

Optimization Transgene Expression of Short Fully Synthesized Lipopeptide-Based Transfection Agent for Non-Viral Gene Delivery Vehicle

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ABSTRACT

Gene therapy using non-viral gene delivery vehicle might be applied for the treatment of viral infection, cancer and genetic disorders. However, the most challenging barrier is the low efficiency of transgene expression. Therefore, optimization of transgene expression is very important and critical to be performed before doing extensive evaluation of the transfection agent. Herein, we present transgene expression optimization data of gene encoding luciferase condensed with a series of short fully synthesized lipopeptide on COS7, HeLa and 293T cells. The complex formation, effect of molar ratio, forced sedimentation, incubation period of the DNA-transfection agent complexes were explored on transfection efficiency. Moreover, the enhancement effect in transfection efficiency of polymer PEI as co-transfection agent in DNA-lipopeptide was also investigated. The results show that the complex particles of the DNA-lipopeptide were formed efficiently in the low ionic strength environment (HEPES Glucose Buffer pH 7.4), and the complex was incubated more than 10 hours before it was transfected onto the cells. Depending on the lipopeptide, the DNA-lipopeptide incubation in 24 hours (at 4-6°C) resulted an increased up to 2-4-fold in the transgene expression. In addition, polymer PEI enhanced the transfection efficiency of the lipopeptide-mediated gene delivery up to 50-fold compared to control. In summary, short and a linear of fully synthesized lipopeptide-based transfection agent facilitated transgene expression in several mammalian cells by optimizing the DNA-lipopeptide complex formation and transfection condition.

Keywords: lipopeptide, complex formation, transfection and transgene expression.

INTRODUCTION

Briefly, gene therapy can be defined as a technique where genetic materials (DNA and RNA) is transferred into somatic cells for therapy purposes which mostly targeted for serious diseases such as cancer, HIV/AIDS, cystic fibrosis and many others [1, 2]. The technique of gene therapy is very challenging and flexible in term of that it may be used alone or in combination with the conventional treatment. The most challenges and barriers in gene therapy approach are the low

clinical efficacy and safety issues. Delivering the gene of interest using viral-based vector is very efficient, however concern regarding safety issue is the major barriers since a non-virulent virus may turn to a wild type virulent. In contrast, non-viral based vector is safe and relatively non-toxic to the host cells, however, the transfer efficiency is very low. There are approximately 2600 gene therapy clinical trials have been approved or in the progress worldwide where most of them are applied for cancer treatment and mainly still at clinical trial phase 1 or 2 [3].

Although there are many vectors have been developed, their efficiencies are still very low especially in non-dividing cells. Its low efficiency is due to physical and biological barriers which hamper and degrade any genetic material and other molecules entering the cells [4-7]. Polyethylenimine (PEI) is probably the most extensively studied for gene delivery vehicle which may be applied individually [8], conjugated with adenovirus element [9] or with other protein virus and other target molecules. In addition to PEI, amphiphatic peptide composing of histidine residue were reported to transfect mammalian cells as efficient as DOTAP and PEI [10]. Recently, peptide-based gene deliveries have also been widely exploited [11-18]. It was reported that peptide composing of histidine and lysine can be used to deliver siRNA [12, 19]. Farkhani et al reviewed a cell-penetrating peptides (CPPs), a relatively short cationic and/or amphiphatic peptides that can be exploited as efficient cellular delivery vectors because of intrinsic capability in entering the cells and mediating uptake of molecular cargo such as pDNA, siRNA, drugs and nanoparticulate pharmaceutical carriers [12]. Meanwhile, Tagalagis et al reported that targeting peptide ligands formed a stable and biologically safe nanovesicle complexes of plasmid DNA or siRNA with particle size of 90-140 nm [19].

Amongst non-viral gene delivery vehicles, lipopeptide-based transfection agent was probably had gained less attention compared to others. However, since lipopeptide is composed of alkyl chain which is hydrophobic and peptide sequence which is hydrophilic region, lipopeptide has interesting feature and recently has also been achieved many attention worldwide to be explored as non-viral gene delivery vehicles or adjuvant [7, 20-25]. We had reported that lipopeptide composing of palmitoyl chain (C-16) and a short amino acid sequence of cysteine, histidine and lysine compacted plasmid DNA into small and stable particle and facilitated gene delivery into mammalian cells [7]. In this study, we optimized the transfection efficiency of short, linear and fully synthesized lipopeptide-based transfection agent on different cell lines of COS7, 293T and HeLa using gene encoding luciferase as a marker. We also exploited the potentiation of PEI in enhancing lipopeptide transfection efficiency by co-transfection to obtained the optimum formula in condensing the plasmid DNA and delivering into the cells.

MATERIALS AND METHODS

Materials

Unless otherwise indicated, all materials used were of analytical or molecular biology grade and were obtained from Sigma Aldrich (NSW, Australia). Cell culture media Dulbecco's Modified Eagle Medium (DMEM), Opti-MEM[®], and Foetal Calf Serum (FCS) were purchased from GIBCO-BRL (Invitrogen Pty. Ltd., VIC, Australia). Plasmid encoding luciferase (pCMVLuc) was purchased from Clontech, NSW, Australia. Cell lines of COS7, HeLa and 293T were kindly provided by Dr. Han Netter (Department of Microbiology, Monash University, Clayton, VIC, Australia). Reporter Lysis Buffer (RLB), Quantilum Recombinant Luciferase (QLR) and luciferase detection kits were purchased from Promega (NSW, Australia). Lipopeptide-based structure transfection reagents were designed and constructed by Auspep Pty. Ltd. (Parkville, VIC, Australia). Commercially available transfection reagents were obtained from different companies: Transfectam[®] (Promega Pty. Ltd., NSW, Australia), Lipofectamine[™] (Invitrogen Pty.

Ltd., VIC, Australia), Poly-L-lysine (PLL) and Polyethylenimine (PEI) were purchased from Sigma Aldrich Pty. Ltd. (NSW, Australia).

