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Saponin conjugation improves p-CRISPR delivery by polyethylenimine modified carbon dots

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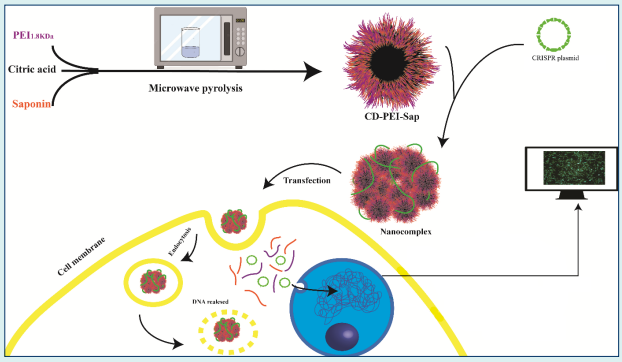
Abstract



Introduction: Non-toxic and efficient delivery platforms are important for gene editing applications. In this report, the preparation of saponin-conjugated low molecular weight polyethyleneimine ($PEI_{1.8kDa}$) modified carbon dots (CD-PEI-Sap) is described.

Methods: Polyethyleneimine modified carbon dots (CD-$PEI_{1.8kDa}$), were synthesized by one-step microwave-mediated pyrolysis of citric acid (CA) and $PEI_{1.8kDa}$ to produce non-toxic fluorescent particles. When saponin was added at this stage followed by microwave treatment a completely clear solution of CD-PEI-Sap was produced.

Results: These 80 nm nanoparticle vectors not only transfected green fluorescent protein plasmid (p-GFP) (3 kb) with 60% expression in human embryonic kidney (HEK 293T) cells, and > 60% in hard to transfect human breast adenocarcinoma cells (MCF7), but could also transfect a large clustered regularly interspaced short palindromic repeat plasmid (p-CRISPR) with 5-fold higher efficiency (50%) than the gold standard reagent, branched PEI MWt 25 kDa (PEI_{25kDa}). Moreover, the CD-PEI-Sap system produced good transfection with a low concentration of p-CRISPR and in the presence of 30% fetal bovine serum (FBS).

Conclusion: This system demonstrated efficient transfection and serum resistance, making it a promising tool for future gene editing research.

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Introduction

Gene editing is accepted as a biomedical revolution that could lead to treatments for both genetic and acquired diseases. Mutations or deletions in specific genes can cause a variety of congenital disorders, while acquired disorders are not generally limited to a single gene.[1] To date, the efficient delivery of gene editing materials to targeted cells has been a major obstacle to researchers, and many new delivery vectors have been explored. These vectors can be divided into viral and non-viral delivery systems. Viral vectors include lentivirus, retrovirus, adeno-associated virus (HFV) and adenovirus (types 2 and 5). Reproducible expression of therapeutic genes can be achieved by viruses. Notwithstanding these advantages, there are some limitations of viruses, such as immunogenicity, toxicity and lack of optimization on an industrial scale.[2] Non-viral platforms have lower pathogenicity, are more economical with easy synthesis giving them a distinct advantage over viral systems. From 2004 to 2013 viral carriers experienced an obvious decline, and non-viral gene therapy came to the fore particularly in clinical trials.[3] The most important problem faced by non-viral carriers is poor transfection efficiency. Scientists have attempted to modify the surface of non-viral vectors using various methods to improve transfection efficiency.

Recently the CRISPR method has revolutionized gene therapy.[4] The award of the Nobel Prize in chemistry 2020 to Emmanuelle Charpentier and Jennifer A. Doudna for their groundbreaking work on CRISPR has sparked a tremendous global interest in this field. The nucleic acids coding for CRISPR/Cas9 can be transferred to targeted cells by viral or non-viral carriers. Delivering CRISPR systems by viral vectors suffers from the same problems as gene therapy,[5] while the loading capacity of viruses is restricted for large plasmids.[6] The most commonly used non-viral carriers for CRISPR/Cas9 can be categorized as cationic liposomes, cationic polymers, lipid nanoparticles (LNPs), gold nanoparticles, extracellular vesicles[7] and carbon dots.[8]

Among various structures, use of carbon dots confers several advantages, due to their biocompatibility, broad excitation spectra for fluorescence, dispersibility in water, ease of synthesis, small size, and the ability for conjugation with diverse functional groups.[9] Many of the currently employed approaches for carbon dot surface modification rely upon various forms of PEI polymer. PEI_{25kDa} is a gold standard in gene delivery.[10] Nevertheless, these polymers show some toxicity due to mitochondrial membrane destabilization which give rise to apoptosis. One way to reduce the toxicity of PEI is to use low molecular weight (LMW) PEI, however LMW PEI shows limited gene transfection efficiency.[11] Hence ways to improve the efficiency of LMW PEI without increasing toxicity are in great demand.

Saponins are phytochemicals that are naturally found in higher plants, marine organisms and microorganisms. Saponins are used in pharmacology due to their anti-inflammatory, anti-viral, immune regulation, cardioprotective and anti-cancer properties.[12,13] Saponins are a large group of glycosides composed of a sugar residue (hydrophilic part) and a triterpenoid (C30) or steroidal (C27) aglycone (lipophilic part).[14] The amphiphilic nature of the saponin molecule makes them capable of binding to cholesterol-rich cell membranes to increase cellular uptake.[15]

In this investigation, we employed a carbon dot based delivery vehicle. The carbon dots were modified by $PEI_{1.8kDa}$ and saponin by incorporating these compounds into the carbon dots using one-step microwave pyrolysis. First, we synthesized the nanoparticles and characterized them by several analysis methods. After complexing with p-GFP (3 kb), we examined the transfection efficiency in various cell lines. Then, we examined the serum stability, toxicity and effect of plasmid concentration on the transfection efficiency by CD-PEI-Sap complexed with p-GFP, and, a heavier plasmid (9 kb) in HEK 293T cells.

