DNA Condensation Studies of Fully Synthesized Lipopeptide-Based Transfection Agent for Gene Delivery Vehicle

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Abstract

The main requirement of transfection agent has to condense DNA in nanoparticle size, protect the DNA from nucleases and other degrading enzymes during its transport in cell cytoplasm and nucleus and should not toxic to target cells. In this research, lipopeptide composed of palmitoyl (C-16) and short peptide sequence have been designed fully synthesized and tested to DNA condensation capability and toxicity. The DNA condensation study was performed using EtBr exclusion assay and cytotoxicity determination was carried out by colorimetric MTT assay. It was revealed that lipopeptide-based transfection agent of Pal-CKKHH and Pal-CKKHH-YGRKKRRQRRR-PKKKRKV condensed DNA molecules efficiently. The lipopeptide was less toxic compared to Lipofectamine and Poly-L-Lysine, that shown by 90% of CHO-K1 cells remained viable when they were treated with 4.36 μM Pal-CKKHH-YGRKKRRQRRR-PKKKRKV. Meanwhile, there were only ~75% and 80% of CHO-K1 viable cells when it was treated with PLL and Lipofectamine®2000, respectively. Moreover, cell viability of HepG2 was ~ 75% after treated with 2.18 μM of Pal-CKKHH-YGRKKRRQRRR-PKKKRKV and decreased to ~65% when the lipopeptide concentration increased to 8.72 □M. In summary, the synthesized lipopeptide condenses DNA molecules efficiently, less toxic than Lipofectamine®2000 and PLL and has possibility to be explored as a non-viral gene delivery vehicle.

Keywords: lipopeptide, gene delivery, cytotoxicity, and DNA condensation study.

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Introduction

Nucleic acid-based therapeutics have emerged and been explored as the next generation agents for treatment and prevention of many diseases including viral infection, cancer, and genetic disorders (Tarwadi *et al.*, 2008; Koloskova *et al.*, 2016). However, the main challenges in delivering nucleic acid-based therapy are the low cellular uptake, enzymatic degradation during the cytoplasmic transport and nuclear cell barrier. Therefore, therapy based on nucleic acid molecules need gene delivery systems which are accurate, efficient and safe. Viral-based gene delivery vehicle is considered very efficient in nucleic acid internalization and facilitating the gene into cell nucleus actively. However, due to the safety reason their applications on gene therapy clinical trials have many regulation and restriction. Meanwhile, non viral-based gene delivery is considered safe for the host cells but the delivery efficiency is relatively low (Wang *et al.*, 2018).

Nowadays, non-vial gene delivery vehicle platform has widely developed due to its safety issues, stability and easiness in commercial production. Several strategies have been applied to deliver and mediate the gene interest into the target cells. It was reported that

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approximately 10^6 DNA molecules were needed to transfect the cells and only ~100 DNA molecules (1/10000) reached the cell nucleus (Tachibana *et al*., 2002). The low level of DNA molecules which enter the cell nucleus due to physical and biological barriers during the transport into the cell nucleus. Amongst the non-viral gene vehicles, cationic lipids and liposome-based gene delivery are the most interesting widely studied molecules being explored for in-vitro transfection. Koloskova and co-workers reported the usage of synthetic cationic amphiphiles with peptide hydrophilic domain as non-viral transfection agent (Koloskova *et al*., 2016). They found that the transfection efficiency was increased if the ornithine was used as amphiphile's polar head. More recently, the physicochemical properties and cytotoxicity of the molecule were found to be dependent on a number of amino acid derivatives in an amphiphilic polar head (Koloskova *et al.,* 2018).

Sequence known as nuclear localization signal (NLS) of PKKKRKV and a sequence of YGRKKQKKRRR known as transactivator of transcription (TAT) have been reported able to increase transgene expression on MCF-7 cells in low level cytotoxicity when it was added to Lipofectamine before transfection process (Doh, 2015). However, the peptide of NLS and TAT has not yet evaluated individually.

