Effect of Tween 20 Inclusion in Oligopeptide-Based Liposome Formulation on Transfection Agent/DNA Particle Size, Complex Stability and Gene Expression

Tarwadi Tarwadi[1](✉), Damai R. Setyawati[1], Sabar Pambudi[1], Prasetyawan Yunianto[2], Rahmana E. Kartasasmita[3], Heni Rachmawati[3,4], and Sukmadjaja Asyarie[3]

[1] Mammalian Cell Culture Laboratory of LAPTIAB-Research Centre for Vaccines and Drugs, National Agency for Research and Innovation (BRIN), Puspiptek Area, South Tangerang, Indonesia
tarw001@brin.go.id

[2] Research Centre for Bulk Pharmaceuticals and Traditional Medicine, National Agency for Research and Innovation (BRIN), Puspiptek Area, South Tangerang, Indonesia

[3] School of Pharmacy, Bandung Institute of Technology, Bandung, Indonesia

[4] Research Centre of Nano Sciences and Nanotechnology, Bandung Institute of Technology, Bandung, Indonesia

Abstract. The main requirement of a transfection agent has to condense DNA efficiently, protect the DNA from nucleases and other degrading enzymes during its transport in a target cell. In this research, Polysorbate 20 which is commercially known as Tween 20 has been supplemented to oligopeptide-based liposome formulation in order to achieve a stable and effective transfection agent in mediating gene expression. Complex particle size and its stability of transfection agent/DNA were determined by dynamic light scattering analysis. Transfection study was carried out using green fluorescence protein (GFP) encoding gene on HepG2 cells. It was revealed that the addition of Tween 20 (0.5% v/v) on liposome formulation reduced the particle size of the liposome/DNA complexes. The addition of Tween 20 in liposome capable to condense DNA molecules effectively. The particle size of the liposome/DNA at charge ratio 1.0 was ~340 nm; meanwhile, Tween 20 increased the zeta potential value at any charge ratio applied. Interestingly, the inclusion of the Tween 20 has also stabilized the liposome as the particle size almost unchanged after the complexes of the liposome/DNA was monitored for 240 h (10 days). Unfortunately, we did not observe that the addition of Tween 20 in the liposome formulation (0.5% v/v) has beneficial for gene expression as the GFP expression level reduced compared to the corresponding oligopeptide. In summary, Tween 20 (0.5% v/v) addition in the liposome formulation has condensed and stabilized the liposome/DNA complex in nanoparticle size, however, it needs further exploration in formulating Tween 20-containing liposome to achieve optimum beneficial in gene expression.

Keywords: Tween 20 · liposome formulation · transfection agent · particle size · nanoparticle

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1 Introduction

Gene delivery vehicle or vector which capable to condense, protect and facilitate gene expression is urgently needed in order to deliver the gene of interest. Due to safety issue, the use of non-viral gene delivery vehicle which well recognized as transfection agent has gained much attention from biomedical research communities [1]. Amongst non-viral gene delivery vehicle, cationic liposome-based transfection agent is the most widely used to deliver a naked or pure plasmid DNA. Liposomes are artificial, spherical vesicles enclosed by one or more phospholipid bilayers having an aqueous core that can be used for the delivery of biologically active molecules [2, 3]. Typical liposome structures are generally formed by self-assembly of dissolved lipid molecules which have hydrophilic head groups and hydrophobic tails to form bilayer membranes and subsequently generate liposome vesicles [4]. Structure of the liposome mimics or resembles biological membranes; therefore, it is also a perfect model to study and explore a function of cell membranes and drug delivery [5, 6]. Many studies have focused on the liposome-based gene delivery vehicle for in-vitro and in-vivo transfection enhancement. As nucleic acid or drug carriers, liposomes have several advantages which include: [1] enhanced drug efficacy and therapeutic index, [2] encapsulation increases liposome stability, [3] non-toxic, flexible, biocompatible, biodegradable, and non-immunogenic, [4] reduced toxicity of the encapsulated drugs, and [5] reduce the exposure of sensitive tissues to toxic drugs [2, 7–11].

