

Multifunctional Nanomedicine Platform for Cancer Specific Delivery of siRNA by Superparamagnetic Iron Oxide Nanoparticles-Dendrimer Complexes

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Abstract: The ability of Superparamagnetic Iron Oxide (SPIO) nanoparticles and Poly(Propyleneimine) generation 5 dendrimers (PPI G5) to cooperatively provoke siRNA complexation was investigated in order to develop a targeted, multifunctional siRNA delivery system for cancer therapy. Poly(ethylene glycol) (PEG) coating and cancer specific targeting moiety (LHRH peptide) have been incorporated into SPIO-PPI G5-siRNA complexes to enhance serum stability and selective internalization by cancer cells. Such a modification of siRNA nanoparticles enhanced its internalization into cancer cells and increased the efficiency of targeted gene suppression *in vitro*. Moreover, the developed siRNA delivery system was capable of sufficiently enhancing *in vivo* antitumor activity of an anticancer drug (Cisplatin). The proposed approach demonstrates potential for the creation of targeted multifunctional nanomedicine platforms with the ability to deliver therapeutic siRNA specifically to cancer cells in order to prevent severe adverse side effects on healthy tissues and *in situ* monitoring of the therapeutic outcome using clinically relevant imaging techniques.

Keywords: SPIO nanoparticles, PPI dendrimer, siRNA, Cisplatin, LHRH peptide, imaging, tumor targeting.

INTRODUCTION

The ability of short interfering RNA (siRNA) to silence specific genes inspired the use of siRNA as a therapeutic agent for a wide spectrum of disorders including cancer, infectious diseases, and metabolic disturbances [1-5]. The main advantages of RNA interference compared to other therapeutic approaches include exceptional specificity of siRNA, high potency of gene silencing, and the ability to target virtually any expressed gene [6, 7]. However, the low penetration ability of naked siRNA into the cellular cytoplasm to induce sequence-specific mRNA degradation represents a primary obstacle limiting the success of siRNA therapy [7-11]. Despite extensive research, an efficient, nontoxic gene delivery approach has not yet been developed. It is recognized that the delivery of the nucleic acid by nanocarriers facilitates the cellular uptake of DNA/siRNA and increases their gene silencing ability [12-14]. Viruses have been studied as gene delivery vectors; however, the immune response elicited by viral capsid proteins represents a major challenge limiting the wide use of this approach [15]. Consequently, considerable interest to the development of nonviral gene delivery vehicles has been generated. In order to provide effective gene silencing, two controversial requirements for

such delivery systems should be satisfied: (1) stability of siRNA carrier complex during its journey in the systemic circulation toward the targeted cells and the protection of the payload against the aggressive biological environment and (2) intracellular availability of the nucleic acids in order to permit desired therapeutic effects within the cells [8, 16-18].

In order to optimize the delivery of siRNA and enhance the efficiency of the treatment, it is highly desirable to employ clinically relevant imaging approaches for *in-situ* monitoring of the disease progression and therapeutic responses [12]. Magnetic Resonance Imaging (MRI) is a powerful tool for non-invasive *in vivo* monitoring due to its high resolution and lack of ionizing radiation [19, 20]. Superparamagnetic Iron Oxide (SPIO) nanoparticles have been widely investigated as MRI contrast agents to enhance images of biological molecules [21, 22]. Moreover, several approaches have been reported for both siRNA and DNA delivery based on SPIO nanoparticles to timely monitor the delivery process and also to evaluate the therapeutic effects [12, 23, 24]. However, these methods have various shortcomings and do not allow a balanced optimization of siRNA compaction, endosomal escape, and dissociation from the nanoparticles. For example, Medarova *et al.* [12] covalently linked siRNA molecules to the SPIO surface and demonstrated the feasibility of using SPIO nanoparticles as MRI enhancers for *in vivo* tracking of tumor uptake and silencing effects of the siRNA. However, siRNA molecules in this study are tethered to the nanoparticles through chemical bonds between the siRNA and SPIO nanoparticles. Consequently, it is highly possible that such chemical conjugations might potentially compromise the

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silencing effects of siRNA. Moreover, a chemical conjugation might also limit the siRNA loading capacity of the SPIO nanoparticles. In addition, cellular uptake of existing SPIO-siRNA complexes is not limited only to the targeted cells. Consequently, such non-targeted complexes can be internalized by virtually any cells in the body. This nonspecific delivery of siRNA can result in serious adverse side effects on healthy tissues and limit clinical applications of this approach [9, 25, 26]. In particular, delivery of anticancer drugs, genes, and imaging agents specifically to primary tumor and distant metastases requires the use of a ligand specific to receptors that are overexpressed in cancer cells [9, 18, 25-27]. Previously, we have shown that many cancer cells overexpress receptors to Luteinizing Hormone-Releasing Hormone (LHRH) [28, 29]. A combination of anticancer drugs and LHRH peptide in one delivery system enhanced the efficacy of chemotherapy and decreased the adverse side effects of treatment to healthy organs [30-32].

In the present study, we focus on the development and characterization of a complex tumor-targeted Drug Delivery System (DDS) for the simultaneous delivery of siRNA and MRI contrast agents (SPIO) specifically to cancer cells. We utilize the ability of small (~5 nm) SPIO nanoparticles to cooperatively form complexes of siRNA with Polypropyleneimine Generation 5 (PPI G5) dendrimers, which are highly branched three-dimensional polymers with defined molecular weight and a large number of peripheral functional groups [18]. To integrate tumor-specific targeting moiety and increase steric stability, the formulated siRNA nanoparticles were modified with heterobifunctional Poly(ethylene glycol) (PEG). The distal end of PEG was coupled with a synthetic analog of LHRH decapeptide as a targeting agent.

MATERIALS AND METHODS

Materials

Polypropyleneimine Tetrahexacontaamine Dendrimer Generation 5 (PPI G5), 2,4,6-Trinitrobenzenesulphonic Acid (TNBSA), oleic acid, 1-octadecene, Poly (Maleic Anhydride-*alt*-1-Octadecene) (PMAO, MW=30,000–50,000 Da), Poly (Diallyldimethylammonium chloride) (PDDA, MW 120,000 Da), microsized iron (III) oxide, Sodium Dodecyl Sulfate (SDS), and (4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid) (*HEPES*) were obtained from Sigma-Aldrich and used without further purification. Ethidium Bromide (EtBr) solution and α -Maleimide- ω -N-hydroxysuccinimide ester Poly(ethylene glycol) (MAL-PEG-NHS) were purchased from Promega (Madison, WI) and NOF Corporation (White Plains, NY), respectively. The sequence of antisense of siRNA targeted to BCL2 mRNA (obtained from Ambion, Austin, TX), was 5'-GUGAAGUCAACAUGCCUGC-dTdT-3' (sense strand) and 5'-GCAGGCAUGUUGA CUU-CAC-dTdT-3' (antisense strand). The non-targeted mock siRNA (negative control) (5'-CCUCGGGCGUGUCU CUUUU-dTdT-3' sense strand and 5'-AAAAGAGCAC AGCCCGAGG -dTdT-3' antisense strand), 5 carboxy-fluorescein (FAM) labeled siRNA were obtained from Applied Biosystems (Ambion, Inc., Foster City, CA). A synthetic analog of LHRH, Lys6-des-Gly10-Pro9-ethylamide (Gln-His-Trp-Ser-Tyr-DLys(DCys)-Leu-Arg-Pro-NH-Et) peptide was synthesized according to our design [29-32]

by Amersham Peptide Co. (Sunnyvale, CA). All other chemicals were purchased from Fisher Scientific (Fairlawn, NJ). Cisplatin (CIS) was purchased from Sigma (St. Louis, MO).

