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Nanoparticle Evaluation of Synthetic Palmitoyl-CKKHH As Transfection Reagent for Non-Viral Gene Delivery Vehicle

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ABSTRACT
Non-viral delivery systems are relatively safe but inefficient in their current form. The main obstacle in using non-viral gene delivery system approach is to transport the gene of interest in cytoplasm and subsequently entering into cell nucleus. In this research, palmityol-CKKHH and its series have been designed and its ability to form nanoparticle of a stable DNA – lipopeptide complex were evaluated to be used as non-viral gene delivery vehicle. The lipopeptide molecules are composed of alkyl chain of palmitoyl (C-16), and amino acid residues of cysteine (C), lysine (K), and histidine (H). The particle size (nm) and zeta potential (ζ) of the complexes were determined with a Zetasizer Nano Series. It was revealed that prolonging incubation time of the complex composing of DNA and Pal-CK_2H_3 (charge ratio of 1.5) more than 2 hours tend to increase the size up to 300 nm. In addition, increasing DNA concentration up to 40 μg (~ 120 nmoles) with lipopeptide (charge ratio of 1.5), the complex size was still relatively stable at less than 400 nm. As the number of lysine residue on lipopeptide is increased, the particle size tends to decreased. However, the particle size is increased as the number of histidine residue on lipopeptide is increased. It was also shown that increasing charge ratio of the nanoparticle complex resulted in an increased zeta potential but lowering the particle size. Transfection efficiency of the nanoparticle on COS-7 had shown that the lipopeptide has potency as non-viral gene delivery vehicle. To conclude, the lipopeptide composing of alkyl chain of palmitoyl and amino acid residues form a nanoparticle and having potential characteristics to be further explored as non-viral gene delivery vehicle.
Keywords: Palmitoyl-CKKHH charge ratio, nanoparticle, particle size and zeta potential.

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I. INTRODUCTION

To-date, several novel non-viral delivery systems have been developed for transfection of dividing cells in research laboratories. However, few have reached clinical trials, because these transfection systems lack the attributes to deliver DNA to the nuclei of non-dividing cells *in vivo*. It has been estimated that approximately 10^6 plasmid DNA molecules are needed for one cell transfection, with only a few hundred reaching the nucleus (Tachibana et al., 2002). This is due to several physical, chemical, and metabolic barriers that restrict delivery to the perinuclear area. This is in addition to the barrier presented by the nuclear membrane, which has been shown to be a major impediment to gene delivery. Successful delivery of DNA expression vectors therefore requires transfection agents capable of evading these barriers in such a way that intact DNA can be delivered to the nucleus *in vivo* as well as *in vitro.*

The first step in design of an effective pharmaceutical DNA delivery system is to produce condensed particles of DNA with a chemically defined transfection agent, which allows cellular uptake and delivery to the cytoplasm (Pouton and Seymour, 1998). Existing transfection agents include cationic lipids, cationic polymers, peptide, or lipopeptide-based vectors. One problem encountered with typical cationic lipid/DNA complexes or polymer/DNA complexes is that they tend to aggregate into highly poly-disperse mixtures, which are difficult to characterise and have very limited

activity *in vivo*. We have investigated monomeric peptide-based non-viral delivery systems, which we believe offer pharmaceutical advantages, such as ease of manufacture, low-cost, and high purity. These agents could also result in improved control of complexation, and can overcome one of the intracellular barriers to DNA delivery, namely escaping the lysosomal degradative pathway. The general structure of these lipopeptides (Figure 1) includes an alkanoyl chain (linked as an amide to the N-terminal amino acid); a cysteine residue (providing a free thiol group, -SH); and short blocks of lysine and histidine residues, the numbers of which can be varied to optimize lipopeptide transfection efficiency.

