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Characterization and In-vitro Evaluation of Positively Charged Oligopeptide as Gene Delivery Vehicle Candidate on BHK-21 Cells

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Abstract

Peptide-based transfection agents have gathered significant interest and are being investigated globally as a possible gene delivery method. The preparation and in-vitro evaluation of oligopeptides composed of GRKKRRQRRR (TAT) and PKKKRKV (NLS) functionalized with the CKKHH sequence as a potential transfection agent is the main goal of this study. Solid-phase peptide synthesis was applied to generate the positively charged of the GRKKRRQRRR-PKKKRKV (OP-1, MW of ~ 2260 Da) and CKKKHH-YGRKKRRQRRR-PKKKRKV (OP-2, MW of ~ 3057 Da) sequences. The oligopeptide's capacity to condense DNA molecules was assessed using the ethidium bromide exclusion and DNA mobility retardation assays. Transmission electron microscopy (TEM) image examination and a particle size analyser were performed to further characterise the oligopeptide/DNA complexes. The MTT assay was used to determine the cytotoxicity, while transgene expression was carried out in BHK-21 cells using a gene encoding green fluorescence protein. Both oligopeptides of OP-1 and OP-02 were able to condense DNA molecules into compacted particles with a size range of 150–220 nm. The transfection agent based on TAT-NLS (OP-1) that functionalized with CKKHH (OP-2) was more efficient in condensing and compressing DNA molecules. Both oligopeptides were comparatively less hazardous to BHK-21 cells than those of poly-L-lysine (PLL), according to the MTT assay result. The oligopeptides' ability to promote GFP-encoding gene transgene expression further emphasises their effectiveness as potential transfection agent candidates. To further increase the positively charged oligopeptide efficacy, more development on the oligopeptide-based transfection agent needs to be explored.

Keywords: Cytotoxicity; DNA condensation; Gene delivery; Nanoparticle; Oligopeptide, Transfection agent.

1. Introduction

Among other qualities and abilities of biological advanced materials, their uniqueness in terms of physicochemical properties-such as their nanoparticle size, stability against enzymatic degradation, and low cytotoxicity-makes them appealing in medical research as drug candidates (Abd El Aty et al., 2023; Ibrahim & Abdel-Aziem, 2019) and gene delivery vehicles (Song et al., 2019; Supe et al., 2023). As transgene gene carriers, synthetic oligopeptide-based gene delivery systems can be designed for DNA condensing, endosomal escaping, and facilitating nuclear uptake to achieve optimum gene delivery (Danielsen & Hansen, 2018; Doh, 2015; Kichler et al., 2019; Koloskova et al., 2018). Oligopeptide-mediated gene transfer is an up-front, safe, and low-risk in immunogenicity for gene delivery vehicle that is attractive when contrasted with viral-based gene delivery carriers due to these attributes. In addition, the oligopeptide-based transfection systems also simultaneously display several features, including DNA condensation ability (Mann et al., 2014), endosomal escape capability (Moulay et al., 2017), facilitating nuclear localization (Yi et al., 2012), and receptor targeting (Todaro et al., 2023).

Amongst the oligopeptides used in gene delivery vehicles are well known as cell-penetrating peptides (CPPs) or protein transduction domains (PTDs). They consist of less than 30 amino acids with the ability to cross the biological membranes in an energy-dependent or energy-independent manner (Guidotti, Brambilla, & Rossi, 2017; Habault & Poyet, 2019). CPPs are probably the most

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exploited oligopeptides for non-viral gene delivery vehicles due to their capability to interact with membrane cells and allow some modifications to enhance gene delivery efficiency (Danielsen & Hansen, 2018; Ho, White, & Pouton, 2018). Several CPPs and CPP-conjugated molecules are undergoing pre-clinical and clinical trials to treat inflammation-related disease, cancers, heart diseases, and age-related diseases (Guidotti, Brambilla, & Rossi, 2017; Habault & Poyet, 2019). Recently, CPPs have also been modified to target specific cells and organelle to reduce side effects as well as to improve cell uptake, achieving an optimal transgene expression (Cerrato & Langel, 2022; Szabó et al., 2022).

Li and co-workers revealed that the functionalization of TAT-NLS to build gene complexes and the introduction of oligohistidine (Hn) sequence into the transfection agent of short peptide sequence (REDV) might increase the endosomal escape rate and accelerate endothelization and angiogenesis (Li et al., 2018). Moreover, it was reported that conjugated the REDV-G-TAT-NLS with poly(lactide-co-glycolide)-g-polyethyleneimine and polyethyleneimine (PLGA-g-PEI) via a hetero-poly(ethylene glycol) spacer (OPSS-PEG-NHS) capable to increase internalization efficiency, endosomal/lysosomal escape, and nucleus location (Li et al., 2019). Guo and colleagues used the CAGW sequence in addition to the REDV motif to create a gene carrier that was conjugated with the PLGA-g-PEG and TAT-NLS sequences to improve endothelial cell internalisation and transfection efficiency (Duo et al., 2019). In addition, the human heterogenous nuclear ribonucleoprotein A1 (hnRNP A1) sequence of ^{263}FGNYNNQSSNF-GPMKGGNFGGRSSGPY^{289} has been investigated for sufficient nuclear import of gene delivery (Lu et al., 2021) or for sequence specific targeting of viral capsid as an alternative to using the classical NLS sequence of PKKKRKV (Chai et al., 2021).

In our previous research, we demonstrated that the CKKHH sequence plus lauryl or palmitoyl alkyl residues in a short linear lipopeptide can interact with importin-α as a potential gene carrier (Tarwadi et al., 2020). It was revealed that lipopeptides containing palmitoyl alkyl and the CKKHH peptide efficiently compacted DNA molecules and promoted greater transgenic expression than corresponding molecule containing CKHH sequence. In this study, to efficiently condense DNA into nanoparticles and promote transgene expression in BHK-21 cells, we have developed a transfection agent that is made up of CKKHH functionalized with TAT (GRKKRRQRRR) and NLS (PKKKRKV) peptide sequences. Moreover, we have also examined the transmission electron microscope (TEM) images and the cytotoxicity of the oligopeptide/DNA complexes.

