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■ RESEARCH ARTICLE

Development of Polymer-assisted Compaction of DNA Nanoparticles for Cancer Treatment

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ABSTRACT: Mutations in Kirsten rat sarcoma virus (KRAS), a protein from the GTPase protein family, are involved in up to 20% of tumors that lead to pancreatic, colorectal, and lung cancers. While it is difficult to directly target KRAS protein with small molecule drugs due to the limited number of active binding sites on KRAS, antisense technology using oligonucleotide drugs has shown promising effects in anti-cancer treatments. To overcome the challenge of natural oligonucleotide intracellular delivery, polymer-assisted compaction of DNA (pacDNA) technology has been developed. pacDNA conjugates are synthesized via click chemistry using an azide functionalized non-cationic PEG (polyethylene glycol)-based nanoparticle and dibenzocyclooctyl (DBCO) conjugated antisense DNA. The pacDNA conjugates showed cytotoxicity in a dose-dependent manner in KRAS-mutated cancer cell lines. In contrast, the PEG-based polymer and naked DNA showed no toxicity as evaluated by a dimethylthiazol-diphenyltetrazolium (MTT) based assay. Furthermore, following protein expression (western blot test) analysis, pacDNA conjugates were shown to downregulate KRAS protein expression; KRAS protein levels in the study were lower than that of the controls. These results indicate that pacDNA comprised of PEG-based polymer and antisense DNA could be potentially effective therapeutics for cancer treatment.

KEYWORDS: Material Science; Nanomaterials; Antisense oligonucleotide; KRAS; PEG; Cancer therapy.

■ Introduction

The human rat sarcoma virus (RAS) gene family is composed of three types, including Neuroblastoma rat sarcoma virus (NRAS), Harvey rat sarcoma virus (HRAS), and Kirsten rat sarcoma virus (KRAS) forms. KRAS is a protein isoform of RAS with the greatest mutability among them. KRAS protein belongs to the GTPase protein family in normal cells, functioning as a signal switch to relay messages from extracellular to intracellular environments.[2] KRAS proteins are inactive when binding to guanosine diphosphate (GDP) but are active when binding to guanosine triphosphate (GTP).[3] KRAS mutations have been discovered to be involved in the pathogenesis of up to 20% of tumors, with their occurrences resulting in high rates of pancreatic, colorectal and lung cancers,[4] which generally have poor responses to standard anti-cancer therapies such as chemotherapy.[5] Due to the limited number of active binding sites in KRAS proteins, using them as a potential direct drug target is challenging.[6] Nonetheless, recent research and development suggest that only a few of potential drug cansdidates can bind directly to specific KRAS conformations.[7]

In 2021, the Food and Drug Administration (FDA) approved the first drug, sotorasib, a small molecule that targets the KRAS mutant protein, G12C, in treating non-small cell lung cancer.[8,9] In 2022, FDA granted accelerated approval to another small molecule, adagrasib, targeting the same KRAS mutant protein.[10] As cancer drug resistance is the principal limiting factor for effective treatment in the clinic, developing different chemical modalities or using cocktail therapy is vital in cancer treatment.[11] Unlike small molecule drugs, antisense oligonucleotide drugs target mRNA of interest and have shown to be a promising anti-cancer treatment.[12] Currently, 15 oligonucleotide drugs have been approved by FDA.[13] Regarded as disruptive therapeutics, oligonucleotide drugs are cost-effective, relatively easy to manufacture, and can target undruggable pathways.[14] AZD4785 is the first anti-KRAS antisense oligonucleotide targeting KRAS mRNA that was put in the clinical study.[15] Unfortunately, the clinical research failed because of lack of efficacy;[16] as oligonucleotides are highly negatively charged and thus pose a challenge for cellular uptake and good distribution. To overcome these difficulties, Professor Zhang's lab at Northeastern University is developing KRAS antisense therapeutics using a platform technology, polymer-assisted compaction of DNA (pacDNA).[17]

pacDNA technology leverages the benefit of both nanoparticles and PEG to deliver a target DNA. Nanoparticles showed great potential as drug carriers; currently, several successful liposome-based nanoparticle drugs or vaccines have been approved by FDA, including COVID-19 mRNA vaccines.[18] Recently, a novel polyethylene glycol (PEG) based nanoparticle vehicle was developed and exhibited great activity in drug delivery. The nanostructure provided mounted DNA moiety with enhanced nuclease stability and improved cellular uptake.[19] Using PEG-based nanoparticles as a novel delivery system shows excellent application potential because PEG is an excipient widely used in drug formulations.[20]

In this study, an antisense DNA oligonucleotide (targeting mouse KRAS mRNA) was conjugated to PEG nanoparticles via non-cleavable bonds. First, the free antisense oligonucleotide was purified with reverse-phase high-performance liquid chromatography (HPLC), and the polymer-DNA conjugate

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was synthesized and thereafter and purified through gel permeation chromatography (GPC). Then, the final pacDNA was characterized by gel electrophoresis. Lastly, the conjugates were evaluated in a cell-based cytotoxicity assay and western blot analysis of KRAS protein levels.