Plasmid Isolation, Lipopeptide Design and Synthesis

The pCMVLuc plasmid encoding luciferase gene was cultivated in *E. coli* strain DH5 α . The culture was grown in Luria Broth (LB) media supplemented with Ampicillin 50 μ g/ml, for 16 hours in 37°C shaking incubator as previously described [7, 26]. The plasmid was isolated using a commercial Qiagen Maxiprep Kit (Qiagen Pty. Ltd., VIC, Australia) in accordance with the supplier's protocol. 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 lipopeptides were synthesized by Auspep Pty. Ltd. (Parkville, Victoria, Australia).

Charge Ratio Determination

Determination of charge ratio (CR) refers as described before where it refers to the number of proton (positive charge of the nitrogen residues) of transfection agent molecules per negative charge of the DNA sugar-phosphate backbone [7]. An average mass of DNA phosphate group (P) of 330 Dalton was used; therefore 1 μ g DNA will be equal to ~ 3 nmoles of anionic phosphate. For Poly-L-Lysine (PLL) solution, an average mass per charge of 128.2 was calculated. For example, to obtain a theoretical charge ratio of 1:1 between PLL and DNA, 1 μ g of DNA (3 nmoles) was mixed to 384.6 ng of PLL (3 nmoles). The term of nitrogen/phosphate (N/P) ratio was attributed to Polyethylenimine (PEI) which under physiological conditions, the nitrogen residue (N⁺) of PEI is only partly protonated. Either C/R or N/P ratio, refer the molar ratio between negative charges of DNA sugar-phosphate backbone and positive charges of the protonated nitrogen residues of transfection agents.

Condensation Assay of DNA-Transfection Agents

In order to investigate the ability of the lipopeptide in condensing DNA, the complex formation was carried out in various concentration of DMSO: 2, 5 and 10% (v/v) by using ethidium bromide (EtBr) exclusion assay as described previously [7]. 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 μ g plasmid DNA and an excess of EtBr (20 μ L; 100 μ g/ μ L) was used to calibrate the spectrofluorometer to 100% fluorescence intensity (λ_{ex} . = 520 nm; λ_{em} . = 610 nm) which was used as reference. For the assays; 50 μ L 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 μ L per well. The samples were left at ambient temperature to stabilize for 2-3 minutes before 20 μ L EtBr solutions were added.

Cell Culture, Complex Formation and Transfection

The mammalian cells (COS7, HeLa and 293T) were cultured in DMEM media supplemented with 10% FCS, 100 units/ml penicillins and 100 μ g/ml streptomycin, grown at 37°C in a humidified

incubator with 5% CO₂. The day before transfection, cells were seeded at 5×10^4 cells/well in the 24-well plates. After reaching a confluence of ~ 60-70%, cells were washed with PBS twice. The media were replaced with Opti-MEM[®] before the complexes of the DNA-transfection agents were added. The complex of DNA-transfection agents were prepared in Opti-MEM[®] or HGB pH 7.4 by diluting DNA and transfection agent separately to obtain a desired charge ratio, mixed and incubated at ambient temperature for a desired period of time: 0.5; 2; 10 and 24 hours. To investigate the effect of HGB pH 7.4 on cell growth, COS7 cells were grown on transfection media composing of HGB pH 7.4 (%) and a proportion of Opti-MEM. After 24-hour growing, cells were harvested, lysed and the total protein content was determined by Bradford Reagent. The transfection efficiencies of DNA-lipo-peptide complex formations carried out in HGB 7.4 were further examined and compared to those carried out in Opti-MEM[®] and DMSO. To further optimize the transfection efficiency of lipopeptide, the transfection time of DNA-lipo-peptide complex was varied for 4, 12 and 24 hours. The transfection efficiency was assayed by adding forced sedimentation or plate centrifugation of 1000 rpm (106 RCF) and 2000 rpm (425 RCF) in Centrifuge 5810 (Eppendorf South Pacific Pty. Ltd., NSW, Australia). The transfection efficiency of the lipopeptides was also optimized in varied charge ratio of 0.5, 1.0, 1.5, 2.0 and 4.0. Finally, the transfection efficiency of the lipopeptide was potentiated by co-transfecting the cells (COS7) with polymer of PEI. Briefly, the confluence cells of ~60 % were transfected with the complexes of 2 µg calf thymus DNA complexed with PEI (N/P ratio 9) and with the particle of DNA-lipo-peptide at a charge ratio of 1.0 before the cells were grown in culture media normally.

Luciferase and Total Protein Detection

After 24 hours incubation, cells were washed with PBS twice then harvested by adding 115 µl/well of Reporter Lysis Buffer (RLB) and incubated for 15 minutes at room temperature. The cells were then scrapped, collected then centrifuged at 13000 g for 2 minutes at 4°C and the supernatants were used for protein and luciferase assays. For luciferase assay, 50 µl of cell supernatant was used to measure the amount of luciferase released from the samples using luciferase detection kit and Quantilum Recombinant Luciferase (QRL) (Promega, NSW, Australia). For protein assay, 50 µl of cell supernatant was used to measure the total protein using Bradford Reagent (Sigma Aldrich, NSW, Australia). All experimental data were plotted and statistically analysed using Graphpad Prism version 5.00 for windows (Graphpad Software, San Diego California USA, www.graphpad.com). Two Ways ANOVA followed by Bonferroni post or paired student's t test were applied where appropriate.

RESULTS AND DISCUSSION

Lipopeptide Construction and Synthesis

In this study, we have constructed a series of a non-viral transfection reagents based on the lipopeptide structures, lauryl- and palmytoyl-CK_xH_y-NH₂. These transfection reagents were designed to pack DNA efficiently, form small complexes, protect the complexes from an enzymatic degradation, and facilitate cytoplasmic transport and cell nucleus uptake. In order to achieve such advantages from the lipopeptide, a series of optimization in the transfection studies have been carried out. Short, linear lipopeptide composing alkyl chain of Lauryl (C-12) or Palmitoyl (C-16), a number of cysteine (C), Lysine (K) and Histidine (H) have been synthesized. Lipopeptide and the controls used in this study is listed in Table 1.