In this study, a series of novel lipopeptide based transfection reagents composing of an alkyl chain (-R) and short peptide containing a number of amino acid residues having thiol group (-SH) of cysteine, neutral and positively charged amino acids under physiological environment pH 7.4 of histidine and lysine have been designed and constructed. For preliminary evaluation, the lipopeptide-based transfection agents were monitored its ability to condense DNA molecules and the cytotoxicity on CHO-K1 and HepG2 cells.

Materials and Methods

Plasmid Isolation and Lipopeptide Synthesis

The plasmid of pCSII-EF-AcGFP was cultivated in *E coli* strain TOP10, and isolated using QIAGEN® QIAprep Maxi Kit (Qiagen Pty. Ltd., Vic, Australia) in accordance with the supplier's protocol. The quantity and purity of the plasmid DNA were determined by spectrophoto-metric analysis at 260 and 280 nm as well as by running the plasmid DNA on 1 % agarose gel electrophoresis after single digestion with restriction enzyme of *Bam*H1 (30 minutes, 90 volt). Purified plasmid DNA was resuspended in Milli-Q water (MQW) and frozen (-20^{o}C) for storage.

The basic structures of designed short linear lipopeptides are composed of an alkyl chain, Cysteine and a number of Histidine and Lysine amino acid residues. The inclusion of an alkyl chain of Palmitoyl (C_{-16}) in the lipopeptide was intended to initiate and provide hydrophobic interactions between the lipopeptide and DNA. The cysteine residue which bears a thiol group (-SH) was intended to produce dimerization in the presence of DNA molecules. Lysine was believed to provide positive charge on the lipopeptide to interact mainly with the negatively charged of sugar-phosphate backbone of the DNA molecule. Histidine was included to buffer the endosome vesicle and escape from endosomal degradation once the complex of the DNA-lipopeptide is taken up by the cells. The lipopeptide was constructed using solid phase peptide syhnthesis method. The commercially available transfection agents of Poly-L Lysine (PLL), Lipofectamine®2000 and Polyethilen-imine (PEI) were used as the controls.

Charge Ratio and N/P Ratio Calculation

The complex formation of DNA and transfection agent (TA) was carried out based on charge ratio or N/P ratio calculation. The charge ratio (+/-) of Lipopeptide/DNA or TA/DNA complexes referred to the number of protonated nitrogen (positively charged) provided by transfection agent per negatively charged nucleotide unit as described previously (Tarwadi *et al.*, 2008).

For the calculation, an average mass of 330 Da per nucleotide was used. To obtain a theoretical charge ratio of 1:1 between Lipofectamine®2000 (3332 Da, $15NH^+$/ molecule) and DNA molecule, 1 μg of DNA (3 nmol) was mixed with 0.666 μg of Lipofectamin®2000 (3/15 nmol) since every one Lipofectamin®2000 molecule (DOPE: DOSPA=1:3) generates 15 NH^+ groups. To obtain a charge ratio of 1:1 between Lipopeptide-A (Pal-CKKHH, 889 Da, $2NH^+$/molecule) and DNA, 1 μg of DNA was mixed with 1.3 μg (3/2 nmol) of Pal-CKKHH since the molecule of Pal-CKKHH generates $2NH^+$ groups. A charge ratio of 1:1 between

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Lipopeptide-B (Pal-CKKHHYGRKKRRQRR-RPKKKRKV, 3296 Da, $15NH^+$/molecule) and DNA, was obtained by mixing 1 μg of DNA with 0.659 μg (3/15 nmol) Pal-CKKHH-YGRKKRRQRRR-PKKKRKV since the molecule generates $15NH^+$ groups. Instead of using charge ratio, complex of cationic polymer PEI with DNA molecules were described in terms of the total nitrogen/ phosphate (N/P) ratio.