In general, although the potential of carbon dot-based non-viral gene delivery systems is significant, most of the current approaches rely on high-molecular-weight PEI to achieve good transfection efficiency, but this also has the cost of increased cytotoxicity. In such situations, increasing the transfection efficiency of low-molecular-weight PEI, which has only low levels of toxicity, could be a promising strategy, and there have been a large number of studies in this direction. Additionally, to the best of our knowledge, the incorporation of bioactive, membrane-interacting molecules such as saponin into carbon-dot-based gene delivery systems has not yet been reported.

In this investigation, we employed a carbon dot-based delivery vehicle, which was modified by attaching $PEI_{1.8kDa}$ and saponin using a one-step microwave pyrolysis reaction. The synthesized nanoparticles were characterized by several analytical methods, and their gene delivery efficiency was evaluated using p-GFP (3 kb) in various cell lines. Then serum stability, cytotoxicity and the effect of plasmid dose on the transfection efficiency by CD-PEI-Sap complexed with p-GFP was investigated. This platform could deliver larger an even larger plasmid (9 kb) into HEK 293T cells. The results revealed that this system significantly enhanced transfection efficiency, and in future may be used in vivo as an effective non-viral gene delivery system (Fig. 1).

Methods and Materials

Materials

Branched $PEI_{1.8kDa}$, Quillaja bark saponin 84510, quinine sulfate (98%), CA, dimethyl sulfoxide (DMSO), hydrochloric acid (HCl) fuming 37% were acquired from Merck, Germany. GenElute™ FFPE RNA/DNA purification plus kit, agarose gel, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit, FBS, Dulbecco's modified Eagle's medium (DMEM, high glucose) were acquired from Sigma-Aldrich, USA. In addition, plasmids PX458 (pSpCas9(BB)-2A-GFP;

9 kb) and pMax-GFP (3 kb) were provided by Addgene (Massachusetts, USA), under catalog number #48138 and #177825, respectively. Noticeably, all chemicals used in the project were of analytical reagent grade.

Methods

Preparation of CD-PEI-Sap

Firstly 1 g of $C_6H_8O_7$, 500 mg of PEI $_{1.8kDa}$ and 250 mg saponin were dissolved in 6 mL of HCL (0.1 N) and mixed on a stirrer until a clear solution was obtained. Then, the reaction was carried out using one-step-pyrolysis in a microwave oven at a power of 720 W for a duration of 300 seconds. Next, 10 mL of ultrapure water was added to the cooled solution in a dialysis bag with a molecular weight cut-off of 1000 Da, and stirred with 600 mL of distilled water for a period of 48 hours. Lastly, the carbon dot powder was obtained by using a freeze dryer.

Characterization and instrumentation

Fourier transform infrared spectroscopy (FT-IR) was carried out using Nicolet AVATAR 370 (Thermo, USA). NMR analysis was used to gain structural information using an AS400-400 MHZ (Qone, Germany) instrument., To confirm the presence of diverse elements, CHN elemental analysis was carried out with a TruSpec elemental analyzer (LECO Corporation, USA).

Optical measurements

To assess the optical properties of the system, the CD-PEI-Sap powder was dissolved in ultrapure water at varying concentrations. For comparison a standard reagent was chosen, quinine sulfate in 0.1 M sulfuric acid. The absorbance of various concentrations of the samples was measured by a UV-Visible spectrophotometer at 340 nm. Several concentrations with absorbance values < 0.1 were selected for measurement of photoluminescence (PL) emission spectra at an excitation wavelength of 340 nm.

Preparation of CD-PEI-Sap or PEI complexes with p-GFP or p-CRISPR

Firstly, the concentration of the plasmid solution was measured using a nano-drop device. Then, CD-PEI-Sap and CD-PEI$_{1.8kDa}$ were dissolved in ultrapure water to provide solutions with 1 mg/mL concentration. Next, equal volumes of CD-PEI-Sap, CD-PEI$_{1.8kDa}$ and p-GFP or p-CRISPR with different concentrations were mixed well by pipetting up and down to obtain the final complexes and kept at room temperature for 30 minutes.

Agarose gel electrophoresis assay

The ability of CD-PEI-Sap and CD-PEI$_{1.8kDa}$ to condense p-GFP was investigated using a gel retardation assay. Various weight ratios of CD-PEI-Sap complexes were obtained after incubation for 30 minutes at room temperature. Subsequently, 2 µL of loading buffer was combined with 8 µL of the complex solution and thoroughly mixed. The solution was loaded onto 1% agarose gel containing 3.5 µL of a safe nucleic acid stain. The gel was electrophoresed in a tank contained 40 mM TAE buffer at 85 V for 25 minutes. Lastly, the fluorescent nucleic acid bands were recognized using a gel documentation system at excitation wavelength of 365 nm.

Dynamic light scattering (DLS) and transmission electron microscope (TEM) measurement

Firstly, DLS was used for assessing the size of the nanoparticles in the CD-PEI-Sap suspension, and after confirming the size was under 100 nm, the best ratio of the complex to plasmid (80:1) was produced. The size was measured by NANOTRAC WAVE ll (Microtrac, USA). Preparation of CD-PEI-Sap suspension started with diluting 1 g of the powder of nanoparticles in 1 mL deionized water and sonicating the resulting mixture. For the complexes, the concentration with the best transfection efficiency was chosen (80:1) and the complex was prepared for observation using Zeiss LEO 906 TEM.

Cell culture for transfection efficiency of p-GFP and p-CRISPR

HEK 293T, rat phaeochromocytoma (PC12), MCF7, and human cervix adenocarcinoma (HeLa) cells were purchased from Iranian Biological Resource Center. The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin- streptomycin (Pen/strep). Culture was carried out at a temperature of 37°C in a humidified atmosphere with 5% carbon dioxide (CO_2).