Table 1. The Lipopeptide and Transfection Reagent Used for Nano Particle Formation and Transfection Studies

Lipopeptide/Transfection Reagent	Molecular Weight (Dalton)	Number of proton/molecule
Pal-CKH ₂	761	1
Pal-CK ₂ H ₂	889	2
Pal-CK ₂ H ₃	1026	2
Pal-CK ₂ H ₄	1163	2
Pal-CK ₂ H ₅	1300	2
Pal-CK ₃ H ₂	1017	3
Pal-CK ₃ H ₃	1154	3
Poly-L-Lysine	128 ^{*)}	1
Transfectam®	1263 ^{**)}	3
Polyethylenimine (PEI)	43 ^{***)}	1
Lipofectamine™	3332 ^{****)}	15

^{*)} Poly-L-Lysine (PLL), molecular relative of 1 unit Lysine 128 Da, 1 N⁺/unit, commercially available.

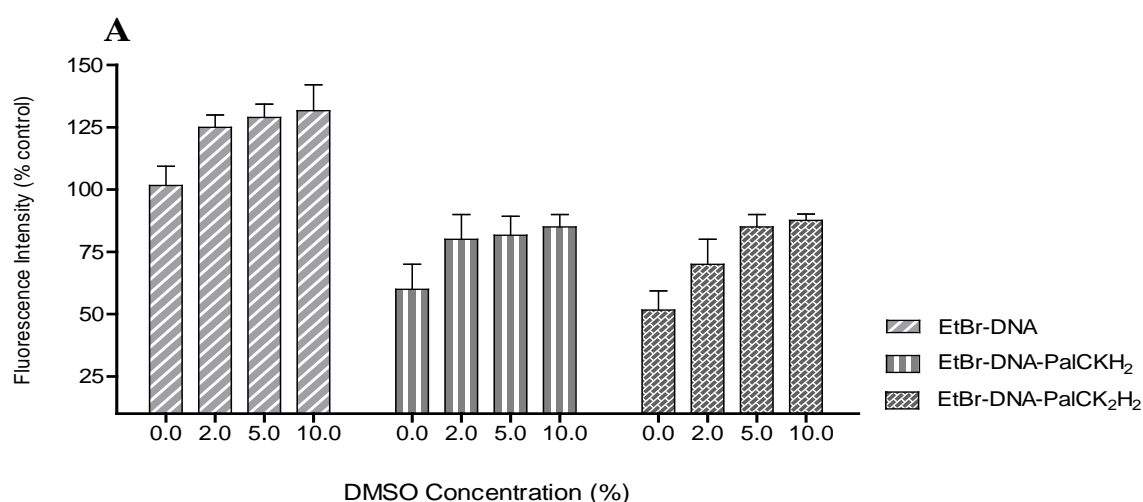
^{**)} Transfectam, molecular relative of 1263 Da, 3N⁺/unit, commercially available.

^{***)} Polyethylenimine (PEI), molecular relative of 1 unit ethylene 43 Da, 1 N⁺/unit, commercially available.

^{****)} Lipofectamine™ (molecular relative of 3332) is composed of 1 molecule of DOPE and 3 molecules of DOSPA, 15 N⁺/molecule, commercially available.

Effect of DMSO Concentration in DNA-Lipopeptide Complex Formation

It was found that the presence of the lipopeptide solvents of DMSO in the complex particles of DNA-lipopeptide could raise problems in the complex formation and transfection. As shown in Figure 1, DMSO had interfered in the complex formation of the DNA-lipopeptide or other transfection agents tested. The efficiency of the lipopeptides of PalCK₂H₂ and PalCKH₂ to condense DNA molecules were statistically different in the presence of 2 % DMSO compared to the controls ($p < 0.001$ for PalCK₂H₂ and $0.001 < p < 0.01$ for PalCKH₂). The data also shown that the fluorescence intensity of the DNA-PalCKH₂ and DNA-PalCK₂H₂ complexes increased as the % DMSO increased in the complex solutions.



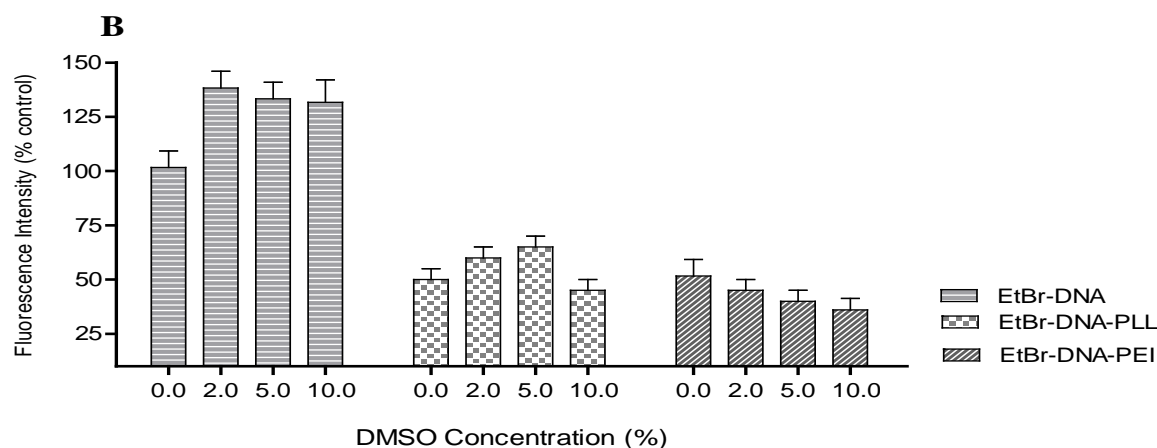


Figure 1. Effect of DMSO concentration in DNA-Lipopeptide complex formation at charge ratio of 1.5

Buffer for DNA-Lipopeptide Complex Formation and Transfection

The effect of HEPES Glucose Buffer (HGB) pH 7.4 on cell growth and transfection efficiency was investigated in COS7 cells. When the cells reached ~60% confluence, the media was replaced with Opti-MEM[®] containing different percentage of HGB pH 7.4. The data shown in Figure 2 suggests that the presence of HGB pH 7.4 during transfection time (~4-5 hours) would only affect the cell growth if there was more than 50 % of HGB pH 7.4 in transfection media.