DNA Condensation Assay

The ability of the transfection agents to condense DNA was evaluated using Ethidium bromide (EtBr) exclusion assay using a plate reader (FluoStar OPTIMA, BMG Lab Tech, Sydney, NSW, Australia) as described previously (Tarwadi *et al.,* 2008). The assay was carried out in 96-well black plates in a series of charge ratios from 0 to 10. Briefly, a sample containing 2 μg plasmid DNA and an excess of EtBr (20 μL; 100 μg/μL) was used to calibrate the spectrofluorometer to 100% fluorescence intensity (λex. = 520 nm; λem. = 610 nm) and used as a reference. For the assays; 50 μL of 60 mM Tris HCl buffer (pH 7.4) was added to each well containing complex of DNA-transfection agent. Milli-Q Water was added to a total volume of 230 μL per well. The samples were incubated at ambient temperature to stabilize for 2-3 minutes before 20 μL EtBr solution was added. Before the fluorescence intensity was measured, the 96-well plate was shaken orbitally for 30 seconds. The relative fluorescence unit (RFU) of the sample was compared to the reference (solution of DNA and EtBr, no transfection agent added) to obtain fluorescence intensity of the sample. To confirm the role thiol group (-SH) of Cysteine moiety in the lipopeptide molecule, the DNA condensation study of Lauryl-CKKH was carried out in the presence of reducing agent dithiothreitol (DTT) 1-5 mM.

Cytotoxicity Assay

Cell viability against transfection agent was determined using colorimetric MTT metabolic assay on CHO-K1 and HepG2 cell lines. Briefly, cells were seeded in a 96-well plate (5 x 10^4 cells/well) in a culture medium (100 μL/well) of DMEM in humidified incubator with 5% CO_2 at 37°C. After reaching approximately 60% confluence, cells were exposed to complex of DNA (1.0 μg)/well and transfection agent with ranging concentration of: 1.09; 2.18; 4.36; 8.72; 17.44 and 34.88 μM. After 24-hour incubation, cells were washed with 1 x PBS and then replaced with fresh media containing 20 μL (5 mg/mL in PBS) of MTT [3-(4,5-dimethyl-thyazol-2-yl)-2,5-diphenyltetrazolium bromide] solution. After incubation for another 4-hour at 37°C, the reaction was terminated by adding 100 μL dimethyl sulfoxide (DMSO)/well and the absorbance intensity was measured by a micro plate reader (Bio-Tek® Instrument, Vermont, USA) at 490 nm. The relative cell viability (%) was expressed as a percentage relative to the untreated control cells (van Meerloo *et al.*, 2011).

Statistical analysis

All experiments of DNA condensation and cytotoxicity studies were carried out in triplicate in each treatment. Data were plotted using GraphPad Prism 5 and analyzed using Two Way Anova followed by Bonferonni Post Test and otherwise stated differently, all cases of significant was set at $p<0.05$.

Results

Lipopeptide Design

The lipopeptide was constructed with hydrophobic region of alkyl chain of lauryl (C-12) or palmitoyl (C-16) and a cysteine residue which has thiol (-SH) group and is aimed for dimerization due to availabilityof its thiol group in the presence of DNA molecules. The lipopeptide was also composed of histidine amino acid residue which was intended for endosomal escape from enzymatic degradation during its transport in cytoplasm. Histidine is a weak base where in the physiologic condition of endosome, it will be protonated causing the endosome swelling and bursting. Moreover, the lipopeptide molecule was also constructed with highly positively charged of lysine and arginine residues to provide ionic interaction with negatively charged of DNA, enhance cell membrane penetration and facilitate nuclear uptake.

The lipopeptides which were explored for this study were synthesized based on solid phase peptide synthesis (SPPS) as listed in Table 1. The oligopeptide molecules were constructed before it was coupled with the

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alkyl chain of lauric acid (C-12) or palmitic acid (C16). Commercial of transfection agents (Lipofectamine, PLL and PEI) and other peptides (pentalysine, oligolysine) were used as the control.

Table 1 Transfection agents (TA) exploited for DNA condensation studies

No	Transfection Agent	MW (Da)	Charge
1	Lau-CKKH	696	2
2	Pal-CKKH	752	2
3	Pal-CKHH	761	1
4	Pal-CKKHH	889	2
5	Pal-CKKHH-YGRKKRRQRRR-PKKKRKV	3296	15
6	Pentalysine (K5)	553	5
7	Oligolysine (K10)	1118	10
8	Poly-L-Lysine (PLL)	128	1
9	PEI (branched)	143	1
10	Lipofectamine^{TM}	3332	15

DNA Condensation Study

Ethidium bromide (EtBr) exclusion assay was used to monitor DNA condensation which was aimed for measuring transfection agent in condensing DNA molecules. In the absence of transfection agent, DNA molecules will intercalate EtBr molecules and results in fluorescence. However, in the present of transfection agents or condensing materials, EtBr will be excluded from the EtBr-DNA interaction thus reducing the fluorescence intensity. Therefore, as the transfection agent concentration increased in the complex solution, the fluorescence intensity of DNA-EtBr will be decreased.