The MTT assay was utilized for measuring viability. HEK 293T cells were cultured in a 96 well plate at a density of 10,000 cells per well. The following day, cells were transfected with various concentrations of nanoparticles. After 24 hours of transfection, 10 µL of MTT solution (5 mg/10 mL PBS) was added to each well. The cells were then incubated for an additional 4 hours to generate formazan crystals. The formazan crystals were dissolved in 100 µL of DMSO and the absorbance was measured at 580 nm using a microplate reader (BioTek USA). The absorbance of non-transfected cells was employed as a control.

In order to assess the effectiveness of plasmid transfection, HEK293T cells were cultured in DMEM supplemented with 10% FBS (v/v) and 1% Pen/strep. The cells were then seeded in a 24-well cell plate at a density of 100,000 cells per well one day prior to transfection. Cells were transfected with different concentrations of nanoparticles (weight ratios) in the absence of FBS and Pen/strep. We used 1 µg of p-GFP (3.5 kb) and 1 µg of p-CRISPR (9 kb) including GFP. The optimum concentration of nanoparticles and 1 µg plasmid were mixed and incubated for 30 min and added to wells. After 4 hours of transfection, medium was exchanged with complete medium. Cells were analyzed using fluorescence microscopy and flow cytometry (BD, USA) 48 hours after transfection. The data obtained from flow cytometry was then analyzed by FlowJo7.6.1 software (Tree Star, USA).

To evaluate the stability of nano-complexes, transfection was performed in presence of different percentages of serum with the optimum concentration of DNA (1 µg). Then complexes were added to medium containing 5, 10, 20 and 30% FBS.

To find the lowest amount of plasmid required we performed transfections with different plasmid concentrations (0.5, 1 and 2 µg).

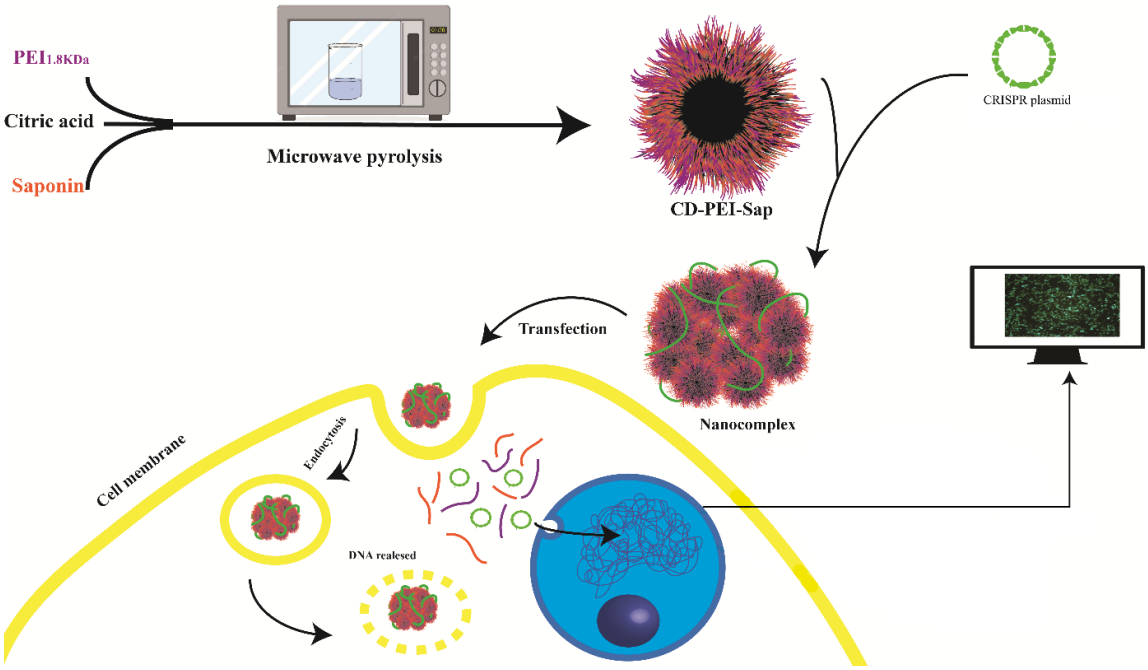

Fig 1. Illustration of CD-PEI-Sap synthesis and its internalization into cells.

Statistical analysis

All data were assessed with 2-way ANOVA with Tukey's multiple comparisons test using GraphPad Prism version 9.3.1 (USA). *P* values lower than 0.05 were taken as statistically significant.

Results and Discussion

Characterization of the CD-PEI-Sap nanoparticles

We initially confirmed the synthesis of the nanoparticles by examining their structure using Fourier FTIR and NMR assays. The FTIR spectra were collected to compare CD-PEI$_{1.8kDa}$ with and without saponin. The peaks at 657.607 cm^{-1}, 719.318 cm^{-1} and 937.235 cm^{-1} in the carbon dot structure disappeared after adding saponin because they were related to the C=C bonds (see Fig. 2A). NMR was performed to characterize the structure of CD-PEI-Sap in a more detailed manner (Fig. 2B). All of the peaks between 3-4 ppm can be attributed to methylene oxygen protons in saponin and confirm the presence of saponin in the structure of CD-PEI-Sap.

When the CD-PEI-Sap powder was added to water it completely dispersed, but it also created a foam. Foaming is a result of the amphiphilic properties of saponin acting as a surfactant agent in the system.[16] Problems could arise in DLS measurements due to foam production, so a low concentration of saponin was employed in DLS measurements to avoid foam generation. As can be observed in Fig. 3A and B, by forming complex, increasing the size of particle could obviously be observed (See Fig. 3C and D).

Elemental analysis

According to Fig. S1, the elemental analysis of CD-PEI-Sap was performed by energy dispersive spectroscopy (EDS) showing 8.97%, 30.86% and 5.80% of nitrogen, carbon and hydrogen, respectively. The nitrogen was due to the presence of PEI$_{1.8kDa}$. This explains the positive zeta potential and improved transfection efficiency of saponin-doped carbon dots.