Superparamagnetic Iron Oxide (SPIO) Nanoparticles Preparation

Iron oxide nanocrystals of 5 nm in diameter were synthesized in organic solvents at high temperature. Typically, microsized iron oxide was mixed with oleic acid, 1-octadecene, and then heated to 320 °C for a certain time to produce monodisperse (5–10% size distribution) iron oxide nanocrystals. The size of nanoparticles was controlled by reaction time, temperature, and the iron oxide and oleic acid concentrations. After the reaction was completed, the mixture was cooled and the iron oxide nanocrystals were precipitated out of 1-octadecene by chloroform/acetone, and then re-dispersed in chloroform. These nanocrystals were highly crystalline and uniform but were not soluble in water due to the hydrophobic oleic acid capping layer. For solubilization of iron oxide nanoparticles in water, we employed a modified previously published method based on forming micelles through amphiphilic polymers (PMAO) for transferring iron oxide nanocrystals from organic solvents into water [33, 34]. The excess of PMAO amphiphilic polymers was removed through ultracentrifugation (600,000 g for 45 min). 5 mg of the PMAO modified iron oxide nanoparticles was added to 20 mL of 10 mg/mL PDDA aqueous solution in 20 mM Tris buffer. The PDDA was allowed to absorb for 20 min under stirring. The formed nanoparticles were purified by the method described above and used for further studies.

Ethidium Bromide Dye Displacement Assay

Fluorescence titration of siRNA/EtBr with the complexation agents were performed as previously described [18]. The complexes were prepared by the intercalation of siRNA with EtBr at 4:1 ratio (siRNA base pairs to EtBr) in water. 1 μ L aliquots of complexation agents were sequentially added to 2 μ M solution of siRNA in 180 μ L water containing EtBr. After each addition, the mixture was stirred and the fluorescence of the solution was measured (590 nm emission; 490 nm excitation). Binding of the complexation agents such as the mixtures of SPIO nanoparticles with PPI G5 dendrimer caused a displacement of bound EtBr, resulting in a decrease in the fluorescence emission intensity. The total amount of complexation agents added to the siRNA solution exceeded 5% of the total volume of the mixture, hence sample dilution factors on the measured fluorescence emission intensity was corrected. All fluorescence measurements were performed using a Cary-Eclipse fluorescence spectrophotometer (Varian, Inc, Palo Alto, CA). The relative fluorescence was based on two independent experiments and calculated using the following equation:

$$\% \text{ Relative FI.} = \frac{\text{Fl. (obs)} - \text{Fl. (EtBr)}}{\text{Fl. (siRNA + EtBr)} - \text{Fl. (EtBr)}} \times 100$$

where Fl. (obs) - fluorescence of siRNA + ethidium bromide + complexation agent; Fl. (EtBr) - fluorescence of ethidium bromide alone; Fl. (siRNA + EtBr) - fluorescence of siRNA + ethidium bromide.

Preparation of SPIO-PPI G5-siRNA Complexes

Prior to the cooperative complexations of siRNA with SPIO nanoparticles and PPI G5, the stock solutions of the mixtures were prepared by adding PPI G5 dendrimer to SPIO nanoparticle solutions with the ratio of primary to the quaternary amines equal to 5:1. The complexes of siRNA with mixture of SPIO and PPI G5 dendrimer were prepared at amine/phosphate ratio (N/P ratio) equal to 0.73. Briefly, siRNA solution was mixed with HEPES buffer (5 mM, pH 7.2) and an appropriate amount of the complexation agents was added. For *in vitro* studies, the final concentration of siRNA in the solution was 4.0 μM . For *in vitro* and *in vivo* studies the final concentrations of siRNA in the solutions were 60 μM and 30 μM , respectively. The samples were vortexed briefly, and the solutions were then incubated at room temperature for 30 min to ensure complex formation.

Modification of SPIO-PPI G5-siRNA Complexes with PEG and LHRH

In order to modify the SPIO-PPI G5-siRNA complexes, NHS-PEG-MAL was reacted with primary amines on the surfaces of the particles in 5 mM HEPES buffer (pH 7.2). The ratio of primary amines to PEG was 10:1. The reaction was carried out for 1 hr at room temperature. PEGylated SPIO-PPI G5-siRNA complexes were then mixed with LHRH peptide dissolved in a HEPES buffer and incubated overnight at 4 °C. The ration of PEG-MAL:LHRH in the reaction mixture was 1:2. The resulting product was dialyzed against deionized water using a Spectra/Pore dialysis membrane with the molecular weight cutoff of 10,000 Da obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA).

Degree of PEGylation

The percentage of amino groups available for PEGylation as well as the decrease in their concentration after the reaction was determined by modified TNBSA assay [18]. Briefly, 180 μL solution of either non-modified or PEGylated SPIO-PPI G5-siRNA complexes was mixed with 4 μL of TNBSA solution (0.03M in water). Absorbance at 420 nm was measured after 30 min incubation at room temperature. All absorption measurements were performed using a Cary-500 fluorescence spectrophotometer (Varian, Inc, Palo Alto, CA). The final concentration of primary amines was calculated using standard curves. Standard curves were prepared by plotting the average blank corrected absorption at 420 nm reading for each standard vs. its concentration in μM .

Agarose Gel Retardation Assay

The complexes of siRNA with mixtures containing SPIO nanoparticles and PPI G5 were prepared as described above. Free siRNA was used as the control. Double-stranded RNA ladder (New England Biolabs Inc., Ipswich, MA) with the smallest base pairs (bp) at 21 bp was used as a size reference. The samples were further diluted with water and electrophoresed in 4 % agarose gel at 100 mV for 60 min in DPBS and stained with EtBr. The gels were digitally photographed and scanned using the Gel Documentation System 920 (Nucleo-Tech, San Mateo, CA). Complexation of siRNA prevented staining of siRNA by EtBr and led to the disappearance of

the siRNA band. Therefore, the fluorescent intensity of the 21 base pair band on the gel disappeared when siRNA was complexed with SPIO nanoparticles and dendrimers.

