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Enhanced transgene expression of branched polyethyleneimine (bPEI-800)-based liposome nanoparticles in HEK-293T cells

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ABSTRACT

Branched low molecular weight PEI (bPEI-800 Da) is capable of condensing DNA molecules effectively. However, it has poor capacity to facilitate gene delivery. In this research, bPEI-800 is formulated with cholesterol, and Tween 20 or Tween 80 to form a liposome in order to obtain an effective transfection agent. The liposome's capacity to condense DNA was assessed using an ethidium bromide exclusion assay. Meanwhile, the ability of the liposome to protect DNA from DNase was evaluated by a DNA mobility shift assay and an enzymatic degradation assay using DNA gel electrophoresis. Transmission electronic microscopy (TEM) analysis was performed to determine the liposome/DNA particle size. Cell viability and transgene expression of the green fluorescence protein (GFP)-encoding gene were evaluated in HEK-293T cells. It was revealed that the *b*PEI-800-based liposome is able to condense DNA into nanoparticles 50–100 nm and protect DNA molecules from DNase I degradation. Furthermore, the *b*PEI-800-based liposome enhanced the transgene expression up to 1.6-fold, as a higher number of GFP-expressing cells were detected compared to *b*PEI-800 alone under the fluorescence microscope. Interestingly, the presence of cholesterol in the liposome formulation also reduced cytotoxicity, as revealed in MTT assay data. In summary, the *b*PEI-800-based liposome allowed condensation of DNA molecules into nanoparticle sizes, and enhanced transgene expression in HEK-293T cells. Further exploration in formulating and evaluating the *b*PEI-800-based liposome in other mammalian cells is needed to achieve a safe and effective transfection agent.

Keywords: Gene expression, Liposome, Polyethyleneimine, Transfection agent.

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1. INTRODUCTION

Gene therapy-based products have been commercially available worldwide, although many barriers and challenges have to be explored and resolved to achieve a safe and effective treatment (Shahryari et al., 2019; Arabi et al., 2022; Hadianamrei and Zhao, 2022). Several methods in gene delivery approaches have been exploited and developed to deliver the target genes based on the virus and non-virus gene vectors (Lesage et al., 2002; Kulkarni et al., 2018; La Manna et al., 2021). Compared to viral vectors, non-viral gene delivery vehicles such as liposomes or cationic polymers of polyethyleneimine (PEI) are generally considered to be safer and less immunogenic upon gene administration,

although they are less effective (Hardee et al., 2017; Arabzadeh et al., 2019). Unlike many viruses, non-viral-based vectors do not possess the mechanisms to target the nucleus of non-dividing cells; consequently, the transfection efficiency is relatively low compared to viral vectors (Patil et al., 2019; Zu and Gao, 2021; Kanvinde et al., 2022). The availability of transfection agent or gene delivery vehicle capable to transfect dividing and non-dividing cells effectively is urgently needed.

Amongst non-viral gene delivery vehicles, the cationic polymer of branched PEI is probably the most exploited and studied (Ahn et al., 2008; Bagai et al., 2014; Wang et al., 2015; Chiper et al., 2017; Ziebarth et al., 2017). Many research groups have explored PEI-based gene vectors due to their low cytotoxicity, capability to transport nucleic acids, and potential for modification (Taranejoo et al., 2015; Wang et al., 2015). Even though there has been serious concern regarding its clinical application due to cytotoxicity, PEI is a cationic polymer that is used as a reference in non-viral gene transfer due to its cationic charge and buffering capacity, which are beneficial in complexing material genetics (Bahadur and Uludağ, 2016). High molecular weight PEIs, including PEI-25K Da and PEI-800K Da, have been shown to be promising gene delivery vectors with high gene transfection efficiency. However, high molecular weight PEI also has drawbacks due to its high cytotoxicity, which hampers clinical application (Xiong et al., 2007; Zhang et al., 2018). In contrast, branched low molecular weight PEI (*b*PEI-800 Da) has good biocompatibility and relatively low cytotoxicity. Nevertheless, it has low DNA condensation and cell uptake capabilities, which reduces transfection efficiency (Zhang et al., 2018). Therefore, various efforts have been explored, including chemical and physical modification (Mahmoudi et al., 2014; Chiper et al., 2017; Dhanya et al., 2018) of the PEIs, to increase the effectiveness of the PEI-based transfection agent, which is capable of condensing and protecting DNA molecules.

In addition to cationic polymers such as PEI, liposome-based gene delivery vehicles have been explored and have been the subject of numerous studies aimed at improving in-vitro and in-vivo transfection (Zylberberg et al., 2017; Elsana et al., 2019). Liposomes are synthetic, spherical vesicles with a hydrophilic core and double layers of phospholipid that can be employed to transport physiologically active substances (Patil and Jadhav, 2014; Sheoran et al., 2022). Although liposomes have several benefits as carriers for nucleic acids or medications, including the potential to lessen the exposure of sensitive tissues to hazardous substances or toxic drugs, their stability is still a problem to be resolved. In a previous study, we found that the addition of Tween 20 to the oligopeptide-based liposome reduced particle size and stabilized the liposome/DNA complexes (Tarwadi et al., 2023). The aim of this research is to generate a PEI-based transfection agent that is both safe and efficient by preparing a series of liposome formulas that are composed of *b*PEI-800, cholesterol, and Tween 20 or Tween 80. Cholesterol was included as a lipid component to increase lipidation and facilitate a fusion between DNA/liposome complexes and the endosomal membrane. Meanwhile, the inclusion of tween was aimed at improving cellular absorption and stabilizing the liposome. The capability of the transfection agent to condense and protect DNA molecules, as well as its cytotoxicity and liposome-mediated transfection system in HEK-293 T cells, were investigated and discussed.

2. MATERIALS AND METHODS

All materials, unless otherwise stated, were purchased from Sigma Aldrich (Sydney, NSW, Australia) and were of analytical grade or molecular biology quality. Fetal calf serum (FCS) and RPMI 1641 cell culture medium were obtained from GIBCO-BRL (Invitrogen Pty. Ltd., VIC, Australia). Human embryonic kidney (HEK-293T) cells were generously donated by Tjandradewi Mosef (Indonesian Institute of Science, Indonesia). The green fluorescent protein (GFP)-encoding plasmid pCSII-EF-AcGFP (9880 bp) was generously provided by the Takeshi Kurosu Laboratory at the Research Institute of Microbial Diseases, Osaka University, Japan.