Solvent of absolute ethanol and dimethyl sulfoxide (DMSO) were used to dilute samples for stock solution (1 mg/ml), therefore those solvents were evaluated in DNA condensation study in case it interfered in DNA complex formation. The ethanol or DMSO was added into DNA-transfection agent complex solution at concentration of 2 or 5% (v/v). In the absence of ethanol, Pal-CKHH condensed DNA efficiently where at charge ratio of 1.0, approximately 40-50% of DNA molecules were condensed ($p<0.05$) as shown in Figure 1 A. Moreover, approximately 30% and 40% of DNA molecules were condensed by Pal-CKKHH and PEI, respectively although it was not significant statistically ($p>0.05$). However, the presence of ethanol in DNA-EtBr complex solution potentiated the fluorescence intensity. The fluorescence intensity of DNA-EtBr complex increased up to 113% and 126% in the present of 2% and 10% ethanol, respectively. Similarly, fluorescence intensity of DNA-EtBr/transfection agent of Pal – CKHH and Pal-CKKHH also increased in the complex solution containing ethanol 2% and 10% v/v. On the contrary, the fluorescence intensity of DNA-EtBr/PEI was not increased in the presence of ethanol in the complex solution. As shown in Figure 1 A, there was a significant different of fluorescence intensity between DNA-EtBr (as reference) complex versus DNA-EtBr/Pal-CKHH ($p<0.01$), DNA-EtBr/Pal-CKKHH ($p<0,005$) and DNA-EtBr/ Pal-CKKHH ($p<0.01$) in the presence of ethanol 2% and 10% (v/v).

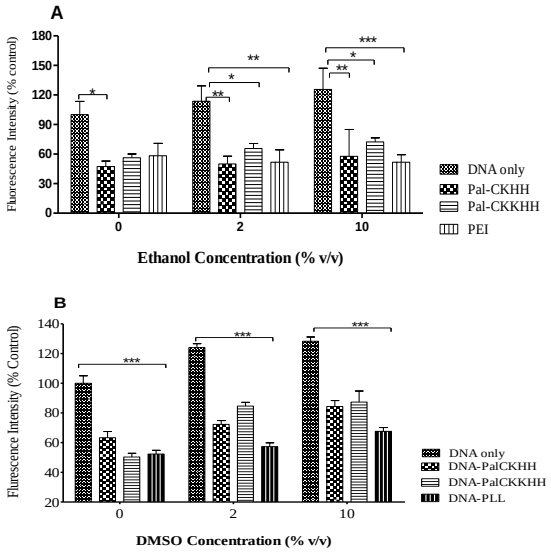

Figure 1. Effect of ethanol (A) and DMSO (B) concentration on EtBr exclusion assay of DNA-transfection agent on charge ratio of 1.0.

Similar to ethanol, the effect of DMSO on EtBr exclusion assay were also evaluated as shown in Figure 1 B. The presence of DMSO in the DNA-EtBr complex potentiated fluorescence intensity slightly as it increased up to 124% and 128% in the present of 2% and 10% DMSO (v/v), respectively. Similar influence was observed on EtBr exclusion assay of Pal-CKHH, Pal-CKKHH and PLL where in the presence of DMSO, the fluorescence intensity increased slightly. This indicated that both DMSO and ethanol (2% and 5% v/v) weakend the interaction DNA-transfection agent in the complex solution as

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the fluorescence intensity increased. However, as shown in Figure 1 B, the transfection agents of Pal-CKHH, Pal-CKKHH and PLL condensed DNA very efficiently in the complex solution without DMSO or in the present of DMSO 2% and 5% (v/v) ($p<0.001$). Based on Figure 1, for working solution of lipopeptide solution, the stock solution (1 mg/ml) was diluted in Hepes Glucose Buffer pH 7.4 to signicantly reduce the amount of DMSO or ethanol in the final solution which was practically far below 0.2%.

