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Research Article

Gene Expression Analysis of Interleukin 8 in NSCLC Cell Lines and Preparation of Non-covalently Functionalized Single-Walled Carbon Nanotubes Conjugated with IL 8 siRNA

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ABSTRACT

Interleukin-8/CXCL8 (IL-8) is a potent chemokine, angiogenic factor, autocrine growth factor with direct growth-promoting effect on many human cancers, and overexpression of IL-8 in carcinomas allows tumor growth and invasion. Ribonucleic acid (RNA) interference is an effective post-transcriptional gene silencing concept, specifically identifying and down-regulating the target gene's expression. Small interfering RNAs (siRNAs) acts as a promising and powerful tool for application in gene therapy. However, siRNA therapies have been impeded by a lack of potential delivery systems. In this scenario, carbon nanotubes are used as an emerging platform for constructing safe, specific, and effective siRNA delivery systems. In this present work, the gene expression of IL-8 was studied in A 549 and H 460 non-small cell lung cancer cells using reverse transcription polymerase chain reaction (RT-PCR), and enzyme-linked immune sorbent assay produced significant levels of IL 8 protein ($9.57 \pm 1.6 \text{ ng mL}^{-1}$ per 10^6 cells and $83.26 \pm 2.8 \text{ ng mL}^{-1}$ per 10^6 cells). Then potential tumor targeting nano delivery system was designed using single-walled carbon nanotubes (SWNTs), non-covalently functionalized with DSPE-PEG 2000 amine polymer to improve the solubility biocompatibility and lower the cytotoxicity. Then these functionalized SWNTs were conjugated to IL 8 siRNA via cleavable disulfide bond using a Sulfo-LC-SPDP cross-linker. The optimized formulation with enhanced biocompatibility and solubility may effectively be used as a nanocarrier for the potential delivery of IL 8 siRNA to tumor target sites.

INTRODUCTION

Non-small cell lung cancer is one of the most common types of lung cancer leading to death that makes up about 80–85% of all cases. Interleukin-8 is a cytokine of the C-X-C chemokine family having heparin-binding proteins which was classified originally as a neutrophil chemoattractant with inflammatory activity.^[1] IL-8 secretion and expression by tumor cells increase the survival rate and proliferation of the cells by autocrine activation. It also promotes angiogenesis and neutrophil infiltration into the tumor.^[2,3] Furthermore, in the same type of lung cancer, IL-8

concentrations also correlates with tumor progression, patient survival, and timing of relapse.^[4] RNA-mediated interference (RNAi) is a conserved biological mechanism in which the double-stranded RNA molecule silences the expression of the protein-coding genes. Recently, RNAi-based therapy is widely used for treating various cancers like lung, liver, breast, etc.^[5,6] RNA interference-based gene silencing has great therapeutic intervention ability in non-small cell lung cancer (NSCLC). RNAi-based therapy for humans can be known through siRNAs which are double-stranded RNA molecules having 21–24 bp in length and are produced from longer dsRNA cleaved by a Dicer.^[7] They

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can be adapted to target specific genes of interest either directly or engineered through various means by degrading targeted cellular compartments upon binding with correct messenger RNA (mRNA) and suppress the expression of target genes.^[8] Carbon nanotubes have created a great interest in the nano medicine field as carriers for drugs and nucleic acid delivery.^[9] SWNTs can be functionalized either non-covalently or covalently.^[10,11] Efficient delivery of siRNA was observed in human T-Cell and Primary cells using carbon nanotubes which were functionalized with amine-terminated polyethylene glycol (PL-PEG 2000-NH₂).^[12]

The present work reports the IL-8 gene expression in H460 and A549 NSCLC cell lines by using RT-PCR and ELISA techniques, and it could be a molecular target for therapeutic intervention. siRNA is a powerful tool and effective method in treating diseases by targeting IL-8 mRNA gene knockdown, henceforth biocompatible IL-8 siRNA conjugated non-covalently functionalized carbon nanotubes were prepared, which may act as potential and convenient carriers for siRNA delivery.

