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RESEARCH PAPER

Preparation and transfection evaluation of modified multifunctional envelope-type nano device -DNA nanocomplexes based on low molecular weight protamine

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ABSTRACT

Objective(s): Gene therapy is a hopeful approach for treatment of a wide range of life threatening disease from infectious and inherited diseases to cancer. Multifunctional Envelope-type Nano Device (MEND) is a new carrier as non-viral genetic vector. Moreover, associating peptide structures with the nuclear localization signals (NLSs), which contains various functional groups enables them to condense DNA and specifically transfer genetic material to the nucleus.

Materials and Methods: In this study, two forms of low molecular weight protamine (LMWP) were used for preparation of MEND carrier. The MEND carriers were then targeted with GE11 ligand to obtain T-MEND structure. The size distribution of the resulting nanoparticles, as well as their transfection efficiency and cytotoxicity, were investigated on the A549 cell line.

Results: Results demonstrated that the size of polyplex carrier's formulation by both peptides (VV45 and VV32) was below 200 nm and MEND formulations were between 200-300 nm. T-MEND formulations contained VV32 and VV45 peptides showed slightly higher transfection than similar MEND formulations. Also, MEND formulation showed increased transfection efficiency compared to similar PD complexes. The result of metabolic activity test showed that MEND lipopolyplex did not represent any remarkable cytotoxicity.

Conclusion: It can be concluded that multifunctional carriers designed based on LMWP are considered as the safe carrier for gene delivery. Presence of protamine and targeted ligand in the nanoparticulate structure did not increase the risk of cytotoxicity of carriers. So, MEND and T-MEND lipoplexes showed low cytotoxicity and acceptable transfection efficiency at the level of PEI 25 kDa.

Keywords: *Cytotoxicity, Gene delivery, Protamine, Transfection*

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INTRODUCTION

Gene therapy is a hopeful approach for the treatment of a wide range of life-threatening disease, from infectious and inherited diseases to cancer [1-4]. The role of gene therapy in cancer treatment is fundamentally based on two strategies, which include the selection of exclusive genetic material expressed in tumour cells, and therapeutic delivery into the host cells in order to destruct or decline tumours at the lesion site [5]. Therefore, a broad spectrum of genetic vectors has been employed as genetic material carriers. Among vectors, viral ones have demonstrated the highest efficiencies by taking advantage of their instinctive potency to deliver nucleic acid based therapeutics in to the tumor cell. While the success

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of viral vectors in gene delivery is non-negligible, their inherent feature to ignite immunogenic reactions and transgene miss-insertion hazardous, etc, has been considered the biggest dilemma for their application. These limitations led to attempts to develop non-viral delivery systems [6, 7].

Non-viral vectors, in comparison with viral ones, are safer approach regarding to their toxicity, and mutagenesis, but their inefficiency and essential requirement for improvements is an inevitable concept [8, 9]. Despite significant development in vector design and production, there are still certain worries that have to be addressed for their translation into the clinics.

Liposomal carriers have demonstrated to be a feasible option for application as a non-viral genetic vector owing to substantial aspects such as high chemical stability, increased capability for nucleic acid loading, a safer profile in cellular evaluation, and controlled release properties. These features contribute to their capacity to enter the clinical settings [10]. Several strategies have been previously employed to design responsive liposomes.

Multifunctional Envelope-type Nano Device (MEND) is a new carrier, have shown strong result as non-viral genetic vector [11, 12]. The multifunctionality of MEND includes structural stabilization of DNA by compaction, topological control of effectors by a lipid membrane, and increased intracellular traffic by these functional moieties [13]. The chemical structure of these devices consist of two compartment, genetic material in free or condensed form with a polycation and a lipid structure surface (envelope). Moreover, they are mostly associated with functional moieties including PEG, targeting ligands, and cell-penetrating peptides (CPPs) to facilitate both *in vitro* and *in vivo* transferring and homing, fusogenic peptides with endosomal escape character in response to pH-sensitivity, and a nuclear localization signal (NLS) for improvement of nuclear delivery [14, 15].

The distinguished role of peptides in gene delivery is inspired by natural-based peptides. Due to different studies, nuclear proteins such as protamine and histones can act a gene delivery vehicles for gene therapy applications [16-18]. However, their immunological challenges should be addressed by various strategies [19]. In one of these approaches, utilizing thermolysin corresponded with an enzymatic digestion of protamine led to the generation of short-chained fragments, known as low molecular weight (LMWP), with a VSRRRRRRGGRRRR sequence [19]. This fragmented sequence, in addition to its ability for DNA condensation, did not cause safety problems [20].