2.1 Liposome Preparation

Liposomes were formed by dissolving 10 µmol of *b*PEI-800 in phosphate buffered saline (PBS) pH 7.4 before being combined with cholesterol and Tween 20 or Tween 80 to achieve the desired *b*PEI-800: cholesterol: tween molar ratios of 100:10:1, 100:50:1, or 100:100:1. Dissolved with or without tween solution in ethanol was injected into bPEI-800 in PBS solution pH 7.4 using a 5 mL syringe with a flow rate of 500 µL/min. The combined solution was subsequently mixed with a magnetic stirrer at 45°C for 30 to 40 min, or until the ethanol solution had evaporated. The resulting mixture was then filtered (0.22 µm) and preserved at 4–6°C for subsequent analysis after being water bath-sonicated for around 20–30 min to produce a transparent liposome.

2.2 Plasmid Isolation

The QIAGEN® QIAprep Midi Kit (Qiagen Pty. Ltd., Vic., Australia) was used to extract the plasmid (pCSII-EF-AcGFP) expressing GFP from *Escherichia coli* TOP 10 in accordance with the kit's handbook. Spectrophotometry at 260 and 280 nm was used to confirm the DNA plasmid's concentration and purity. By performing a single digestion for 60 min at 37°C with the *Eco*RI restriction enzyme, the plasmid identification was verified. This was followed by a 25-min evaluation using gel electrophoresis at 110 volts. The isolated DNA was resuspended in nuclease-free water (NFW) and preserved at -20°C.

2.3 Gel Mobility Shift Assay

The gel mobility shift assay was used to examine the affinity of the DNA and the *b*PEI-800-based liposome, as

previously described (Elsana et al., 2019). Briefly, the DNA plasmid (400 ng) was mixed with the *b*PEI-800 or the liposomes to achieve a mass ratio of 0.5, 1.0, 2.0, 3.0 and 4.0, then incubated at 25°C for 30 min. The complex of DNA and liposomes was placed into microtubes containing HEPES Glucose Buffer pH 7.4 (HGB pH 7.4, 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and 5.13% w/v glucose). Loading buffer (2 µL) was added to the transfection agent/DNA solution (10 µL) and mixed well in the microtube. The mixture solution was run on a 1% agarose gel electrophoresis at 110 volts for 30 min. The DNA bands were visualised at a 320 nm transilluminator.

2.4 Enzymatic Degradation Assay

The stability of transfection agent/DNA complexes to enzymatic degradation was carried out by incubating the complexes with DNase I solution (Turbo DNase, Ambion, VIC, Australia), as previously described (Tarwadi et al., 2008). Briefly, the plasmid DNA (1 µg/µL) and transfection agents were dissolved separately in 12.5 µL of HGB pH 7.4 in a series mass ratio of 0.5; 1.0; 2.0; 3.0; and 4.0. The mixed solutions were combined and incubated for 15 min at 37°C in the presence of DNase I (1 U/µg DNA), then they were run on 1% agarose gel electrophoresis at 110 volts for 30 min. The DNA bands were visualised at a 320 nm transilluminator.

2.5 DNA Condensation Assay

The Ethidium bromide (EtBr) exclusion assay is used to determine the DNA condensation ability of the transfection agents. The assay is performed in a 96-well black plate using a FluoStar OPTIMA plate reader (BMG Lab Tech, Sydney, NSW, Australia) by measuring the relative fluorescence unit (RFU) of transfection agent/DNA complexes at l-excitation = 520 nm and l-emission = 610 nm, as previously described (Tarwadi et al., 2008; Tarwadi et el., 2020). Plasmid DNA (500 ng) was diluted in HGB pH 7.4 with an excess of EtBr (20 µL; 100 mg/mL) and used as the reference or standard value for calculating fluorescence intensity (100% RFU) in the absence of a transfection agent. The 500 ng DNA solution was put into each well, and then 50 µL of the HGB pH 7.4 was added. Then, the liposomes or the *b*PEI-800 transfection agent was combined with the DNA serial mass ratios of 0.5, 1.0, 2.0, 3.0 and 4.0. After 10 min of 25°C incubation, 20 µL of 100 µg/mL EtBr was added. The well plate was then shaken orbitally for 30 sec before the measurement. To calculate the fluorescence intensity, the RFU of the sample was compared with that of the reference, the RFU value of the sample without the transfection agent.

2.6 TEM Analysis

The transfection agent of *b*PEI-800 and the *b*PEI-800-based liposome/DNA complexes were prepared for TEM image analysis using UranyLess procedure, as previously described (Paris et al., 2023). Briefly, a drop of sample containing DNA diluted in HGB, pH 7.4 (10 µg/250 µL) and corresponding transfection agent in 250 µL HGB pH 7.4 to achieve a mass ratio of 4.0 was placed on EMR carbon coated grid (Microscopy Solution, Sheffield, South Yorkshire, UK), incubated for 2 min at room temperature. The grid was then placed upside down on the UranyLess drop (EMS, Hatfield, UK) for 2 min before it was blotted using filter paper to remove to water content. Subsequently, grid-containing sample was allowed to dry at ambient temperature for another ~ 2 h. Finally, samples were imaged using HT7700-SS transmission electron microscope using accelerating voltage at 100 kV (Hitachi High Technology, Japan).

2.7 Transfection Study

A transfection study was performed in a 96-well plate, and GFP expression was observed under an Axiovert 40 CFL fluorescence microscope (Carl Zeiss Microscopy GmbH, Germany) after 24 h transfection, as previously described (Hidayat et al., 2023). The HEK-293T cells were grown (1.0×10^5 cells/mL) in RPMI 1640 medium enriched with 10% FCS and 100 units/mL penicillin-streptomycin in a humidified incubator with 5% CO_2 at 37°C in order to evaluate the GFP expression. The cells were rinsed with PBS when they had reached around 60% confluence, and the media was replaced with 100 mL of the RPMI 1640 media without FCS supplement per well before the transfection solution was added. A 25 mL HGB pH 7.4 microtube containing 300 ng plasmid DNA and a transfection agent solution were combined to create the transfection solution, which had a transfection agent/DNA mass ratio of 0.5, 2.0 and 4.0. Before applying to cells, the transfection agent/DNA complex solution was incubated at 25°C for approximately 10 min. The cells were grown for a 24-h transfection period. Then, using a fluorescence microscope, the Axiovert 40 CFL (Carl Zeiss Microscopy GmbH, Germany), the expression of the GFP in cells was observed. The mean fluorescence intensity (MFI) of the GFP-expressing cells that were transfected by *b*PEI800 and *b*PEI-800-based liposome were further analyzed using ImageJ software.