MATERIALS AND METHODS

Hipco single-walled carbon nanotubes (SWNTs) were purchased from Nopo nanotechnologies, Bangalore. The diameter and length of the SWNTs were ~0.6–1.2 nm and ~400–1000 nm, respectively, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG 2000 amine) was purchased from Avanti polar lipids Inc., Alabama, sulfosuccinimidyl 6-(3'-(2-pyridyldithio) propionamido) hexanoate (Sulfo-LC-SPDP) from Sigma, Hydrochloric acid (HCl), Nitric acid (HNO₃) and ultra pure water.

Non Small Cell Lung Cancer Cell Lines and Cell Culture

NCI H460 and A549 NSCLC cell lines (purchased from NCCS, Pune) were cultured in RPMI 1640 medium (Himedia), which was supplemented with 10% Fetal Bovine Serum (FBS) (Himedia), 100U/mL of Penicillin G sodium (Sigma) and 100µg/mL of Streptomycin (Sigma) at 37°C in CO₂ incubator with humidified 5% CO₂ and 95% air.

mRNA Expression Analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The expression of mRNA for IL 8 was determined by using RT-PCR. A 2 µg portion of total RNA was used to synthesize cDNA (Sigma) following PCR amplification. For each pair of primers in a reaction volume of 40 µL using 20 U of Reverse Transcriptase (Sigma), 2 µg of oligo (dT) primer, 40U of RNase inhibitor, 2 nmol of each dNTP and 1X buffer (50 mM Tris-HCl, 8 mM MgCl₂, 40 mM KCl, 1-mM dithiothreitol, pH 8.3). Now at 45°C, the reaction was incubated for 50 minutes. Now to the 10µL of the above product, 1U of Taq Polymerase, 0.2 nmol of each dNTP, 0.1 µg of both forward and reverse PCR primers,

and 1X PCR buffer were added and made upto a volume of 50µL using nuclease-free water. The primers for IL-8 were sense, 5'-ATG ACT TCC AAG CTG GCC GTG GCT-3'; antisense, 5'-TCT CAG CCC TCT TCA AAA ACT TCT-3', the GAPDH primers are sense, 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'; antisense, 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'. 35 PCR cycles were conducted in accordance with the below-mentioned temperature and time profile. In PCR, with an initial denaturation at 94°C for 3 minutes, primer annealing at 58°C for 40 seconds, primer extension at 72°C for 1-minutes, and a final extension of 72°C for 6 minutes. Then the PCR products obtained were subjected to electrophoresis on a 2% agarose gel, and bands were visualized by Ethidium bromide staining, and images were obtained in Gel Doc imager system.

Quantitative Estimation of IL 8 Protein Expression by Enzyme-linked Immunosorbent Assay (ELISA)

The IL 8 protein expression was determined by ELISA kit (Invitrogen). To ELISA plate, capture antibody in coating buffer (100 µL) was added and incubated at 4°C for overnight and washed. Then blocked the wells by adding 200 µL of 1X ELISA diluent and incubated for 1h at room temperature and washed. 100 µL of 1X ELISA diluent was added to the wells leaving the first wells empty. Then 200 µL of standard (Human IL 8 Standard as per manufacturer protocol) concentrations were added to the first empty wells A1/A2 and then transferred 100 µL of standard from A1/A2 to wells B1/ B2 and the contents were mixed well. Now 100 µL of the sample was added and incubated at room temperature for 2 hours. After 3 washes, 100 µL of diluted detection antibody (biotin-conjugated anti-human IL-8 antibody) was added and incubated for 1-hour at room temperature and again washed for 3 times. Then 100 µL of Avidin-horseradish peroxidase (HRP) was added and incubated for 30 minutes at room temperature. Then 100 µL of 1X TMB was added (Tetramethylbenzidine) and incubated for 15 minutes at room temperature. Finally, 50 µL of stop solution was added, and the degree of color that had been generated was determined by measuring the optical density at 450 nm in ELISA plate reader (Thermoscientific).