Structural similarity of LMWP to that of the broadly used protein transduction domain (PTD) peptide, TAT, is creating a good potential for application in cell mediated cargo delivery. Compared to TAT, the safety profile of LMWP showed no significant toxicity in animal studies. Moreover, chemically synthesized LMWP can be manufactured in large quantities from its natural precursor, protamine. Therefore, it has a substantial advantage in cost effectiveness [21]. Associating peptide structures with the nuclear localization signals (NLSs), which contain various functional groups, enables them to condense DNA and specifically transfer genetic material to the nucleus [22]. In our previous work, two forms of LMWP repeat, in linear and branch form, were synthesized and incorporated in to 10X-histidine tag (H10) for proton sponge effect favor and a simian virus 40 (SV40) large T antigen (PKKKRRV) block as an NLS for nuclear delivery. After that, LMWP-based peptides were assessed in the form of peptide/DNA (PD) and liposome/peptide/DNA (LPD) for non-viral gene delivery. Based on our findings, LPD structures represented acceptable results and a better improvement in transfection efficacy compared to PD structures [23].

In another point of view, one of the surface overexpressed receptors on cancerous cells is the epidermal growth factor receptor (EGFR). The dodecapeptide GE11 (YHWYGYTPQNVI) is an EGFR ligand that was earliest explored by Li *et al*. in 2005 and its significant affinity for tumor cells was confirmed in the *ex vitro* and *in vivo* investigations [24]. The GE11 sequence was obtained by the phage display technique, which effectively optimized gene delivery to a wide spectrum of tumor based cells *in vivo* and *ex vivo* without activating EGFR. In many studies, GE11 has been used as a ligand to target EGFR on the surface of cancer cells, and GE11 ligand effectively increases the efficiency of drug or gene transfer to cells that express EGFR at a high level [25]. Herein, based on our previous experience, we aim to build two forms of LMWP to obtain MEND targeted carrier. The prepared MEND carrier was targeted with the GE11 ligand to evaluate its efficiency in

transfection of the A549 cell line in comparison with PD carriers.

MATERIALS AND METHODS

Materials

Cholesteryl hemisuccinate (CHEMS), dioleoylphosphatidylethanolamine (DOPE), and DSPE-PEG (2000)-MAL were provided from Avanti Polar Lipids (USA). Methanol and chloroform were purchased from Merck (Germany). Fetal bovine serum (FBS) and penicillin/streptomycin were from Gibco (Gaithersburg, MD, USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Biowest (France). Cholesterol, [3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide] (MTT) and branched polyethylenimine (25 kDa) were obtained from Sigma-Aldrich (Munich, Germany). VV45 linear peptide, VV32 branched peptide and GE11 targeting ligand were designed and custom synthesized respectively by China peptides Co., Ltd (VV45) and Pepmic Co., Ltd (VV32, GE11) (China).

Preparation of plasmid DNA

Transformed *Escherichia coli* bacterial strain DH5α was exploited for Plasmid DNA (pDNA) encoding (pGFP) purification. Amplification media was selective Luria-Bertani (LB) medium and purification was carried out with QIAGEN Mega Plasmid kit (Qiagen, Hilden, Germany). The purity of the extracted pDNA was confirmed by measuring the A260/A280 ratio by NanoDrop (NanoDrop 2000 spectrophotometers).

Preparation of the peptide stock solution and polyplexes

Acetate buffer (5 mM, pH=5.5) was the solution of both linear VV45 and branched peptides VV32. All the stocks were provided in optimum concentration of 1 mg/ml. To avoid peptide denaturation due to freeze/thaw cycles, aliquots were prepared and stored at -20 ◦C.

Polyplexes were fabricated by using VV45 and VV32 peptides in different amounts and pGFP plasmid. Different concentrations of peptides as a carrier (C) were separately diluted into 50 µl of HBG buffer (HEPES-buffered glucose, 20 mM HEPES, 5% glucose, pH 7.4) and mixed with 50 µl solutions of the plasmid (pEGFP) (P) in the same solvent (4 µg/50 µl). Different weight ratios (C/P) ranging from 0.5:1 to 10:1 were prepared and incubated at room temperature for 20 min to form polyplexes (polycation/DNA complexes). The prepared polyplexes were used to obtain the most acceptable C/P ratio using the gel retardation assay.