Polysorbate 20 is a non-ionic surfactant, chemically as polyoxyethylene-sorbitan-20-monolaurate ($C_{58}H_{114}O_{26}$, molecular weight 1228) is commercially known as Tween 20, has been used as an excipient in pharmaceutical applications to stabilize emulsions and suspensions [12]. Tween 20, which has fatty acid ester moiety and a long polyoxyethylene chain, has been used primarily as a water-in-oil emulsifier [13]. Tween 20 has also been explored for a stabilizing agent in topical pharmaceutical formulations in drug delivery systems as well as in mediating cellular uptake and gene expression [14]. The aims of this research are to prepare and evaluate the effect of Tween 20 in liposome-based transfection agent in order to achieve a stable and effective in mediating gene expression. Complex particle size of transfection agent/DNA, cytotoxicity and gene expression mediated by the liposome were evaluated in order to achieve effective and safe gene delivery vehicle.

2 Materials and Methods

2.1 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 fetal calf serum (FCS) were purchased from GIBCO-BRL (Invitrogen Pty. Ltd., VIC, Australia). HepG2 was a kind gift from Tjandradewi Mosef (Indonesian Institute of Science, Indonesia). The plasmid encoding fluorescence protein of pCSII-EF-AcGFP (9880 bp), was kindly gifted

from Takeshi Kurosu Laboratory (Research Institute of Microbial Diseases, Osaka University, Japan). EMR carbon support films and the UranyLess-E.M. stains were purchased from Microscopy Solution (EMS, Hatfield, UK). Commercially available transfection reagents were obtained from different companies: 1,2 Dioleoyl-sn-glycero-3-phosphatidylethanol-amine (DOPE) was purchased from Avanti Polar Lipids, Inc. (VIC, Australia), Poly-L-Lysine (PLL) was purchased from Sigma Aldrich Pty. Ltd. (NSW, Australia). Oligopeptide of GRKKRRQRRRPKKRKV and CKKHH- GRKKRRQRRRPKKRKV (OP-1 and OP-2) were synthesized in Peter Doherty Institute-Melbourne University, VIC, Australia.

2.2 DNA Isolation

Plasmid pCSII-EF-AcGFP was maintained in E. coli DH5 and isolated using QIAGEN® QIAprep Maxi Kit (Qiagen Pty. Ltd., Vic, Australia) under the supplier's protocol. The quantity and purity of the plasmid DNA were determined by spectrophotometric analysis at 260 and 280 nm as well as by running the plasmid DNA on 1% agarose gel electrophoresis after single digestion with a restriction enzyme of BamH1 (30 min, 90 V). The purified plasmid DNA was resuspended in Milli-Q water (MQW) and frozen (–20 °C) for storage.

2.3 Liposome Preparation

The oligopeptides of OP-1 and OP-2 were formulated with DOPE in molar ratio 1:1, 1:2, and 2:1 to form liposome. Briefly, 2260 μg (OP-1), 3060 μg (OP-2), respectively diluted in HGB pH 7.4 and mixed with the DOPE solution (1 mg/mL) to obtain a molar ratio of the transfection agent: DOPE: 1:1, 1:2 and 2:1. After the thin lipid layer was formed, the amount of the HGB pH 7.4 was added to achieve a liposome concentration of 1 mg/mL. Water bath sonication process was carried out to achieve transparent solution (~2 h) then a portion of the liposome (1 mg/mL) was mixed with 0.5% (v/v) Tween 20. The liposome solution with and without Tween 20 was then filtered (0.22 mm) and stored at 4 °C before it was used in transfection studies.