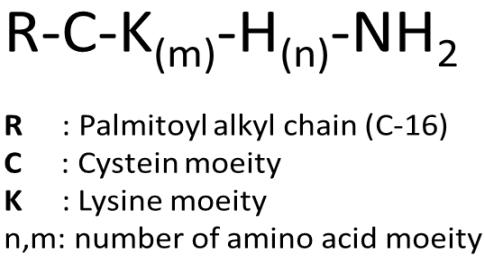


Figure 1. General structure of lipopeptide based transfection reagents

The alkanoyl side chain is included to provide a hydrophobic effect which promotes DNA condensation. Cysteine was included to facilitate dimerization of the lipopeptide molecules in the presence of DNA templates in manner analogous to the strategy used in previous work (Blessing *et al*., 1998; Dauty *et al*., 2001; Lleres *et al*., 2001). The positively charged amino acid, lysine, was used to: (a) provide an initial ionic interaction between lipopeptide and the negatively charged DNA phosphate backbone, and (b) compact DNA molecules into small and stable particles. The size and charge of particles are critical to cell uptake and intracellular trafficking (Pelisek *et al*., 2006; Ross and Hui, 1999a). A number of histidine moieties were also included in the lipopeptide structure to provide an endosomal escape mechanism, which prevents lysosomal enzymatic degradation (Kumar *et al*., 2003; Midoux and Monsigny, 1999; Pichon *et al*., 2000). This is analogous to the use of imidazole-containing compounds as an endosome-lytic agent (Ihm *et al*., 2003; Pack *et al*., 2000). The weakly basic histidine residues were designed to behave in a way that is analogous to the 'proton sponge effect' that is thought to contribute to the transfection efficiency of PEI (Florea *et al*., 2002). However, the imidazole functional group was chosen for its specific *p*Ka value (~6.0), which we believe is a better strategy than to rely on the broad spectrum of *p*Ka values present in PEI, which is the result of various degrees of coulombic repulsion within the polymer. The typical of lipopeptide transfection reagent structure used in this study is presented in Figure 2. The particle size, zeta potential and poly-dispersity of lipopeptide-DNA complex was evaluated. The effect of inclusion of histidine residues in the lipopeptide on the particle size of DNA complexes was found to be opposite to that of lysine. Furthermore, *in vitro* transfection studies in COS-7 cells revealed that the efficiency of delivery of the luciferase encoding plasmid, pCMV-Luc, mediated by lipopeptide construct was much higher than poly-L-lysine (PLL), which lacks an endosomal escape mechanism, and was comparable to that of branched poly-ethylenimine (PEI) (Tarwadi *et al.*, 2008).

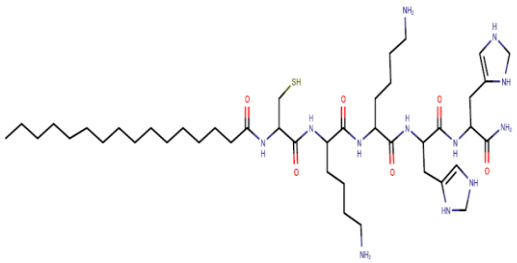


Figure 2. Typically structures of the lipopeptide-based transfection agents composed of palmitoyl chain, cysteine, 2 molecules of lysine and histidine (Pal-CK_2H_2).

II. MATERIALS AND METHODS

Plasmid Isolation

The pCMVluc plasmid encoding luciferase gene (Clontech, NSW, Australia), was cultivated in *Eschericia coli* strain DH5α. The culture was grown in Luria Broth (LB) media supplemented with Ampicillin 50 μg/ml, for 16 hours in 37^{o}C shaking incubator [Jiang *et al*., 1998]. The plasmid was isolated using a commercial Qiagen Maxiprep kit (Qiagen Pty. Ltd., VIC, Australia) in accordance with the supplier's protocol. The quantity and purity of the plasmid DNA was determined by spectrophotometric analysis at 260 and 280 nm as well as by running the plasmid on 0.8 % agarose gel electrophoresis (1 hour, 90 volt). Purified plasmid DNA was resuspended in Milli-Q water (MQW) and frozen (-20^{o}C) for storage.

Lipopeptide Construction

The basic structures of designed 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. The presence of 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 moiety was included in the lipopeptide to be able to buffer the endosome vesicle and escape from endosomal degradation once the complex of the DNA-lipopeptide is taken up by the cells and transported in cytoplasm. Furthermore, the number of lysine and histidine was varied to optimize the lipopeptide transfection efficiency. The lipopeptides were constructed by Auspep Pty. Ltd. (Parkville, Victoria, Australia). The purities of the products were confirmed by High Performance Liquid Chromatography (HPLC) and were $\geq$ 95%. The products molecular weights were confirmed by mass spectral analysis. The lipopeptide and transfection reagents used in this study are listed in Table 1. Polyethelenimine (Sigma Aldrich, VIC., Australia) and Lipofectamine® (Invitrogen, VIC., Australia).