2. Materials and Methods

2.1. Materials

All materials were obtained from Sigma Aldrich (Sydney, NSW, Australia) and were of molecular biology or analytical grade, unless otherwise stated. The suppliers of cell culture media of Dulbecco's Modified Eagle Medium (DMEM) and foetal calf serum (FCS) were GIBCO-BRL (Invitrogen Pty. Ltd., VIC, Australia). The Baby Hamster Kidney (BHK-21, ATCC CCL-10) cells were kindly donated by Tedjo Sasmono, PhD of the Eijkman Research Centre for Molecular Biology, Indonesia. The green fluorescent protein (GFP)-encoding plasmid pCSII-EF-AcGFP (9880 bp) was generously provided by the Laboratory of the Research Institute of Microbial Diseases via Prof Kurosu, Osaka University, Japan. We use PLL (Sydney, NSW, Australia) as positive control in this experiment because PLL is capable of condensing DNA effectively and has been used for transfection agent. In addition, PLL relatively has low cytotoxicity and highly positive charged that resembles the oligopeptide. These reasons made us choose PLL as the control.

2.2. Peptide synthesis

The oligopeptides of GRKKRRQRRR-PKKKRKV (OP-1) and CKKHH-YGRKKRRQRRR-PKKKRKV (OP-2) sequences were synthesized using an automated solid-phase synthesizer of a CEM Liberty Microwave peptide synthesizer (DKSH, Hallam, VIC, Australia) with 0.5 mmol of pre-loaded Tentagel S-RAM resin. The activating, coupling, de-protecting, washing, and final de-protecting of amino acids involved in the oligopeptide synthesis process were performed using the Liberty® software linked to the equipment as described previously (Zeng et al., 2016).

2.3. Plasmid DNA isolation

Following the supplier's instructions, the plasmid pCSII-EF-AcGFP, was extracted and kept in *E. coli* DH5α using the QIAGEN® QIAprep Maxi Kit (Qiagen Pty. Ltd., Vic, Australia). The amount and purity of the plasmid DNA were evaluated by spectrophotometric measurement at 260 and 280 nm and electrophoresis on a 1% agarose gel after a single digestion with *Bam*H1 (30 minutes, 90 volts) for the plasmid confirmation. The purified plasmid DNA was frozen at -20 °C and redissolved in Milli-Q water (MQW) for storage.

2.4. Charge and N/P ratio

Based on previous descriptions, the charge ratio (CR, +/-) reflects the number positively charged protonated nitrogen atoms of transfection agent per negatively charged nucleotide unit (Maiti et al., 2018; Tarwadi et al., 2008). For the purposes of the calculations, each nucleotide was given a mean mass of 330 Da. To achieve a charge ratio OP-1/DNA of 1.0, 522 ng of OP-1 (3/13 nmol, MW 2260) and 1000 ng of DNA plasmid (~ 3 nmol) were mixed. Since OP-1 has 13 positively charged amino acids that come from 6 (six) number of lysine (K) and 7 (seven) arginine (R) residues, every molecule of OP-1 generates 13 NH_3^+ groups in physiological pH (HGB pH 7.4). A similar calculation was performed for OP-2, as the oligopeptide OP-2 generates 15 NH_3^+ groups per molecule (8K and 7R). This resulted in a mixture of 1000 ng DNA and 611 ng OP-2 (MW 3057) to obtain a CR of 1.0. The nitrogen/phosphate (N/P) ratio was ascertained for the cationic polymer of PLL, which has a lysine unit (MW of 128), rather than the charge ratio.

2.5. DNA condensation assay

The transfection agent capacity of the oligopeptides or control PLL to condense DNA was assessed using the Ethidium bromide (EtBr) exclusion assay in 96-well black plates, as previously described (Tarwadi et al., 2020).

Briefly, 50 µL of HEPES Glucose Buffer (HGB,15 mM HEPES and 5.13% w/v glucose) pH 7.4 was used to dilute two micrograms (2 µg) of plasmid DNA, with an excess of EtBr (20 µL; 100 mg/mL). After that, the spectrophotometer was calibrated using this mixture to determine the fluorescence intensity, which was measured at λ-excitation = 485 nm and λ-emission = 590 nm. Serial mass ratios of 1, 2, 3, 4, 5, 10, and 20 were used to combine the plasmid DNA with OP-1, OP-2, or PLL. Into each well, MQW was added to obtain a total volume of 230 µL. After allowing the samples to stabilise for ~ 10 minutes at room temperature, 20 µL of EtBr solution (100 mg/mL) was added. Before measuring the fluorescence intensity in a plate reader (FluoSTAR OPTIMA, BMG Lab Tech, Sydney, NSW, Australia), the 96-well plate was orbitally shaken for 30 seconds. The relative fluorescence unit (RFU) of the sample and the reference solution, which is the solution without the transfection agent, were compared to ascertain the intensity of the sample's fluorescence.

2.6. 2.6 DNA mobility shift assay

The DNA mobility shift or DNA retardation experiment was used to determine the oligopeptide's affinity to DNA molecules, as previously described (Tarwadi et al., 2008). Briefly, 10 µL of HGB pH 7.4 was used to dissolve the DNA plasmid (1 µg) and the transfection agents of OP-1, OP-2, and PLL separately. The two solutions were then mixed to produce sample with charge ratios of 0.4, 0.8, 1.6, 3.2, 6.4, or 12.8. The mixture was then incubated for 30 minutes at room temperature, or 25 ^{o}C. After loading the complex samples (10 µL) with 2 µL of loading buffer, the samples were electrophoresed on a 1% agarose gel at 110 volts for 30 minutes, and the DNA bands were detected using a 320 nm transilluminator.

2.7. Complex stability from enzymatic degradation

To assess the transfection agent/DNA complexes' resistance to enzymatic degradation, the complexes were exposed to DNase I (Turbo DNase, Ambion, VIC, Australia). To put it briefly, 1 µg of DNA plasmid and the transfection agents for OP-1, OP-2, and PLL were dissolved in 10 µL of HGB pH 7.4. The charge ratios of 0.1, 0.4, 0.8, 1.6, 3.2, 6.4, or 12.8 were then achieved by mixing the DNA and transfection agent solutions. Prior to being subjected to a 30-minute run on a 1% agarose gel electrophoresis at 110 volts, the combined solutions were incubated for 15 minutes at 37 ^{o}C with 1 unit/µg DNA of DNase I. The presence of DNA bands on the agarose gel was then determined using transilluminations at 320 nm.