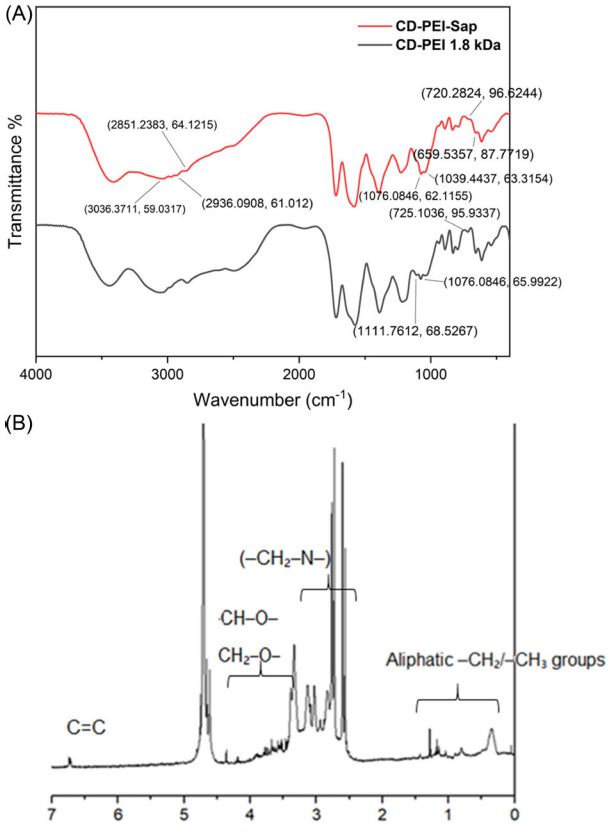

Fig. 2. FTIR and NMR spectroscopy. A) FTIR spectra for CD-PEI$_{1.8kDa}$ and CD-PEI-Sap and the difference between peaks at various wavelengths. B) NMR spectra of the CD-PEI-Sap structure

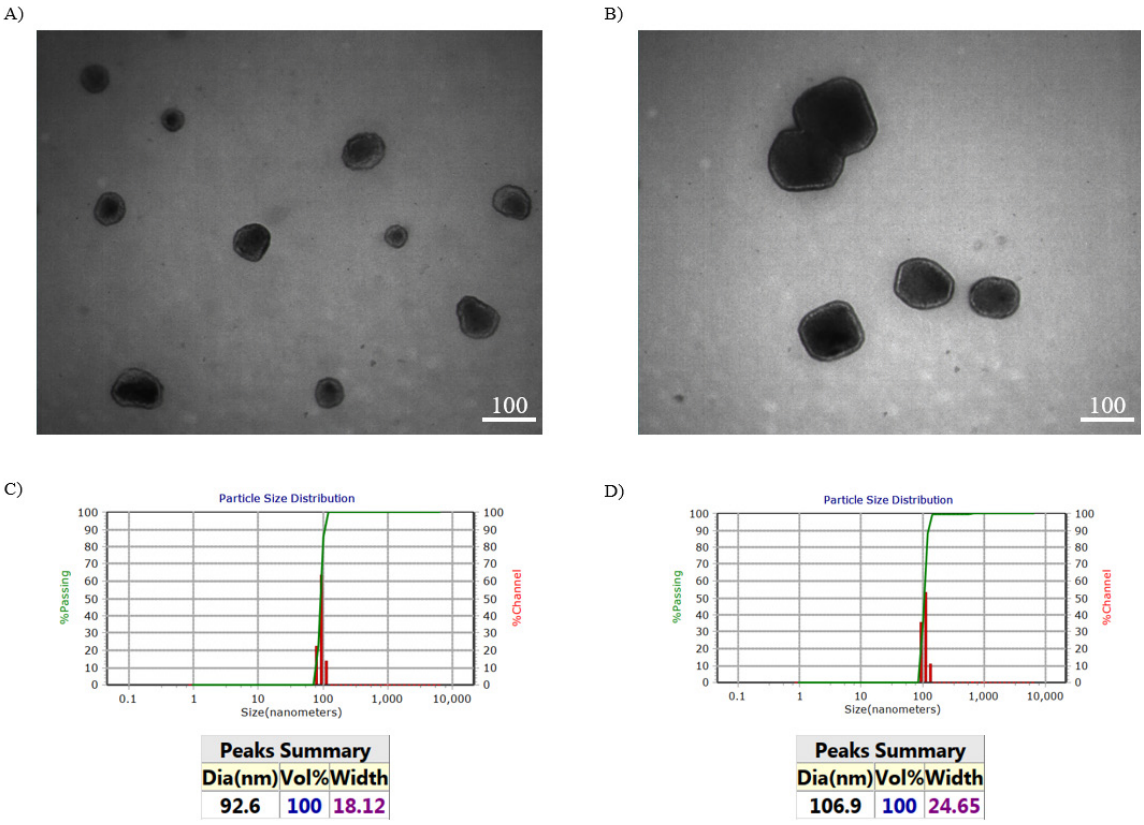

Fig. 3. Morphological and size characterization of CD–PEI–Sap nanoparticles and their plasmid complexes. A) TEM image of CD-PEI-Sap nanoparticles showing a spherical morphology of approximately 90 nm diameter. B) TEM image of CD-PEI-Sap complex with plasmid prepared at a nanoparticle-to-plasmid weight ratio of 80:1, which provided the highest transfection efficiency with an observed average size of approximately 110 nm. C) DLS results of the characterized CD-PEI-Sap particles. D) DLS of the plasmid CD-PEI-Sap complex. It is noteworthy that the TEM and DLS results were in good agreement with each other.