2.8 Cytotoxicity Determination

Cell viability in the presence of the transfection agent was evaluated on HEK-293T cells using the colorimetric MTT metabolic assay, as previously described (Tarwadi et al., 2018; Hidayat et al., 2023). Briefly, cells (1.0×10^5 cells/mL) were seeded in a 96-well plate, and the transfection agent was added at a dosage of 0.2 mg/mL or 1.6 mg/mL into each well once the cells had reached approximately 60% confluence. After the cells had been incubated with the transfection agent for 24 h, 1 x PBS was used to wash them, and the medium was then changed to fresh RPMI 1640 media containing 20 µL of MTT solution (5 mg/mL in PBS). The MTT assay was stopped by adding 100 µL sodium

dodecyl sulfate (SDS) per well after the cells were cultured for an additional 4 h at 37°C. The absorbance density (OD) was quantified in a plate reader (Bio-Tek® Instrument, Vermont, USA) at 570 nm. The proportion (%) of untreated cells was used as a control to represent cell viability (van Meerloo et al., 2011).

2.9 Statistical Analysis

Each treatment in all experiments of DNA condensation and cytotoxicity studies were carried out in triplicate. Data were analyzed using Wilcoxon signed-rank test analysis and all cases of significance were set at $p < 0.05$.

3. RESULTS AND DISCUSSION

We have prepared liposomes using the ethanol injection method by formulating *b*PEI-800, polysorbate 20 (Tween 20), or polysorbate 80 (Tween 80) to obtain the transfection agents as listed in Table 1. Cholesterol addition in the liposome formula is intended to improve transfection efficiency, decrease cytotoxicity, and subsequently enhance cell uptake and transgene expression; meanwhile, polysorbate or tween inclusion is projected to stabilize the liposome structure.

Table 1. Sample formula of *b*PEI-800-based liposome for gene delivery study

No	*b*PEI-800 (µmol)	Cholesterol (µmol)	Tween 20 (µmol)	Tween 80 (µmol)
1.	10	1	-	-
2.	10	1	0.1	-
3.	10	1	-	0.1
4.	10	5	-	-
5.	10	5	0.1	-
6.	10	5	-	0.1
7.	10	10	-	-
8.	10	10	0.1	-
9.	10	10	-	0.1

3.1 Physicochemical Characterization

The *b*PEI-800-derived liposomes were evaluated based on the DNA mobility shift assay, EtBr exclusion assay, particle size and TEM image analysis, for their physicochemical characterization. As a lipophilic compound, cholesterol supports *b*PEI-800 in condensing DNA molecules. As shown in Fig. 1, *b*PEI-800 was able to condense DNA molecules completely at a mass ratio of 2.0 or higher (Fig. 1 (a-1)). The presence of 1 µmol of cholesterol in the *b*PEI-800-based liposome formula tends increase the effectivity of the transfection agent to condense DNA, as the DNA bands were detected at a mass ratio of 0.5; however, it was not observed at a liposome/DNA mass ratio of 1.0 or higher. In comparison to *b*PEI-800 alone or *b*PEI-800 formulated with cholesterol only, the liposome formula with tween resulted in more condensed DNA, as shown in Figs. 1 (b-3) and (b-4).

An EtBr exclusion assay was used to further assess the liposome's capacity to condense DNA. The presence of transfection agents in the transfection agent/DNA complexes hampers the ability of EtBr molecules to intercalate between the DNA strands, resulting in a reduced fluorescence intensity. Therefore, an increased mass ratio in the transfection agent/DNA complex significantly reduced fluorescence intensity, as shown in Figs. 2 and 3. An increased cholesterol concentration in the liposome formula was not significantly followed by a reduced fluorescence intensity when it was applied to condense DNA (Fig. 2). It seems that the cholesterol/*b*PEI-800 molar ratio of 1/10 resulted in an effective DNA condensation with reduced fluorescence intensity to only 60% compared to a higher cholesterol concentration in the liposome formula (molar ratio cholesterol/*b*PEI-800 of 1) that accounted for 70%. A higher cholesterol concentration in the liposome formula did not significantly correspond to a lower fluorescence intensity.

It appears that a *b*PEI-800/cholesterol molar ratio of 10 (*b*PEI-800: cholesterol = 1:10) generated an efficient DNA condensation; as observed at a mass ratio liposome/DNA of 4, the fluorescence intensity was ~ 60%. Meanwhile, at the same mass ratio of 4, with a higher proportion of cholesterol (*b*PEI-800: cholesterol = 2:1 and *b*PEI-800: cholesterol = 1:1), the fluorescence intensity was ~ 65% and ~ 70%, respectively. This implied that an increased cholesterol molar ratio in the liposome formula tend to decrease its capability of condensing DNA molecules.

The presence of cholesterol in the liposome-based transfection agents tend to increase the fluorescence intensity, which implied it reduced their capabilities to condense DNA molecules ($p < 0.0001$). However, as the mass ratio of the transfection agent to DNA increased, the fluorescence intensity decreased, indicating that more EtBr molecules were excluded from the DNA due to the presence of the transfection agent. Based on Wilcoxon signed-rank test, except of *b*PEI-800/cholesterol with a molar ratio of 1, there was a statistically different ($p < 0.0001$) of the transfection agent of the *b*PEI-800-based liposomes in condensing DNA molecules compared to *b*PEI-800 alone. In contrast to the results of the DNA mobility shift assay (Figs. 1 (b-3) and (b-4)), inclusion of Tween-20 or Tween-80 in the *b*PEI-800-based liposome formula behaved differently in the DNA condensation study. The presence of Tween-20 in *b*PEI-800-based liposome (*b*PEI-800: cholesterol: Tween-20 = 100:100:1) resulted in a higher fluorescence intensity compared to *b*PEI-800 alone ($p < 0.001$). This indicates that the proportion of Tween-20 in the liposome formula did not change its ability in condensing DNA molecules. In contrast to Tween-20, there was no statistically different in condensing DNA between *b*PEI-800-based liposome with Tween-80 inclusion compared to *b*PEI-800 alone ($p < 0.05$) (Fig. 3). This implied that the