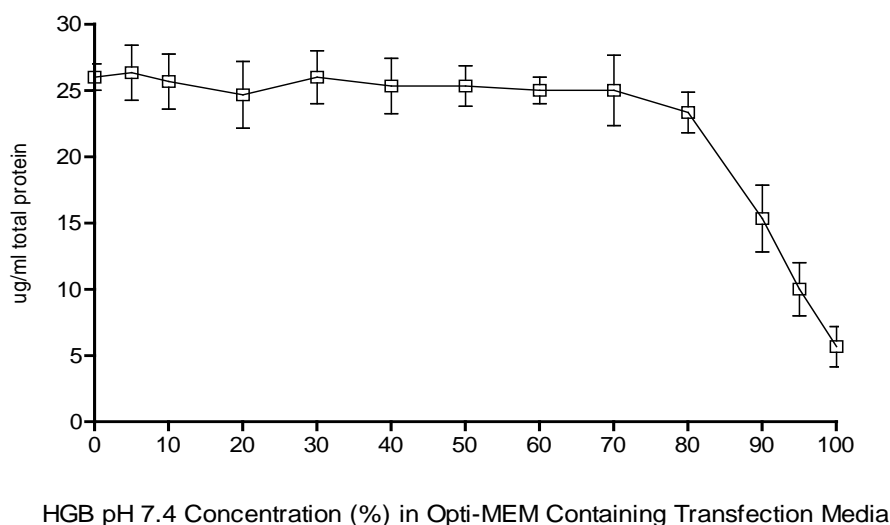


Figure 2. Effect of HGB pH 7.4 on COS7 Cell Growth

The efficiencies of HGB pH 7.4 on the DNA-lipopeptide complex formations and their transfection efficiencies were further examined and compared to those carried out in Opti-MEM[®]. The data shown in Figure 3 implies that the transfection efficiency of the lipopeptide PalCK₃H₂-mediated gene delivery slightly improved when the DNA complex formation was prepared in HGB pH 7.4 rather than in Opti-MEM[®]. There was no significant different in the transfection efficiency of the PEI-mediated gene delivery when the complex of DNA-PEI was prepared in HGB pH 7.4 or Opti-MEM[®]. However, the transfection efficiency of the Transfectam[®] and Lipofectamine[™]-mediated gene delivery prepared in Opti-MEM[®] were higher compared to those prepared in HGB pH 7.4. The buffer probably provides the most suitable ionic strength for the lipopeptide to form complexes with DNA molecules as the ionic strength plays crucial role in the DNA complex formation [27].

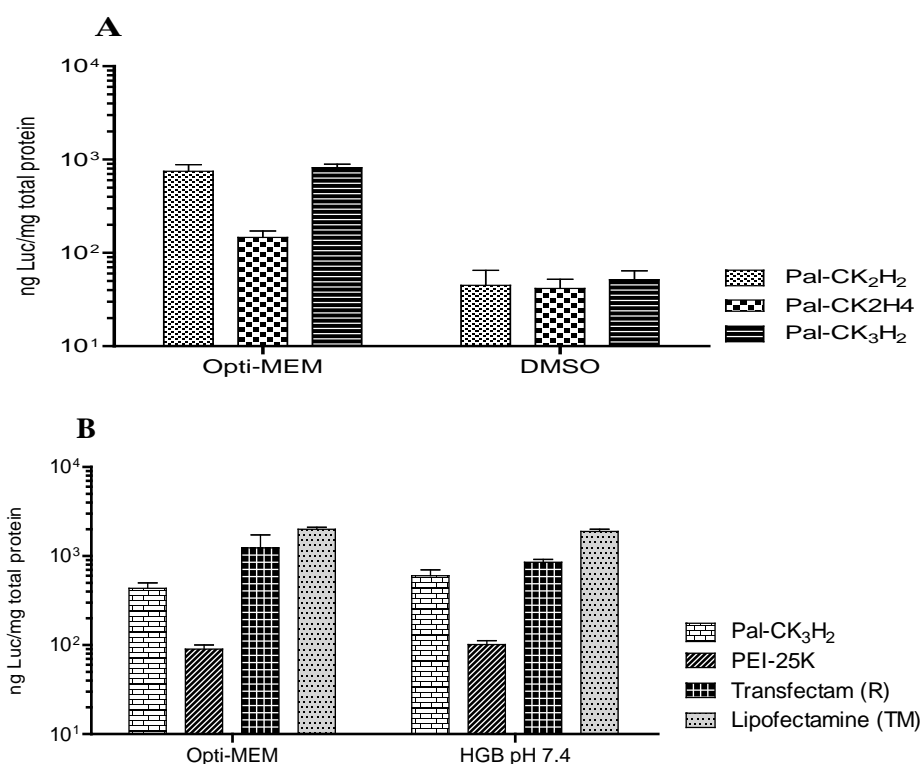


Figure 3. Transfection efficiency of complex DNA-transfection agents of charge ratio 1.5 on COS7 cells: (A) Lipopeptide prepared in Opti-MEM and DMSO; (B) Lipopeptide and transfection agents prepared in Opti-MEM and HGB pH 7.4.