2.8. Particle size and zeta potential determination

The transfection agent/DNA complexes' mean particle size (nm) and zeta potential (□) values were determined using the Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK). The mean particle sizes were measured using a 1.5 mL disposable cuvette, and the zeta potential values were determined using a folding capillary cell/Smoluchowski. Separately, 5 µg of plasmid DNA and the transfection agents of OP-1, OP-2, or PLL were dissolved in 500 µL of HGB pH 7.4. Two solutions were then combined to produce charge ratios of 1.0,2.0,3.0,4.0,5.0, and 10.0. Following the transfection agent/DNA complex formation, which took 20–30 minutes to develop, particle size and zeta potential values were assessed. To evaluate the stability of the particle size, the transfection agent/DNA particle sizes were monitored for 4 hours, 190 hours (8 days), 310 hours (13 days), and 480 hours (20 days). The complexes were maintained between point measurements at 4–8 ^{o}C in a refrigerator.

2.9. Transmission electron microscopy (TEM) analysis

A sample of the oligopeptide/DNA complex was dropped onto an EMR carbon-coated grid (EMS, Hatfield, UK), and it was then left to incubate for one to two minutes at room temperature. After two minutes of the grid being upside down on the UranyLess drop, any leftover solution was blotted out with filter paper. Subsequently, a sample containing a grid was left to dry for two hours at room temperature. Samples were examined using a Hitachi High Technology, Japan, HT7700-SS TEM device with an accelerating voltage range of 60–100 kV.

2.10. Cytotoxicity assay

The 3- (4 , 5-dimethyl-2-thiazolyl) – 2 , 5 - diphenyltetrazolium bromide (MTT) metabolic assay was used to assess the viability of the cells against the transfection agents in Baby Hamster Kidney (BHK-21) cells as previously described (Hidayat et al., 2023; Ibrahim & Abdel-Aziem, 2019). In brief, 5 x 10^4 cells/well were seeded in triplicate into a 96-well plate using DMEM supplemented with 10% foetal bovine serum (FBS) and 100 units/mL penicillin-streptomycin. The cells were cultured for 24 hours at 37 ^{o}C in an incubator with 5% CO_2. After the confluency of the cells was approximately 60%, 1.0 µg of DNA plasmid was complexed with the transfection agent in 25 µL HGB pH 7.4 at charge ratios of 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0. The complexes were then inserted into the cells, incubated for 24 hours. The culture media were changed with basal media containing 20 µL of MTT solution (5 mg/mL in PBS), then kept at 37 ^{o}C for a further 4 hours in 5% CO_2 incubator. To stop the process, 100 µL of sodium dodecyl sulphate (10% SDS) was applied to each well in the 96-well plate. Afterwards, the well plate was wrapped with aluminium foil and incubated for about 24 hours in a dark setting to dissolve formazan crystal formed. Using a plate reader equipment (Bio-Tek® Instrument, Vermont, USA) set at 570 nm, the absorbance density (OD) of dissolved formazan crystals were determined. Relative cell viability (%) was expressed as the percentage of treated cells relative to control cells.

2.11. Transfection study

A gene expressing green fluorescent protein (GFP) was used to evaluate the transfection investigation in Baby Hamster Kidney (BHK-21) cells in the 96-well plate. To summarise, 10,000 cells/well were seeded into DMEM media containing 10% FCS and 100 units/mL penicillin-streptomycin. The well plate was placed into humidified incubator and maintained at 37 ^{o}C with 5% CO_2. Once the cells had reached around 60% confluence, they were transfected with 100 µL of fresh basal culture media/well after a PBS wash. To generate a transfection agent/DNA mass ratio of 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, or 12.0, the corresponding transfection agent solution was added dropwise into a microtube that contained 300 ng plasmid DNA (pCSII-EF-AcGFP) in 25 µL of HGB pH 7.4. The transfection agent/DNA solution was left to incubate at room temperature for 10-15 minutes before the complex was added to the cells. Subsequently, the cells were further

grown at 37 ^{o}C in a humidified incubator containing 5% CO_2. Finally, the expression of the green fluorescence protein was observed using an Axiovert 40 CFL fluorescent microscope (Carl Zeiss Microscopy GmbH, Germany) after 24-hours incubation.

3. Results

3.1. Design and synthesis of oligopeptides

The goal of the GRKKRRQRRR-PKKKRKV (OP-1) oligopeptide is to represent nuclear localisation sequence (NLS) and trans-activator transcription (TAT). The lengthier oligopeptide sequence made up of CKKHH-YGRKKRRQRRR-PKKKRKV (OP-2) was obtained by incorporating the peptide sequence of CKKHH into the oligopeptide of OP-1. However, we also include amino acid of tyrosine (Y) in the OP-2, simply to mimic an amino acid sequence trans activating transcription (TAT) of YGRKKRRQRRR. The sequence of TAT has been explored intensively in gene delivery as cellular uptake enhancer (Zhang et al., 2023; Zhang et al., 2014) and mediates in crossing the cell membranes (Kim, 2024; Yang & Hinner, 2015). However, the function of individual amino acid of tyrosine still needs to be further exploited.

The purpose of the oligopeptide-based transfection agent design is to bind, compress, and shield DNA molecules from enzyme destruction during gene transfer process. The cysteine (-C-) amino acid inclusion in the OP-2 molecules is intended to facilitate dimerization, enhance the oligopeptides' ability to condense DNA molecules effectively. To promote endosomal escape by the proton sponge effect at a low pH inside endosomal vesicles, two histidine (-H-H-) were also included (Arabzadeh et al., 2019). Before attaining successful transfection, it was anticipated that histidine residues, which supply weakly basic groups, would become protonated in the endosome and promote endosomal escape in a way similar to those of PEI (Belguise-Valladier & Behr, 2001). We utilized the CKKHH sequence as well as peptide sequences rich in lysine (K) and arginine (R) residues from the nuclear localisation sequence (PKKKRKV) and trans-activator of transcription (YGRKKRRQRRR-PKKKRKV) to facilitate and improve cellular and nuclear absorption (**Figure 1**).