To evaluate the capability of lipopeptide and other transfection agents in condensing DNA molecules, the EtBr exclusion assay were performed as shown in Figure 2.

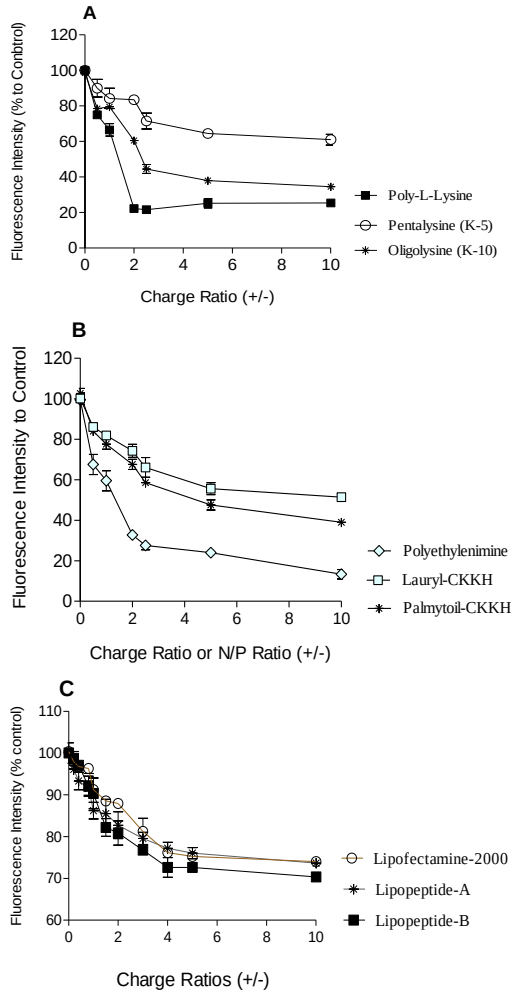

Figure 2. EtBr exclusion assay of DNA-lysine based molecules (A), transfection agent of Polyethylenimine, Lau-CKKH and Pal-CKKH (B), and Lipofectamine, Lipopeptide-A:Pal-CKKHH and Lipopeptide-B:Pal-CKKHH-YGRKKRRQRRR-PKKKRKV (C).

The interaction between DNA molecules and transfection agent is mainly based on ionic and hydrophobic interaction. Molecules of positively charge such as amino acid of Lysine (K) or Arginine (R) will interact and condense efficiently to the negatively charge phosphate backbond of DNA. As shown in Figure 2 A, three molecules based on lysine (Pentalysine, Oligolysine and Poly-L-lysine) have been evaluated in condensing DNA. Compared to other molecule-based lysine, Poly-L-lysine (PLL) was the most efficient transfection agent in condensing DNA molecules as its fluorescence intensity of DNA-EtBr/PLL was the lowest demonstrating that the amount of excluded EtBr by PLL is the highest. Similarly, the amount of EtBr molecule by Pentalysine (K-5) is smaller compared to Oligolysine (K-10). Thus, longer positively charged sequence of the lysine-based complexing agent performs better in condensing DNA. In addition to positively charge, hydrophobic portion of transfection agent such as lipopeptide was very important as shown in Figure 2 B. Palmytoyl-CKKHH (C-16) condensed DNA molecules more efficiently compared to Lauryl-CKKHH (C-12) where in every charge ratio, the fluorescence intensity of DNA-EtBr/ Palmytoil-CKKHH was lower than DNA-EtBr/Lau-CKKHH.

Finally, the ability of short lipopeptide (Lipopeptide A = Pal-CKKHH) in condensing DNA molecules was compared with the long lipopeptide (Lipopeptide B = Pal-CKKHH-YGRKKRRQRRR-PKKKRKV) as shown in Figure 2 C. Based on EtBr exclusion assay, Lipopeptide B was slightly more efficient than Lipopeptide A and Lipofectamine®2000 in excluding EtBr molecules from DNA-EtBr complex solution where at charge ratio of 4.0, about ~30% DNA molecules were condensed.