Incubation Time of DNA-Lipopeptide Complexes

In the presence of DNA, the lipopeptide was expected to generate dimer molecules of (Pal-CK_xHy-NH₂)₂/DNA by oxidative reaction. However, it was unclear how long the monomeric state of the lipopeptide turned into a dimer state. To investigate and optimize the time needed to form such complexes, the DNA-lipopeptide particles were formed in a different incubation time.

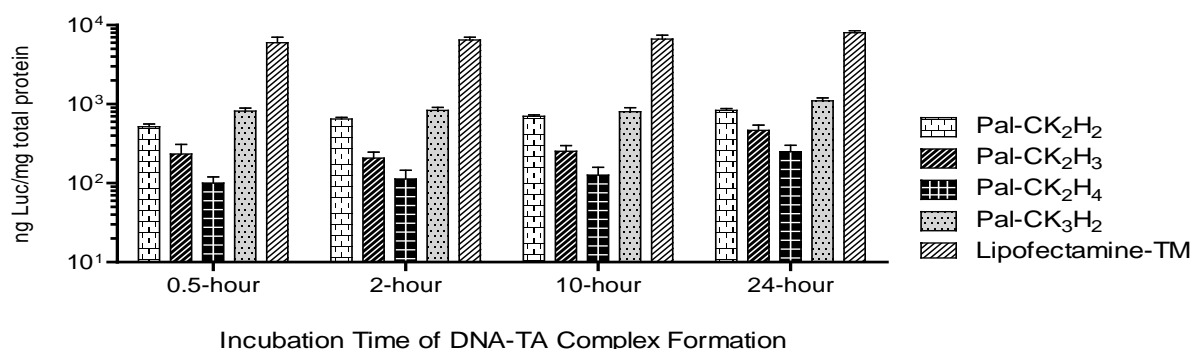


Figure 4. Transfection efficiency on COS7 cells prepared in different incubation time in complex formation.

According to Figure 4, there was no significant difference in transfection efficiency of the DNA-lipopeptides (PalCK₂H₂, PalCK₂H₃, PalCK₂H₄ and PalCK₃H₂) complexes incubated in 30 minutes, 2 hours and 10 hours. However, when the DNA-lipopeptide complex particles were incubated in 24 hours (4-6°C), an increase of the transfection efficiency was observed. Indeed, the transfection efficiency of the complexes incubated in 24 hours increased 2-fold (PalCK₃H₂) and 4-fold

(PalCK₂H₃) compared to those incubated in 30 minutes. The DNA-lipopeptide complex incubation for 24 hours was carried out in the 4-6°C to avoid contamination. The data in Figure 4 suggests that to achieve efficient transfection, it required more than 10 hours time incubation for the complexes. This fact was in contrast with Blessing et al. who reported that the complex formation of dimerized cationic amphiphile guanidinocysteine N-decylamide, C₁₀-C^{G+} was formed within 5 hours in an aerobic condition in the presence of DNA [28]. This discrepancy of the incubation time needed to form such stable complex probably was due to the difference in alkyl chain lengths. Our lipopeptide has hydrophobic portion of Palmytoyl (C₁₆) chain whereas Blessing et al. used decylamide (C₁₀).

Prolonged Transfection Time Period and Forced Sedimentation

Minimum amount of media provides more opportunity to the complex of the DNA-lipopeptide to interact. This fact leads to the idea that sedimentation of the DNA-lipopeptide complex by gravitational force may increase the transfection efficiency. The cells were subsequently subjected to gentle centrifugation of 1000 RPM (106 RCF) and 2000 RPM (425 RCF) after transfection before it was grown normally (Figure 5).

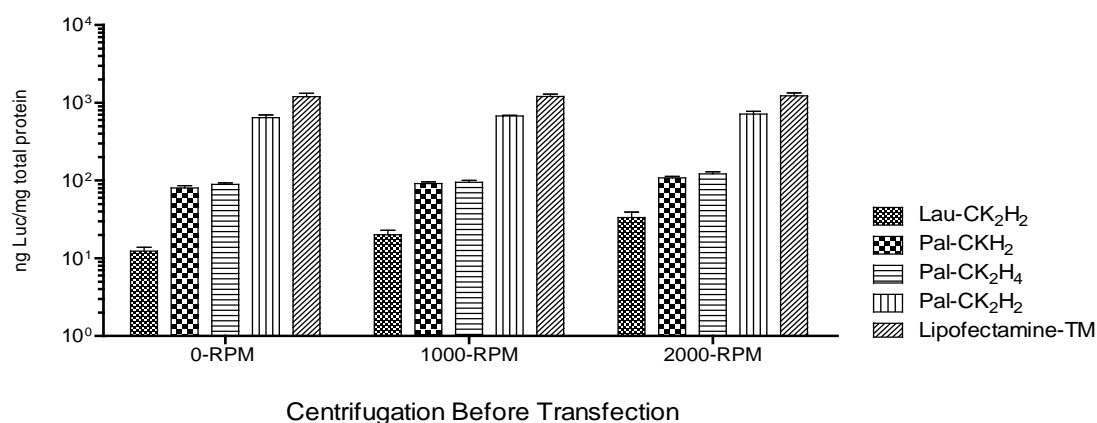


Figure 5. Transfection efficiency on COS7 after plate centrifugation performed at 1000 RPM (106 RCF) and 2000 RPM (425 RCF).

There was no statistically significant difference of the centrifugation performed in the transfection efficiency compared to the controls in LauCK₂H₂, PalCKH₂ and PalCK₂H₄-mediated gene deliveries ($p > 0.05$). However, the effect of centrifugation force at 1000 or 2000 rpm on transfection efficiency mediated by PalCK₂H₂ was different compared to the control ($p < 0.001$). Direct contact of the DNA-lipopeptide complexes with the cells was improved by giving a gentle centrifugation. This treatment provides significant effect in particle sedimentation especially for small DNA complex particles as it is limited by the Brownian motion as retroviruses do [29]. Boussif and co-workers reported that after gentle centrifugation of the complex particles on to the cells, the transfection efficiencies of the Transfectam[®] and PEI improved significantly [30]. The transfection efficiencies of the PalCK₂H₂ were improved 1.5-fold and 2.0-fold after gentle centrifugation for 2 minutes at 1000 rpm and 2000 rpm, respectively (Figure 5). It is generally accepted that a longer transfection time of transfection may lead to higher transfection efficiency. We have conducted several transfection experiments in which we varied the transfection time (4, 12 and 20 hours) to provide the direct contact of the complexes with the cells thus optimize the transfection efficiency as shown in Figure 6.