2.4 Determination of Particle Size and Zeta Potential

The mean particle sizes and zeta potential values of the oligopeptides of OP-1 (MW: 2260) and OP-2 (MW: 3057)-based liposome/DNA complex particles were determined at charge ratio 1.0, 4.0, 8.0, 16.0, and 32.0. Briefly, 5 μg DNA plasmid in 500 μL HGB pH 7.4 with the transfection agents of OP-1: DOPE (1:1); OP-1: DOPE (1:2); OP-1: DOPE (2:1); OP-2: DOPE (1:1); OP-2: DOPE (2:1); and OP-2: DOPE (2:1) in 500 μL HGB pH 7.4, respectively to obtain a certain charge ratio. Following incubation for ~30 min, the particle size of the complexes was determined. Similarly, the oligopeptide-based liposome containing Tween 20/DNA complexes of the OP-1: DOPE: Tween 20 (1:1:0.5%), OP-1: DOPE: Tween 20 (1:2: 0.5%), OP-1: DOPE: Tween 20 (2:1:0.5%), OP-2: DOPE: Tween 20 (1:1:0.5%), OP-2: DOPE: Tween 20 (2:1:0.5%), and OP-2: DOPE: Tween 20 (2:1: 0.5%) in 500 μL HGB pH 7.4, respectively were also measured

their mean particle sizes to evaluate the effect of 0.5% (v/v) Tween 20 addition in the oligopeptide-based liposome/DNA complexes. For charge ratio calculation, molecular weight (MW) of the liposome was referred to the molecular weight of lipopeptide or oligopeptide.

2.5 GFP Expression

Transfection of HepG2 cells were performed in 96-well plate using 300 ng DNA (pCSII-EF-AcGFP)/well mixed with oligopeptide of OP-2, liposome-based OP-2 which was formulated with Tween 20, and PLL on HepG2 cells to achieve charge ratios of 2.0, 4.0, 6.0, and 8.0. The transfection solution was prepared by adding DNA solution in 12.5 L HGB pH 7.4 mixed in drop wise manner with respective transfection agent solution in equal volume of HGB pH 7.4. The complex solution was incubated at room temperature for at least 5 min before the transfection process. To study the GFP expression, the cells were cultured (10,000 cells/well) in DMEM supplemented with 10% FCS, 100 unit/mL penicillin, and 100 mg/mL streptomycin in humidified incubator with 5% CO_2 at 37 °C. After reaching ~60% confluence, the cells were washed with PBS, and the media was replaced with 100 L fresh basal culture media/well before adding a transfection solution. Subsequently, the cells were incubated in a humidified incubator with 5% CO_2, at 37 °C, then viewed under fluorescence microscope Axiovert 40 CFL (Carl Zeiss Microscopy GmbH, Germany) after 24-h transfection. After a 24-h transfection process, the green fluorescence protein expression was visualized and compared qualitatively.

2.6 Statistical Analysis

Each treatment in all experiments of DNA condensation, particle size and zeta potential analysis, and GFP expression studies were carried out in triplicate. Data were plotted using GraphPad Prism 5 and analyzed using One Way Anova followed by Dunnett's Post Test, and unless otherwise stated, all cases of significance were set at $p < 0.05$.

3 Results

3.1 Liposome Particle Size and Stability

The addition of DOPE was intended to increase the lipidation degree of the oligopeptide, meanwhile, Tween 20 (0.5%, v/v) was included to stabilize the liposome solution. As revealed in Fig. 1, as the DOPE molar ratio in the liposome formulation increased (OP-1: DOPE = 1:2), the particle size of the liposome/DNA at charge ratio 1.0 increased from ~600 nm to ~900 nm. However, as the OP-1 molar ratio increased in the liposome preparation (OP-1: DOPE = 2:1), the particle size of the liposome/DNA at charge ratio 1.0 dropped to ~335 nm (Fig. 1A). Interestingly, the OP-1-based liposome formulated with Tween 20 (0.5% v/v) had compact the complex particle size (~200 nm to ~400 nm), including for those with a higher portion of the lipid (OP-1: DOPE: Tween 20 = 1:2:0.5%) as revealed in Fig. 1B.

Like the oligopeptide of OP-1, the particle size of the longer oligopeptide of OP-2/DNA was smaller when Tween 20 (0.5% v/v) was added to the liposome (Fig. 2).