Non-covalent Functionalization of SWNTs using DSPE-PEG 2000 Amine

Single walled carbon nanotubes were non-covalently functionalized and activated by following the method of Zhuang Liu.^[13] Firstly SWNTs were purified by acid treatment method where SWNTs were reacted with a mixture of HCl and HNO₃ in 1:3 (v/v) ratio in an ultrasonic water bath for 4 hours. Filter the solution and washed using distilled water until the filtrate collected obtained a pH of 7. Then the SWNTs were oven-dried at 105°C. Purified SWNTs and DSPE-PEG 2000 amine were taken in a ratio of 1:4, and 5 mL of DEPC water was added to 20 mL scintillation glass vial and mixed. Then subjected

for sonication using ultrasonic bath sonicator (PCi analytics) for 90 minutes by maintaining the temperature not exceeding $20 \pm 3^\circ\text{C}$. Now the SWNTs suspension was subjected for centrifugation using ultracentrifuge at 4°C , 23,000rpm for 6 hours and supernatant was collected and stored at 4°C . The morphology of the sample was studied using FE-SEM (Field Emission Scanning Electron Microscopy-Zeiss, Supra55)

Conjugation of sulfo-LC-SPDP Cross-linker to Functionalized SWNTs (Activation of SWNTs)

To 500 μL of functionalized SWNTs, 0.5 mg of sulfo-LC-SPDP cross-linker and 50 μL of 10X phosphate buffer saline (PBS) were added and incubated at room temperature for 2 hours. Then the excess of sulfo-LC-SPDP was removed using Amicon centrifugal filter (MWCO-100 KDa) at 10,000 rpm for 8–10 minutes by adding 4 mL of DEPC water. The washing was repeated 5 to 6 times until the final volume is upto 50 μL . Then the sample was analysed using In Photonics Raman Spectrometer Model (DV420-OE) at 532 nm laser wavelength.

Small Interfering RNA Purification and Conjugation of siRNA to Activated SWNTs

The IL 8 gene silencing siRNA duplex, 5'-GGG UGCA-GAGGUUGUGGAGAdTdT-3' and 5'-UCUCCACAA CCCUCUG-CACCCdTdT-3' was selected after validating its specificity to IL 8 by BLAST. The sequences were purchased from Santa cruz. IL 8 siRNA and was modified with 5' thio in order to attach with Sulfo-LC-SPDP cross-linker. DTT treated siRNA was purified using the NAP-5 Column (GE Healthcare). Different ratios of activated SWNTs: siRNA were mixed by gentle vortexing. The SWNT-PEG-NH₂-S-S-/siRNA complexes were formed by incubating 0 to 5 μg of activated SWNTs with 50 nM siRNA at different mass ratios (1:1 to 9:1) in serum-free RPMI 1640 medium on ice for 1-hour. The conjugation of siRNA to SWNT-PEG- NH₂-S-S-complexes was evaluated by Agarose gel retardation assay. The particle size and zeta potential for SWNT-PEG-NH₂-S-S-/siRNA complexes were measured using Zeta sizer Nano ZS 90 system (Malvern).

Agarose Gel Retardation Assay

The siRNA condensed by activated SWNTs were evaluated by Agarose gel retardation assay. The different mass ratios

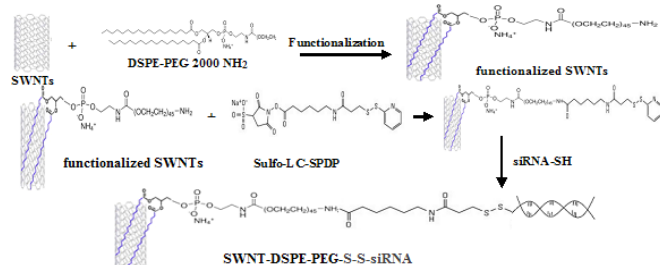


Fig 1: Schematic representation of the preparation of siRNA/SWNT-DSPE-PEG-S-S-siRNA complexes

of activated SWNTs: siRNA were resolved by 2% agarose gel using 1XTAE buffer at 100V for 25 minutes. siRNA bands were visualized using Ethidium bromide staining and images were photographed under UV light using Gel Doc imager system (BioRad).

RESULTS AND DISCUSSION

mRNA and Protein Expression Analysis of IL 8 by RT-PCR and ELISA

Human NSCLC cell lines constitutively produce IL-8, which is shown as an important mitogenic factor. Studies reported that lung tumor proliferation was inhibited by exogenous rIL-8 and cancer-derived IL-8 in A549 and four other lung cancer cell lines by autocrine and paracrine pathways^[14] correlated with the growth rate in two human NSCLC cells lines A549 and Calu-1 in SCID mice.^[15] IL-8 promoted the growth of human lung cancer by its angiogenic properties. In the present study, the NSCLC cell lines H460 and A549 cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS in humidified 5% CO₂, 95% air 37°C as shown in Figs 2A and B, and were cultured in a time-dependent manner. The conditioned serum-free media was harvested at indicated times and assessed for the presence of IL-8.