Evaluation of LHRH Peptide Reaction with SPIO-PPI G5-siRNA Complexes

Determination of the presence of LHRH peptide on the surface of SPIO-PPI G5-siRNA complexes was performed using Bicinchoninic Acid (BCA) protein assay (Pierce, Rockford, IL). The BCA method employs the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium. The combination of Bicinchoninic acid and Cu^{+1} creates a purple-colored product that absorbs at 562 nm. The amount of product formed is dependent upon the amount of protein in the sample. The spectra of the product corresponding to free LHRH and SPIO-PPI G5-siRNA-PEG-LHRH complexes have well defined absorbance maximum around 560 nm corresponding to the absorbance of the BCA/copper complex. These complexes are formed as a result of the reaction of BCA reagent with the cuprous cation produced from the reduction of Cu^{+2} to Cu^{+1} by the LHRH peptide [18, 35]. The absorbance maximum was absent in the assay spectra of the non-targeted complexes that are not modified with LHRH. Briefly, 20 μL of the test solution was mixed with 200 μL of working reagent and left to react for 30 min at 37 °C. The solution then was incubated at room temperature for 10 min and the absorbance was measured at 562 nm.

Dynamic Light Scattering

Dynamic Light Scattering (DLS) studies were performed using the Dawn EOS multi-angle light scattering spectrometer modified with a QELS attachment (Wyatt Technology Corp., Santa Barbara, CA). Data were collected at an angle of 108° using an avalanche photodiode and an optical fiber and processed with the Wyatt QELS software (regularization analysis). The duration of each DLS measurement was 60 s. Each light scattering experiment consisted of a minimum of 5 measurements.

Atomic Force Microscopy

In order to obtain Atomic Force Microscope (AFM) images of formulated complexes, 5 μL aliquots of SPIO-PPI G5-siRNA solutions were deposited on a freshly cleaved mica surface. After 5 min of incubation, the surface was rinsed with several drops of nanopure water (Barnstead), and dried under a flow of dry nitrogen. AFM images were obtained using Nanoscope IIIA AFM (Digital Instruments, Santa Barbara, CA) in a tapping mode, operating in ambient air. A 125 μm long rectangular silicon cantilever/tip assembly was used with a spring constant of 40 Nm^{-1} , resonance frequency of 315–352 kHz, and tip radius of 5–10 nm. The applied frequency was set on the lower side of the resonance frequency. The image was generated by a change in amplitude of the free oscillation of the cantilever as it interacted with the sample. The height differences on the surface are indicated by the color code, lighter regions indicating an increase in the height of the complexes. The height and outer diameter of formulated complexes were measured using the Nanoscope software.

Cell Lines

Two cancer cell lines with a different level of expression of LHRH receptors were used. Human LHRH positive A549 lung carcinoma cells and SKOV-3 LHRH negative ovarian cancer cells were obtained from the ATTC (Manassas, VA, USA). In addition, A549 human lung adenocarcinoma epithelial cell line transfected with luciferase was purchased from Xenogen Bioscience, (Cranbury, NJ). Cells were cultured in RPMI 1640 medium (Sigma Chemical Co., Louis, MO) supplemented with 10% fetal bovine serum (Fisher Chemicals, Fairlawn, NJ). Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂ (v/v) in air. All of the experiments were performed on the cells in exponential growth phase.

Cellular Internalization of siRNA

Cellular internalization of FAM-labeled siRNA complexes were analyzed by fluorescence (Olympus America Inc., Melville, NY) and confocal (Leica Microsystems Inc., Bannockburn, IL) microscopes as previously described [2, 18, 35]. To assess cellular internalization and localization of siRNA, ten optical sections, known as a z-series, were scanned sequentially by a confocal microscope along the vertical (z) axis from the top to the bottom of the cell. Prior to the visualization, A549 and SKOV-3 cells were plated (20,000 cells/well) in 6-well tissue culture plate. The cells were treated with different formulations for 24 hrs. The concentration of siRNA was 0.25 μM. After 24 hrs of treatment cells were washed three times with phosphate buffered saline (PBS) and 1 mL of fresh medium was added to each well.

In Vitro Cytotoxicity

The cellular cytotoxicity of the formulated siRNA complexes was assessed using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously [14, 18, 36, 37]. Briefly, A549 cells were separately incubated in 96-well plate with different concentrations of the studied formulations, which resulted in a total of seven separate series of experiments: (1) Control (fresh media); (2) Mixture of 5 nm SPIO nanoparticles and PPI G5 dendrimers; (3) 5 nm SPIO-PPI G5-siRNA complexes; (4) 5 nm SPIO-PPI G5-siRNA-PEG-LHRH complexes; (5) CIS; (6) Mixture of CIS and 5 nm SPIO-PPI G5-siRNA complexes and (7) Mixture of CIS and 5 nm SPIO-PPI G5-siRNA-PEG-LHRH complexes. Control cells received an equivalent volume of fresh media. The duration of incubation was 24 hrs. On the basis of these measurements, cellular viability was calculated for each formulation concentration.

Gene Expression

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used for the analysis of the expression of genes encoding BCL2 protein as previously described [37-39]. RNA was isolated after 24 h incubation of cancer cells with modified and non-modified SPIO-PPI G5-siRNA complexes, using an RNeasy kit (Qiagen, Valencia, CA). First strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ) with 4 mg of total cellular RNA and 100 ng of random hexa-

deoxynucleotide primer (Amersham Bioscience, Piscataway, NJ). After synthesis, the reaction mixture was immediately subjected to polymerase chain reaction, which was carried out using the GenAmp PCR System 2400 (Perkin-Elmer, Shelton, CT). The following pairs of primers were used: *BCL2* – GGA TTG TGG CCT TCT TTG AG (sense), CCA AAC TGA GCA GAG TCT TC (antisense); β_2 -*microglobulin* (β_2 -*m*, internal standard) – ACC CCC ACT GAA AAA GAT GA (sense), ATC TTC AAA CCT CCA TGA TG (antisense). PCR products were separated in 4% NuSieve 3:1 Reliant agarose gels in 1×TBE buffer (0.089 M Tris/Borate, 0.002 M EDTA, pH 8.3; Research Organic Inc., Cleveland OH) by submarine gel electrophoresis. The gels were stained with EtBr, digitally photographed, and scanned using the Gel Documentation System 920 (NucleoTech, San Mateo, CA). Gene expression was calculated as the ratio of mean band intensity of analyzed RT-PCR product (*BCL2*) to that of the internal standard (β_2 -*m*).

In Vivo Study

NCR nude mice (female, ~6 weeks, 20 g) were purchased from Taconic Farms, Inc. (Germantown, NY). We used a previously described animal model of human cancer xenografts [18, 30, 32]. Briefly, A549 human cancer cells transfected with luciferase (5×10^6) were subcutaneously transplanted into the flanks of female athymic *nu/nu* mice. According to the approved institutional animal use protocol, the tumors were measured by a caliper every other day and their volumes were calculated as $d^2 \times D/2$ where d and D are the shortest and longest diameter of the tumor in mm, respectively. When the tumor reached a mean size of 50 mm³, mice were divided into seven groups and injected intratumorally 3 times within 10 days with 150 μL of the following formulations: (1) saline (control); (2) free non-bound LHRH; (3) free non-bound siRNA; (4) free non-bound CIS; (5) Mixture of CIS and SPIO-PPI G5; (6) Mixture of CIS and SPIO-PPI G5-siRNA; and (7) Mixture of CIS and SPIO-PPI G5-siRNA-PEG-LHRH complexes. The concentrations of CIS and siRNA in the formulations were 2.5 mg/kg and 30 μM, respectively. Changes in tumor size were monitored by real-time bioluminescence in anesthetized animals by IVIS imaging system (Xenogen Bioscience, Cranbury, NJ).