Effect of thiol group (-SH) of lipopeptide on complex formation

The inclusion of cysteine moiety in the lipopeptide is crucial since the thiol group of the cysteine in the presence of DNA template dimerizes and collapses the DNA molecules into a small, compact particle. This was confirmed when the DNA condensation was carried out in the presence of the reducing agent, Dithiothreitol (DTT). In the presence of DTT, the lipopeptide could not form a dimeric state to condense and trap the DNA molecules efficiently as shown in Figure 3. The data showed that the lipopeptide of Lau-CKKH

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failed to condense DNA in the presence of reducing agent Dithiothreitol (DTT). The fluorescence intensity of the DNA-EtBr/ Lau-CKKH complex particles went up to 110 % in the presence of 1 mM DTT compared to the control. The intensity increased further to approximately125 %, when the complex of DNA-EtBr/Lau-CKKH was incubated in the presence of 2 mM DTT.

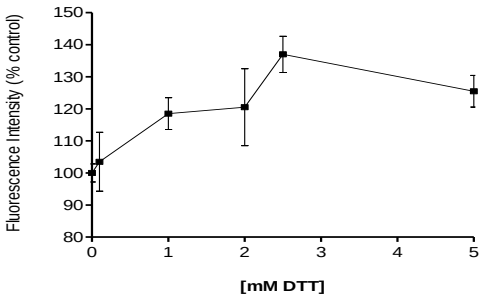

Figure 3. DNA condensation study of Lauryl-CKKH in the presence of reducing agent DTT in DNA-EtBr/Lau-CKKH complex solution.

Discussion

In designing the transfection agent, it was reported the relationship between formulation of cationic phosphonolipids/DNA complexes and lung transfection efficiency (Floch *et al.*, 2000; Loisel *et al.*, 2001). They found that the most important parameters for the cationic phosphonolipid transfection activity were: (i) structure of the cationic phosphonolipid, (ii) the lipid to DNA charge ratio and (iii) the inclusion of co-lipid in the formulation of transfection reagent. Aliphatic chain lengths of the cationic phosphonolipids also had an influence on transfection efficiency and there was a relationship between transfection efficiency and the chemical structures of cationic phosphonolipids. They reported that oleyl acyl chains of phosphono-lipids ($C_{18:1}$) were more efficient than their analogues, myristyl acyl chains ($C_{14:0}$) on transfection activity. Drubruel and co-workers have also designed and evaluated synthetic vectors of cationic polymethacrylates (Dubruel *et al*., 2004; Dubruel *et al.*, 2003). They discovered that the chemical composition and molecular weight of the polymers had influence DNA condensation process where the amount of the polymers needed to condense DNA increased linearly with their molecular weights. Other research groups reported the synthesized multivalent cationic lipids known as lipopolyamines or lipospermines by varying the number, nature and location of charges on the head group (Blessing *et al.,* 1998; Dauty *et al.*, 2001).

To obtain a lipopeptide-based transfection agent which has capability in condensing the DNA molecules effectively, a series of transfection reagents based on the lipopeptide structure has been designed and synthezised. Our rational designs of the lipopeptide constructs are based on the following characteristics:

(1) The lipopeptides have to condense DNA molecules efficiently into small size particles. The inclusion of relatively short alkyl chain of Lauryl (C_{12}) or palmytoil (C_{16}) initiates hydrophobic interactions between DNA and the lipopeptide.

(2) The lipopeptides have to bear positively charged amino acid molecules. This provides the transfection agent with the ability to condense the DNA molecules efficiently at a lower risk of toxicity to the cells. As the molar ratio of the transfection agent to DNA molecule increases, so does the transfection agent's toxicity to the cells which eventually results in a low transfection efficiency (Lesage *et al*., 2002; Slimani *et al.*, 2006). The inclusion of lysine and arginine moieties as positively charged molecule in the lipopeptide was intended to increase lipopeptide-DNA interactions through ionic bonding. This neutralizes anionic phosphate of DNA resulting in a positively charged net of the complex DNA-lipopeptide particles. The positively charged of lipopeptide-DNA complexes have ability to interact with the cell surface polyanions and facilitating the complex entry into the cells.