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_2H_2	889	2
Pal-CK_2H_3	1026	2
Pal-CK_2H_4	1163	2
Pal-CK_2H_5	1300	2
Pal-CK_3H_2	1017	3
Pal-CK_3H_3	1154	3
Polyethylenimine (PEI)	43[*)]	1
Lipofectamine®	3332[**)]	15

[*)] Polyethylenimine (PEI), molecular relative of 1 unit ethylene 43 Da, 1 N^+/unit, commercially available.
[**)] Lipofectamine™ (moleculare relative of 3332) is composed of 1 molecule of DOPE and 3 molecules of DOSPA, 15 N^+/molecule, commercially available.

Charge Ratio (N/P) of Lipopeptide/DNA Determination

The charge ratio (C/R) refers to the number of proton (positive charge of the nitrogen residues) of transfection reagent (including lipopeptide) molecules per negative charge of the DNA sugar-phosphate backbone. An average mass of DNA phosphate group (P) of 330 Dalton was used; therefore 1 µg DNA will be equal to 3 nanomoles 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 µg of DNA (3 nanomoles) was mixed to 384.6 ng of PLL (3 nanomoles). Similar calculations were performed to obtain other charge ratios. Rather than using charge ratio, the term of nitrogen/phosphate (N/P) ratio was attributed to the cationic polymer of Polyethylenimine (PEI) which under physiological conditions the nitrogen residue (N^+) of PEI only partly protonated. However, in general both terms, 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 reagents.

Particle Sizing And Zeta Potential

The mean particle size (nm) and zeta potential (ζ) of the DNA-lipopeptide complexes were determined with a Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK). Calibration of particle sizes was carried out with 60 nm $\pm$ 2.7 nm NIST/Nanosphere™ (Duke Scientific Corp. Palo Alto, CA, USA) standard polystyrene spheres. For zeta potential calibration, the -50 mV $\pm$ 5 mV Zeta Potential Standard Transfer was used. The DNA-lipopeptide/transfection agent samples were prepared in HEPES glucose buffer pH 7.4. The mean particle sizes were measured at 25 ^oC using disposable cuvettes (1.5 mL), the zeta potentials were determined at 25 ^oC using the folded capillary cell/Smoluchowski cell (Malvern Instrument, UK).

To study the stability to aggregation of the DNA-lipopeptide complex, 40 µg of DNA (pCMV-Luc) was complexed in separate cuvettes, with each of Pal-CK_2H_2, Pal-CK_2H_3, Pal-CK_3H_2 or Pal-CK_3H_3 (at a charge ratio of 1.5) in a total of 500 µL of HEPES glucose buffer, pH 7.4. There were 3 (three) methods to prepare the complex of DNA-Lipopetide to obtain desired charge ratio: (i) method 1: amount of lipopeptide was added into microtube containing 500 µl DNA solution in HEPES Glucose Buffer pH 7.4, (ii) method 2: amount of DNA was diluted in 250 µl HGB pH 7.4 then added in drop wise manner into 250 µl HGB pH 7.4 containing lipopeptide and (iii) method 3: amount of DNA was added into microtube containing 500 µl lipopeptide solution in HGB pH 7.4 to obtain desired charge ratio. The mean particle sizes were measured at 0.05, 0.5, 1, 2, 8, 30, 60 and 120 hours following complex formation. The effect of increased DNA concentration on particle

aggregation, at a charge ratio of 1.5, was also studied. Amount of DNA (2.5, 5, 10, 15, 20 or 40 µg) was diluted with 250 µL HGB pH 7.4 and then added, in dropwise manner, into 250 µL HGB pH 7.4 containing Pal-CK_3H_2, Pal-CK_3H_3, Polyethylenimine (PEI) or Lipofectamine, to obtain a final DNA concentration of 7.6, 15.2, 30.4, 45.6, 60.8 or 121.6 nM respectively. The effect of increasing charge ratio on particle size and zeta potential was also studied. This was achieved by diluting 5 µg DNA in 250 µL of HGB pH pH 7.4., and added in drop wise into 250 µL HGB pH 7.4 in a microtube containing Pal-CK_2H, Pal-CK_2H_2, Pal-CK_3H_2, or Pal-CK_3H_3 to obtain charge ratios of 0.5, 0.75, 1.0, 2.0, 3.0, 4.0 or 5.0, respectively.