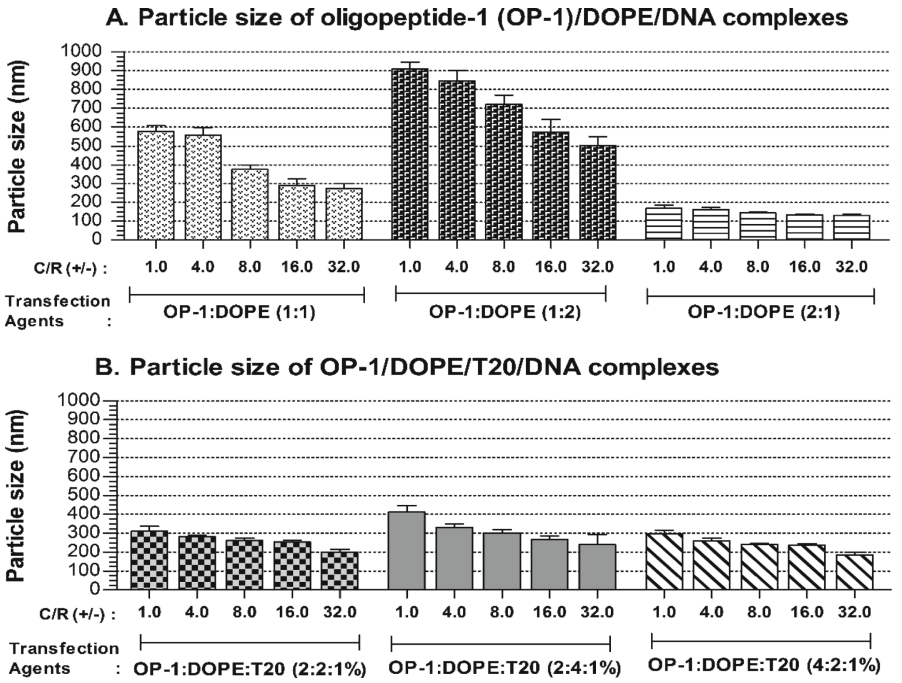

Fig. 1. Particle size (nm) of the liposome-based OP-1/DNA in HGB 7.4 at charge ratios of 1.0–32.0: A. OP-1/DOPE/DNA; B. OP-1/DOPE/Tween 20/DNA. OP-1: GRKKRRQRRR-PKKKRKV. Data are represented as mean ± SD (n = 3).

Addition of Tween 20 in the OP-2-based liposome significantly reduced the particle size of the liposome/DNA complexes, whereas at charge ratio 1.0 from ~552 nm (Fig. 2A) to ~347 nm (Fig. 2B).

Although the portion of DOPE in the liposome formulation increased (OP-2: DOPE = 1:2), the addition Tween 20 (0.5% v/v) reduced the particle size of the complexes (Fig. 2). It was revealed that particle size of liposome/DNA complexes at charge ratio 1.0 reduced from ~875 nm (OP-2: DOPE = 1:2) to approximately 468 nm when Tween 20 was included (OP-2: DOPE: Tween 20 = 1:2: 0.5%). Interestingly, when the molar ratio of OP-2 increased (OP-2: DOPE = 2:1), addition Tween 20 (0.5% v/v) in the liposome formulation made the particle size of the liposome/DNA complexes almost unchanged in the range of charge ratios: 1.0–32.0 (~213 nm to ~173 nm). The addition of Tween 20 in the liposome formulation decreased the particle size of the liposome/DNA; however, it increased the zeta potential value at any charge ratio applied (Fig. 3). It was observed that at charge ratio 4.0, the zeta potential value of the liposome/DNA increased from ~5 ± 1.7 mV (OP-1: DOPE = 2:1) to 13.7 ± 3.8 mV (OP-1: DOPE: Tween 20 = 2:1:0.5%) as shown in Fig. 3A.