Optical properties of CD-PEI-Sap

In order to investigate the optical properties of CD-PEI-Sap, the PL intensity was measured over a range of emission wavelengths with an exact excitation wavelength (340 nm). Based on the obtained graph, it can be seen that the PL of CD-PEI-Sap was slightly lower compared to CD-PEI $_{1.8kDa}$. This reduction in PL may be attributed to the quenching effect caused by the addition of saponin to the nanoparticle surface. The UV-VIS spectra for CD-PEI $_{1.8kDa}$ and CD-PEI-Sap in different concentrations are provided in Fig. S2A and S2B.

Evaluation of CD-PEI-Sap binding with p-CRISPR

A gel electrophoresis assay was used to measure the ability of the nanoparticles to condense p-CRISPR in order to confirm our hypothesis about the interaction of amine groups on the positively charged surface-modified carbon dots and the nucleic acid phosphate groups on the p-CRISPR. According to Fig. 4, as the weight ratio (carrier/p-CRISPR) increased, the electrophoretic mobility of the plasmid decreased. Moreover, CD-PEI-Sap interacted with the plasmid even at 0.5x weight ratio. However, with non-modified CD-PEI $_{1.8kDa}$ the mobility did not decrease until the weight ratio reached 2x.

Cytotoxicity assay

Maintaining a balance between transfection efficiency and cytotoxicity plays a crucial role in the development of a useful gene delivery vehicle. The MTT assay was exploited to assess effects of the nanovectors on HEK 293T cell viability after a 24 hours incubation. The results showed slight toxicity ~20% after exposure to the highest concentration of CD-PEI-Sap (150 w/w), while less than 50% of the cells were viable when exposed to 18 N/P PEI$_{25kDa}$. As shown in Fig. 5 the cell viability of CD-PEI-Sap was similar to PEI$_{1.8kDa}$, but CD-PEI$_{1.8kDa}$ demonstrated higher cell viability in HEK 293T cells. As previous studies pointed out, low molecular weight PEI shows less toxicity due to reduced aggregation and adherence on the cell surface.[17] Additionally, saponin has been employed as an agent for induction of apoptosis and necrosis in EL4 cells or apoptosis in SKOV3 cells.[18] Hence, using this molecule in a gene delivery vehicle might raise concerns about its toxicity. However, results from other studies showed low toxicity of chitosan-saponin nanoparticles in avian cells even at high doses[19] which is consistent with our present results. Noticeably, PEI$_{25kDa}$ was evaluated using the N/P ratio, which is the standard parameter reflecting the number of protonatable amines associated with DNA phosphates and mostly, is employed for reporting polycation-mediated gene transfection efficiency. In transfection studies, this substance is conventionally reported in terms of N/P ratio.

In contrast, as for PEI$_{1.8kDa}$ and its CD-modified derivatives, the effective amine content per unit mass differs substantially due to molecular weight differences

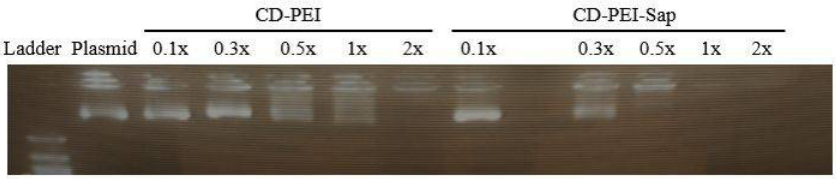

Fig. 4. Gel retardation analysis of CD-PEI 1.8kDa/p-CRISPR and CD-PEI-Sap/p-CRISPR complexes at various weight ratios. Progressive retardation of plasmid migration with increasing weight ratio indicates improved electrostatic interactions between the positively charged amine groups on the nanoparticle surface and the negatively charged phosphate groups of p-CRISPR. CD–PEI–Sap exhibited effective plasmid binding at a low weight ratio of 0.5:1, whereas CD–$PEI_{1.8kDa}$ required a higher weight ratio (≥2:1) to significantly decline plasmid mobility, demonstrating improved DNA condensation efficiency following saponin incorporation.

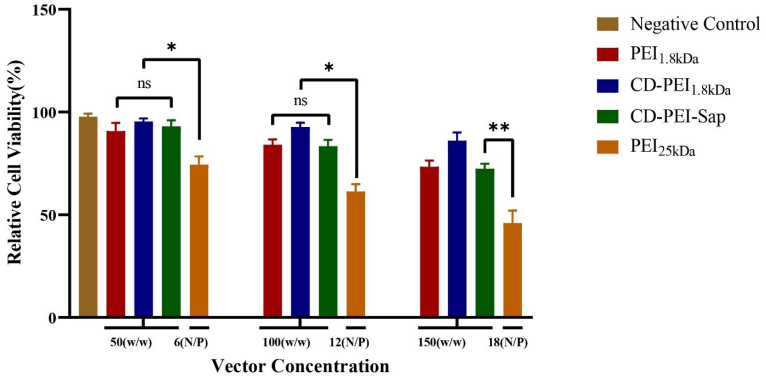

Fig. 5. Cytotoxicity in HEK 293T cells. The cytotoxicity of $PEI_{1.8\ kDa}$, CD–$PEI_{1.8\ kDa}$, CD–PEI–Sap nanoparticles, and PEI_{25kDa} was assessed in HEK 293T cells using the MTT assay following 24 h incubation. Cell viability was expressed as a percentage relative to untreated control cells. CD–PEI–Sap exhibited only mild cytotoxicity (~20% reduction in viability) at the highest tested carrier-to-plasmid ratio (150 w/w), comparable to $PEI_{1.8\ kDa}$, however, $PEI_{25\ kDa}$ resulted in pronounced cytotoxicity, with cell viability dropping below 50% at an N/P ratio of 18. These results demonstrate that incorporation of saponin into LMW PEI–modified carbon dots does not remarkably increase cytotoxicity while maintaining a favorable safety profile compared to high-molecular-weight PEI.

and chemical modifications. In such systems, especially when reporting carbon dot-based nanoparticles, transfection efficiency is commonly provided as polymer/DNA weight ratio (w/w), depending on structure. Therefore, polymer concentration (w/w) was employed instead of N/P ratio to allow accurate comparison of their dose-dependent behavior among modified polymers with different amine densities.