Mammalian Cell Culture Transfection

The mammalian cells of COS7 (African Kidney Green Monkey Cell lines) were cultured in DMEM media supplemented with 10 % FCS, 100 units/ml penicillins and 100 µg/ml streptomycin in T flasks. Cells were grown at 37^{o}C in a humidified incubator with 5 % CO_2. The day before transfection, cells were seeded at 5 x 10^4 cells/well in the 24-well plates. After reaching a confluency of ~ 60-70 %, cells were washed with PBS twice. The media were replaced with Opti-MEM® before the complexes of 5 µg DNA-transfection reagents were added (charge ratio of 1.5). Cells were harvested and centrifuged at 13000 g for 2 minutes at 4^{o}C. For luciferase assay, 50 µl of cell supernatant was used to measure the amount of luciferase released from the samples using luciferase detection kit (Promega, NSW, Australia). The Quantilum Recombinant Luciferase (QRL) (Promega, NSW, Australia) was used as a standard for luciferase assays. For protein assay, 50 µl of cell supernatant was used to measure the total protein using Bradford Reagent (Sigma Aldrich, NSW, Australia). Bovine serum albumin (BSA) was used as a standard for protein assays.

III. RESULTS AND DISCUSSION

1. Particle Size Distribution Of DNA-Lipopeptide Complexes In HEPES Glucose Buffer Ph 7.4

One might expect that the method used to mix the transfection agent and DNA would have an influence on particle morphology, and the subsequent transfection efficiency. As many academic laboratories involved in transfection studies, we did not have large enough quantities of reagents to consider investigating the manufacturing process in detail, i.e. making use of larger volumes and using mechanical mixing devices. We did investigate three different ways of mixing the dissolved reagents (see methods above) to explore the degree to which the particle size was dependent on method of mixing. As shown in Figure 3, the DNA-lipopeptide complex preparation either using method-2 (Figure 3 A) or method-3 (Figure 3 B) was relatively stable until more than 30 hours. The particle size of lipopeptide-DNA complexes prepared with method-1 which formed an aggregation is not shown.