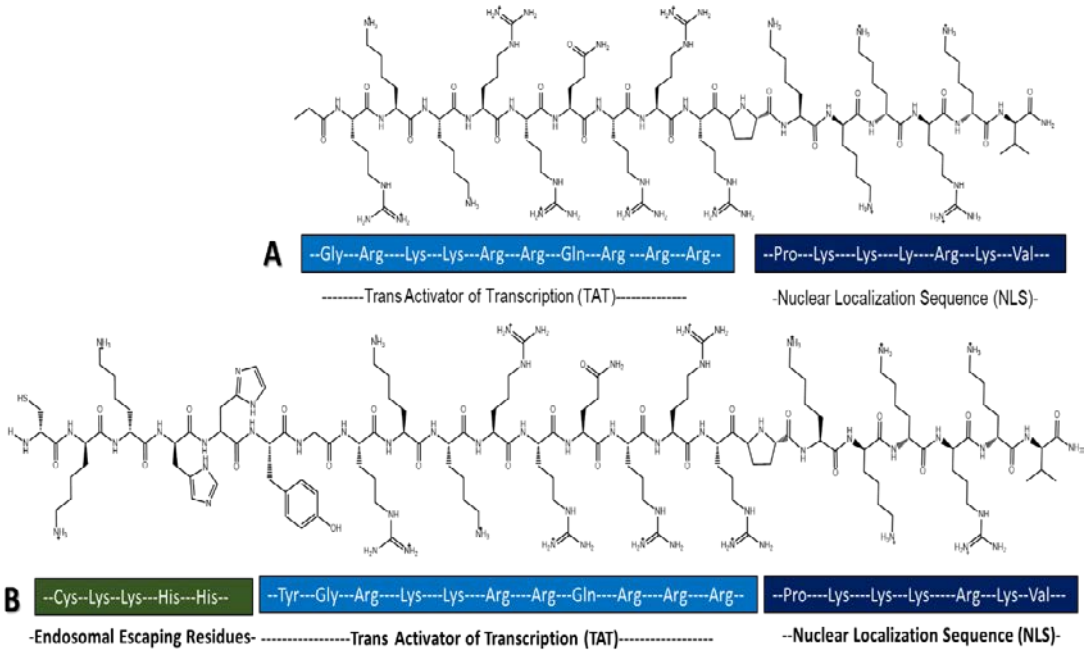

Figure 1. Representative molecular structures of positively charged oligopeptide-based transfection agents: (A). GRKKRRQRRR-PKKKRKV (OP-1) and (B). CKKHH-YGRKKRRQRRR-PKKKRKV (OP-2).

The purpose of the positively charged arginine (-R-) and lysine (-K-) residues inclusion was to facilitate transgene expression, create a compact particle, and provide ionic contact with negatively charged DNA. It has been previously observed that transfection agents' histidine-rich amphipathic peptides may facilitate the effective delivery of DNA molecules in mammalian cells (Kichler et al., 2013). The Fmoc solid-phase peptide synthesis (SPPS) method was used to synthesise the oligopeptides of OP-1 and OP-2, and LC-MS data was used to confirm their molecular structures (**Figure 2**). The molecular size of the oligopeptide of OP-1 was found to be ~ 2260 Da, which was calculated from the m/z values, as shown by the observed m/z peaks at 566.2 (4+), 754.6 (3+), and 1131.2 (2+). Concurrently, the OP-2's LC-MS spectra revealed m/z at 612.6 (5+), 765.4 (4+), and 1020.1 (3+), which supported the molecular size of ~ 3057 Da.

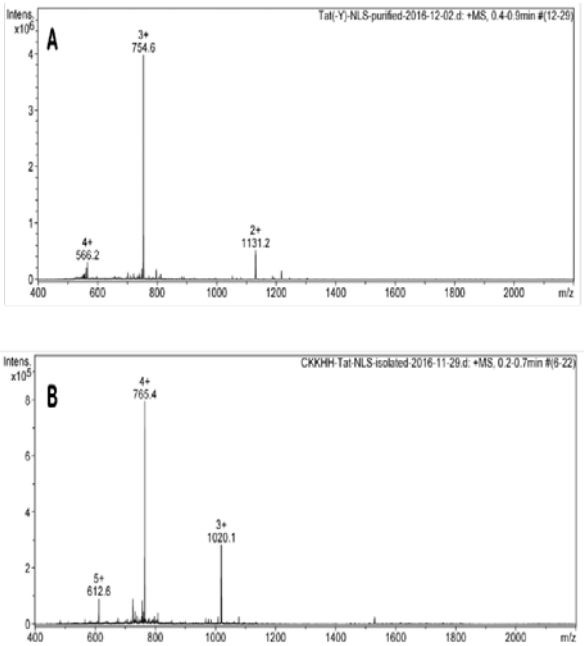

Figure 2. Liquid chromatography-mass spectroscopy (LC-MS) spectra of (A) OP-1 that has specific signals at m/z: 566.2 (4+), 754.6 (3+), and 1131.2 (2+), (B). OP-2 that has specific signals at m/z: 612.6 (5+), 765.4 (4+), and 1020.1 (3+).

3.2. Physicochemical characterization

The EtBr exclusion experiment was used to test the oligopeptides' ability to condense DNA molecules. EtBr creates fluorescence after intercalating between DNA strands with ease. When a transfection agent attaches or condenses DNA molecules, some EtBr will be omitted or excluded from the DNA molecules, resulting in a reduced intensity of fluorescence (Blessing, Remy, & Behr, 1998; Chen et al., 2016). The fluorescence intensity of the oligopeptide/DNA diminishes as the concentration of the transfection agent increases (Figure 3).

Figure 3. The binding studies of oligopeptides and the PLL (as the control) with DNA molecules were performed by EtBr exclusion assay at charge ratio 0-10. The samples' fluorescence intensity was measured at 485 nm for excitation and 590 nm for emission. The data are displayed as mean ± SD (n=3).