Statistical Analysis

Data were analyzed using descriptive statistics, single-factor analysis of variance (ANOVA), and presented as mean values ± standard deviation (SD). Ten animals were used in each group of *in vivo* experiments. The comparison among groups was performed by the independent sample Student's t-test. The difference between variants was considered significant if $P < 0.05$.

RESULTS

Mixture of SPIO Nanoparticles and PPI G5 as a Carrier for siRNA Delivery

siRNA complexes were prepared with a mixture of SPIO nanoparticles and PPI G5 dendrimers, which introduced functional primary amino groups on the surfaces of formulated siRNA complexes for their further modification Fig. (1). PPI dendrimers were covered with PEG polymers and

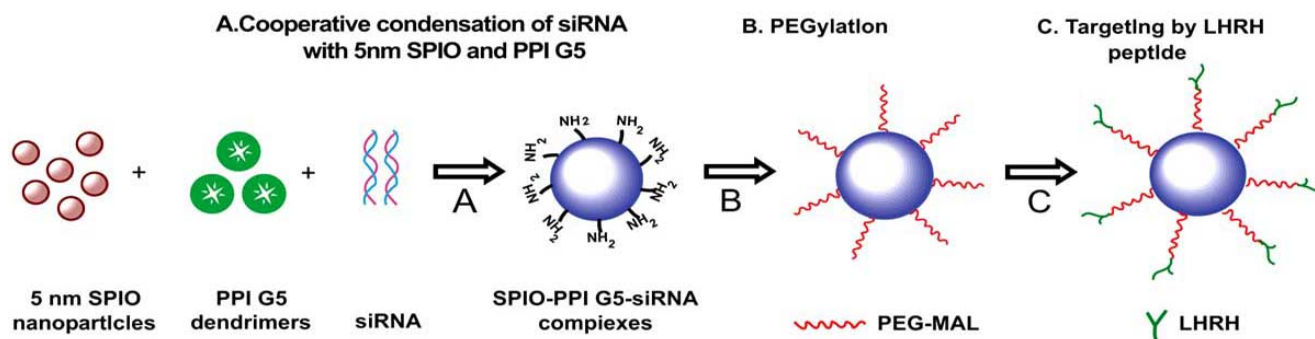


Fig. (1). The preparation of tumor-targeted, stable siRNA nanoparticles. (A) Cooperative condensation of siRNA with 5 nm SPIO nanoparticles and PPI G5 dendrimers. (B) PEGylation. (C) Conjugation of LHRH peptide to the distal end of the PEG layer.

LHRH peptide as a targeting moiety specific to cancer cells was conjugated to the distal end of the polymer. The efficiency of 5 nm and 10 nm SPIO nanoparticles to cooperatively provoke siRNA complexation with PPI G5 was studied by ethidium bromide dye displacement assay. The ratio of free amines (nitrogen) on PPI dendrimers and SPIO nanoparticles to phosphate on the siRNA (N/P ratio) was employed to quantify the efficacy of cooperative siRNA condensation. Quantitative analysis of the mixture's complexation efficiency reveals that the mixture of 5 nm SPIO and PPI G5 is more effective in provoking siRNA complexation (the apparent end point of complexation of N/P ratio = 0.73) than PPI G5 dendrimer alone (N/P ratio = 1.13) and 10 nm SPIO with PPI G5 (N/P ratio = 1.3) Fig. (2A) end points of complexation are denoted as circles in the insert. Therefore, the mixture of 5 nm SPIO and PPI G5 was the most effective complexation agent and was employed for the development of multifunctional nanomedicine platform for cancer specific delivery of siRNA. Agarose gel retardation assay was additionally involved to confirm the formation of 5 nm SPIO-PPI G5-siRNA complexes formed at N/P ratio which represented the apparent complexation end point obtained from ethidium bromide dye displacement assay. It was found that a complete binding of siRNA (without the presence of a trailing band) with the mixtures of 5 nm SPIO and PPI G5 dendrimers was observed in comparison to free siRNA Fig. (2B).

Characterization of siRNA Complexes

DLS measurements at a 108° scattering angle were used to estimate the apparent hydrodynamic diameters of the resulting siRNA complexes. The results of DLS measurements demonstrate that the average diameter of 5 nm SPIO-PPI G5-siRNA complexes was 169.8 ± 28.4 nm Fig. (3A). In addition to DLS measurement, the formation of nanosized siRNA complexes has further been confirmed by AFM Fig. (3B). AFM analysis verified that 5 nm SPIO nanoparticles cooperatively with PPI G5 dendrimers could effectively produce complexes with siRNA leading to the formation of discrete particles with an average diameter of 214.3 ± 53.1 nm. The differences in the size of nanoparticles probably reflect the differences in a sample preparation for the size measurements by two different methods. DLS was performed on nanoparticles in a fully hydrated state in solution, whereas AFM studies were carried out on samples dried to the mica

surface, which resulted in flattening of the nanoparticles on the mica surface during the drying process [40].

PEGylation and *In Vitro* Transfection of siRNA Complexes

The PEGylation of SPIO-PPI G5-siRNA complexes was carried out by coupling of linear MAL-PEG-NHS to the amino groups on the surface of the complexes, which were introduced by PPI G5 dendrimers. The availability of the primary amines in the structure of the prepared siRNA complexes before PEGylation as well as the decrease in their concentration after PEGylation has been estimated by the TNBSA assay. The result reveals that the degree of PEGylation was 70% for SPIO-PPI G5-siRNA complexes.

To examine the influence of nanoparticles coating on their cellular uptake, PEGylated and non-PEGylated siRNA complexes were incubated with A549 cancer cells in a fresh medium. Fluorescence microscopy studies revealed the fact that PEG modification of SPIO-PPI G5-siRNA complexes enhance their sterical stability and prevent the aggregation of complexes that was abundant in non-PEGylated complexes Fig. (4 compare panels A and B). On the other hand, non-PEGylated complexes provided for an effective delivery of labeled siRNA into the cells Fig. (4A). As expected, PEGylation of the siRNA complexes decreased their internalization by cancer cells.