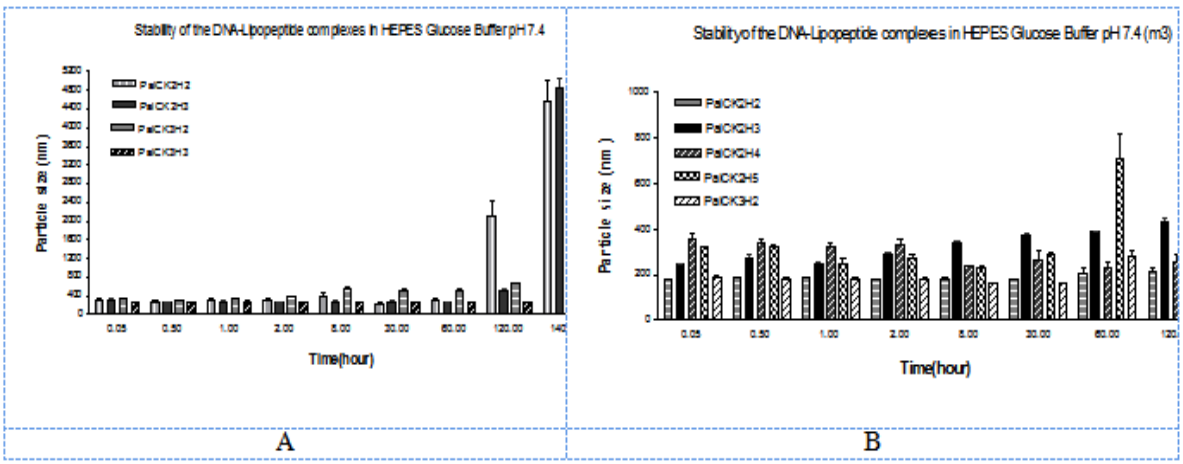


Figure 3. The complex stability of DNA-lipopeptide at charge ratio 1.5 (DNA = 5 µg) formed in Hepes Glucose Buffer (HGB) pH 7.4. **A**. Forty µg of DNA was diluted in 250 µl HGB pH 7.4, then it was dropped wise into 250 µl solution of lipopeptide (method-2), B. Forty µg of DNA was diluted in 500 µl HGB pH 7.4 containing lipopeptide (method-3); The particle sizes were measured using Nanosizer (Malvern, UK) over the time. Data are represented as mean ± SD of triplicate measurements (n=3).

2. Effect of DNA concentration on particle size

Lipofectamine and PEI are the most transfection reagents used for in vitro non viral gene delivery vehicles. However, as it is shown in Table 2, although the charge ratio of transfection reagents and

DNA is kept constant (CR 1.5 and N/P 9 for PEI), the particle size of the complex is getting bigger as DNA concentration is increased. This is not the case for lipopeptide, especially for **Pal-CK_3H_2** and **Pal-CK_3H_3** where although the DNA concentration was increased up to more than 120 nmoles, their particle sizes are relatively stable at approximately 200 – 400 nm.

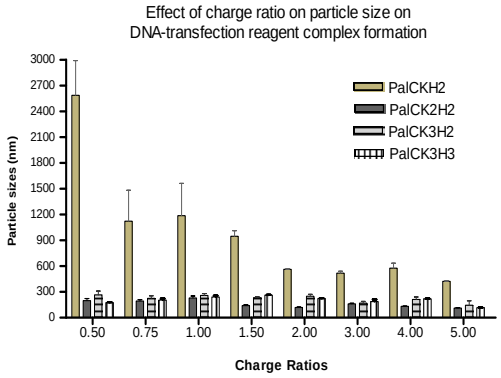
Table 2. Effect of DNA concentration on the particle size (nm) of DNA-transfection reagent complexes at charge ratio 1.5 in 500 µl HEPES Glucose Buffer (HGB) pH 7.4

[DNA], nmoles	Particle sizes (nm), mean ± standard deviation*) on Charge Ratio 1.5 at pH 7.4			
	$PalCK_3H_2$	$PalCK_3H_3$	PEI (N/P) 9	Lipofectamine
7.60	355 ± 10.7	224 ± 8.8	493 ± 3.9	855 ± 10.0
15.20	305 ± 2.6	266 + 33.8	553 ± 22.9	2138 ± 573.2
30.40	313 ± 17.6	226 ± 13.2	877 ± 11.5	2480 ± 23.5
45.60	255 + 9.6	184 ± 2.2	4369 ± 426.9	4590 ± 29.9
60.80	400 ± 4.9	184 ± 0.9	6319 ± 1033.1	5663 ± 1574.9
121.60	397 ± 28.6	275 ± 10.2	9359 ± 591.8	8490 ± 69.8

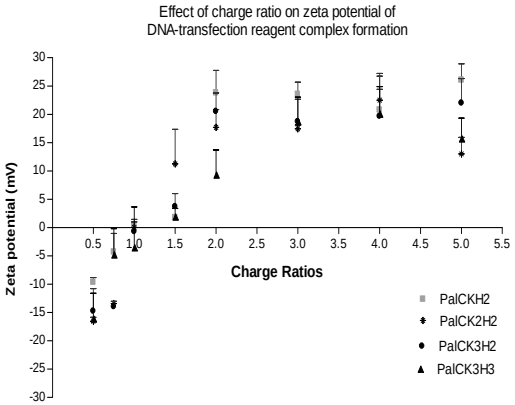
Note: Effect of DNA concentration on the particle size of DNA-transfection reagent complexes at charge ratio 1.5. DNA of 2.5, 5.0, 10.0, 15.0, 20.0, or 40.0 µg was diluted in 250 µl HGB pH 7.4 and then added into 250 µl HGB pH 7.4 containing transfection reagents to achieve charge ratio of 1.5 to obtain 7.6, 15.2, 30.40, 45.60, 60.80 or 121.60 nmoles DNA (method-2). The particle sizes were measured using Nanosizer (Malvern, UK). Data are represented as mean ± SD of triplicate measurements (n=3).