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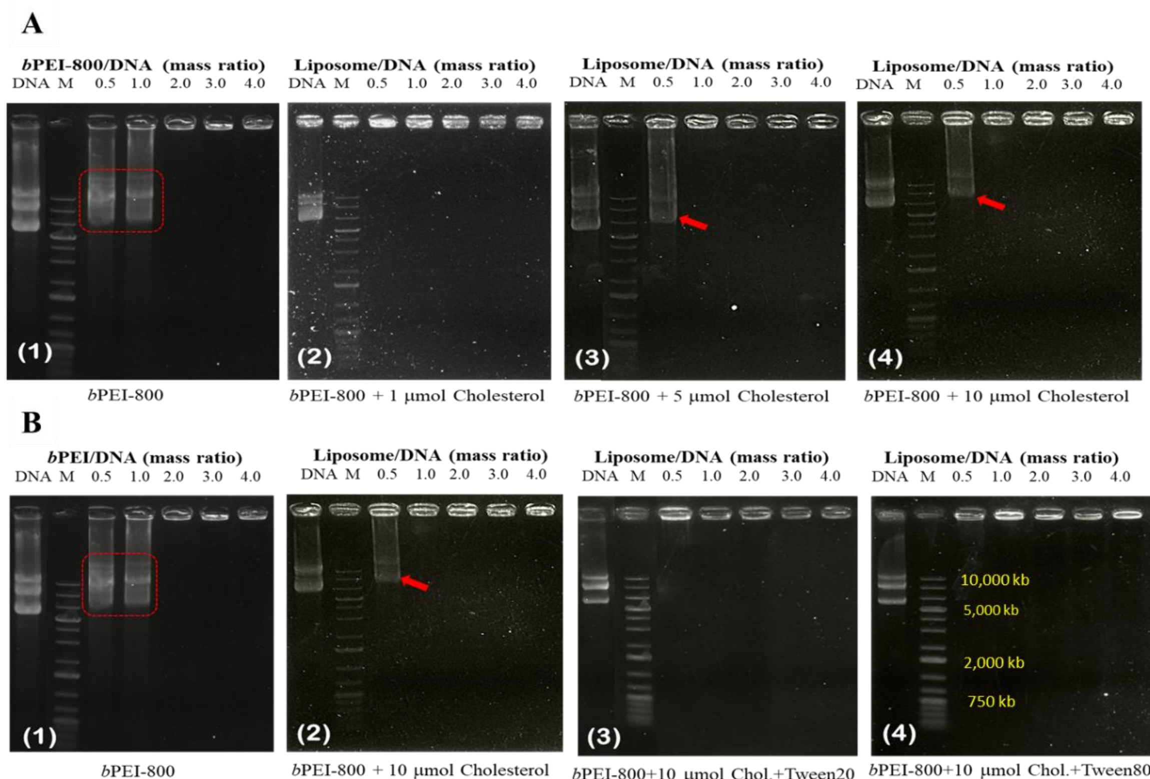

Fig. 1. DNA mobility shift assay of liposome/DNA as (A) a function of cholesterol concentration, and (B) tween inclusion in the liposome formula. The red rectangles and arrows indicate free DNA molecules that were detected in the agarose electrophoresis analysis. DNA marker of 1 kb DNA Ladder was used with size of (kb): 10,000; 8,000; 6,000; 5,000; 4,000; 3.000; 2,500; 2,000; 1,500; 1,000, 750, 500 and 250 kb

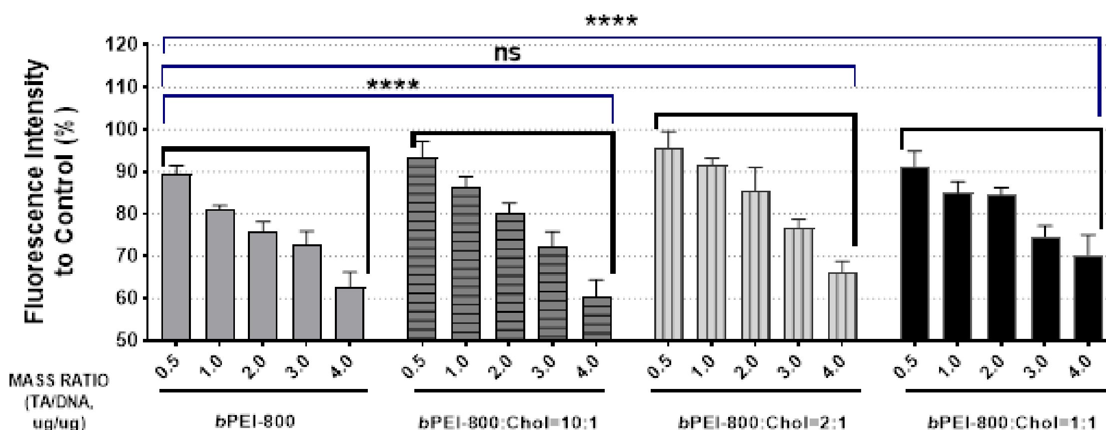

Fig. 2. Effect of cholesterol concentration in the *b*PEI-800-based liposome formula analysis by DNA condensation study using EtBr exclusion assay. Data are represented as mean ± SD (n = 3) and were analyzed using Wilcoxon signed-rank test analysis. All cases of significance were set at $p < 0.05$

inclusion of Tween-80 increased liposome's capability comparable to *b*PEI-800 alone in condensing DNA molecules.

The *b*PEI-800-based liposomes were further evaluated for their ability to protect DNA molecules from DNase degradation. In the presence of DNase, the DNA molecules were completely degraded, as demonstrated by the presence of smeared DNA bands. The *b*PEI-800 was capable of partly protecting DNA molecules, as some degraded DNA bands were still observed at a *b*PEI-800/DNA mass ratio of 4.0. However, as the cholesterol concentration in the liposome formula increased, the degraded DNA bands became thinner (Fig. 4 (a)), indicating that cholesterol contributed to DNA protection from enzymatic degradation. The degraded DNA bands were not detected in Fig. 4 (b-4),

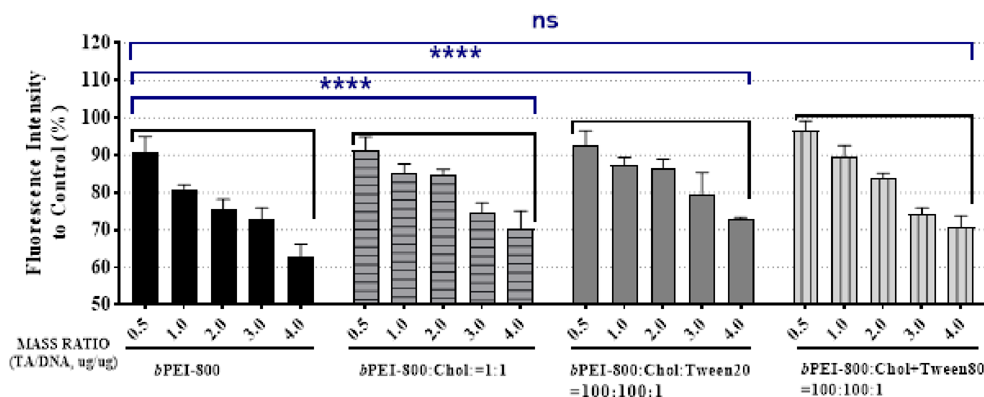

Fig. 3. DNA condensation study of the effect of Tween 20 and Tween 80 inclusion in the liposome formulation by EtBr exclusion assay. Data are represented as mean ± SD (n = 3) and were analyzed using Wilcoxon signed-rank test analysis. All cases of significance were set at $p < 0.05$