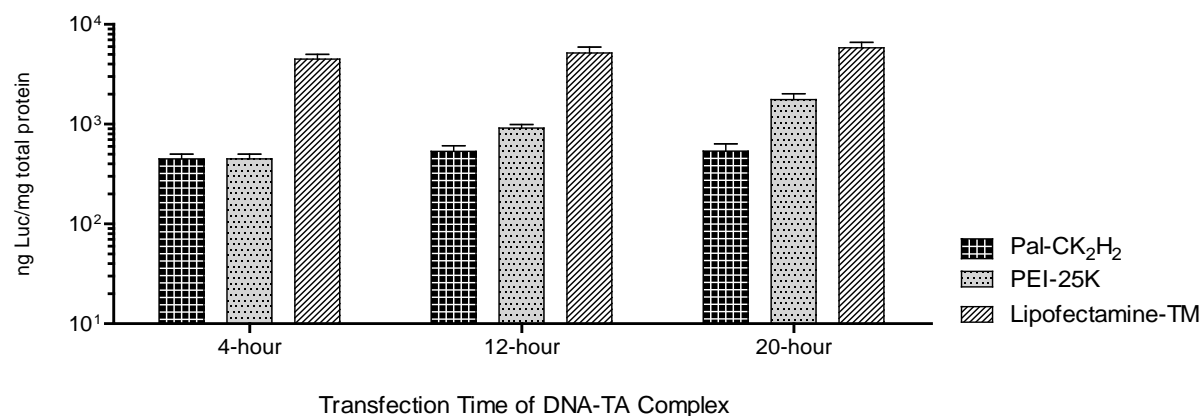


Figure 6. Transfection efficiency of transfection agent on COS7 cells were performed in different transfection period.

There was no statistically significant difference in the transfection efficiencies of PalCK₂H₂ and Lipofectamine-mediated gene deliveries performed in 12 or 20 hours compare to 4 hours transfection time ($p > 0.05$). However, there was ~ 2.5-fold improvement in transfection efficiency of PEI mediated-gene delivery when the transfection was carried out in 12 hours compared to 4 hours. The data shown in Figure 6 also suggests that there was about 6-fold increase in the transfection efficiency of PEI-mediated gene delivery when the transfection time was extended up to 20 hours compared to 4 hours as listed in Table 2. A longer transfection time of PalCK₂H₂ up to 20 hours did not increase the transgene expression significantly. During the contact time of the complexes with the cells, the particles of the PalCK₂H₂-DNA probably floated around during transfection time. This is probably because the sizes of the DNA-lipopeptide complex particles did not make any aggregation and relatively small. The complex particles of the DNA-lipopeptide were formed under low ionic strength of HGB pH 7.4 possibly produce the particle complexes in small sizes as those reported in TfPEI-DNA complexes [31].

Table 2. Effect of transfection time of lipopeptide PalCK₂H₂ on COS7 cells

Transfection Reagents	Luciferase expression ng/mg protein (mean \pm SD)			2 Ways ANOVA with Bonferroni post-test, p values	
	4 hours	12 hours	20 hours	4h vs 12h	4h vs 20h
PalCK ₂ H ₂	313 \pm 10.27	325 \pm 17.3	315 \pm 10.8	NS	NS
PEI	295 \pm 14.72	800 \pm 40.82	1817 \pm 131.2	$p < 0.01$	$p < 0.001$
Lipofectamine TM	2933 \pm 124.7	3000 \pm 216	3200 \pm 216	NS	NS

NS: not significant, $p < 0.01$ = very significant, $p < 0.001$ = extremely significant. The data were obtained from experiment, $n=3$ shown in Figure 6.

Effect of Charge Ratio DNA-Transfection Agent on Transfection Efficiency

Generally, an increase molar ratio of transfection agent to DNA enhances the transfection efficiency. To investigate the effect of an increase molar ratio of lipopeptide-DNA on the lipopeptide-mediated transfection efficiency, the cell lines of 293T, COS7 and HeLa were transfected with complex particles of the DNA-lipopeptides of PalCK₂H₂, PalCK₂H₄ or PalCK₃H₂ prepared in HGB pH 7.4 with charge ratios of 0.5, 1.0, 1.5, 2.0 and 4.0, respectively. The data in Figure 7 shows that 293T cells are the easiest cells to be transfected, since it results the highest

transfection efficiency. The optimum transfection efficiency of the lipopeptide PalCK₂H₂ and PalCK₃H₂ mediated-gene delivery were achieved at a molar ratio of 1.5 in all cells.