Methods

Oligonucleotide:

The 16-mer KRAS antisense oligonucleotide (ASO) targets mouse KRAS mRNA, and the sequence is 5'-CAT GTA AAT ATA GCC C-3'. The dibenzocyclooctyl (DBCO)-conjugated 16-mer KRAS ASO was synthesized in collaboration with coworkers from Professor Zhang's lab using standard solid-phase phosphoramidite methodology in an Applied Biosystems 391 DNA synthesizer. First, 5'-DBCO was incorporated into the sequence using a commercially available 5'-DBCO-TEG phosphoramidite. Phosphoramidites and supplies for DNA synthesis were purchased from Glen Research Co. All DNA strands were cleaved and deprotected from the CpG support using aqueous ammonia at room temperature for 24 hours. Finally, the crude oligonucleotide mixture was purified through reverse-phase HPLC.

Synthesis of PEG Conjugated Polymer:

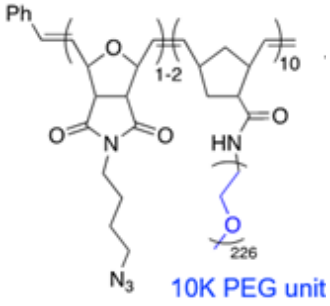

Figure 1: Chemical Structure of PEG-conjugated polymer.

The PEG conjugated polymer (Figure 1) was synthesized in collaboration with coworkers from Professor Zhang's lab according to a previously reported method. The polymer was purified by dialysis and lyophilized for future use.

A General Method for pacDNA Synthesis:

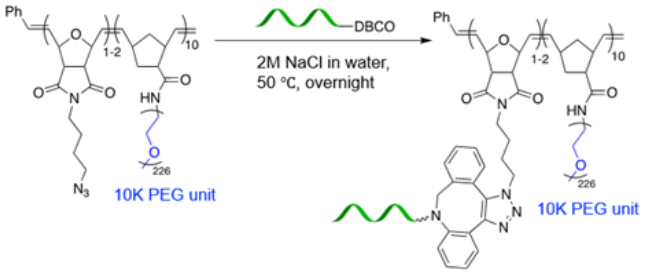

Figure 2: Reaction to produce pacDNA.

Azide-modified brush polymers were dissolved in water to make a 50 μM solution. DBCO-modified DNA (100 nmol) was dissolved in 1 mL azide-brush polymer solution, and ~160 mg of NaCl was added to make a final concentration of 2 M NaCl solution. The mixture was incubated while shaking at 50°C for 16 hours on an Eppendorf Thermomixer. The mixture was purified through GPC with a mobile phase of 0.1 M $NaNO_3$. The purification fractions containing the conjugate were collected. After pooling and concentration, the solution was then run through NAP 25 column for desalting. The final solution was lyophilized to form a white powder (Figure 2) and analyzed via gel electrophoresis.

MTT Cytotoxicity Assay:

Free DNA, pacDNA, and polymer were evaluated individually by a dimethylthiazol-diphenyltetrazolium (MTT) cytotoxicity assay in two different cell lines: mouse KRAS cell lines D658 and K273.[16] 1.0 x 10^4 cells were placed into 96-well plates which were preloaded with 180 μL DMEM medium per well. The plate was then incubated for 24 hrs at 37 °C before free DNA, pacDNA, and the polymer was added to make a final volume of 200 μL. The testing concentration of free DNA, pacDNA and polymer were at 0.1 μM, 0.25 μM, 0.5 μM, 1 μM, 2 μM, 3 μM, 5 μM, 10 μM. Phosphate buffered saline (PBS) was used as the control and added to a 96-well plate. After incubation for 48 hours at 37 °C, MTT stock solution (20 μL, 5 mg/mL) in PBS was added to each well. The plate was then incubated for additional four hours at 37 °C. After removing the medium with unreacted MTT, 200-μL dimethylsulfoxide (DMSO) solution was added to each well to dissolve the purple crystals. The plate was then put in a microplate reader to detect the absorbance at 490 nm.