Transfection efficiency

Transfection procedures often require a balance between high gene delivery efficiency and controlled toxicity in cells. Furthermore, a successful carrier should decrease cellular toxicity while promoting high transfection capacity. In order to examine the in vitro transfection efficiency, the p-GFP expressing 3 kb plasmid was employed as a reporter gene. Initially, this plasmid was transfected into HEK 293T using carbon dots and $PEI_{1.8kDa}$ preparations Transfections were carried out at different weight ratios, as shown in Figs. 6-8, CD-PEI-Sap showed a transfection efficiency up to 75% in its optimal weight ratio of 80:1, but PEI_{25kDa} used as a gold standard for transfection, only showed 45% efficiency. The reason for the high transfection ability of our nanoparticles can be attributed to saponin residues acting as efficient membrane-permeating agents and to forming complexes with cholesterol molecules in the cell membrane. These effects tend to increase the cell uptake of the nanoparticles.[20] The affinity of saponin to cholesterol depends on the specific type of aglycone in the saponin structure.[21,22] In the present case we used Quillaja bark saponin 84510.[23] Moreover, the length of the saponin nanoparticle incubation time will affect pore development and recovery.[24] Since transfection of the 3 kb plasmid into the easy-to-transfect HEK 293T cells gave encouraging results, other cell lines such as HELA, PC12 and MCF7 were investigated to extend the possible applications of the nanoparticles. The efficiency of the system was significantly higher than CD-$PEI_{1.8kDa}$ and PEI_{25kDa} in all the different cell lines. In the case of MCF7 (a hard to transfect cell) the transfection efficiency was even higher than HEK 293T cells, suggesting the platform may have potential for breast cancer related studies.

In vitro transfection procedures are usually performed in the absence of serum.[25] Since serum (both bovine and human) contains large amounts of enzymes, the transfer of genetic material in living organisms requires protection from nucleases, so a vector that could protect the genetic material against serum enzymes is desirable. On the other hand, serum may also cause nano-complex instability.[26] It is possible that serum components can react with the nano-complex to increase its size, thus reducing cell uptake.[27] The transfection ability of the nanoparticles was assessed at increasing concentrations of serum (0%, 10%, 20%, 30%) by measuring p-GFP expression in HEK 293T cells. As shown in Fig. S3, significant transfection efficiency of CD-PEI-Sap was maintained, even at high serum levels. In contrast, when the serum level was increased, the transfection efficiency of PEI_{25kDa} was decreased.