3. Effect of Charge Ratio on particle size and zeta potential

The present of amino acid of lysine in lipopeptide is very crucial in compacting the DNA as shown in Figure 4.A. Compared to other lipopeptides which have 2 or 3 lysine molecules, the particle size of DNA-$PalCKH_2$ is the largest even in an increased charge ratio up to 5. This is because, the lipopeptide has only one lysine molecule which has not efficient enough to condense the DNA molecules which have negatively charge. All lipopeptide molecules which have 2 or 3 lysine molecules condensed DNA molecules efficiently at low charge ratio. However, an increased charge ratio up to 5 is not followed by dramatic decreased in particle size. Meanwhile, increasing charge ratio will be followed by increasing zeta potential. As shown in Figure 4. B, at charge ratio of 0.5, the zeta potential value is negative. In this point, the particle of DNA-lipopeptide would not be taken up by the cells as cell surface is also negatively charge. However, it should be considered that if the particle is too positively charge, it would bind to plasma protein as a result it would be cleared from blood circulation very fast. In practice, particle complex of DNA-transfection reagent is usually designed to be positively charge but should not exceed + 20 mV in zeta potential value.



(A)



(B)

Figure 4. **A**. Effect of charge ratio on particle size of the DNA-lipopeptide complexes. Five µg of DNA was diluted in 250 µl HGB pH 7.4 then added in drop wise manner into 250 µl HGB pH 7.4 containing lipopeptide (method-2). **B.** Effect of charge ratio on zeta potential of DNA-transfection reagent complexes. Five µg of DNA was diluted in 250 µl HGB pH 7.4 then added in drop wise manner into 250 µl HGB pH 7.4 containing lipopeptide (method-2) to obtain 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0,

or 5.0 charge ratios. The particle sizes and zeta potentials were measured using Nanosizer (Malvern, UK). Data are represented as mean ± SD of triplicate measurements (n=3).

4. Effect of Histidine and Lysine Inclusion on Lipopeptide Ability to Compact DNA Molecules

The histidine inclusion in lipopeptide structure is aimed to provide the endosomal escaping ability, since it has a weak base characteristic. However, due to the bulkiness of the histidine, this inclusion resulted in an increased in particle size as shown in Table 3. It is very obvious that as number of histidine is increasing, it is followed by increasing particle size (Pal-CK_2H_2 < Pal-CK_2H_3 < Pal-CK_2H_4). The increasing size of the particle is also followed by increasing polydispersity index (PDI), meaning that the particle formed was tend to aggregate as the PDI value is getting bigger. In contrast, the zeta potential value is decreased as the number of histidine is increased (Pal-CK_2H_2 > Pal-CK_2H_3 > Pal-CK_2H_4). Inclusion histidine more than 4, causing the particle is forming sediment, as the particle size is too big to be measured with the Zetasizer Nano ZS (Malvern, UK).

Table 3. Effect of histidine and lysine inclusion in lipopeptide structures on mean particle size, zeta potential and polydispersity of DNA-lipopeptide complexes at a charge ratio of 1.5 in HGB pH 7.4

Lipopeptide	Particle size (nm) mean ± SD	PDI mean ± SD	Zeta potential (mV) mean ± SD
Pal-CKH_2	688 ± 27.8	0.77 ± 0.05	3.57 ± 1.67
Pal-CK_2H_2	240 ± 4.1	0.31 ± 0.03	13.17 ± 0.74
Pal-CK_2H_3	254 ± 6.2	0.35 ± 0.04	5.44 ± 0.28
Pal-CK_2H_4	724 ± 317.0	0.67 ± 0.18	1.53 ± 0. 25
Pal-CK_2H_5	**Sedimentation***	-	-
Pal-CK3H3	**247 ± 3.3**	**0.27 ± 0.001**	**6.28 ± 0.48**

Complexes were formed by diluting 40 µg DNA (~243 nM), data are presented as mean ± SD of triplicate measurements (n = 3).

*) Sedimentation; the complex solution quickly formed aggregates that sedimented, therefore their mean particle sizes and other particle properties could not be measured with the Zetasizer Nano ZS (Malvern, UK).