Evaluation of DNA binding and neutralization by gel retardation assay

Gel retardation assay was implemented to assess the potency of the vectors to compact the pDNA. In order to perform the evaluation peptide/ pDNA complexes (polyplexes) were former prepared loaded into a 1% (w/v) agarose gel in TBE buffer 1X (Tris base 10.8 g, boric acid 5.5 g, disodium EDTA 0.75 g and water) containing gel red dye**.** Electrophoresis was performed at 70 V for 45 min and observed with the gel documentation Imaging System (Gel Doc).

Conjugation of GE11 to DSPE-PEG (2000)-MAL

In order to bind the GE11 ligand to DSPE-PEG (2000)-MAL, first DSPE-PEG (2000)-MAL was dissolved in dry chloroform and GE11 in anhydrous DMSO. They were added together in a ratio of 1:1.2 (lipid:peptide). The reaction was performed in the dark in a 28 ◦C incubator under argon gas with rotation of the reaction mixture with a magnetic stirrer for 48 hr. To confirm the binding of the peptide to the lipid, TLC was performed at 0, 24 and 48 hr intervals to remove the DSPE-PEG-MAL lipid stain in the reaction on the TLC paper.

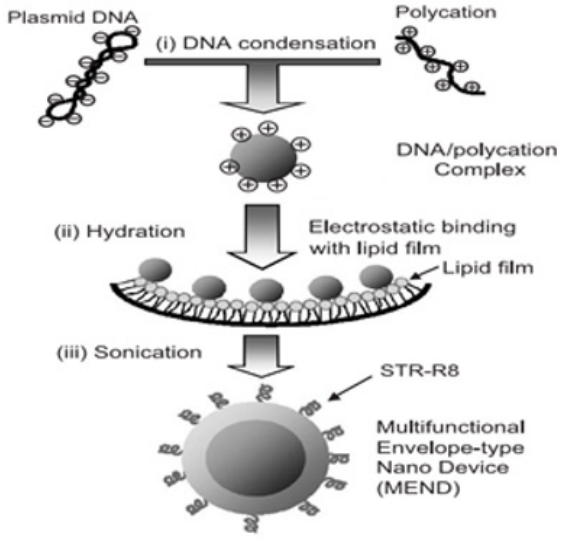
After the completion of the reaction, the sample was placed in a rotary for 5 hr to remove chloroform, and then argon gas was applied to the sample for 15 min to completely remove the chloroform. Otherwise, the sample will not freeze due to the presence of chloroform. Then the sample was placed in the freezer to freeze well, and then the frozen sample was connected to a refrigerated freeze-dryer for 24 hr. Freeze dryer was used to remove DMSO. Finally, the lyophilized powder was dissolved in 1 ml of sodium chloride solution and transferred into a 12 kDa dialysis bag, and dialyzed in a normal saline buffer for 4 hr. HPLC analysis confirmed that most of the DSPE-PEG-MAL molecules were conjugated with the ligands after this reaction. After freeze-drying, the resulting powder was dissolved with 1 ml of 150 mM sodium chloride solution with a little heat, and then this solution was poured into a 12 kD dialysis bag and dialyzed in sodium chloride medium for 2 hr.

Preparation of MEND and T-MEND formulations

This formulation was prepared according to Kogure methodology (1), and the concentration of lipids in this formulation is 0.55 mM. Considering that the total concentration of lipids in a volume of 500 µl is 275 nmol, the amount of each lipid was calculated based on its ratio and molecular weight. According to the results of our previous project using the same peptides to prepare LPD formulations for transfection (2), carrier-to-plasmid (C/P) ratio 6 of peptide complex (DPC)/ DNA was used to prepare MEND formulations. The calculated amounts of lipids were poured into the bottom of a test tube, and while spinning, the solvent evaporated under argon gas to form a lipid layer. After that, the necessary amounts of peptide and DNA were dissolved in HEPES buffer to prepare C/P 6, and the peptide solution was added to the plasmid solution and mixed slowly, and kept at room temperature for 15 min to form a complex. The lipid film was hydrated with a DPC solution for 15 min. It was then sonicated for 2 min at 40 °C in a water bath and an extra one minute with a probe sonicator while the sample was on ice. For preparing targeted MEND liposomes (T-MEND), 192 µl of DSPE-PEG-GE11 conjugated micellar solution was added to 1 ml of MEND liposome solution and placed at room temperature for one hour to allow the conjugated targeting ligand to enter the liposomal bilayer (Schematic 1).

Characterization of polyplexes and lipopolyplexes

The zeta potential and size distribution of complexes were measured with Zetasizer Nano ZS (Malvern Instruments, UK) at different C/P ratios. The measurements were performed three times for each sample, and the results are presented as mean ± SD.