At a charge ratio of 5.0, the fluorescence intensities of the DNA molecules condensed by OP-1 and OP-2 oligopeptides were approximately 50%; however, at charge ratio of 10, their fluorescence intensities decreased up to ~45% and ~40%, respectively. The data shows that a longer amino acid sequence of OP-2 (which consists of 23 amino acids) condenses DNA more efficiently than OP-1 (which consists of 17 amino acids). Both oligopeptides, along with the control PLL, displayed similar physicochemical characteristics in condensing DNA molecules. Fascinatingly, when the ratio of the transfection agents to DNA molecules increased from 10 to 20, the fluorescence intensities remained almost constant. This suggests that EtBr still has access to intercalate between the double strands of DNA molecules to produce fluorescence even though the transfection agent's concentration increased two-fold from the charge ratio of 10 to 20. The ability of the oligopeptide to condense and protect DNA molecules were further evaluated by agarose gel electrophoresis experiments. Based on the DNA mobility shift assay, the oligopeptides of OP-1 (13+) and OP-2 (15+) were considered strong DNA condensers, as there were no DNA bands observed at a low charge ratio of 0.4 (Figures 4A and 4B).

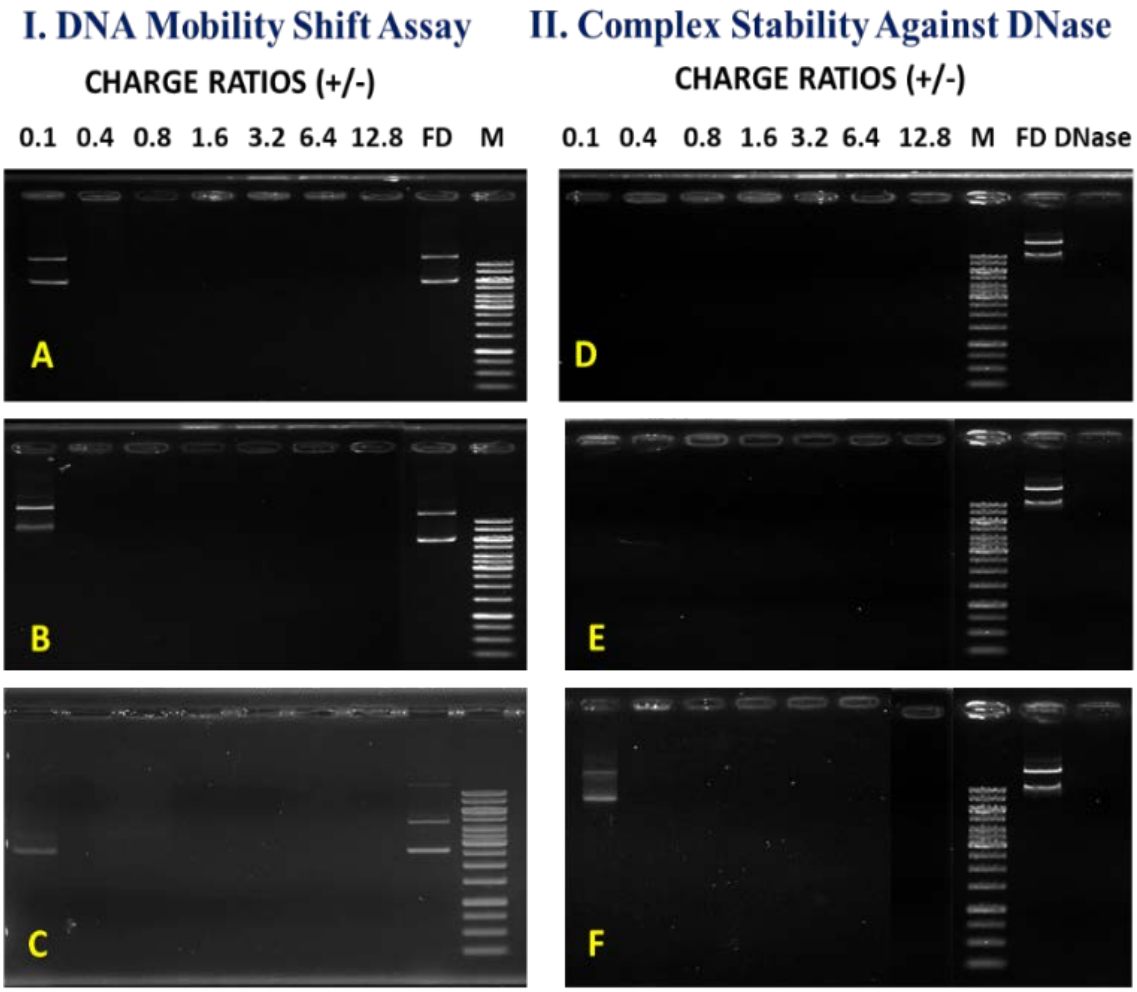

Figure 4. Gel mobility shift assays (I) and complex stability against DNase (II) of the oligopeptide/DNA performed in agarose gel electrophoresis: A/D. OP-1, B/E. OP-2, and C/F. PLL. M: DNA marker, 1 kb ladder, FD: free DNA molecules without transfection agent. DNase: DNA + DNase without transfection agent. PLL was used as the control.

The capability of the oligopeptides to condense DNA molecules was comparable to that of the cationic polymer PLL. The DNA bands were clearly detected on agarose gel from un-complexed, free DNA plasmid (lane FD) as shown (Figures 4A-4C). However, as the DNA molecules were complexed by both OP-1 and OP-2 or PLL, the DNA molecules were retarded in the agarose gel wells. This indicates that oligopeptides are very efficient in condensing DNA molecules, comparable to the control of cationic polymer PLL. The oligopeptide of OP-1 and OP-2 were further evaluated by incubating the oligopeptide/DNA complexes in the presence of DNase. In the absence of oligopeptide, the free DNA plasmid (lane DNase) was fully degraded as observed (Figures 4D-4F). The ability of the oligopeptides to protect DNA molecules from DNase degradation was also similar to that of the PLL. The smeared DNA bands were un-detected in agarose gels as the DNA molecules were protected by the oligopeptide and the control PLL. The complex of the oligopeptide/DNA was further evaluated by measuring their particle sizes and zeta potential values (Figure 5).