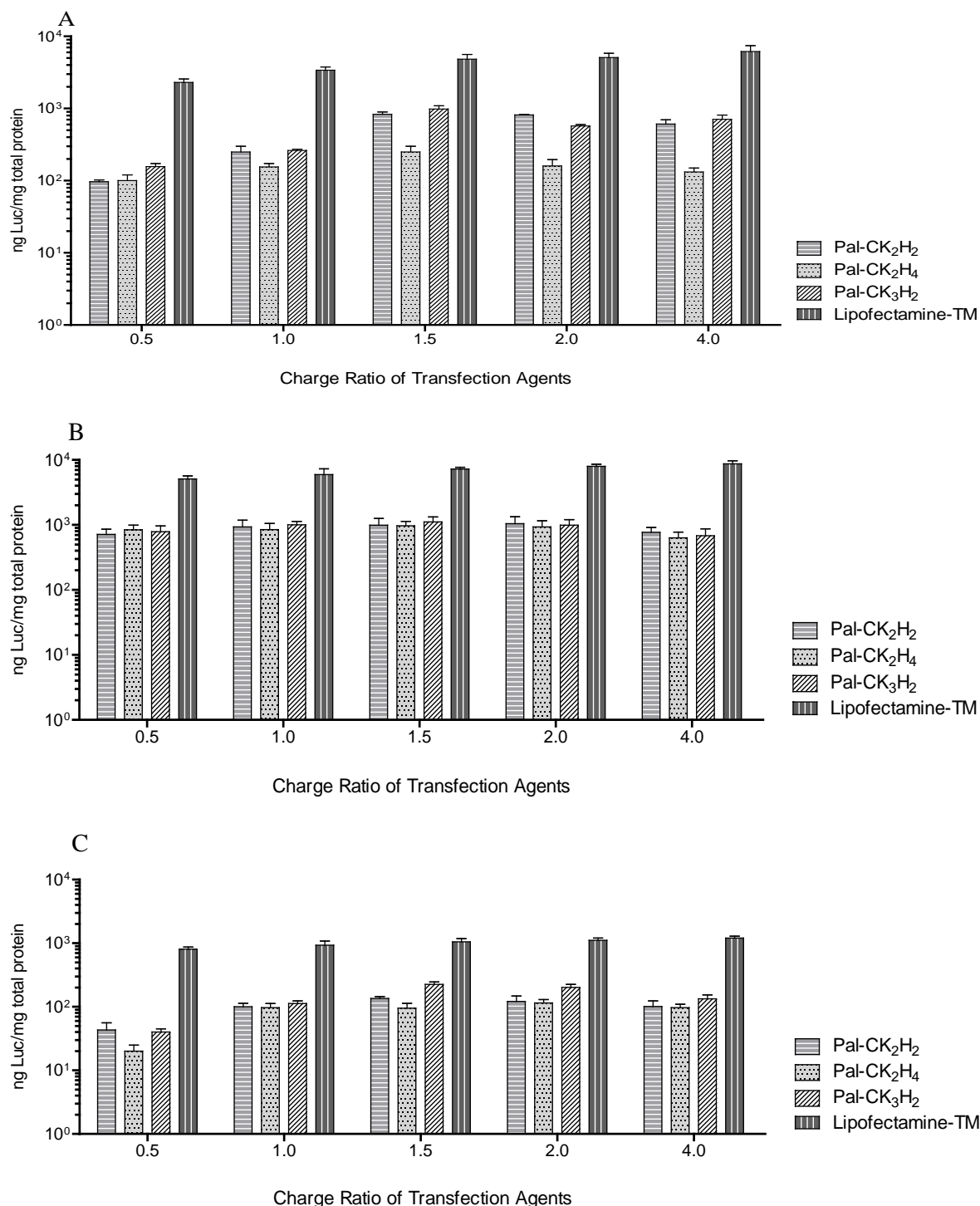


Figure 7 Transfection efficiency of lipopeptide-DNA complexes were performed in different charge ratio on: (A) COS7, (B) 293T and (C) HeLa cells.

In contrast to the lipopeptide, an increase of charge ratio up to 4.0 of LipofectamineTM to DNA tends to increase the transfection efficiency of LipofectamineTM-mediated gene delivery in COS7, HeLa and 293T cells. There were statistically significant difference in the transfection efficiencies of the lipopeptide PalCK₂H₂ and PalCK₃H₂-mediated gene deliveries in the COS7 cells performed in different charge ratio. The data in Figure 7.B shows that the effect of charge ratio of the lipopeptide to DNA on 293T cells were similar to COS7 cells where after reaching the highest

transfection efficiency at a charge ratio of 1.5. Amongst cell lines tested, HeLa cells which, originated from human cervix epithelial carcinoma cells, were the hardest cells to be transfected since it gave the lowest transfection efficiency compared to 293T cells (human embryonic cell lines) and COS7 cells (African green monkey kidney cell lines). It remains unclear why the transfection efficiency of the selected lipopeptides of PalCK₂H₂, PalCK₂H₄ and PalCK₃H₂ decreased at charge ratio more than 2.0. We assume that the decreased in the transfection efficiency in higher charge ratio probably due to the toxic effect of the lipopeptide to the cells as generally shown by cationic lipids or lipopeptide-based liposomes [32]. An increase in charge ratio of the lipopeptide condenses DNA more efficiently and subsequently provides more protection to DNA molecules from nuclease degradation. However, an increase in the molar ratio of the lipopeptide to DNA probably also means an inhibition effect in the cell growth which results in a decrease in the level of the luciferase expression.

Enhancement Transfection Efficiency by Polyethylenimine (PEI)

To increase the compactness of the DNA-lipopeptide complex particles, the cationic polymer Polyethylenimine (PEI) was co-transfected at an N/P ratio of 9 into COS7 cells. The data shown in Figure 8 suggests that the transfection efficiency of the lipopeptides increased by 10-50-fold in the presence of PEI.