The mRNA levels of IL 8 in H 460 and A 549 NSCLC cells were analyzed by RT-PCR. After amplification, the RT-PCR products were subjected to run on 2% agarose gel electrophoresis. Fig. 2C shows that a band of IL 8 mRNA was observed at 291bp, and a band at 593 bp was observed for control GAPDH. In controls without a template, non-specific PCR products were observed, indicating no DNA contamination. The mean cycle threshold (Ct) values for IL 8 gene and GAPDH gene were obtained as shown in Table 1. Lower the Ct values indicate the higher gene expression representing the IL 8 gene over expression in H 460 and A 549 cell lines. The quantitative estimation of protein levels of IL 8 gene was done by using ELISA. After

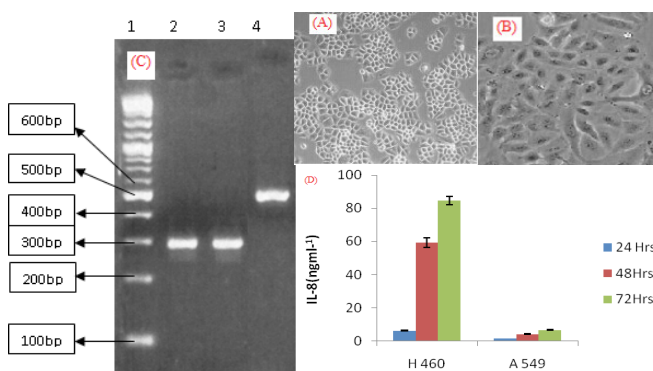


Fig 2: (A) H460 cell culture (B) A549 Cell culture. (C) Lane 1- Ladder, IL-8 mRNA band was observed at 291bp (Lane 2 H460 Lane 3 A 549), and band at 593bp (Lane 4) was observed for control GAPDH. (D) Medium was collected after 1×10^6 cells cultured in RPMI medium for 24, 48, and 72hours. Each bar indicates the mean $\pm$ Standard error of 3 determinations.



24 hours, H460 cells produced 6.24 ngmL^{-1} per 10^6 cells, and A549 cells produced 1.86 ngmL^{-1} per 10^6 cells. After 48 hours, IL-8 levels were 59.38 ngmL^{-1} per 10^6 cells in H460, and 4.06 ngmL^{-1} per 10^6 cells in A549 cells, and after 72 hours, levels were further increased to 83.26 ngmL^{-1} per 10^6 cells in H460, and 9.57 ngmL^{-1} per 10^6 cells in A549 cells, respectively, as shown in Fig 2D.^[16,17] It was observed that the H460 and A549 cells produced moderate to significantly higher levels of IL-8 protein, indicating that the IL 8 plays key role in cell proliferation and can be preferred as a target gene in lung cancer therapy.

Non-covalent Functionalization of Single-walled Carbon Nanotubes

Schematic representation of the functionalization and preparation of SWNT-PEG-NH₂-S-S-siRNA complexes was shown in Fig. 1. The functionalized SWNTs dispersion was characterized by FE-SEM. FE-SEM analysis showed that the size of the SWNTs was shortened to an average of $110 \pm 10 \text{ nm}$ as shown in Figs 3A and B. The EDAX analysis (Fig. 3c, Tables 2 and 3) showed that the carbon weight % decreased from 98.87 to 61.76 and the introduction of other new atoms (N, O, P) indicating the SWNTs were functionalized with polymer. So from this analysis, it was confirmed that there is a reduction in the size of SWNTs, and they were non-covalently functionalized by using the polymer DSPE-PEG 2000 amine.