In order to evaluate the biological activity of the delivered siRNA, the siRNA targeted to BCL2 mRNA was used in the present study. Fig. (5) shows the expression of the BCL2 gene in A549 and SKOV-3 human cancer cells treated with siRNA delivered by different SPIO-PPI G5 complexes. The suppression of BCL2 mRNA by the PEGylated complexes was substantially lower when compared with the corresponding non-PEGylated system (lines 2 and 3). The sufficient decrease in gene silencing activity of the PEGylated complexes corroborates with cellular internalization data. To exclude nonspecific effects on gene expression by SPIO-PPI G5 complexes alone without bound siRNA, we examined whether the mixtures of SPIO nanoparticles with dendrimers could impact the BCL2 gene expression. RT-PCR analysis demonstrated that the employed siRNA delivery systems did not induce statistically significant changes in the expression of BCL2 mRNA in A549 cancer cells at the studied concentrations Fig. (5, line 5). Similarly, a mocked siRNA duplex with a scrambled sequence having no significant homology

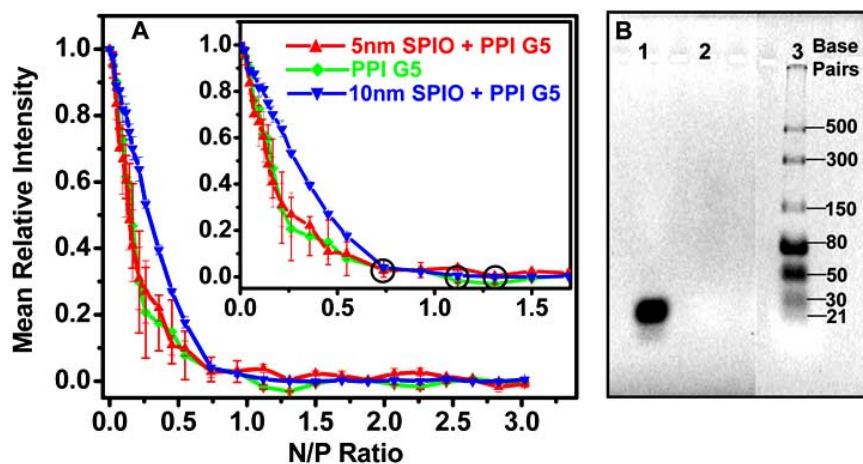


Fig. (2). (A) siRNA complexation efficiency of SPIO nanoparticles and PPI G5 dendrimer evaluated by the ethidium bromide dye displacement assay. Figure inset shows enlarged portions of the curves in the vicinity of N/P ratios which represent the apparent ends of complexation. The circles in the enlarged curves highlight the N/P ratios corresponding to the apparent end of siRNA complexation by different complexation agents. Means \pm SD are shown. (B) Typical agarose gel electrophoresis image representing siRNA complexation efficiency by mixture of 5 nm SPIO nanoparticles and PPI G5. The gel is stained with ethidium bromide. (1) Free siRNA (control); (2) Mixture of 5 nm SPIO with PPI G5 and (3) Double stranded RNA ladder.

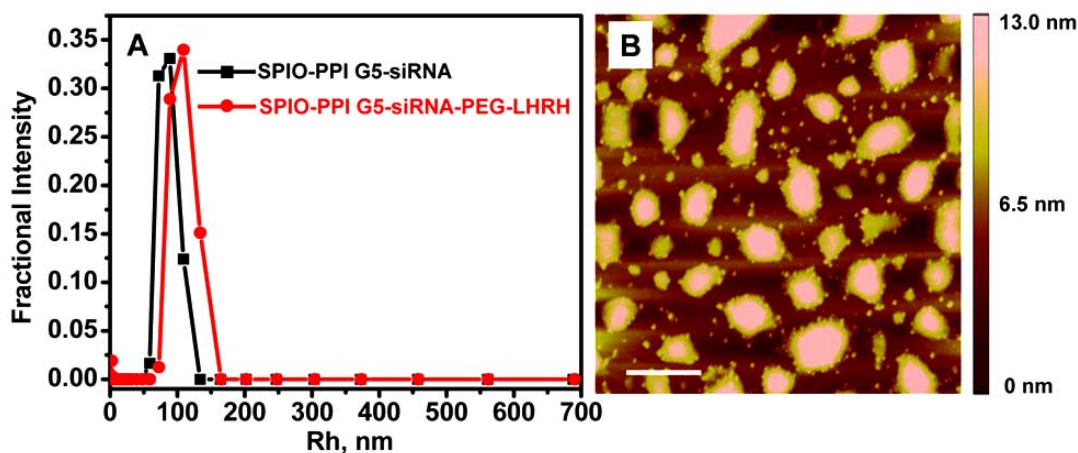


Fig. (3). (A) The representative curves of the size distribution of 5 nm SPIO-PPI G5-siRNA complexes prior and after modification with PEG and targeting LHRH peptide measured by DLS. Rh is hydrodynamic radius. (B) Representative atomic force microscope image of the nanoparticles formed as the result of siRNA complexation with mixture of SPIO and PPI G5. Scale bar is equal to 400 nm.

to any known gene sequences was used in this series of the experiments as a negative control. RT-PCR data demonstrated that complexes with such mocked siRNA did not show any statistically significant inhibition of BCL2 mRNA expression confirming the specificity of BCL2 mRNA functional knock-down Fig. (5, line 6).

Specific Targeting of SPIO-PPI G5-siRNA Complexes to Cancer Cells by LHRH Peptide

In order to conjugate a targeting moiety (LHRH decapeptide) to the siRNA nanoparticles, the maleimide group at the distal end of the PEG-chain was coupled to thiol group presented by cysteine residue in modified LHRH sequence. The presence of LHRH peptide on the complex surface was confirmed by Bicinchoninic Acid (BCA) protein assay (Thermo Fisher Scientific Inc., Rockford, IL) according to manufacture protocol (data not shown). As shown in Fig. (3A) DLS measurements reveal that the diameter of modified SPIO-PPI

G5-siRNA complexes was 212.0 ± 35.6 nm, respectively. The increase in the size of the modified siRNA complexes compared to nonmodified ones could be explained by the presence of the polymer layer on the surface of siRNA complexes [18].

In vitro studies were performed to characterize the influence of LHRH peptide as a targeting moiety on the uptake and intracellular activity of the entrapped siRNA. The fluorescence microscopy images demonstrated a sufficient increase in the intracellular internalization of LHRH-targeted complexes by A549 cancer cells which overexpress LHRH receptors Fig. (4 C, D). In contrast, cellular uptake of tumor-targeted siRNA complex in LHRH negative SKOV-3 cells was substantially less when compared with non-targeted complexes Fig. (4 D). These experiments confirmed that the targeted shielded nanoparticles indeed delivered the siRNA specifically to the cancer cells, which overexpress the targeting receptors.