Likewise, the addition of Tween 20 (0.5 v/v) in the liposome-based OP-2 increased the zeta potential value of the liposome/DNA at any charge ratio applied. It was noticeable that the addition of Tween 20 in liposome at charge ratio 4.0, the zeta potential increased from 8.1 ± 1.5 mV (OP-2: DOPE = 2:1) to 13 ± 1.7 mV (OP-2: DOPE:

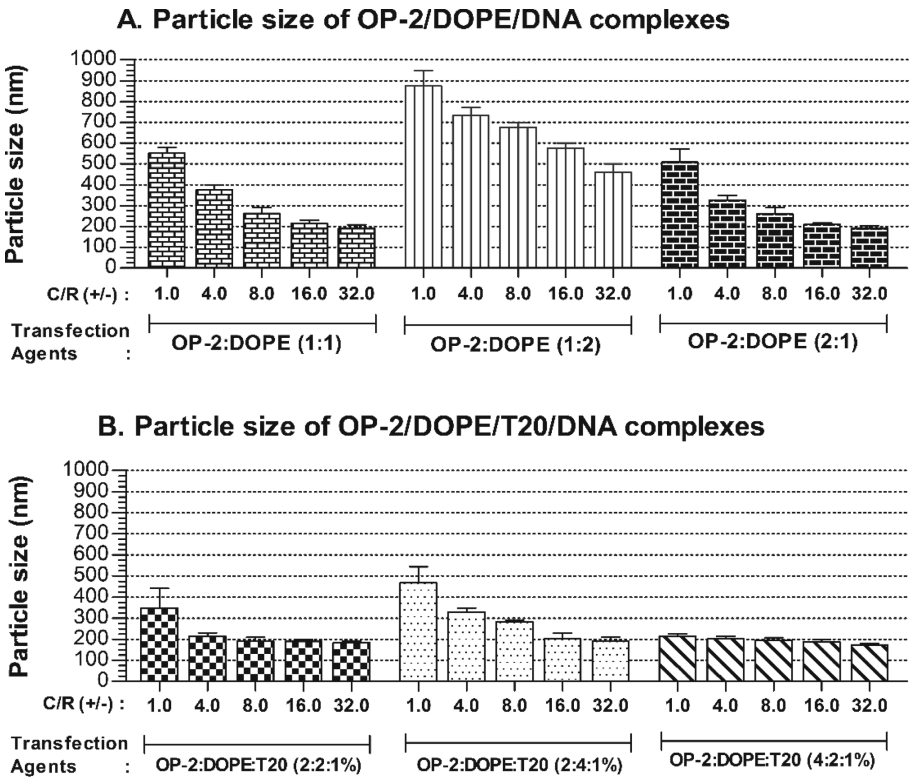

Fig. 2. Particle size (nm) of the OP-2-based liposome in HGB pH 7.4 at charge ratios of 1.0–32.0: A. OP-2: DOPE/DNA; B. OP-2: DOPE: Tween 20/DNA. OP-2: YGRKKRRQRRR-PKKKRKV. Data are represented as mean ± SD (n = 3).

Tween 20 = 2:1:0.5%) as shown in Fig. 3B. To further evaluate the effect of Tween 20 on liposome stability, the complexes were monitored for ~240 h, and the particle sizes were measured (Fig. 4). As the incubation time of the complex increased, the particle size of the complexes enlarged for both liposomes (Fig. 4A). After 240-h incubation time of the liposome/DNA complexes, the particle size of the OP-1: DOPE (1:1)/DNA increased from ~500 nm to ~600 nm. Meanwhile, the particle size of the OP-2-based liposome/DNA increased from ~350 nm to ~500 nm after 240-h complex formation.

Interestingly, the addition of Tween 20 (0.55 v/v) in the liposome formulation stabilizes the complex particle size of even though the complexes had been incubated up to 240-h (Fig. 4B). The particle size of the OP-1: DOPE: Tween 20/DNA complexes were around ~263 nm up to ~283 nm; meanwhile, the complexes of the OP-2: DOPE: Tween 20/DNA had narrow particle size distribution of ~233 nm up to 243 nm.