The complex particle sizes of the OP-1 and OP-2 with DNA molecules were comparable to the PLL/DNA complexes, approximately 110-200 nm. However, based on Wilcoxon signed-rank test analysis, there was a statistically different (p<0.05) of the complex particle size between PLL, OP-1, and OP2 (Figure 5A). As the charge ratio of the oligopeptide to DNA increases, the zeta potential (ζ) value also increases. Theoretically, as the charge ratio increases, the positive value will continue to rise. We discovered that when the oligopeptide/DNA charge ratio was 3.0, the zeta potential value was nearly negative and there was not statistically different amongst the transfection agent of OP-1, OP-2 and PLL (Figure 5B). Interestingly, the particle size of the PLL or OP-1/DNA at charge ratio of 1.5 that were incubated for up to 480 hours (20 days), remained nearly unchanged, suggesting that there was no evidence of PLL/DNA or OP-1/DNA aggregation (Figure 5C). Compared to the complexes of PLL/DNA and OP-1/DNA, OP-2/DNA behaved differently as the particle size dropped from ~150 nm to ~100 nm as it was incubated up to 20-days (480 hours).

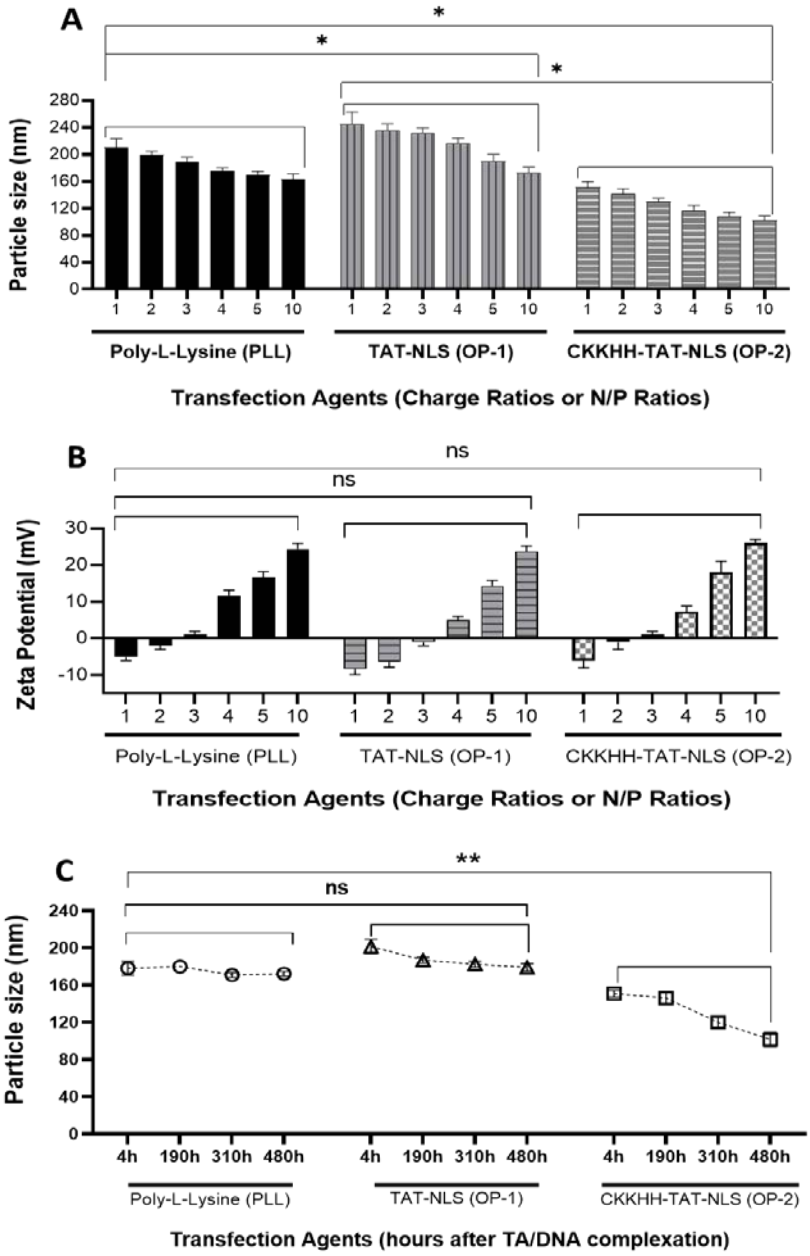

Figure 5. Particle size (nm), zeta potential value, and oligopeptide/DNA complex stability in HGB pH 7.4: (A) particle size of transfection agent/DNA at charge (N/P) ratios of 1.0-10.0, (B) zeta potential values of transfection agent/DNA at charge ratios of 1.0-10.0, and (C) particle size stability of the transfection agent/DNA up to 480 hours after the complex formation at a charge ratio of 1.5. Data are triplicates, expressed as mean ± SD and subjected to Wilcoxon signed-rank test analysis.

Transmission electron microscopy (TEM) image analysis was used to further characterise the particle size and morphology of the oligopeptide/DNA complexes (Figure 6). The complexes of OP-1/DNA and OP-2/DNA at charge ratio 4.0 resulted in compacted particles with diameters of 100-150 nm (Figures 6D-6E).