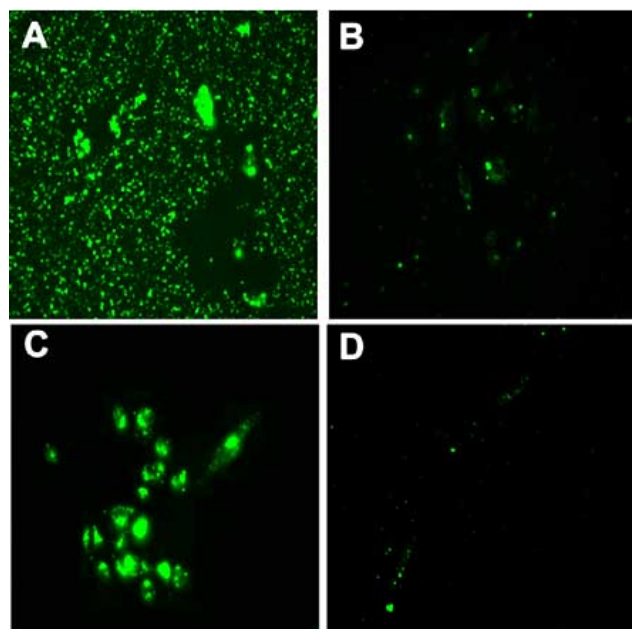


Fig. (4). Representative fluorescence microscopic images of (A) SPIO-PPI G5-siRNA; (B) SPIO-PPI G5-siRNA-PEG and (C, D) SPIO-PPI G5-siRNA-PEG-LHRH complexes after 24 h of incubation with LHRH-positive A549 (A-C) and LHRH-negative SKOV-3 (D) cancer cells. siRNA was labeled with 6-FAM Green.

Theoretically, the formulated siRNA complexes could adhere to the surface of LHRH-positive cancer cells and erroneously be visualized on microscopic images as internalized within the cell. To exclude such errors, we analyzed the distribution of LHRH targeted siRNA complexes in different cellular layers from the upper to the lower surfaces of the cell using confocal fluorescent microscopy. In these experiments, the formulated complexes with FAM labeled siRNA were incubated with human A549 cancer cells. The cells were subjected to confocal microscopy. The z-section of single cells transfected with the modified complexes, formed by complexation of siRNA with the mixture of SPIO nanoparticles and PPI G5 dendrimer, and showed their homogeneous and uniform distribution in all layers of the cell from the top surface to the bottom Fig. (6).

To assess the ability of LHRH targeted complexes not only to deliver siRNA but knockdown targeted gene expression, we prepared complexes with BCL2-specific siRNA. In this series of the experiments, both LHRH positive (A549) and negative (SKOV-3) cancer cells were treated with the prepared siRNA complexes for 24 hrs. The RT-PCR data obtained revealed that the LHRH modification of siRNA complexes restore the knockdown activity for siRNA complexes, which was decreased after PEGylation Fig. (5, line 4). On the other hand, the silencing effect of the BCL2-targeted siRNA was not significant in LHRH-negative cancer cells Fig. (5, line 7), which is in good agreement with the siRNA cellular internalization result represented in Fig. (4). Therefore, as expected, LHRH peptide proved its capability to target effectively the siRNA complexes to the specific receptors in the plasma membrane of cancer cells.

In Vitro Cytotoxicity of siRNA Complexes

The influence of the formulated siRNA delivery systems on cell viability was investigated in A549 human lung cancer cell line by the MTT assay. Fig. (7A) shows the average data from three different experiments with increasing concentration of the complexes. One can see that over 95% average cell viability was observed for both 5 nm SPIO-PPI G5 and 5nm SPIO-PPI G5-siRNA delivery systems at the concentrations used for *in vitro* and *in vivo* experiments of the present study. At a maximum available concentration, the mean cell viabilities for the targeted SPIO-PPI G5-PEG-LHRH complex was 85% compared with that of the control, respectively.

Co-Delivery of siRNA and an Anticancer Drug

The ability of the developed siRNA delivery system to enhance efficiency of a chemotherapeutic drug such as CIS was evaluated in the current study both *in vitro* and *in vivo*. The cellular cytotoxicity of Cisplatin alone and in combination with non-targeted SPIO-PPI G5-siRNA or targeted SPIO-PPI G5-siRNA-PEG-LHRH delivery systems was assessed using a modified MTT assay. Data in Fig. (7B) shows that cytotoxicity of CIS against multidrug resistant human cancer cells was sufficiently enhanced in the presence of non-targeted or LHRH targeted siRNA delivery vectors. The maximum enhancement of anticancer activity of CIS

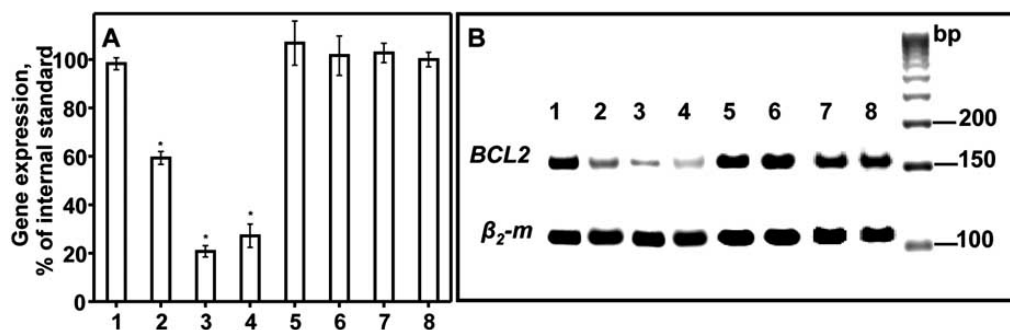


Fig. (5). Typical image of RT-PCR products and expression of the *BCL2* gene in human A549 (1-6) and SKOV-3 (7, 8) cancer cells. A549 cells were treated with (1) medium (control); (2) SPIO-PPI G5-siRNA-PEG; (3) SPIO-PPI G5-siRNA; (4) SPIO-PPI G5-siRNA-PEG-LHRH; (5) SPIO-PPI G5; (6) SPIO-PPI G5-siRNA (scrambled). SKOV-3 cancer cells were treated with (7) SPIO-PPI G5-siRNA-PEG-LHRH and (8) medium (control). Gene expression was calculated as a ratio of band intensity of studied gene to that in internal standard (β_2 -m, β_2 -microglobulin). Means \pm SD are shown.