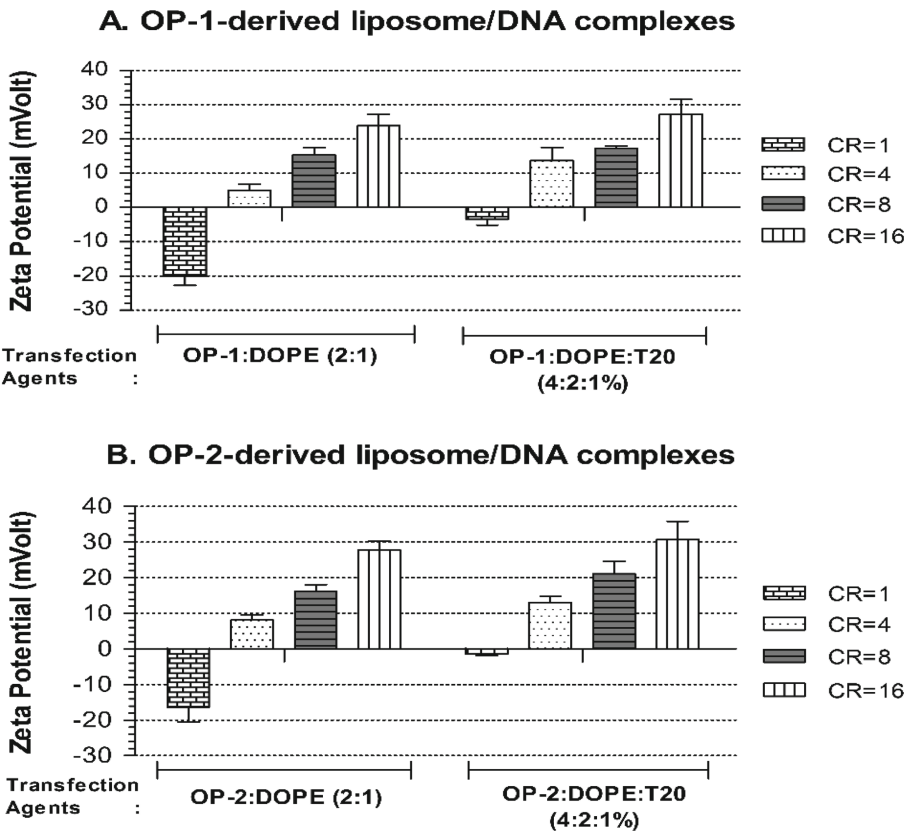

Fig. 3. Zeta potential (mV) of the complexes DNA/oligopeptide-based liposome formulated with and without Tween 20 in HGB pH 7.4 at charge ratio 1.0–16.0: A. the liposome of OP-1: DOPE = 2:1/DNA and the OP-1: DOPE: Tween 20 (2:1:0.5%)/DNA. B. the liposome of OP-2: DOPE = 2:1)/DNA and OP-2: DOPE: Tween 20 (2:1:0.5%)/DNA. OP-1: GRKKRRQRRR-PKKKRKV; OP-2: CKKHH-YGRKKRRQRRR-PKKKRKV. Data are represented as mean + SD (n = 3).

3.2 GFP Transgene Expression

The GFP encoding gene (pCSII-EF-AcGFP) was complexed using oligopeptides (OP-1 and OP-2) and oligopeptide-based liposomes to evaluate fluorescence expressed cells qualitatively. The OP-2, PLL, and OP-2-based liposomes in mediating GFP expression on HepG2 cells were detected clearly (Fig. 5). Unfortunately, Tween 20 addition (0.5%, v/v) in the liposome formulation reduced GFP expression in HepG2 cells. The presence of Tween 20 in the liposome formulation decreased the cell population and subsequently decreased GFP expression. Qualitatively. as the charge ratio of the transfection agent/DNA increased, the cell population decreased as shown in Fig. 5.