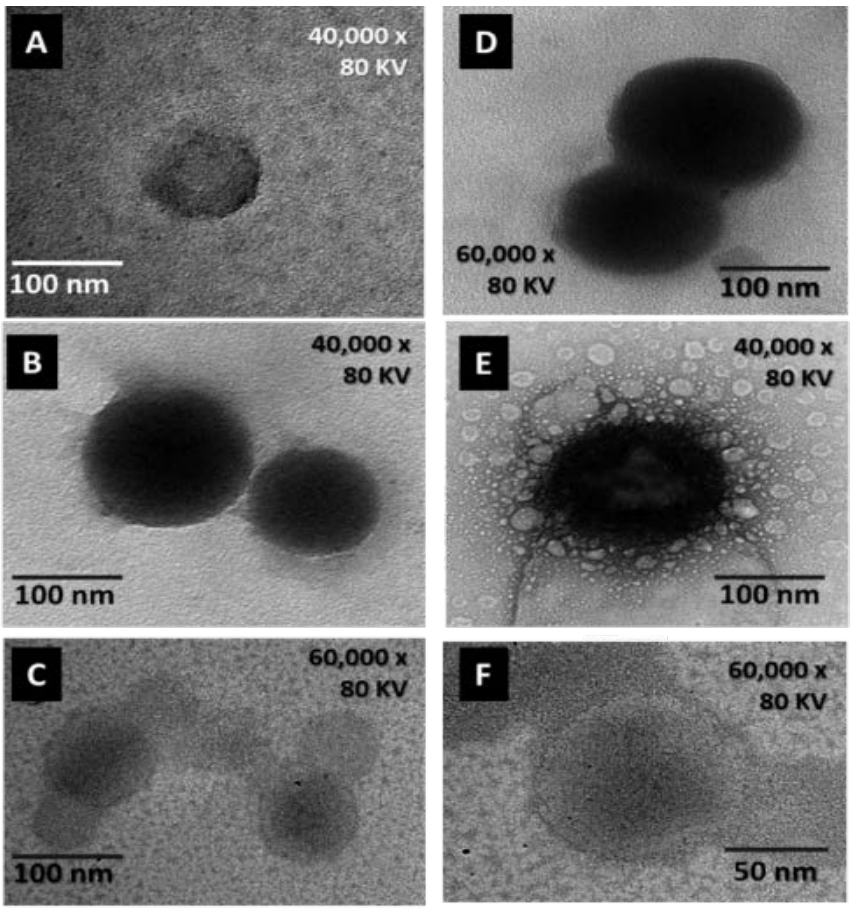

Figure 6. TEM images of the oligopeptide-based transfection agent and the oligopeptide/DNA complexes in HGB pH 7.4 at a charge ratio 4.0. (A) OP-1, (B) OP-2, (C) PLL, (D) OP-1/DNA, (E) OP-2/DNA, and (F) PLL/DNA. PLL was used as the control.

The individual nanoparticle was clearly observed, and no aggregation was detected as the oligopeptide was diluted in HGB pH 7.4 or complexed with DNA molecules. Based on TEM analysis, the oligopeptide/DNA complex particle size was comparable to PLL/DNA (100-150 nm).

3.3. Biological activity evaluation

Following a 24-hour growth period, the MTT assay was used to assess the viability of the oligopeptide/DNA complexes on BHK-21 cells at charge ratios of 1, 2, 4, 8. 16, and 32. These charge ratio values are equivalent with sample concentration of 10, 20, 40, 80, 160, and 320 µg/mL or ppm. The findings showed that the charge ratio of the oligopeptide/DNA has a significant impact on the BHK-21 cell viability (Figure 7). The oligopeptides are less toxic compared to BHK-21 cells compared to PLL based on the Wilcoxon signed-rank test analysis (p<0.01). Furthermore, it was evident that when BHK-21 cells were treated with PLL at charge (N/P) ratios > 8.0 or above 80 ppm, over 40% of the cells were inhibited; in contrast, only around 20% of the cells were inhibited by OP-1 and OP-2 at the same concentration.

Figure 7. Cytotoxicity assay of the OP-1, OP-2, and PLL at charge ratio 1.0-32.0 in HGB pH 7.4 on BHK-21 cells. PLL was used as the control. Data are displayed as mean ± SD (n=3) and subjected to Wilcoxon signed-rank test analysis.

The oligopeptides were further evaluated to transfect BHK-21 cells (**Figure 8**). The GFP-expressing cells were detected at the oligopeptide/DNA charge ratios of 2.0, 4.0, and 6.0. However, when the charge ratio increased further beyond 6.0, the GFP-expressing cells were hardly detectable. Although the GFP-expressing cells mediated by the oligopeptides are relatively higher than those mediated by PLL, the transfection enhancement was not significantly improved. It might be due to the presence of

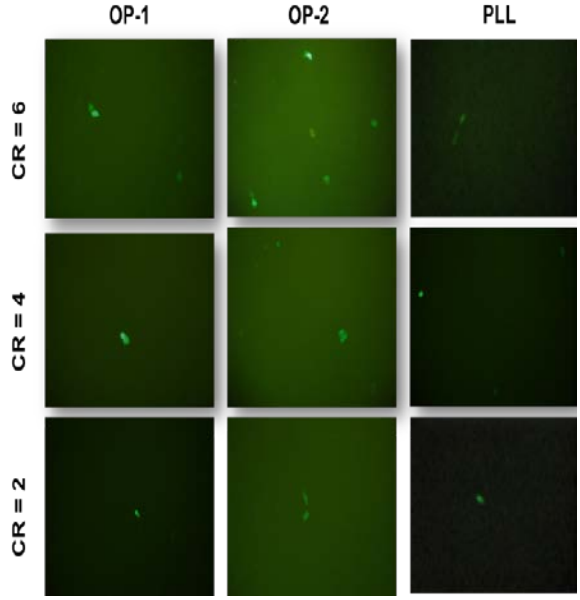

Figure 8. GFP expression of 24-hours post transfection in BHK-21 cells with plasmid DNA encoding GFP gene condensed with OP-1, OP-2, and PLL at charge ratios of 2.0, 4.0, and 6.0. PLL was used as the control.

4. Discussion

We have successfully demonstrated that the oligopeptides of GRKKRRQRRR-PKKKRKV (OP-1) and CKKKHH-YGRKKRRQRRR-PKKKRKV (OP-2) with molecular sizes of ~ 2260 Da and ~3057 Da, respectively (Figures 1 and 2). This means, the molecular weight of the OP-1 can be calculated as follows: = (566.2 x 4)-4 = 2260.8 Da, or (754.6 x 3)-3=2260.8 Da, or (1131.2 x 2)-2 = 2260, 4 Da. Similarly, based on LC-MS data, the molecular weight of the OP-2 was calculated as follows: = (612.6 x 5)-5 = 3058 Da, or (765.4 x 4)-4=3057.6 Da, or (1020.1 x 3)-3 = 3057.3 Da. Both oligopeptides were capable of condensing and protecting DNA molecules efficiently (Figures 3) and forming nanoparticle sizes of 100-220 nm as revealed by TEM image analysis (Figure 6). Moreover, by visualizing the DNase-treated samples from agarose gel electrophoresis, we have demonstrated that our oligopeptides were able to protect DNA molecules from enzymatic degradation (Figure 4). Instead of visualizing DNA bands on agarose gel, He *et al*. quantified the effectiveness of the DNA protection by measuring the optical density (OD260) after incubating the samples in the DNase solution (He et al., 2013). Meanwhile, Fihurka and co-workers have employed the EtBr exclusion assay to determine the undigested DNA of the samples after incubation with DNase solution (Fihurka, Sanchez-Ramos, & Sava, 2018). They found that as the transfection agent 13 or 15 positively charged amino acids (OP-1 or OP-2) which provide very strong ionic interaction between the oligopeptides and the DNA molecules. As the charge ratio of the oligopeptide and DNA increases, the complexes of oligopeptide/DNA are more compacted than those of low charge ratio. This compacted oligopeptide/DNA complex limited the release of the DNA molecule from the complex to be further transcribed into functional mRNA and, as a result, decreased the number of GFP-expressing cells. condensed DNA molecules efficiently to form compacted nanoparticles, the release of DNA from transfection agents was very slow. This implies that a very strong interaction between the transfection agent and the DNA molecules might result in unfavorable conditions.