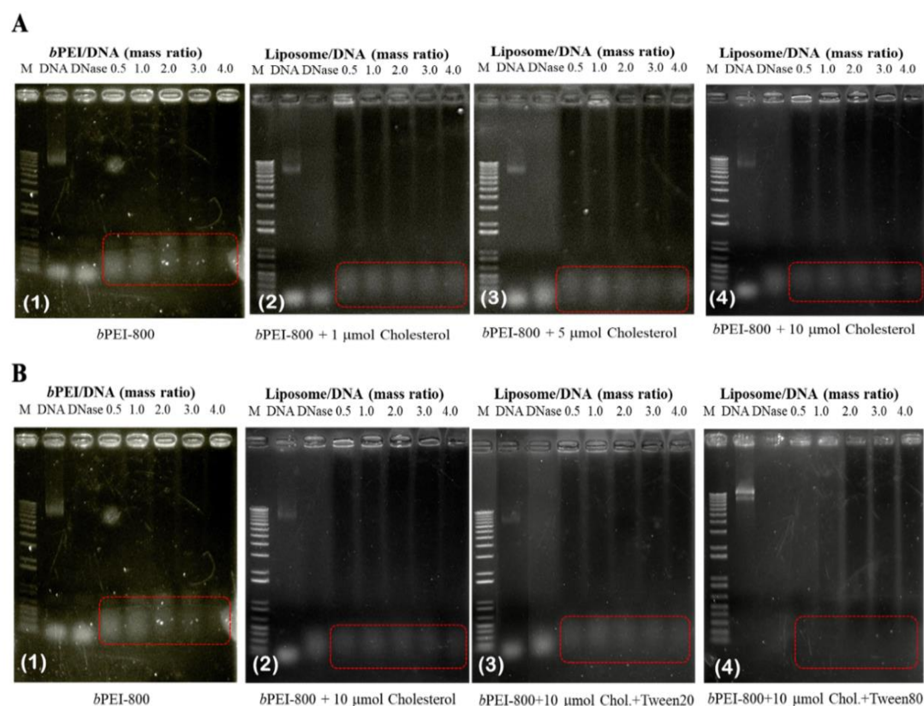

Fig. 4. Enzymatic degradation of the liposome/DNA complex by DNase I as a function of cholesterol concentration (A) and tween inclusion in the liposome formula (B) The red rectangles indicate free DNA molecules that were detected in the agarose electrophoresis analysis. DNA marker of 1 kb DNA Ladder was used with size of (kb): 10,000; 8,000; 6,000; 5,000; 4,000; 3,000; 2,500; 1,000; 750; 500 and 250 kb

suggesting that the presence of Tween 80 in the liposome formulation provided more DNA protection from DNase degradation compared to other liposome formulas.

3.2 Nanoparticle Analysis

We have further characterized the transfection agents by measuring the particle size of the *b*PEI-800/DNA and the *b*PEI-800-based liposome/DNA complexes using dynamic light scattering and transmission electronic microscope image analysis. Based on dynamic light scattering measurement, the mean particle sizes of the complexes were approximately 300–400 nm (data are not shown). However, TEM image analysis has shown that the complex particle sizes of the *b*PEI-800/DNA and the *b*PEI-800-based liposome/DNA were significantly smaller of 50–100 nm (Fig. 5). We speculate that the TEM image analysis reflects the actual size of the particles. The particle size discrepancy between the dynamic light scattering measurements and TEM image analysis is probably due to the homogeneity level of the complex solution. Some aggregation of the transfection agent/DNA complexes may occur and contribute to the mean particle size of the *b*PEI-800/DNA

and *b*PEI-800-based liposome/DNA complexes when it was measured by the dynamic light scattering apparatus.

It was observed that the particle size of *b*PEI-800/DNA complexes at mass ratio of 4 was relatively homogenous with the sizes between 50–100 nm (Fig. 5 (a)). TEM image analysis has also shown that the particle size was slightly increased when the DNA was complexed with the *b*PEI-800-based liposome composed of *b*PEI-800: cholesterol = 1:1 (Fig. 5 (b)). Interestingly, the particle size of the liposome/DNA was almost unchanged when the DNA was complexed with the *b*PEI-800-based liposome with an increased *b*PEI-800 proportion or Tween 80 inclusion (Figs. 5 (c) and (d)). Apparently, addition of 1% of Tween in the *b*PEI-800-based liposome did not significantly influence the physicochemical characterization of the liposome.

Our finding is in agreement with Arabzadeh and co-workers report that low molecular weight protamine peptide (LMWP: VSRRRRRRGGRRRR, MW of 1880 g/mol) formulated with cholesterol formed a compacted particle capable of condensing the DNA molecules effectively with a particle size of 150 nm (Arabzadeh et al., 2019). However, we found that the presence of cholesterol in the *b*PEI-800-based liposome reduced the DNA condensation ability of the cationic polymer *b*PEI-800 when packing DNA molecules. Apparently, the excess amount of cholesterol in the liposome lessens the DNA/liposome interaction and might increase capability of the EtBr to intercalate between the DNA strands, which leads to an increased fluorescence intensity as shown in Fig. 3.

To improve cellular absorption and gene expression, polysorbate, also known commercially as tween, has been used as an emulsifier in cholesterol-based liposomes (Tarwadi et al., 2023). As previously reported, the presence of tween in the liposome formula stabilized the liposome and caused a reduction in the particle size of the liposome/DNA complex (Zylberberg et al., 2017). We obtained evidence that tween inclusion in the liposome formula has increased liposome capability in complexing DNA molecules, as revealed by the DNA mobility shift assay, where at a low mass ratio of the liposome/DNA of 1.0, the DNA molecules were retained in the agarose gel, as the DNA band was not detected (Figs. 1 (b-3) and (b-4)).

Tween-80 (MW of 1310 g/mol) inclusion in the *b*PEI-based liposome enables the liposome to condense DNA molecules comparable to *b*PEI-800 alone (Fig. 3). However, we are unable to show a similar result in the DNA condensation study, where in the presence of Tween-20 (MW of 1227.5 g/mol) in the *b*PEI-800-based liposome, the fluorescence intensity did not decrease as a sign of more condensed DNA compared to *b*PEI-800 alone. Further research is required to clarify why the DNA condensation data from *b*PEI-800-based liposome composing of Tween-20 and Tween-80 resulted differently. However, we speculate the discrepancy between Tween-20 and Tween-80 in DNA condensation effectiveness probably due to the differences in the molecular weights and the length of aliphatic tails in the emulsifiers. We propose the hypothetic interaction between the liposome components of *b*PEI-800, cholesterol, and tween in forming nanoparticle size with DNA molecules (Fig. 6).

3.3 Transgene Expression

The GFP transgene expression was studied in HEK-293T cells using transfection agent/DNA mass ratios of 0.5, 2.0 and 4.0. It was discovered that as the transfection agent concentration increased, the number of GFP-expressing cells visible under a fluorescence microscope increased. This suggested that the transfection efficiency increased as the mass ratio of the *b*PEI-800/DNA or the liposome/DNA increased (Fig. 7).