Western Blot Analysis:

2.0 x 10^5 cells were placed into 24-well plates, which were preloaded with Roswell Park Memorial Institute (RPMI) medium and incubated overnight at 37 °C with 5% carbon dioxide. Then, free DNA, pacDNA, polymer (10 μM, 500 μL final volume) serum-free medium, and PBS control were added to each well and incubated for 4 hours before the medium was replaced with a complete medium. The 24-well plates were then incubated for 68 hours. Following the manufacturer's protocol (Cell Signaling Technology, Inc), the cells were harvested and lysed with lysis buffer. The extracted protein was then quantified using a BCA kit (Thermo Fisher), and an equal amount of protein (5 ug per lane) was loaded onto sodium dodecyl sulfate (SDS) gel. After gel electrophoresis and electrotransfer to a nitrocellulose membrane, the membrane was incubated with 3% bovine serum albumin (BSA) in Tris buffer with 0.05% Tween-20 for 1 hour at room temperature. After that, the membrane was incubated with primary antibodies overnight at 4°C. The membrane was then washed 3 times and incubated with secondary antibodies for 1 hour at room temperature. Finally, the bands were visualized after incubation with chemiluminescence (ECL western blotting substrate, Thermo Scientific). ImageJ software was used to quantify western blot images by comparing the protein KRAS with that of the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Results and Discussion

DBCO-DNA Purification:

The oligonucleotide crude after solid-phase synthesis was purified by reverse phase HPLC (acetonitrile and 0.1 M triethylammonium acetate (TEAA)). The elution of desired product was fractionalized and collected based on UV absorbance at both 260 nm and 310 nm shown in Figure 3. DNA

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had an absorbance at 260 nm, while unique DBCO moiety had an absorbance at 310 nm. Therefore, the 310 nm trace indicated a successful DNA strand and signaled that the correct product was generated.

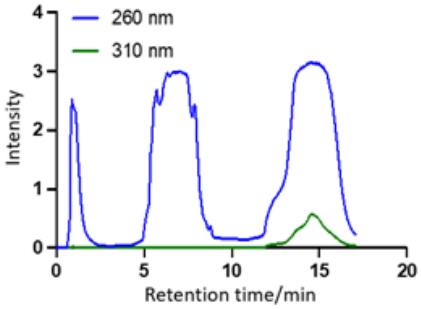

Figure 3: HPLC Chromatogram of DBCO-DNA Purification.

Separation and analysis of pacDNA conjugate:

The pacDNA conjugate was then purified and analyzed by GPC. From Figure 4, pacDNA with a larger size was eluted earlier than unreacted DNA with a smaller size. The conversion of conjugation is around 85% (based on the GPC peak area ratio).

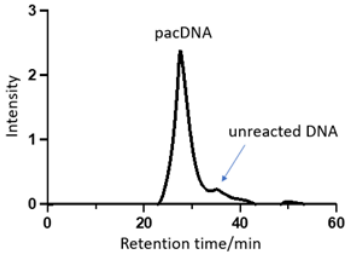

Figure 4: GPC Chromatogram of purified pacDNA conjugate.

Agarose Gel Confirmation:

The purified pacDNA was loaded onto agarose gel with free DNA as control. From Figure 5, after ethyl bromide staining, free DNA and pacDNA showed different locations on the gel, which further confirmed the success of the click reaction and the generation of desired pacDNA conjugate with a significantly larger size.

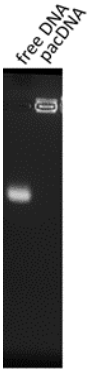

Figure 5: Ethyl bromide-stained agarose gel.

MTT Assay:

MTT assay is a robust cell proliferation assay that can measure cell viability after treatment. The assay utilizes the yellow tetrazolium (3-(4,5-dimethylthiazolyl-2) - 2,5-diphenyltetrazolium bromide. This tetrazolium salt (MTT) can be reduced by dehydrogenase enzymes in living cells and change the yellow-colored solution to a purple crystal soluble in DMSO; this color change can be quantified by spectrophotometric means.

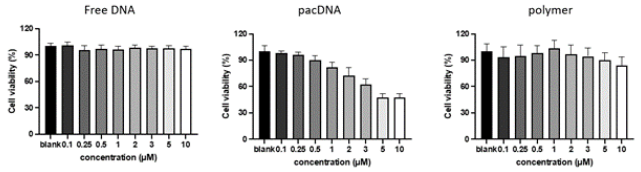

Figure 6: MTT assay of free DNA, pacDNA, polymer in mouse KRAS cell line D658.