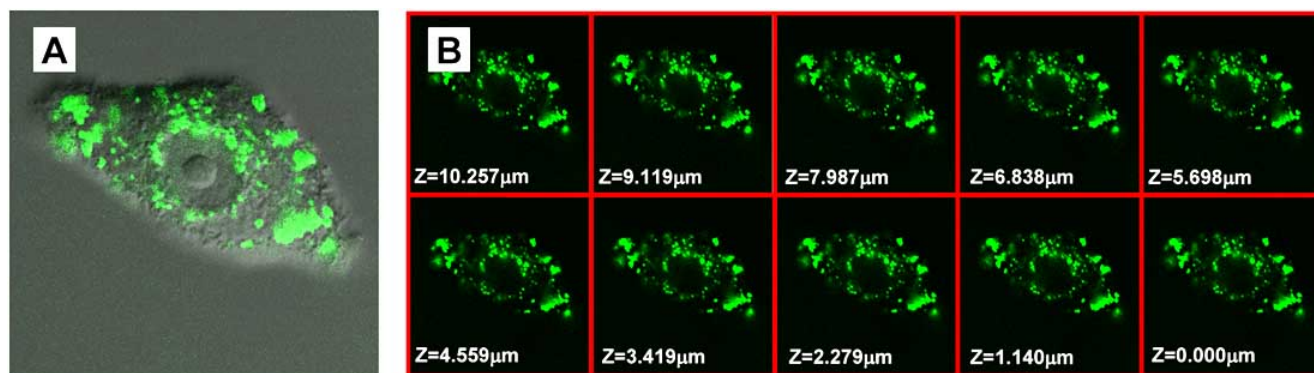


Fig. (6). Representative confocal microscopy images (light + fluorescence) of A549 human cancer cells incubated for 24 h with SPIO-PPI G5-siRNA-PEG-LHRH (A) and z-series from the top ($z=10.257 \mu\text{m}$) to the bottom ($z=0 \mu\text{m}$) of the single cell (B).

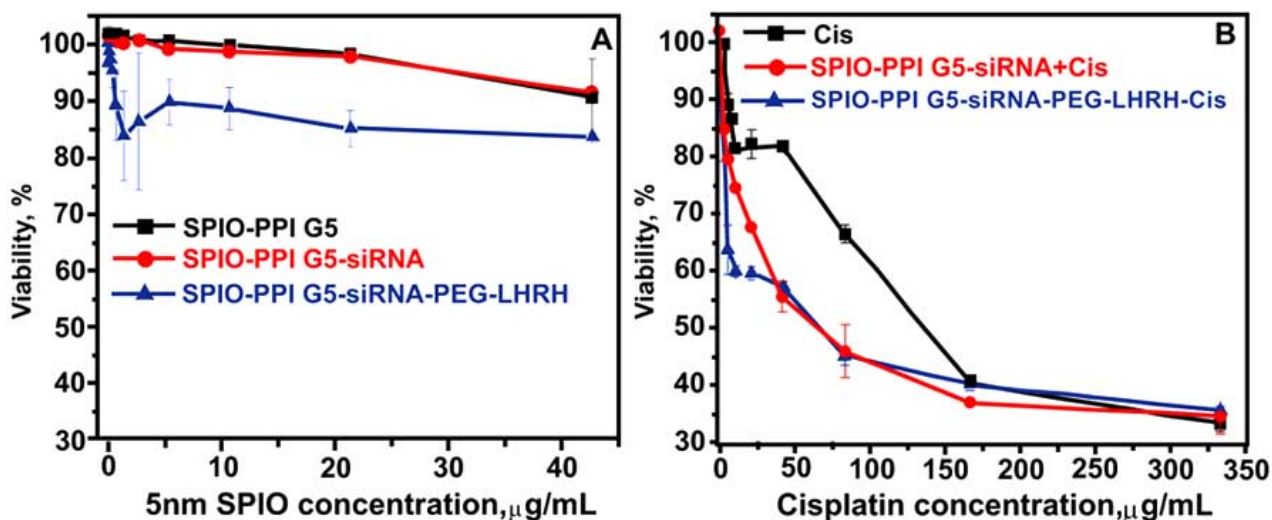


Fig. (7). Cytotoxicity of (A) SPIO-PPI G5; SPIO-PPI G5-siRNA; SPIO-PPI G5-siRNA-PEG-LHRH complexes and (B) Cisplatin; SPIO-PPI G5-siRNA + CIS; SPIO-PPI G5-siRNA-PEG-LHRH + CIS against A549 human cancer cells. Means \pm SD are shown.

was observed at the lower concentration range of the drug ($1 \mu\text{g/mL} - 150 \mu\text{g/mL}$).

Antitumor Activity

Antitumor activities of the proposed formulations with corresponding controls were studied *in vivo* using subcutaneous xenograft model of human cancer. The progression of tumor growth was monitored by an IVIS imaging system and by measuring the tumor volume Fig. (8) upper and bottom panels, respectively. It was found that free LHRH and non-conjugated naked siRNA did not significantly influence the growth of the tumor Fig. (8 bottom panel, curves 2-3). Free CIS limited the growth of the tumor at the last day of the treatment on 36.2 % when compared with untreated control Fig. (8 image 4, curve 4). Simultaneous delivery of CIS and SPIO-PPI-G5 dendrimer complex slightly increased the anti-tumor activity of the drug Fig. (8 image 5, curve 5). The suppression of cellular antiapoptotic defense by siRNA targeted to BCL2 mRNA, delivered by SPIO-PPI G5-siRNA complex simultaneously with CIS significantly enhanced the antitumor activity of the drug. In fact, the tumor volume decreased on 67.5 % after the combinatorial treatment when compared with untreated control Fig. (8 image 6, curve 6).

Targeting of siRNA complexes specifically to the tumor by LHRH peptide led to the further enhancement of the anti-tumor activity of CIS. The tumor volume decreased on 75.5% when compared with untreated control Fig. (8 image 7, curve 7).

DISCUSSION

Previously, we developed multifunctional nonviral vector for siRNA delivery based on SPIO nanoparticles modified with PDDA and PMAO, which contain quaternary ammonium and carboxylic functional groups on the periphery for siRNA condensation and endosomal release. These SPIO nanoparticles demonstrated high efficiency to form complexes with siRNA and to facilitate their internalization by the cancer cells. Cellular uptake of such SPIO-siRNA complexes most probably occurred by adsorptive-mediated endocytosis, which is triggered by electrostatic interactions between the negatively charged plasma membrane and the positively charged complexes. Targeting of the SPIO-siRNA complexes specifically to cancer cells by incorporating a ligand to the receptors overexpressed in the plasma membrane of cancer cells can offer at least three advantages. First, it switches the mechanism of cellular internalization

toward more efficient receptor-mediated endocytosis. Second, specific targeting to cancer cells prevents rapid clearance of the siRNA cationic complexes by liver, spleen, and kidney after systemic administration [18, 41]. Third, the delivery of therapeutic payload specifically to cancer cells limits adverse side effects of the treatment on healthy organs by changing its organ distribution toward the preferential accumulation in the tumors [18, 30, 31]. Consequently, in the present study we developed and characterized tumor-targeted superparamagnetic iron oxide nanoparticles-dendrimer complexes for simultaneous delivery specifically to tumor cells of siRNA and MRI-contrast agents. Therefore, the proposed complex multifunctional drug delivery platform can be used for simultaneous suppression of cellular resistance by siRNA and MRI imaging of the system itself, and primary tumor or metastases. Experimental data show the following advantages of the proposed delivery system.