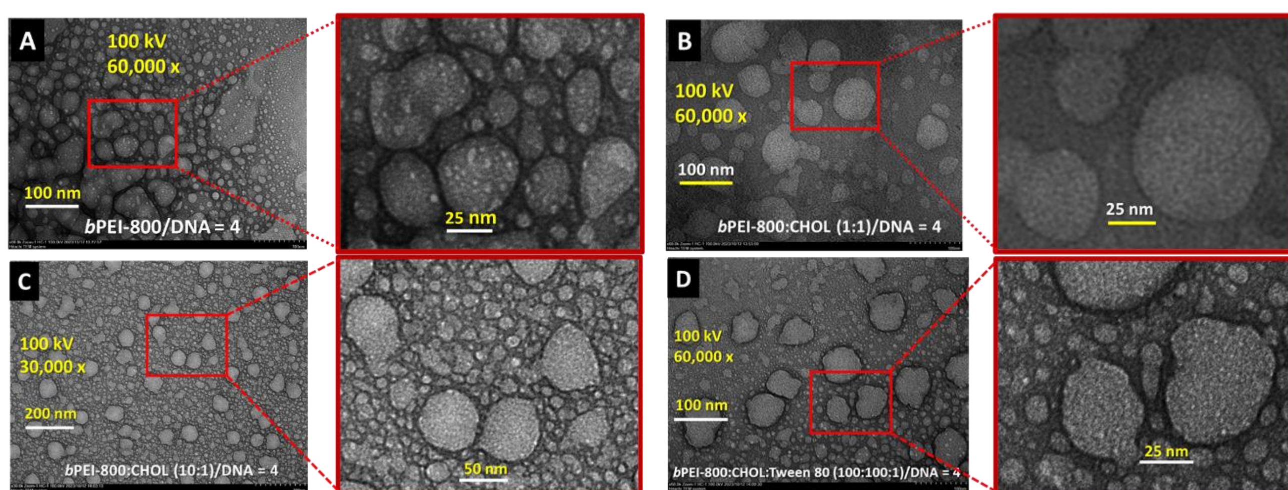

Fig. 5. TEM images of the *b*PEI-800/DNA and *b*PEI-800-based liposome/DNA complexes in HGB pH 7.4 at mass ratio 4.0: (A) *b*PEI-800/DNA complexes, (B) *b*PEI-800: cholesterol = 1:1/DNA complexes, (C) *b*PEI-800: cholesterol = 10:1/DNA complexes, (D). *b*PEI-800: cholesterol: Tween 80 = 100:100:1/DNA complexes. The images were visualized using the HT7700-SS TEM apparatus with voltage set at 100 kV

Fig. 6. Hypothetic and schematic interaction of the DNA molecules with liposome components of *b*PEI-800, cholesterol, Tween-20, or Tween-800. The neutral lipid of cholesterol inclusion in the liposome might increase the particle size of the liposome/DNA. Meanwhile, as emulsifier with hydrophilic and hydrophobic parts, Tween-20 or Tween-80 increases the compactness of the complexes resulted in more condensed nanoparticles

Fig. 7. Transgene expression of GFP encoding gene in HEK-293T cells mediated by *b*PEI-800 and *b*PEI-800-based liposomes that were observed under the fluorescent microscope. The images were further assayed using ImageJ software

Interestingly, the GFP-expressing cells were significantly enhanced when the cells were transfected with *b*PEI-800 formulated with cholesterol. Furthermore, as the molar ratio of the cholesterol/DNA increased in the liposome formula, the observed GFP-expressing cells were also increased (Figs. 7 (F), (I) and (L)). It implied that as the molar ratio of the cholesterol to the *b*PEI-800 increased from 1:10, 1:2 and 1:1, higher GFP-expressing cells were observed. The GFP transgene expression was also clearly detected when the cells were transfected with the liposome/DNA at a low mass ratio of 0.5 (Fig. 7 (J)). These results were supported by the value of the MFI of the GFP-expressing cells (Table 2). The MFI of the GFP-expressing cells increased from 22.5 to 51.2 as the mass ratio of the *b*PEI-800/DNA increased from 0.5 to 4.0. Interestingly, when the HEK-293T cells were transfected with the *b*PEI-800-based liposome (molar ratio of the *b*PEI-800/cholesterol = 2) at mass ratio of the liposome/DNA of 2.0, the MFI value increased from 27.5 to 44.0 (1.6-fold). Similarly, the transgene expression of GFP-encoding gene increased from 51.2 to 75.0 MFI (1.46-fold) as the cells were transfected with the liposome (molar ratio of *b*PEI-800/cholesterol = 1) at a mass ratio of the liposome/DNA of 4. These indicate that cholesterol inclusion in the *b*PEI-800-based liposome formula is capable of increasing transgene expression in HEK-293T cells, as shown in Table 2, in which the transgene expression enhancement of the liposome compared to the corresponding *b*PEI-800 is presented in the brackets.

Inclusion of Tween 20 and Tween 80 in the *b*PEI-800/cholesterol formula was not further significantly increase the GFP transgene expression in HEK-293T cells (Fig. 8). The number of GFP-expressing cells that correlate with GFP transgene expression facilitated by the liposome with and without tween inclusion in Figs. 8 F, 8I and 8L are comparable. The fluorescence intensity of the GFP-expressing cells transfected with *b*PEI-800-based liposome with and without tween inclusion were further analyzed using ImageJ software as listed (Table 3). There is no difference in facilitating GFP expression in HEK- 293T cells for the liposome with Tween-20 or Tween-80 inclusion in charge ratios of 0.5–2.0. Compared to liposome without tween inclusion (*b*PEI-800: cholesterol: 1:1), the MFI value was slightly drop from 75.0 to 71.0 (*b*PEI-800: cholesterol: Tween 20 = 100:100:1) or 73.0 (*b*PEI-800: cholesterol: Tween 80 = 100:100:1). Nevertheless, the *b*PEI-800-based liposome containing tween is still capable in enhancing transgene expression up to 1.43-fold compared to *b*PEI-800 alone (Table 3).

Table 2. Cholesterol inclusion in the *b*PEI-800-based liposome on GFP transgene expression

Mass ratio TA/DNA (mg/mg)	MFI values of GFP-expressing cells mediated by transfection agents (Transgene expression enhancement of the liposome compared to *b*PEI-800)			
	*b*PEI-800	*b*PEI-800: Chol = 1:10	*b*PEI-800: Chol = 2:1	*b*PEI-800: Chol = 1:1
0.5	22.5	26.0 (1.15)	30.0 (1.33)	30.0 (1.33)
2.0	27.5	24.0 (0.87)	44.0 (1.60)	34.1 (1.24)
4.0	51.2	61.0 (1.19)	60.0 (1.18)	75.0 (1.46)

MFI: mean fluorescence intensity.