Spectrum Processing

Peak possibly omitted: 4.517 keV, Processing option: All elements analyzed (Normalised) Number of iterations = 7

Conjugation of Sulfo-LC-SPDP Cross-linker to Functionalized SWNTs

Conjugation of Sulfo-LC-SPDP cross-linker to functionalized SWNTs was analyzed by Raman spectroscopy. From the Spectrum, the D-band indicates the disorganization in the hexagonal framework of the carbon nanotubes. The relative intensities of D-band and G-band (I_D/I_G) indicate the defects introduced upon functionalization of SWNTs. D-band peak at 1300 cm^{-1} and G-band peaks at 1586 cm^{-1} was observed, and I_D/I_G of COOH functionalized SWNTs

(after purification) showed 0.7, I_D/I_G of NH₂- functionalized SWNTs showed 1.1 and I_D/I_G of NH₂-functionalized SWNTs linked with Sulfo-LC-SPDP showed 1.3 as represented in Fig 4C indicating increased disorder in the structure of SWNTs which confirms the functionalization and cross-linking.

Characterization of siRNA Loaded SWNTs

The conjugation of IL 8 siRNA to SWNT-PEG-NH₂-S-S-complexes by electrostatic interaction was studied by agarose gel shift assay. The amine group of functionalized SWNTs was linked to Sulfo-LC-SPDP cross-linker via NHS-ester, and on the other side, pyridyl disulfides groups react with sulfhydryls of siRNA to form cleavable disulfide bonds, thus retarding the mobility of negatively charged siRNA. Compared with oligo IL 8 siRNA (positive control), siRNA bands decreased with an increase in mass ratios of SWNT-PEG-NH₂-S-S-siRNA complexes from 1:1 to 9:1, suggesting that the siRNA movement was impeded gradually,^[18] and completely disappeared when the mass ratio was 8:1, indicating complexes formed were not able to migrate in the gel and were retained in the well representing the complete interaction of siRNA with activated SWNTs as shown in Fig 4A. These results show activated SWNTs binds with siRNA and form stable complexes.

Further, the SWNT-PEG-NH₂-S-S-siRNA complexes at a mass ratio of 8:1 was successfully condensed into a nanostructure and confirmed by using TEM as shown in Fig. 4B. The shape of SWNT was observed to be

Table 1: mRNA expression levels of IL 8 gene in H 460 and A 549 cell lines

Cell line	Mean Ct values	
	IL 8 gene	GAPDH gene (Control gene)
H 460	18.4 ± 0.4	16.2 ± 0.9
A 549	18.8 ± 0.3	16.8 ± 0.5

Table 2: Represents the standards of the elements

C	CaCO ₃
N	Not defined
O	SiO ₂
P	GaP
Fe	Fe
Cl	KCl

Table 3: Shows weight% and atomic% of various elements in the functionalized SWNTs

Element	Weight%	Atomic%
C K	61.76	72.24
O K	24.32	19.25
N K	12.98	8.09
P K	0.94	0.42
Total	100.0	

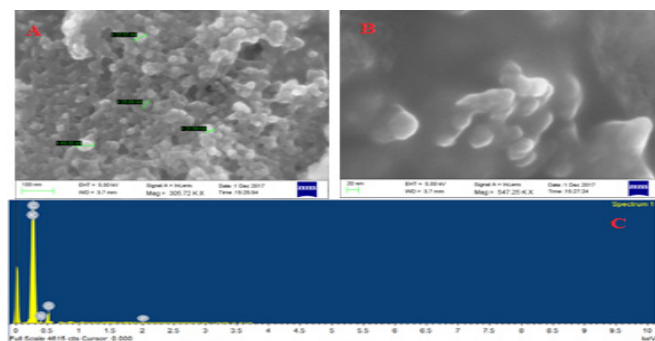


Fig 3: A) FESEM Analysis of a sample at 305.72KX, B) FESEM Analysis of sample at 547.25KX, C) EDAX image representing the presence of different elements.