5. Transfection Efficiency of DNA-Lipopetide on COS7 Cell Lines

To date, Lipofectamine (TM) is regarded as the golden standard for in vitro transfection. As shown in Figure 5, the highest transfection efficiency is given by Lipofectamin (TM), where it was insensitive to mixing method and was significantly more effective than PEI or the most effective Pal-CK_mH_n lipopeptide. However, it should be noted that the transfection efficiency of lipopeptide is comparable to those given by PEI. It was clear that method 2 and 3 gave better transfection efficiency results. Nevertheless, the complex of DNA-lipopeptide (charge ratio of 1.5, DNA = 5 µg) prepared by method 1 is undesirable since this particle tend to aggregate (data not shown). We speculate that higher transfection efficiency of particle complex prepared by method 1 compared to method 3 is due to the particle complex of DNA-transfection reagents enter the COS7 cells when these cells are dividing. It might not happen when transfection process is carried out in non-dividing cells where the aggregate particles will not be easy to enter the cell nucleus.

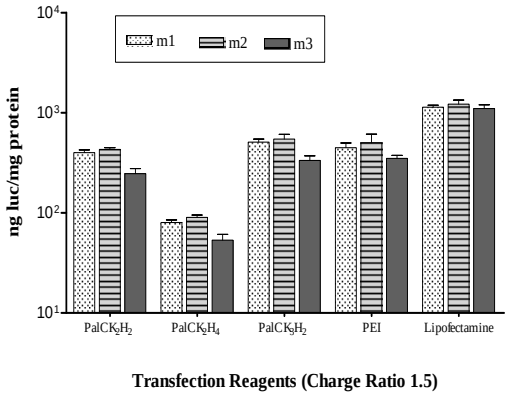


Figure 5. Transfection efficiency of DNA - lipopeptide particle on COS-7 at charge ratio 1.5 (DNA = 5 µg) by 3 different particle preparations. Method-1: Amount of transfection reagent is added into DNA solution, Method-2: DNA was diluted in 250 µl HGB pH 7.4, then it was dropped wise into 250 µl solution of lipopeptide, Method-3: DNA was diluted in 500 µl HGB pH 7.4 containing lipopeptide. Data are represented as mean ± SD of triplicate measurements (n=3).

As shown in Figure 5, the transfection efficiency of lipopeptide bearing 3 lysine residues (Pal-CK_3H_2) is higher slightly compared to those lipopeptide bearing 2 lysine residues (Pal-CK_2H_2). Furthermore, the transfection efficiency of Pal-CK_3H_2 is comparable to PEI. However, it is very

obvious that the transfection efficiency of Pal-CK_2H_4 is significantly lower than Pal-CK_2H_2 and Pal-CK_3H_2. It suggested that inclusion more histidine residues on lipopeptide make the transfection reagent less efficient partly due to the enlarging particle size and increasing polydispersity of the complex lipopeptide-DNA which hinder the cell uptake and subsequent gene delivery process.

IV. CONCLUSION AND FURTHER DIRECTION

The Palmitoyl-based lipopeptides have ability to make a nanoparticle when it was complexed with DNA plasmid. The lipopeptide has potency to be used as an efficient gene delivery vehicle by further optimizing the structure of lipopeptide. The addition of lysine residue on lipopeptide decreases the size of the complex, meanwhile addition of histidine residue tend to increase the size of DNA-lipopeptide complexes as well as its zeta potential. The present of histidine residue on lipopeptide provide the complex to escape from endosomal degradation before it enters the cell nucleus. The transfection efficiency of palmitoyl-based lipopeptide is comparable to that of branched PEI (poly-etyleneimine).

The present of alkyl chain and amino acid residues have to be optimum in condensing DNA molecules in compact size yet it should release the DNA molecules before entering cell nucleus. For further research, in order the complex of lipopeptide-DNA to enter cell nucleus especially in non-dividing cells, the DNA should be coupled or complexed with a sequences such as nuclear localization sequence derived from Cytomegalovirus (CMV) or trans-activating transcriptional activator (TAT) from human immunodeficiency virus 1 (HIV-1) to improve the cell uptake.

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