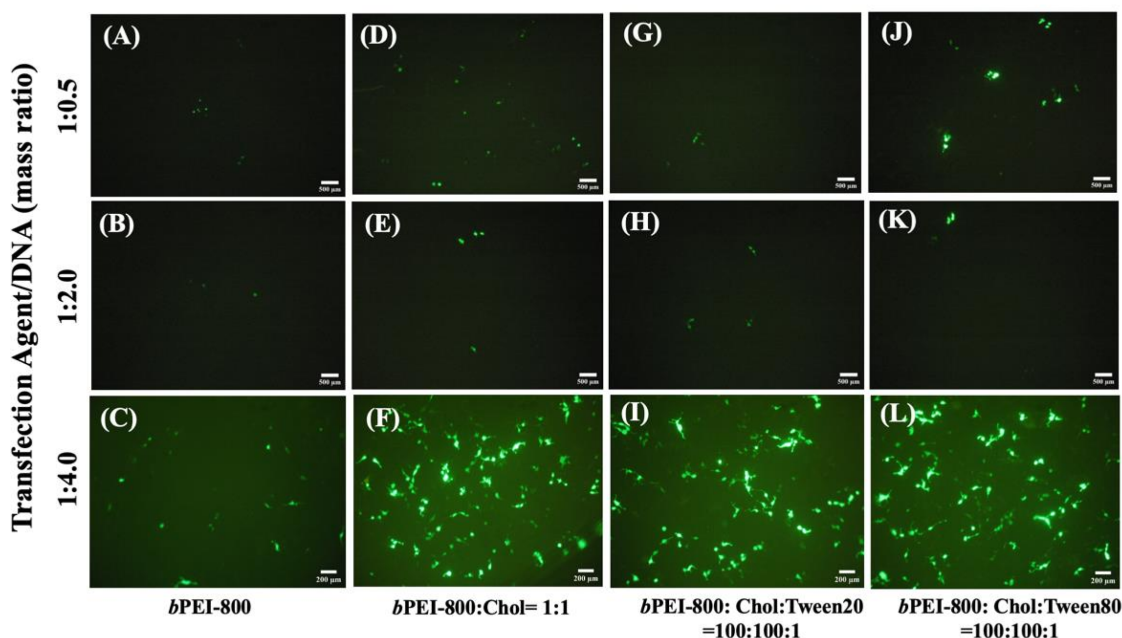

Fig. 8. Effect of Tween 20 and Tween 80 inclusion in the *b*PEI-800-based liposome formulation on transgene expression in HEK-293T cells that were observed the fluorescent microscope. The images were further assayed using ImageJ software

Table 3. Tween inclusion in the *b*PEI-800-based liposome on GFP transgene expression

Mass ratio TA/DNA (mg/mg)	MFI values of GFP-expressing cells mediated by transfection agents (Transgene expression enhancement of the liposome compared to *b*PEI-800)			
	*b*PEI-800	*b*PEI-800: Chol = 1:1	*b*PEI-800: Chol: Tw20 = 100:100:1	*b*PEI-800: Chol: Tw80 = 100:100:1
0.5	22.5	30.0 (1.33)	30.0 (1.33)	27.0 (1.20)
2.0	27.5	34.1 (1.24)	27.0 (0.98)	25.0 (0.91)
4.0	51.2	75.0 (1.46)	71.0 (1.39)	73.0 (1.43)

MFI: mean fluorescence intensity.

It was reported that the addition of cholesterol to low-transfecting DOTAP/DOPC complexes resulted in a 10-fold increase in transgene expression (Zidovska et al., 2009). They speculated that although cholesterol alone could not account for the increased transfection efficiency, it might promote fusion between cationic lipid/DNA complexes and ionic endosomal membranes to facilitate DNA release and hence increase transfection efficiency. Similarly, Pinnapireddy et al. (2017) have reported that when cholesterol was formulated with other lipids such as DPPC and DOPE to coat branched PEI (MW of 25,000) and linear PEI (MW of 22,000), the luciferase transgene expression increased significantly compared to those without coated lipid formulas due to cholesterol's contribution to liposomal complex stability. Inclusion of cholesterol components in the liposome formulation of cationic peptide (K16-GACYGLPHKFCG) was also explored in order to achieve optimal transfection in-vitro and in-vivo (Grant-Serroukh et al., 2022). In addition, cholesterol is also to be believed capable of improving transgene expression by protecting the DNA molecules from DNase degradation in the cells and has been shown to be effective in reducing the binding of serum proteins to the lipoplexes (Pozzi et al., 2012). This finding is in agreement with Fig. 4, where in the presence of an increased cholesterol concentration in the liposome formula, degraded DNA bands were less detected.

Previously, we reported that an inclusion of Tween 20 in the liposome formulation of cationic oligopeptide/DOPE has formed a stable and compacted particle size of 260–280 nm; however, the transgene expression facilitated by the liposome of oligopeptide/DOPE/Tween 20 in HepG2 cells decreased significantly compared to those without Tween 20 inclusion (Tarwadi et al., 2023). In contrast to those that have been reported, the inclusion of Tween 20 and Tween 80 in the *b*PEI-800/cholesterol in this research did not decrease GFP transgene expression in HEK-293T cells (Fig. 8). The number of GFP-expressing cells that correlate with the GFP transgene expression facilitated by the liposome with and without tween inclusion is comparable. Huang et al. (2011) have reported that Tween 20 in the DC-cholesterol-based liposome might mediate cellular uptake and increased transgene expression in COS-7 cells. It needs further investigation to clarify this discrepancy's result on the effect of Tween 20 and Tween 80 inclusion-based liposome transfection agents on mediated transgene expression. Exploring other biomaterials as sources of non-viral gene delivery vehicles based on liposome formula is urgently needed. Bulbake and co-workers reported the use of cationic liposome and PAMAM dendrimer to enhance cellular uptake of unstable anticancer siRNA in MCF-7 and MDA-MB-231 cells (Bulbake et al., 2020). Recently, instead of preparing PEI-based liposome, Liu and colleagues grafting PEI molecules with silver nanoparticle to form stable and compact sizes of 25–100 nm as a safe and efficient gene carrier (Liu et al., 2022).