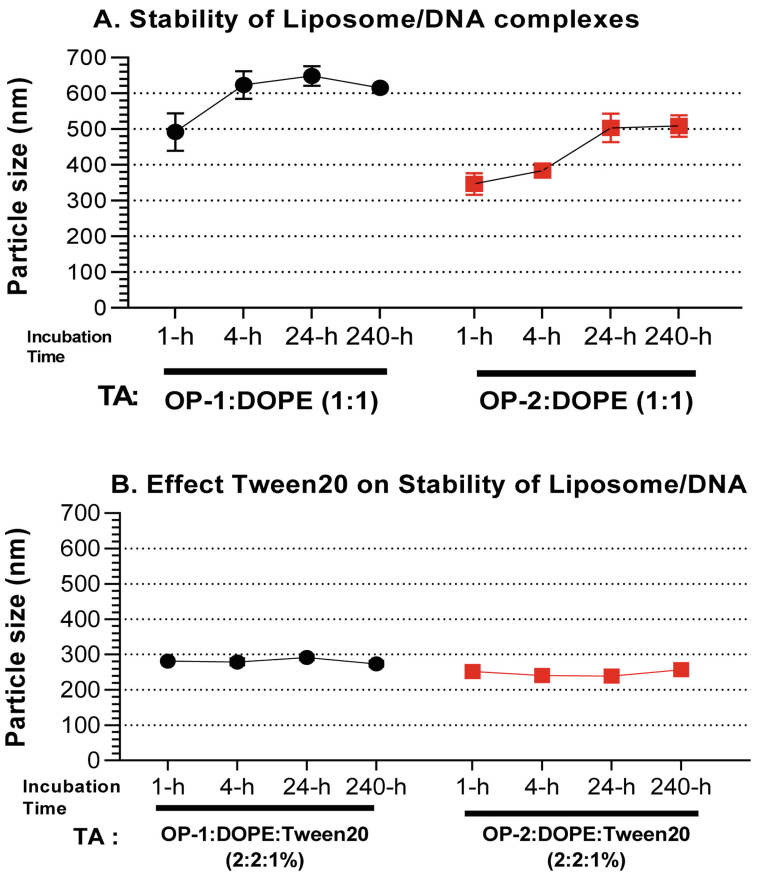

Fig. 4. Particle size stability up to 240-h after liposome/DNA complex formation in HGB pH 7.4 at charge ratio 4.0: A. liposome/DNA; B. Tween 20-containing liposome/DNA. Data are represented as mean + SD (n = 3). OP-1: GRKKRRQRRR-PKKKRKV; OP-2: CKKHH-YGRKKRRQRRR-PKKKRKV.

4 Discussion

Cationic peptides including oligopeptide having sequence of Trans-Activator of Transcription (TAT), GRKKRRQRRR, and nuclear localization sequence (NLS), PKKKRKV capable of condensing DNA molecules hence can be utilize for gene delivery vehicle. These peptides have been formulated with the neutral lipid of DOPE (Dioleoylphosphatidyl ethanolamine) due to its capability in destabilizing bilayer membranes and promoting membrane fusion [15, 16]. It was reported that DOPE also facilitates a decrease of the surface potential by losing the binding of the cationic lipid to the DNA molecule resulting in DNA dissociation from the complex particle and eventually increasing the transfection efficiency [17, 18]. We have also formulated the oligopeptide and lipopeptide with Polyoxyethylene [20] sorbitol monolaurate (Tween 20) to stabilize the liposome. Tween 20 has been reported in drug vector formulation for nano-carriers,

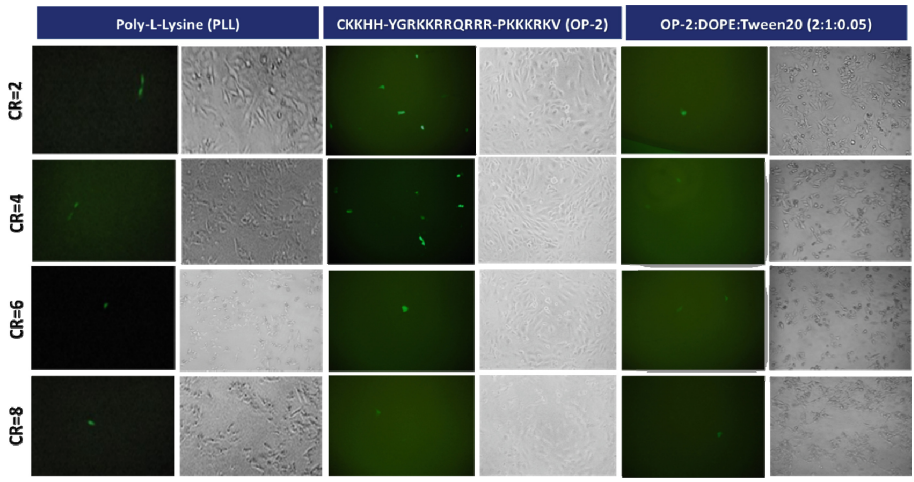

Fig. 5. GFP expression on HepG2 cells after 24-h post-transfection with GFP gene (pCSII-EF-AcGFP) condensed with oligopeptide OP-2 and the oligopeptide-based liposome (OP-2:DOPE: Tween 20 (2:1:0.5%) at charge ratios of 2, 4, 6, and 8. PLL was used as the control.