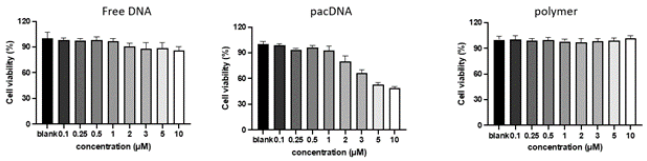

Figure 7: MTT assay of free DNA, pacDNA, polymer in mouse KRAS cell line K273.

In Figure 6 and Figure 7, both free DNA and polymer had over 90% cell viability in mouse KRAS cell lines D658 and K273, whereas, in the case of pacDNA, cell viability decreased depending on the concentration of pacDNA. The cell viability leveled off when pacDNA concentration increased from 0.1 to 10 μM. At 10 μM, the cell viability was below 50% in both cell lines, which confirmed the toxicity against the KRAS-mutated cancer cell line.

Western Blot Analysis:

Western blot is an antibody-based technology to detect proteins of interest, particularly proteins of low abundance.[22] From Figure 8, pacDNA showed inhibition of KRAS protein in both D658 and K273 cell lines, while free DNA and polymer showed similar band intensity as blank. GAPDH, which was a housekeeping protein, was used as a loading cont

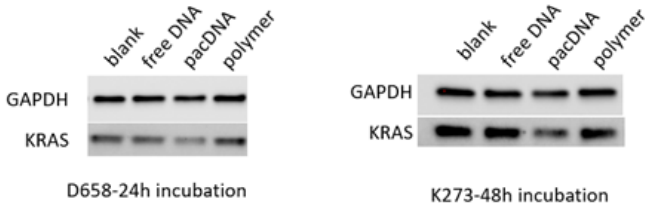

Figure 8: Western blot analysis of 10 μM free DNA, pacDNA, and polymer in both D658 and K273 cell lines.

Discussion

Although two small molecule drugs targeting mutated KRAS have been approved by the FDA most recently, they only target G12C mutated KRAS. Conversely, oligonucleotides offer an expanded range of targeting options for various types of mutations found in the KRAS protein, implying more significant potential as an anti-KRAS therapy for cancer treatment. The major challenge in oligonucleotide application is their low transfection efficiency due to the negative charge of the DNA backbone. Therefore, it is necessary to depend on a delivery vehicle to overcome the barrier of intracellular delivery. Traditional cationic drug delivery carriers for oligonucleotides generally have toxicity issues[23] and are unsafe to

use in anti-cancer therapy. The utilization of non-cationic bottlebrush polymer comprised of biocompatible PEG polymer is shown to be both safe and effective in delivering oligonucleotides. In this study, pacDNA conjugates were synthesized via click chemistry of DBCO-conjugated antisense DNA and PEG-based polymer. The HPLC analysis showed successfully synthesized DBCO-DNA strands. The DNA-polymer conjugates were then subjected to GPC purification and gel electrophoresis analysis as pacDNA had a larger size and was eluted earlier. In contrast, the DBCO-labeled DNA was smaller and was eluted later. Therefore, a desired pacDNA conjugate was prepared.

The MTT cell cytotoxicity assay was tested in both D658 and K273 cell lines, PEG-based polymer and naked DNA showed no toxicity, and the cell viability was above 90% even at the highest concentration (10 μM), in contrast, pacDNA conjugates showed cytotoxicity in a dose-dependent manner. The cell viability leveled off when increasing dosages; at the highest 10 μM concentration, both cell lines showed less than 50% cell viability. The cell cytotoxicity results showed that the pacDNA could inhibit the growth of KRAS-mutated cell lines. In contrast, the components in pacDNA did not have nonspecific toxicities against those cell lines.

The pacDNA conjugates inhibition of KRAS protein expression was tested in the Western blot analysis. KRAS protein expression levels were much lower in the pacDNA-treated group than in the control groups. The GAPDH housekeeping gene confirmed the identical protein loading between groups, proving the downregulation of KRAS protein in the pacDNA treatment group is the decrease of KRAS protein expression level. The cellular assays demonstrated that pacDNA could be a promising candidate for further study as anti-KRAS cancer therapy.

Conclusion

In this study, pacDNA conjugate comprised of PEG-based polymer and antisense DNA has been successfully prepared. Furthermore, the pacDNA conjugate showed cytotoxicity in a dose-dependent manner in two mouse mutated-KRAS cell lines D658 and K273. In addition, it offered to downregulate KRAS protein expression based on Western Blot analysis. These results demonstrate that pacDNA comprised of PEG-based polymer and antisense DNA could be potentially promising effective therapeutics in cancer treatment.

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