3.4 Cell Viability

Cholesterol is regarded the most suitable component for liposome component for gene delivery since it increases lipidation, increases and stimulates cell uptake (Zhang et al., 2021). We demonstrated that cell viability of HEK-293T was higher in the presence of *b*PEI-800-based liposome compared to the corresponding bPEI-800 alone (Fig. 9). It was observed that the cationic polymer of *b*PEI-800 has relatively low cytotoxicity on HEK-293T cells, as at 1.6 µg/µL (1600 ppm), the cell viability was 60-70% compared to control (Fig. 9 (a)). Interestingly, when the *b*PEI-800 was formulated with cholesterol to form a liposome, the cell viability increased significantly ($p < 0.05$). It was observed that at the transfection agent concentration of 0.2 µg/mL (200 ppm), the cell viability of HEK-293T increased from 80% to 90% compared to the control when *b*PEI-800 was formulated with cholesterol (10:1). Remarkably, the cell viability was approximately 100% when the *b*PEI-800 was formulated in higher cholesterol molar ratio (*b*PEI-800: cholesterol = 2:1). However, as the molar ratio of cholesterol further increased (*b*PEI-800: cholesterol = 1:1) the cell viability slightly decreased to ~ 90% compared to the control (Fig. 9 (b)).

The cell viability of the HEK-293T cells was comparable in the presence or absence of Tween 20 or Tween 80 in the liposome formula (Fig. 9 (c)). The presence of the tween in the *b*PEI-800-based liposome formula (*b*PEI-800: cholesterol: tween = 100:100:1) did not significantly change the cell viability. It was observed that at a transfection agent concentration of 0.2 µg/µL, the cell viability was ~ 90% compared to the control cells, regardless of the presence or absence of tween in the liposome formula.

Generally, linear PEI has low cytotoxicity compared to *b*PEI. In addition, low molecular weight PEI also has lower cytotoxicity compared to the higher molecular weight PEI, as previously reported (Xiong et al., 2007; Kafil and Omidi, 2011; Taranejoo et al., 2015). The presence of Tween 20 or Tween 80 in the *b*PEI-800-based liposome formula (*b*PEI-

800: cholesterol: Tween = 100: 100: 1) in this research has demonstrated low cytotoxicity, despite reports that the surfactant non-ionic of tween has hemolytic effect on red blood cells (Kafil and Omidi, 2011) and promoted the generation of the biologically active complement products in normal human serum and plasma that may cause an inflammation (Weiszhár et al., 2012).

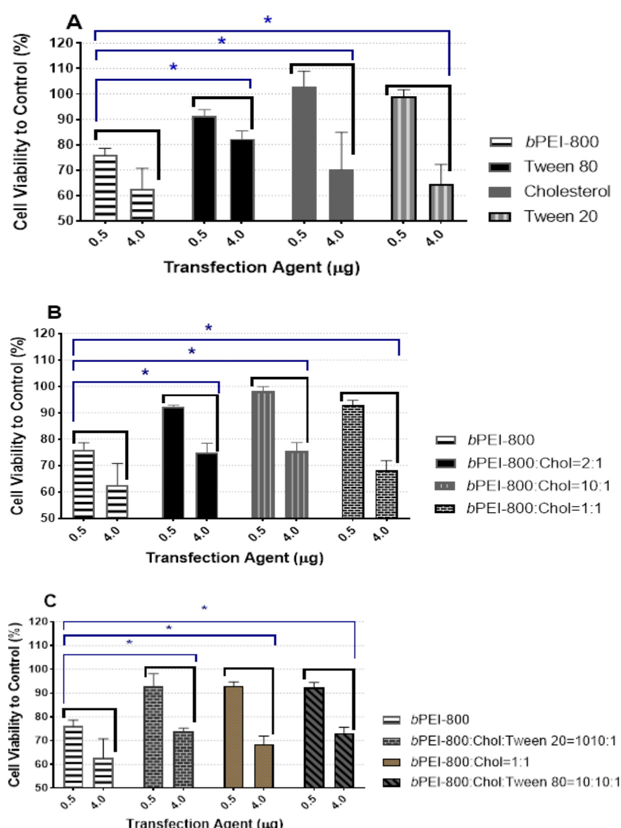

Fig. 9. Cell viability study of *b*PEI-800 and *b*PEI-800-based liposomes in the presence and absence of Tween 20 and Tween 80. Data are represented as mean ± SD (n = 3) and were analyzed using Wilcoxon signed-rank test analysis. All cases of significance were set at $p < 0.05$

As expected, the cholesterol component in the *b*PEI-800-based liposome increased transfection efficiency. Meanwhile, tween inclusion in the liposome formula was intended to stabilize and condense DNA effectively. Tween inclusion in the liposome formula has shown able to condense DNA molecules effectively (Fig. 1) and protect them from DNase degradation, as shown in agarose gel analysis (Fig. 3). However, the DNA condensation study failed to show that Tween 20 or Tween 80 inclusion in the *b*PEI-800-based liposome was more effective at condensing DNA compared to the liposome without tween addition. It needs further physicochemical characterization, such as particle size distribution and transmission electron microscopy (TEM) image analysis to confirm the effect of tween inclusion on liposome structure stability and compactness. Remarkably, the cholesterol component in the *b*PEI-800-based liposome significantly increased GFP transgene expression in HEK-293T (Figs. 7 and 8). The utilization of other sources of lipid such as DOPE or functionalization of TAT peptide sequence in preparing liposome may also increase the effectiveness of a non-viral gene delivery vehicle (Ebrahimian et al., 2022; Moreira et al., 2023). Nevertheless, transfection studies need to be further performed in other mammalian cells, including primary cells, to optimize the cytotoxicity and effectivity of the liposome-based transfection agent.

4. CONCLUSION

We have formulated *b*PEI-800-based liposomes with and without Tween 20 or Tween 80 inclusion to achieve transfection agents with enhanced transgene expression and low cytotoxicity. We have succeeded in revealing that the *b*PEI-800-based liposomes are capable of condensing and protecting DNA molecules from enzymatic degradation and generate homogenous nanoparticle sizes as revealed by TEM image analysis. The *b*PEI-800-based liposome significantly improved transgene expression of GFP in HEK-293T cells, as more GFP-expressing cells were detected under the fluorescence microscope. Interestingly, the presence of cholesterol in the liposome formulation also reduced cytotoxicity, as revealed in MTT assay analysis. In summary, the presence of cholesterol in the *b*PEI-800-based liposome is capable of condensing DNA molecules and enhancing transgene expression in HEK-293T cells. Nevertheless, further exploration in formulating the *b*PEI-800-based liposome in other cells is needed to achieve a safe, stable, and effective transfection agent.

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AUTHOR CONTRIBUTION

Retno Lestari: conceptualization, supervision, and analysis; Bismi Yasinta Maharani: data curation, methodology, and analysis; Damai Ria Setyawati: data curation and methodology; Etik Mardliyati: data curation and analysis; Ankan Dutta Chowdhury: review and data analysis; Tarwadi: conceptualization, data curation, formal analysis, writing original draft, supervision, editing, final revision.

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