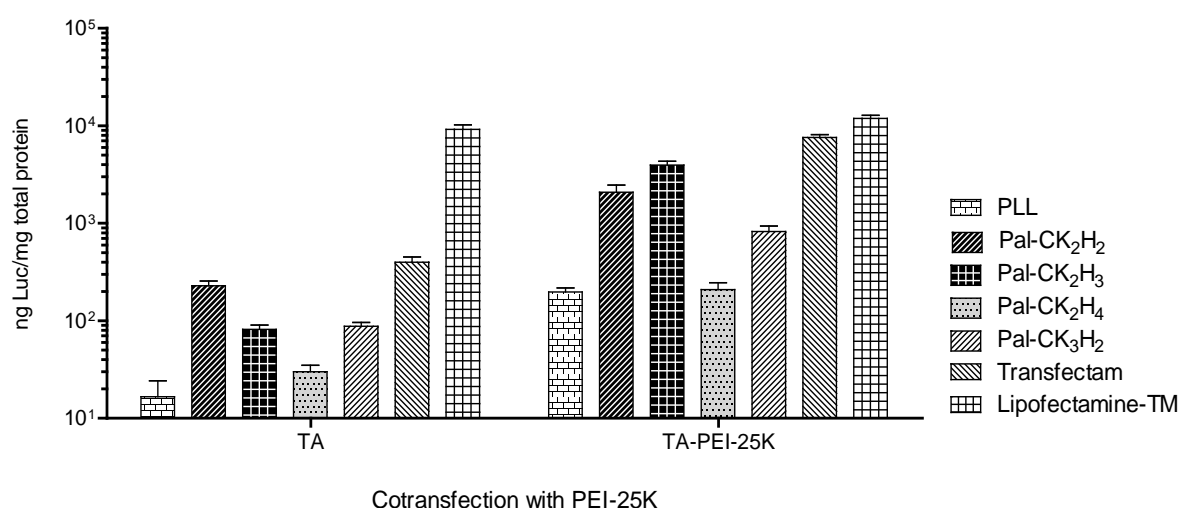


Figure 8. Transfection efficiency of the lipopeptide and transfection agents was enhanced by PEI (N/P of 9.0) on COS7 cells. Data are represented as mean \pm SD of triplicate determinations (n=3). TA = transfection agent.

Polyethylenimine (PEI), is a versatile non-viral vector, able to deliver genetic materials to the cell nucleus and probably the most cationic polymer used as a transfection reagent. It has been studied by many groups [33-42]. The high density of the primary, secondary and tertiary amino groups of PEI exhibits protonation only in every 3rd or 4th nitrogen atom at pH 7.0. This provides significant buffering capacity over wide pH ranges [33, 40]. We have exploited these PEI properties to increase the transfection efficiency of the lipopeptide by mixing the complex of the DNA-lipopeptide with a calf thymus (CT) DNA previously condensed by PEI at an N/P of 9. This formed ternary complexes of the DNA-lipopeptide-PEI particles. As shown in Figure 8, PEI co-transfection on the lipopeptide PalCK₂H₂, PalCK₂H₃, PalCK₂H₄ and PalCK₃H₂ increased the transfection efficiencies up to 6-20-fold higher compared to their controls. It was reported that the transfection efficiency of the cationic liposome improved by 2-28-fold using cationic polymer poly-l-lysine (PLL) in a number of cell lines *in vitro* [43]. The mechanism by which co-polymer such as PEI, PLL or H-K addition in the transfection system improves the transgene expression

has not been fully understood. However, there are several possibilities that may explain the enhanced transgene expression of PEI co-polymer on the lipopeptide mediated-gene delivery. *Firstly*, the co-polymer may reduce the complex particle size and provide an excess of cationic charge resulted in an enhanced cell uptake. It was reported that using an electron microscopic, the complex particles of the liposome-poly-l-lysine-DNA was around 100 nm which was small enough to be taken up by endocytosis [43]. It was also reported that highly branched PEI was able to condense large molecules of DNA to produce a homogenous spherical particle size of 100 nm [44, 45]. *Secondly*, co-polymer PEI may provide additional protection to the complexes of the DNA-lipopeptide from enzymatic degradation and physical damage. Addition of the co-polymer to transfection system may provide more protection and guard the super-coiled DNA conformation from physical and enzymatic degradation which lead in enhanced transgene expression. Complex particles of the DNA-lipopeptide-PEI might be routed and taken up by both clathrin- and caveolae-mediated endocytosis [46]. Taken together the PEI effect on the particle complex sizes, DNA complex protection and intracellular trafficking may explain why the addition of the co-polymer PEI enhanced the lipopeptide transfection more than 50-fold.

Table 3. Co-polymer PEI transfection on lipopeptides mediated-gene deliveries in COS7 cells.

Transfection Agents	ng Luciferase/mg total protein (mean \pm SD)		Paired Student's <i>t</i> Test, <i>p</i> values
	TA (CR of 1)	TA (CR of 1) + PEI (N/P of 9)	
PLL	16.6 \pm 7.6	197 \pm 20.6	<i>p</i> < 0.01
Pal-CK ₂ H ₂	229 \pm 26.56	2083 \pm 382	<i>P</i> < 0.01
Pal-CK ₂ H ₃	81.7 \pm 8.5	3958 \pm 364	<i>P</i> < 0.01
Pal-CK ₂ H ₄	30 \pm 5	208 \pm 38	<i>P</i> < 0.001
Pal-CK ₃ H ₂	87.6 \pm 8.7	825 \pm 114	<i>P</i> < 0.01
Transfectam®	398 \pm 52	7583 \pm 520	<i>P</i> < 0.01
Lipofectamine-TM	9166 \pm 1040	11916 \pm 877	<i>P</i> > 0.05

TA = Transfection Agent, SD = standard deviation, N/P = nitrogen/phosphate, CR = charge ratio, *p* > 0.05 = not significant (ns), *p* < 0.01 = very significant, *p* < 0.001 = extremely significant.

CONCLUSION

The complex particles of the DNA-lipopeptide were formed efficiently in the low ionic strength environment of HEPES Glucose Buffer pH 7.4. The optimum charge ratio of the lipopeptide mediated-gene delivery to DNA was 1.5-2.0 in HeLa, COS7 and 293T cell transfections. The driving force of gentle centrifugation at 2000 rpm (425 RCF) for 2 minutes had doubled the transfection efficiency of the lipopeptide compared to the controls. There was no significant different in the lipopeptide mediated-gene delivery by extending the transfection time up to 20-hours compared to 4-hours transfection time. Co-polymer PEI enhanced the transfection efficiency of the lipopeptide-mediated gene delivery up to 50-fold probably by interfering particle complex sizes, protecting the DNA from enzymatic degradation and involving in the intracellular trafficking through endocytic pathways.

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