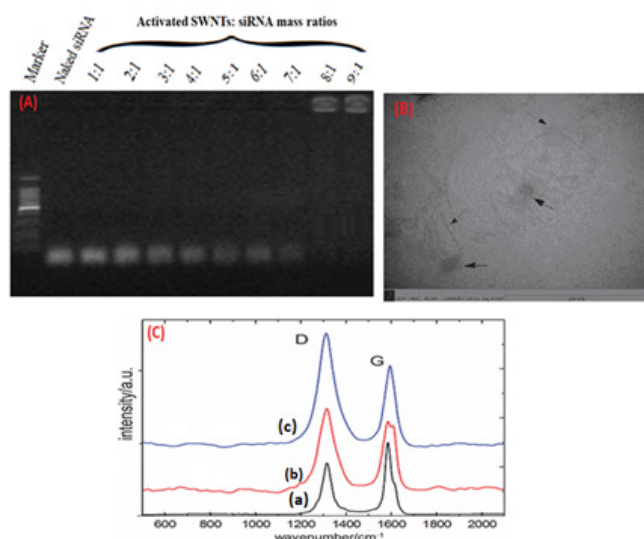


Fig. 4 (A) Binding capacity of activated SWNTs towards siRNAs and agarose gel electrophoresis at varying activated SWNTs/siRNA complexes mass ratios (B) TEM image of siRNA:SWNT-PEG-S-S- and the scale bar is 200nm (C) Raman spectra of SWNTs (a) Raman spectrum acquired from COOH functionalized SWNTs, $I_D/I_G = 0.7$; (b) Raman spectrum acquired from NH_2 -functionalized SWNTs, $I_D/I_G = 1.1$; (c) Raman spectrum acquired from NH_2 -functionalized SWNTs linked with Sulfo-LC-SPDP, $I_D/I_G = 1.3$.

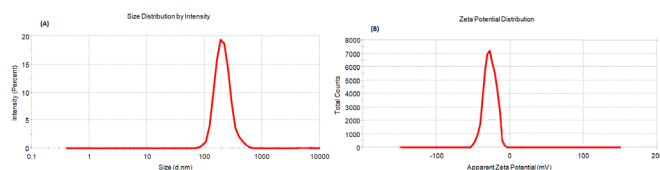


Fig:5 A) Size distribution of activated SWNTs/siRNA complexes B) Zeta potential of activated SWNTs/siRNA complexes

nanofibrous, and the SWNTs were coated with DSPE-PEG 2000 amine Fig. 4B, arrows). Increased electron-rich areas (dark) were observed because of siRNA condensation. The results obtained are consistent with the previous data. The degree of complexation between siRNA and ammonium functionalized carbon nanotubes prepared using 1,3 dipolar cycloaddition reaction shows the formation of electron-rich areas on the surface of nanotubes.^[19]

Particle Size and Zeta Potential of IL 8 siRNA/SWNT-PEG-NH₂-S-S- Complexes

The size and zeta potential of 8:1 mass ratio IL 8 siRNA/SWNT-PEG-NH₂-S-S- complexes were studied at 25°C. The size distribution was between 152.4 ± 1.8 nm to 163 ± 2.6 nm as shown in Fig. 5A, and the zeta potential was found to be -25.3mV as shown in Fig. 5B. Therefore the stability may be maintained in IL 8 siRNA/-S-S-NH₂-SWNTs complexes by electrostatic repulsion contributed by siRNA and activated SWNTs. The non-covalently functionalized SWNTs with PC and PVP showed 170.4 ± 8.5 nm particle size and -12.5 ± 1.1 mV of zeta potential.^[20]

CONCLUSION

In summary, the IL-8 gene expression in NCI H460 and A549 NSCLC cell lines was reported by RT-PCR and ELISA. The expression of IL-8 was significantly increased from moderate to high levels showing the mitogenic role of IL-8 in NSCLC cell growth, and the H460 cells were found to produce higher levels of IL-8 than A549 cells. Non-covalently functionalized SWNTs were prepared as a nanocarrier for safe and effective targeted delivery of IL 8 siRNA into the A 549 and NCI H 460 lung cancer cell lines. Biochemical modification of complexes increased the potency, stability, solubility, and biocompatibility with biological fluids demonstrating that this non-viral vector was suitable for efficient siRNA delivery. Non-covalent functionalization of SWNTs with DSPE-PEG 2000 amine minimized the defects on the surface of the SWNTs and resulted in aqueous uniform dispersion by hydrophobic interaction. Further, the functionalized SWNTs were activated by conjugating with the cross-linker Sulfo-LC-SPDP to prepare SWNT-PEG-NH₂-S-S- conjugates. This allows the conjugation of siRNA molecule in a defined manner, avoiding the dimer and polymer formation. The optimized formulation with potential biocompatibility and solubility can be promptly used in the biological environment and can be used as an efficient delivery system for siRNA to target and suppress the disease-causing genes selectively.

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