which shows a growing interest in drug delivery vehicles [19, 20]. The stability of the oligopeptide-based liposome/DNA (Fig. 4A) declined compared to the oligopeptide/DNA complexes (data not shown). Apparently, as the neutral lipid, DOPE has a minor role in the ionic interaction between the oligopeptide-based transfection agent and the DNA molecules; hence it tends to increase the transfection agent/DNA size.

Polysorbate 20 or commercially known as Tween 20 has fatty acid ester moiety and a long polyoxyethylene chain, has been used primarily as a water-in-oil emulsifier [13]. Tween 20 also been explored for a stabilizing agent in topical pharmaceutical formulations in drug delivery systems [21, 22]. Furthermore, Tween 20 had been also utilized in a gene transfer study by formulating the polysorbate with cationic cholesterol using a film hydration method and exhibited high efficiency in mediating cellular uptake and gene expression [14]. As it was expected, Tween 20 significantly reduced the particle sizes of the oligopeptide-based liposome/DNA complexes (Fig. 1 and Fig. 2). In addition, the inclusion of Tween 20 has also stabilized the liposomes as the particle sizes of the liposome/DNA complexes almost unchanged (Fig. 4). Lu and co-workers have shown the particle size of the Au nanoparticle decorated graphene oxide nano-sheets using Tween 20 (AuNPs/Tween 20/GO) composites was ~5–20 nm [23]. Meanwhile, Huang *et al.* have shown the particle size of the complex Tween 20-containing niosome/DNA was ~160 nm [14].

It was expected that the compacted and stable particle size of the Tween 20 containing-liposome/DNA would be beneficial for the gene delivery and facilitated more gene expression. Conversely, we have found the conflicted result as the Tween 20 inclusion in the liposome formulation reduced GFP transgene expression compared to the corresponding oligopeptide without Tween 20 (Fig. 5). The green dots that corresponded to GFP expressing HepG2 cells mediated by the Tween 20-containing liposome (OP-2:

DOPE: Tween 20 = 2:1:0.5%) significantly lesser than the corresponding oligopeptide. We have not convinced yet why the presence of Tween 20 in the transfection system has reduced the GFP expression dramatically. However, we speculated that the reduced amount of the GFP expression on HepG2 cells was due to Tween 20 cytotoxicity. Amongst non-ionic surfactants of the polyethylene glycol sorbitan esters tested on biological membrane, Tween 20 has the highest hemolytic effect on red blood cells than Tween 40, 60, 80 [24]. Weiszhar and co-workers showed the Tween 20, as well as Cremophore-EL and Tween 80, promoted the generation of the biologically active complement products in normal human serum and plasma that may cause inflammation [25]. We have formulated 0.5% v/v Tween 20 in the liposome formulation, and it would be equal to ~0.5–1.0 μL/mL of the Tween 20 in the transfection systems. The Tween 20 inclusion in the liposome formulation has significantly reduced the HepG2 cell viability.

5 Conclusion

Tween 20 has been utilized for liposome-based oligopeptide and capable to reduced particle size of the complex liposome/DNA. The presence of Tween 20 in liposome also stabilized the complexes over 240-h. Unfortunately, inclusion Tween 20 in liposome formulation failed to enhance gene expression probably due to cytotoxicity of Tween 20 molecule to the cells. Although Tween 20 has been utilized in many pharmaceutical formulations, we suggested to reduce the Tween 20 concentration in the liposome formulation or explore other non-ionic surfactants in order to obtain a safe and efficient transfection agent for *in-vitro* and *in-vivo* experiment purposes.

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