantages, such as relatively high miRNA transfection efficiency and low cytotoxicity. Data will introduce miRNA-based molecular therapy approach to the diagnosis of leukemia. These findings need to be supported by preclinical studies in animal models, which might improve in turn treatment protocols in clinical applications. This new delivery system with PEG–PEI nanocomplexes provides a means of improving such future investigations.

Funding

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References


Table Legends

Table 1. Copolymer amounts and synthesis efficiencies

**Abbreviations:** MW, molecular weight; Da, Dalton

Table 2: Particle size distribution (PSD), polydispersity index (PDI) and zeta potential (ζ) results of the formulations

**Abbreviations:** F1, PEI(1800)-g-PEG(550)3+mir-150; T3, PEI(1800)-g-PEG(1100)2+mir-150, T9, PEI(25K)-g-PEG(5K)6+mir-150; nm, nanometer; mV, milivolt

Figure Legends

**Figure 1:** Thermal analysis curves of T1, T3 and T9

**Abbreviations:** T1, PEI(1800)-g-PEG(550)3; T3, PEI(1800)-g-PEG(1100)2, T9, PEI(25K)-g-PEG(5K)6

**Figure 2:** miRNA bands in gel

**Notes:** Lane 1: Marker 50bp; Lane 2: naked miR-150; Lane 3: 100:1 μg/ml:nM [PEI(1800)-g-PEG(550)3:miRNA-150, N/P 26]; Lane 4: 10:1 μg/ml:nM (N/P 2.6); Lane 5: 1:1 μg/ml:nM (N/P 0.26); Lane 6: 100:1 μg/ml:nM [PEI(1800)-g-PEG(1100)2:miRNA-150, N/P 24]; Lane 7: 10:1 μg/ml:nM (N/P 2.4); Lane 8: 1:1 μg/ml:nM (N/P 0.24); Lane 9: 100:1 μg/ml:nM [PEI(25K)-g-PEG(5K)6:miRNA-150, N/P 22]; Lane 10: 10:1 μg/ml:nM (N/P 2.2); Lane 11: 1:1 μg/ml:nM (N/P 0.22);

**Abbreviations:** PEG-PEI, polyethylene glycol-polyethyleneimine; N/P, ratio of PEG-PEI amino groups; miR-150 phosphate groups

**Figure 3:** Fluorescence microscope image of transfection of T1 nanoparticle (400×)

**Notes:** Transfection of K562 T1+siGLO (A); KU812 T1+ siGLO (B); NCI-BL2347 T1+ siGLO (C)
Abbreviations: T1, PEI(1800)-g-PEG(550)3; siGLO, Red transfection indicator control

Figure 4. Viability of leukemia cells after treatment with formulations

Notes: Cytotoxicity of K562 T1 (A); K562 T1+siGLO (B); KU812 T1 (C), KU812 T1+ siGLO (D); NCI-BL2347 T1 (E); NCI-BL2347 T1+ siGLO (F)

Abbreviations: T1, PEI(1800)-g-PEG(550)3; T3, PEI(1800)-g-PEG(1100)2, T9, PEI(25K)-g-PEG(5K)6; siGLO, RNA-induced silencing complex (RISC)-free control

Figure 5. Expression analysis of nanoparticle-mediated miR-150 transfection in chronic myeloid leukemia cell models and control cells via qRT-PCR

Figure 6. Transfection efficiencies of nanoparticle in chronic myeloid leukemia cell models
Table 1. Copolymer amounts and synthesis efficiencies

<table>
<thead>
<tr>
<th>Copolymer Code</th>
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<th>PEG (MW)</th>
<th>Efficiency (%)</th>
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</table>

Abbreviation: MW, molecular weight; Da, Dalton

Table 2: Particle size distribution (PSD), polydispersity index (PDI) and zeta potential (ζ) results of the formulations

<table>
<thead>
<tr>
<th>Code of the formulation</th>
<th>PSD ± SD (nm)</th>
<th>PDI ± SD</th>
<th>Zeta potential ± SD (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>160.8 ± 11</td>
<td>0.442 ± 0.03</td>
<td>8.9 ± 0.67</td>
</tr>
<tr>
<td>F2</td>
<td>219.4 ± 14</td>
<td>0.490 ± 0.02</td>
<td>5.6 ± 0.68</td>
</tr>
<tr>
<td>F3</td>
<td>252.6 ± 17</td>
<td>0.496 ± 0.05</td>
<td>14.8 ± 0.26</td>
</tr>
</tbody>
</table>

Abbreviation: F1, PEI(1800)-g-PEG(550)_3+mir-150; F2, PEI(1800)-g-PEG(1100)_2+mir-150, F3, PEI(25K)-g-PEG(5K)_6+mir-150; nm, nanometer; mV, milivolt
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6