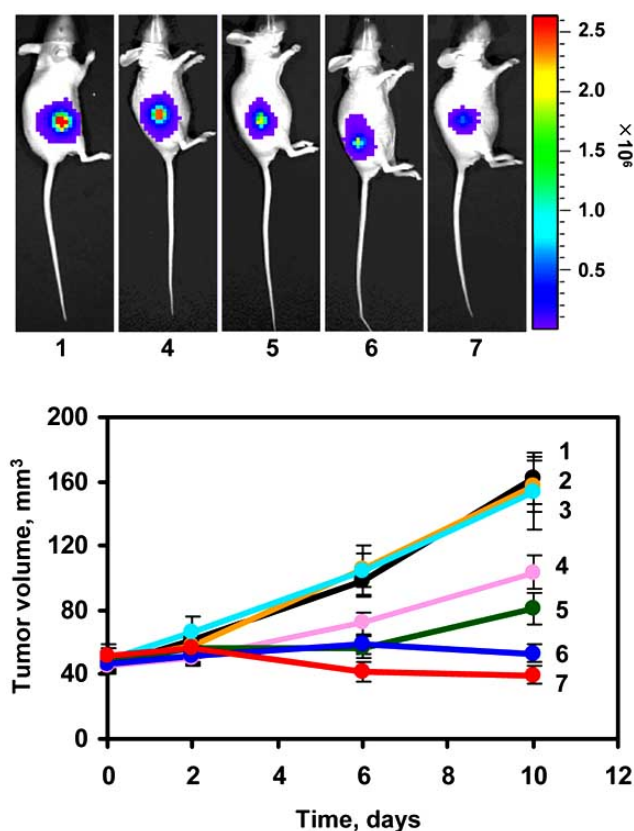


Fig. (8). Antitumor activity of different formulations. Upper panel: typical bioluminescent images of mice bearing subcutaneous tumor xenografts of human A549 cancer cells transfected with luciferase. Images were taken using the IVIS imaging system (Xenogen) in anesthetized animals at the end of the experiment. Bottom panel: Changes of tumor volume during the treatment. Mice were treated 3 times within 10 days with the following formulations: (1) Control (saline); (2) LHRH; (3) siRNA; (4) CIS; (5) SPIO-PPI-G5 + CIS; (6) SPIO-PPI-G5-siRNA + CIS; (7) LHRH-PEG-SPIO-PPI-siRNA + CIS. Means \pm SD are shown.

It is well-known that siRNA complexes should have an optimal size and proper shape for effective gene delivery because that often governs the transfection efficiency, cytotoxicity, and tissue targeting of an entire system *in vivo* [41].

Generally, in order to enable its effective penetration into tissue, the size of gene delivery vehicles should not exceed 250 nm [42], although the optimal size of the particles is still under debate. Direct measurements by several independent approaches determined that the size of complexes developed in the present study was approximately 200 nm (with complexed siRNA). The nanoparticles were compact and close to spherical shape. Consequently, based on our previous data, one could expect that such dendrimer-based systems will provide for an efficient delivery of its payload into cancer cells [13, 18, 35]. Further investigations confirmed this suggestion.

Cytotoxicity of gene transfection vectors including viral vectors, inorganic nanoparticles, cationic liposomes, and polymeric cations is a major barrier for their efficient use for the delivery of therapeutic genes [15]. Recently, Omid *et al.* [43] demonstrated that PPI dendrimers can intrinsically alter the expression of many endogenous genes, the nature and extent of which were dependent on the dendrimer generation, and cell type. Although cytotoxicity of a nanocarrier itself is not an issue for the delivery of anticancer drugs with much more higher cytotoxicity, we found that the proposed targeted and nontargeted SPIO-PPI G5 vehicles alone and in combination with siRNA possessed low cytotoxicity. Moreover, a mixture of SPIO with PPI G5 dendrimers alone without siRNA did not influence the expression of the targeted *BCL2* gene. Such low toxicity of the modified siRNA complexes makes them attractive for *in vivo* delivery of non-toxic compounds. Consequently, similar drug delivery systems can be used for applications other than cancer chemotherapy.

It is known that siRNA complexes are usually easily opsonized and removed from the circulation long prior to completion of their main function [44, 45]. Chemical modification of siRNA delivery vector with certain synthetic polymers, such as PEG, is the most frequent way to increase the *in vivo* longevity in the systemic circulation of siRNA delivery vectors. The layer of hydrophilic polymer (in most cases PEG) sterically hinders interactions of blood components with the positively charged surface of siRNA complexes and enhances their stability in the blood stream [18, 46, 47]. However, simultaneously while improving the pharmacokinetics, PEGylation usually limits cellular internalization *in vivo* (stealth effect) [18]. It is known that neutral surface charge of PEGylated siRNA complexes limits their interactions with a negatively charged cell membrane when compared with positively charged non-modified siRNA complexes [18]. To overcome these obstacles, the modification of sterically stabilized siRNA delivery carriers with cell targeting ligands is usually used in order to enhance its transfection activity [18, 35]. The different targeting moieties including, galactose, folate, RGD-peptide and antibodies were examined for the delivery of DNA and siRNA [9, 46]. Recently, we found that the receptor for LHRH is overexpressed in many types of human cancer cells and was not detectably expressed in healthy human visceral organs [28, 29]. Furthermore, our previous findings show that the use of the LHRH peptide for the targeting of a polymeric anticancer drug delivery system to cancer cells substantially limits its adverse side effects on healthy tissues and significantly enhances the antitumor efficacy of the anticancer drug [31].

Therefore, based on these results we selected the synthetic analog of LHRH peptide as a targeting moiety to enhance the internalization of the developed siRNA delivery system specifically by cancer cells. The results of the *in vitro* and *in vivo* experiments of this tumor-targeted system showed that an incorporation of LHRH peptide substantially improved cellular internalization of siRNA, increased its transfection efficiency, and enhanced the antitumor activity of drug.

Our data clearly show that the combinatorial delivery of siRNA with anticancer drug substantially enhanced the efficiency of chemotherapy leading to the more significant limitation of the tumor growth. Therefore, it is important to deliver siRNA inside tumor cells simultaneously with an anticancer drug. The delivery of siRNA requires an appropriate carrier because naked siRNA is unstable in the blood stream and poorly penetrates cells. The proposed in the present research delivery system significantly improves the stability of siRNA in plasma and provides for its efficient cellular internalization. In addition, an incorporation of a tumor-targeting moiety (LHRH peptide) into the DDS permits the delivery of siRNA specifically into tumor cells further enhancing anti-tumor effects of the drug and limiting adverse side effects of the treatment on healthy organs [30-32].

CONCLUSIONS

In summary, the designed siRNA delivery vector based on SPIO nanoparticles modified with PPI G5 dendrimers and PEG combines the cell-targeted selectivity with the specificity of siRNA. The modular chemical design of the proposed system allows for the substitution of used cancer targeting moiety with other ligands, or combinations of ligands, to selectively target other type of cancer cells. The results obtained open new perspectives for the development of targeted multifunctional siRNA delivery vectors capable of *in-situ* monitoring of therapeutic responses of the RNA interference.

ACKNOWLEDGEMENTS

The research was supported in part by NIH R01 CA100098, R01 CA111766, R01 CA138533, R41 CA130986 and NSF CBET-0933966 grants.

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