In order to investigate the effect of plasmid concentration

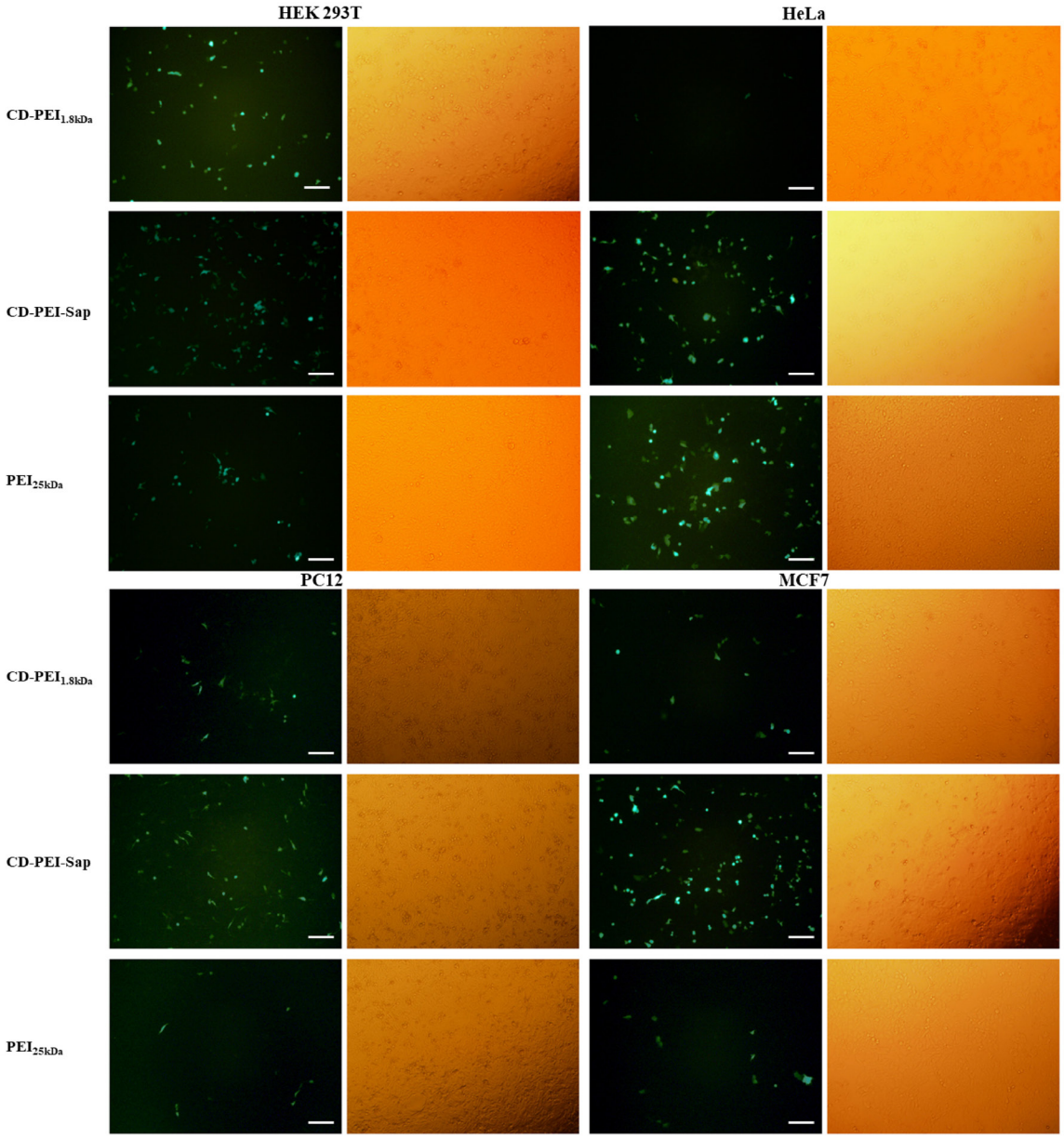

Fig. 6. Transfection efficiency of p-GFP in diverse cell lines (HEK 293T, HeLa, PC12, MCF7). Fluorescence microscopy images showing the expression of GFP after plasmid transfection by CD-PEI $_{1.8kDa}$, CD-PEI-Sap and PEI $_{25kDa}$ in various cell lines including HEK 293T, HeLa, PC12 and MCF7 cells. Images were acquired 48 h post-transfection; scale bar = 100 µm.

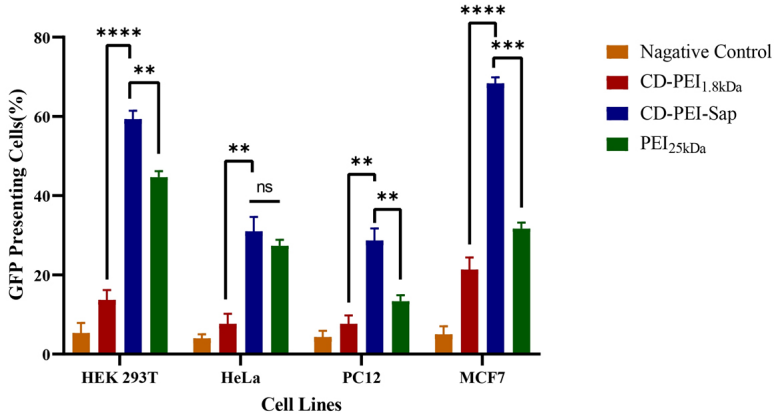

Fig. 7. Transfection efficiency of p-GFP in diverse cell lines (HEK 293T, HeLa, PC12, MCF7). The proportion of GFP expressing cells after transfection with carrier/plasmid (3 kb) complexes using CD-PEI $_{1.8kDa}$, CD-PEI-Sap and PEI $_{25kDa}$ in HEK 293T, HeLa, PC12 and MCF7 cell lines was assessed by flow cytometry under the same conditions. Transfections were performed using 1 µg of p-GFP plasmid at the optimized carrier-to-plasmid weight ratios. CD–PEI–Sap produced significantly higher transfection efficiency in all tested cell lines compared to CD–PEI $_{1.8kDa}$ and PEI $_{25kDa}$, demonstrating enhanced gene delivery performance.