The physicochemical characteristics of the oligopeptides in condensing, binding, and protecting DNA molecules as well as the particle size were comparable to PLL. Interestingly, we found that our oligopeptides that compose of CKKHH, TAT, and NLS (OP-2) are capable of condensing DNA molecules more effectively than the conjugated TAT-NLS compound only (OP-1) as more compacted particle size was achieved (Figure 5A). Previously, it was reported that the conjugate TAT-NLS condensed DNA molecules at N/P ratios of 5.0 (Yi et al., 2012) or mass ratio 2.0-3.0 (Li et al., 2018). In our research, the DNA molecules were condensed effectively even at a very low mass ratio of 0.4 (Figure 4). The presence of 13 or 15 positive charges from lysine and arginine in the OP-1 or OP-2 provides very strong ionic interaction between the oligopeptides and the negative charges of the DNA molecules. This interaction creates compact nanoparticles of the oligopeptides composed of TAT and NLS sequences as revealed by particle size and TEM analysis of the oligopeptide/DNA complex. Both oligopeptides are effective at condensing DNA into nanoparticle sizes of approximately 200 nm. OP-2 has more capability to condense DNA molecular effectively as shown in Figure 3 and Figure 5, where DNA condensation and DNA complex formation facilitated by OP-2 was tighter and smaller compared to OP-1. These physicochemical characteristics of OP-2 might come from longer amino acid sequence (23 > 17 amino acids), higher positively charge (15 > 13 charges), and inclusion of cysteine residue (-C-). Longer amino acid sequence and higher positively charges of the oligopeptide enhance ionic interaction between positively charged oligopeptide and the negatively charged DNA molecules. Meanwhile, cysteine has been reported capable of promoting complexation by the formation of disulfide bonds (Dauty et al., 2001; Tarwadi, 2018) that might enhance the compactness of the oligopeptide/DNA particles.

Homogenous particle and complex stability of the oligopeptide/DNA are beneficial for the transgene expression since they are influence cellular uptake and cytoplasmic delivery (Sang et al., 2015). However, for transgene expression to occur, DNA molecules are expected to be released safely from the carrier prior to gene transcription. Therefore, it should be kept in mind that the interaction between the gene of interests and the carriers must be flexible enough, protected from enzymatic degradation, and capable of penetrating nucleus membrane before resulting transgene expression. Two (2) histidine residues in the CKKHH sequence of OP-2 were expected to enhance transgene expression as the histidine capable of

facilitating endosomal escape. Our Findings in transfection study on BHK-21 cells have showed that there was only a minor transfection enhancement as the number of GFP-expressing cells slightly increased as cells were treated with OP-2 compared to the control PLL (Figure 8). It is interesting to note that although both oligopeptides are capable of condensing and protecting DNA molecules from enzymatic degradation during cellular transport (Figure 4), the transgene expression facilitated by the oligopeptides was still moderate. We speculate this phenomenon might be due to very tight ionic interaction between the oligopeptides and DNA molecules that made it difficult for RNA Polymerase to initiate transcription process and hampered gene expression. Nevertheless, our oligopeptides are relatively non-toxic as the viability of BHK-21 cells was ~ 80% compared to control, although cells were treated with oligopeptides at a charge ratio of 12.0 that gives a chance to interfere and damage the cell membranes. To achieve safe and efficient non-viral gene delivery vehicles, these transfection agents composing of CKKHH, TAT, and NLS could be further explored by formulating the oligopeptides with lipids or other components.

5. Conclusion

We have successfully demonstrated that inclusion of CKKHH functionalized positively charged amino acid of lysine and arginine in TAT and NLS sequence is capable of condensing and compacting DNA molecules to form nanoparticle sizes (100-220 nm) as revealed by DNA condensation, DNA shift mobility, particle size and TEM image analysis. Based on MTT assays, both oligopeptide sequences of GRKKRRQRRR-PKKKRKV (OP-1) and CKKKHH-YGRKKRRQRRR-PKKKRKV (OP-2) with molecular sizes of ~ 2260 Da and ~3057 Da, respectively, have low cytotoxicity in BHK-21 cells. However, a very strong ionic interaction between DNA molecules and the oligopeptide hampered DNA molecules released from the complexes and in turn limited the transgene expression. Therefore, further exploration to optimize the oligopeptide sequence or modulate the ionic interaction of the positively charged amino acids and the negatively charged DNA molecules is needed by formulating the oligopeptide with lipids or other compounds to increase transfection agent efficiency.

Conflict of Interest

All authors declare that there is no conflict of interests.

Author Contribution

Damai Ria Setyawati: data curation and analysis; Sjaikhurrizal El Muttaqien: data analysis and manuscript correction, Irvan Faizal: data analysis and manuscript correction, Asep Saepudin: data curation and analysis, Efrida Martius: data analysis and manuscript preparation, Indira Putri Negari: data analysis and manuscript preparation, Stefi Mitra Wahyuningsih: data curation and analysis; Neni Nurainy: data curation and review; Mulyoto Pangestu: formal analysis and review; Tarwadi: conceptualization, data curation, formal analysis, writing original draft, supervision, editing, and final revision.

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