(3) The transfection agent of lipopeptides have to facilitate the transport of DNA-lipopeptide complexes into the cell cytoplasm. Unprotected DNA molecules are very unstable in the cytoplasm due to enzymatic degradation. Study of the intracellular processing of the cationic lipid-DNA complexes by an electron microscopy has revealed that DNA was released into cytoplasm from an early endosomal compartment (Blessing *et al.*,

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1998; Friend *et al.*, 1996; Zhou & Huang, 1994). The inclusion of histidine in the lipopeptide was designed to provide an escape mechanism from endosomal degradation. This is because histidine is a weak basic amino acid (pI 7.59), which under physiological condition (pH 7.4) would be almost neutral and only partially protonated. However, under endosomal conditions of pH 5.5-6.0, histidine will have higher degree of protonation. Additional protonation of the molecule inside the endosome compartment results in an influx of chloride counter ions (Belguise-Valladier & Behr, 2001; Blessing *et al*., 1998). This osmotic effect causes the rupture and burst of the endosome and results in the release of the DNA plasmid-lipopeptide complexes in the cytoplasm. This is considered to be one of the key steps in the transfection processes (Pouton & Seymour, 1998).

(4). The transfection agent of lipopeptide should also include a Cysteine molecule which acts as a source of thiol group, which as a result of a spontaneous oxidative reaction stabilizes the DNA complex particles (Blessing et al., 1998). Based on these characteristics, a series of lipopeptide molecules were designed and synthesized. The lipopeptide constructs had a varying length of an alkyl side chain (lauryl or palmitoyl), one Cysteine, a number of positively charged amino acid, Lysine, Arginine and a weak basic amino acid Histidine.

The capability of lipopeptide in condensing DNA was monitored by Ethidium bromide (EtBr) fluorescence inhibition which is also known as Ethidium bromide exclusion assay or DNA condensation assay (Bloomfield, 1996; Mishra, *et al.* 2016). It was reported that inhibition of EtBr/DNA fluorescence was not due to fluorescence quenching but due to the expulsion or displacement of EtBr molecules by complexion agent from the DNA structure during complexation (Parker *et al*., 1995; Szumilak *et al.*, 2016). The EtBr exclusion assay basically monitors the DNA collapse into particles as a result of the DNA condensation by cationic polymer or other condensing agents.

The inclusion of an alkyl chain lauryl (C-12) or palmitoyl (C-16) on the lipopeptide molecule probably functioned for initiation of a hydrophobic interactions between DNA-lipopeptide to result a compacted particles and hydrated the complexes to render more stable particles. In this regard, longer hydrophobic portion of palmitoyl (C_{16}) on the lipopeptide molecule might provide a stronger hydration and hydrophobic interaction compared to lauryl side chain (C_{12}) to result more compact and stable particles of DNA-lipopeptide as shown in Figure 2 B.

The inclusion of positively charged lysine in lipopeptide was aimed to provide ionic interaction with negatively charged of DNA molecules. However, as shown in Figure 2A, an ionic interaction was not sufficient to condense DNA as both pentalysine (K-5) and oligolysine (K-10) less effective compared to PLL in excluding EtBr. Cationic polymer of poly-l-lysin (PLL) condensed DNA molecules very efficiently as reported earlier (Jeong & Park, 2002; Mannisto et al., 2002). The high efficiency of PLL in condensing DNA was probably due to the entrapment process of a highly branched PLL besides an ionic interaction between the lysine unit of PLL and phosphate backbone of the DNA molecules.

Like PLL, polyethylenimine (PEI) which is composed of ethylene monomer has ability to condense DNA molecules very efficiently compared to lipopeptide of Lauryl-CKKHH and Pal-CKKHH as shown in Figure 2 B. Such characteristic of PEI in excluding EtBr is due to its polymeric nature (Petersen et al., 2002; Rudolph et al., 2002). As transfection agent, the most important properties of PEI are: (i) its high cationic charge density as every third atom is potentially protonable amino nitrogen, (ii) capability to buffer below physiological pH and (iii) capability to buffer over a very broad of pH range (Katebi *et al.*, 2018; Malik *et al*., 2018; Wightman *et al*., 2001). The data shown in Figure 2 B and 2 C suggests that the highly branched PEI molecules condensed DNA more efficiently than Lipofectamine™. Despite having 15 positively charged protons (N^+) per molecule, Lipofectamine did not condense DNA as efficiently as PEI. In fact, according to Figure 2, at an N/P ratio of 2.0, PEI condensed ~70 % of DNA molecules; whereas Lipofectamine™ only condensed ~10-15 % at the same molar ratio. As a comparison, the lipopeptide Pal-CKKHH condensed DNA slightly higher compared to Lipofectamine™.