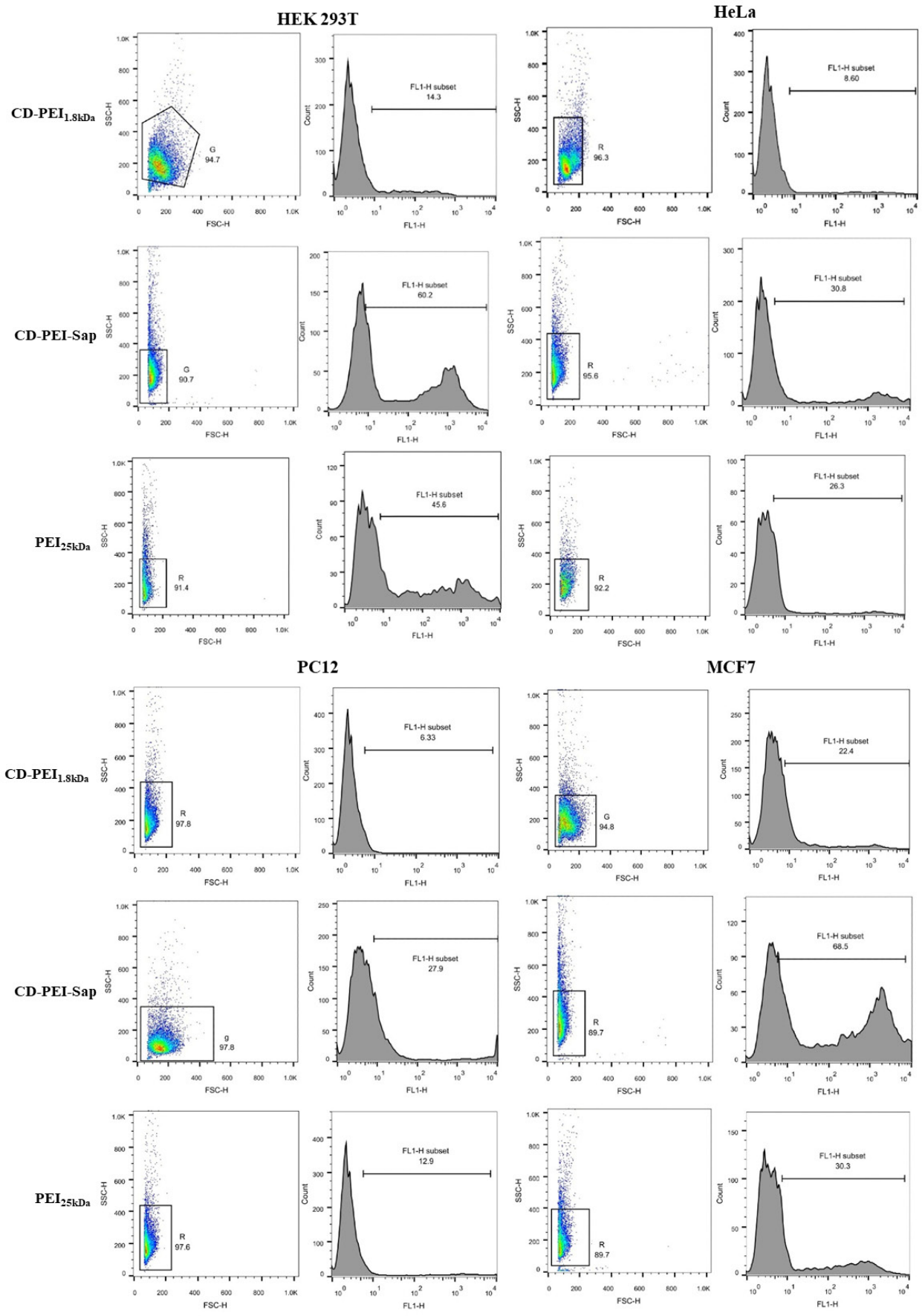

Fig. 8 . Transfection efficiency of p-GFP in diverse cell lines (HEK 293T, HeLa, PC12, MCF7). Average flow cytometry data of all cell lines. Transfections were performed using 1 µg of p-GFP plasmid at the optimized carrier-to-plasmid weight ratios. CD–PEI–Sap produced significantly higher transfection efficiency in all tested cell lines compared to CD–$PEI_{1.8kDa}$ and PEI_{25kDa}, demonstrating enhanced gene delivery performance.

on the transfection efficiency of the nanostructure, different concentrations were used (0.5, 1, 2 µg). In terms of transfection efficiency, the modified nanoparticles provided 70% transfection at a p-CRISPR (3 kb) dose of 0.5 µg (Fig. S4). This was roughly twice as high as the gold standard PEI_{25kDa}. The excellent transfection efficiency of CD-PEI-Sap could be ascribed to its high affinity for cholesterol molecules on the membrane surface owing to its saponin content.[21] This novel nanovector with good performance at high serum concentration and low plasmid quantity could be a promising system for further studies.

Delivery of the large p-CRISPR plasmid into cells

The p-CRISPR system can edit DNA with greater precision than existing technologies. Simplicity, versatility, efficiency, programmability are advantages which p-CRISPR offers over other gene editing tools.[28] Despite the promising advantages of CRISPR, some major drawbacks still exist. Off target effects are defined as unplanned cleavage and mutations at non-specific sections of the DNA.[29] In addition, the substantial size of the p-CRISPR system poses challenges in its delivery and gives rise to increased cell death.[30] The transfection efficiency of the CRISPR plasmid also expressing GFP by the nanoparticles was evaluated. Although the size of the p-CRISPR was 9 kb, which is large enough to provide difficulties in delivery, the CD-PEI-Sap transfection efficiency was more than 50%. Notably, this value was significantly higher than that from PEI_{25kDa} used as a gold standard, and even with increasing plasmid concentration, the efficiency remained unchanged, showing its capability for delivering large cargos (Fig. S5).

Conclusion

In this study, a novel nanostructure was designed and synthesized using an affordable, one step microwave-assisted pyrolysis procedure. The nanostructure was created by combining CA (carbon source), branched $PEI_{1.8kDa}$ (nitrogen source and plasmid condensing agent) and saponin (internalization agent) was added for surface modification during the pyrolysis process. The resulting nanocarrier demonstrated considerably higher transfection efficiency and lower cellular toxicity in HEK 293T and other cell lines in comparison with PEI_{25kDa} as a gold standard transfection reagent. CD-PEI-Sap preserved high transfection efficiency at various serum concentrations and at low p-CRISPR doses, however the gold standard PEI_{25kDa} showed lower efficiency under those conditions. The main conclusion to be drawn is that saponin as a surfactant[22] with amphiphilic properties boosts the transfection efficiency because it binds to cholesterol moieties on the cell surface and encourages internalization into cells. Other properties of the saponin structure such as its anti-inflammatory, hypo-cholesterolemic, vasoprotective, anti-bacterial, anti-fungal and anti-parasitic properties[31] could make it an outstanding candidate for gene delivery purposes. In addition, its effectiveness even at high concentrations of serum, proved its superiority to more established alternative gene delivery vehicles. Furthermore, the use of saponin as a surface modification agent, not only provided high efficiency, but also imparted significant serum resistance.

Research Highlights

What is the current knowledge?

- Efficient, safe delivery of gene-editing systems remains a major limitation in gene and CRISPR therapy.
- Viral vectors offer high expression but suffer from immunogenicity, toxicity, and limited cargo capacity.
- Non-viral carriers are safer and economical but generally exhibit low transfection efficiency.
- High-molecular-weight PEI enhances transfection but induces cytotoxicity; low-molecular-weight PEI is safer but less efficient.
- Carbon dots are promising non-viral vectors due to biocompatibility, small size, and facile surface functionalization.

What is new here?

- A novel carbon dot delivery system co-modified with PEI1.8 kDa and saponin was developed.
- Saponin functionalization significantly enhanced cellular uptake and transfection efficiency with low toxicity.
- The CD–PEI–Sap system demonstrated good serum stability across multiple cell lines.
- Efficient delivery of both small (3 kb) and large (9 kb) plasmids was achieved, highlighting broad applicability.

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Competing Interests

The authors have no conflict of interest.

Data Availability Statement

The data supporting the findings of this study are available within the article and its Supplementary file 1.

Declaration of AI-assisted Tools in the Writing Procedure

No AI tools have been used for writing this article.

Ethical Approval

This study was approved by the Ethics Committee of Iran University of Medical Sciences (Ethics approval code: IR.IUMS.REC.1402.791). The research did not involve human participants or animal subjects and was limited to in vitro experiments.

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Supplementary files

Supplementary file 1 contains Figures S1-S5.

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