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To aid nuclear transport, Ritter and co-workers reported a novel non-viral gene delivery vector consisting of a tetramer of the nuclear localization signal from SV40 large T-antigen (Ritter *et al*., 2003). They found that the 4.4-kDa lysine-rich peptide (NLS) bound and compacted the DNA by electrostatic interactions and formed stable polyplexes. Instead of having rich in lysine residues, Rajagopalan and co-workers reported a sequence rich arginine (R) of the 19-amino acid of MRRAHHRRRRASHRRMRGG was able to bind and condense DNA plasmid (Rajagopalan et al., 2007). This sequence with 12 basic amino acids resulted in positive charges on the protein and enhanced the cationic lipid-mediated transfection. Others reported a basic domain of trans activator transcription (TAT) sequence was responsible for the cellular and nuclear uptake (Wadia & Dowdy, 2003). This uptake was contributed by a nuclear localization sequence (NLS: PKKKRKV) and YGRKKRRQRRR (TAT) sequences. Based on previously reported transfection agent, the lipopeptide composing of Palmytoil (C-16), sequence of CKKHH which has ability in condensing DNA molecules and escaping from endosomal degradation, further inclusion of TAT and NLS was constructed (Lipopeptide-B) and evaluated in EtBr exclusion assay. As shown in Figure 2 C, Lipopeptide-B which has 15 positive charges in total condensed DNA molecules slightly more efficient than Pal-CKKHH (Lipopeptide-A) and Lipofectamine-2000 where at charge ratio of 2.0, approximately 20% DNA molecules had been condensed. Therefore, the inclusion of lysine and arginine residues on the lipopeptide molecule was also expected to mediate cellular and nuclear uptake besides conferring the ability of the lipopeptide to condense DNA molecules efficiently.

Cysteine with sulphur containing R-groups has a pI of 5.02 (pKa_1 = 1.71, pKa_2 = 10.78, pKa_3 = 8.33). This amino acid is therefore slightly anionic under a physiological pH of 7.4 in which the DNA-lipopeptide complex is formed. Cysteine is often involved in the electron-transfer reactions since it is easily oxidized to form cystine molecules in which the two residues of cysteine are joined together by the disulfide bond. The presence of thiol group of cysteine in the lipopeptide molecule is believed to provide disulfide bond between 2 cysteine molecules. This is expected to increase the stability and the compactness of the DNA-lipopeptide complex.

In the presence of DNA template, the lipopeptide bearing cysteine is likely to produce a dimerization as if it had a double alkyl chains. This is the main advantage of having cysteine on the lipopeptide structure since most of the single alkyl chain is less efficient to condense DNA and is generally regarded as toxic to cells. Because of the toxicity effect, the use of single alkyl chain in the gene therapy has been very restricted (Nayerossadat, *et al*. 2012). The data shown in Figure 3 suggests that as the reducing agent concentration of 1,4- Dithiothreitol (DTT) increased, the fluorescence intensity also increased. This implies that in the presence of such reducing agent, the DNA condensing ability of the lipopeptide was retarded. This is also evidence that the lipopeptide (of Lau-CKKH) has dimerized in order to condense DNA molecules efficiently. When the ability of Lau-CKKH to produce dimerization was disrupted by the presence of DTT in complex solution, the lipopeptide could not condense DNA efficiently. DNA was then released from the complex particles and resulted in an increase in the fluorescence intensity. Based on the EtBr exclusion study result, the fully synthesized of lipopeptide-based transfection agents condensed DNA molecules efficiently. However, in order to explore the lipopeptide for transfection agent, further in-vitro evaluation including complex particle stability, particle size characterization and transfection efficiency on mammalian cells are urgently needed.

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