Schematic 1. The schematic presentation of chemical representation of MEND structure formation

Cell culture

A549 (ATCC CCL-185) Human lung adenocarcinoma cell line was used for cellular evaluation. Cultured media as RPMI1640 supplemented with 10% fetal bovine serum, penicillin at 100 U/ml, and streptomycin at 100 µg/ml. Cells were incubated at the condition of 37 °C under 5% CO_2.

Evaluation of transfection efficiency

A549 cells were seeded at a density of 8×10^4 cells/well in 24-well plates 24 hr before transfection tests. Polyplexes and lipopolyplexes in C/P ratios of 1, 3, and 6 were performed and exposed to the cells. After 4 hr of incubation at 37 °C, the medium was removed and replaced with a fresh complete medium, and the cells were further incubated for 24 hr.

A fluorescent microscope was employed to monitor and record the fluorescence intensity (Digital bio Juli, Korea). Prior to conducting a flowcytometry analysis, each well's cells were individually extracted using a trypsin-EDTA solution after being cleaned with PBS. Following the neutralization of the trypsin by the added medium, the cell suspension was centrifuged at 5000 rpm for 10 min at 4 °C in order to isolate the cellular pellet. After that, supernatant discarded and the cell residue was washed with 1 mL cold PBS. Finally, the cells were suspended with 1 ml cold PBS for visualization assessment. The GFP fluorescent intensity was recorded by BD Accuri C6 flow cytometer equipped with a 533/30 nm laser in the FL1 channel (BD Biosciences, San Jose, CA, USA). The data analysis was performed by Flowjo 7.6.1 software.

Evaluation of cytotoxicity

In order to assess the vitality of the cells, A549 cells (1×10^4 cells/well) were sown for a whole day in 96-well plates. After being exposed to the cells, the previously generated C/P ratios were cultured in fresh medium (without FBS) for 4 hr. After optimised 4 hr of incubation, supplemented medium was exchanged and the cells were incubated for 24 hr. 10 µl of an MTT

dye solution (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide) (5 mg/ml) in PBS solution was added to each well and incubated for 4 hr at 37 °C. Finally, the medium was aspirated off and replaced with 100 µl of dimethyl sulfoxide (DMSO) for each well. The optical absorbance was measured using the microplate reader (Biotech Instruments Inc., Winooski, VT, USA) at 570/630 nm.

Statistical analysis

Data are reported as the mean±standard deviation (SD) triplicates. The significance level was determined using one-way ANOVA with a P-value<0.05 or less as the significance level.

RESULT AND DISCUSSION

LMWP with a typical sequence of VSRRRRRRGGRRRRC is manufactured with the enzymatic hydrolysis of natural protamine. Protamine is considered a FDA nontoxic cell-penetrating peptide, and the powerful protein transduction domain (PTD) peptide [26]. LMWP act is so similar to TAT protein, the classic HIV transcriptional activator that identified as an assistant of delivery vehicles for cellular penetration without antigenicity and mutagenicity. PTD-mediated cellular entry does not utilize regular transcellular pathways such as receptor-mediated endocytosis; therefore, PTD-mediated cell internalization does not damage and rupture cellular membrane [27, 28]. Due to the Tabor *et al.* study, branched lipopolyplexes that contained branched arginine-containing peptides showed better transfection efficacy compared to linear ones [29].

In our previous study, two multifunctional peptides were manufactured based on two consecutive linear or branched LMWP with a histidine group (H10) followed by an NLS (PKKRKV) block. LPD complexes were fabricated by both peptides, VV32 and VV45, branched and linear peptides respectively. The condensation ability of both peptides was also examined by gel electrophoresis assay, and the result showed that VV45 and VV32 peptides could compact the pDNA in the C/P ratios above 1 [23].

These peptides are used in the MEND system for the specific binding of the carrier to the target cells, from the targeted peptides to the specific markers of the cells. Using these designed and synthesized peptides, polyplex formulations and MEND formulations were prepared by the LPD and Kogure methodology, and their physicochemical properties, transfection efficiency, and toxicity were investigated.

For the preparation of lipopolyplex structure by MEND method, DOPE and CHEMS lipids were applied in a ratio of 9:2. On the other hand, T-MEND formulations were fabricated from MEND particles, which were targeted by the DSPE-PEG-GE11 conjugate.

In order to check the percentage of reaction efficiency and purity of DSPE-PEG-GE11 conjugate, HPLC analysis was performed. Using the line equation of the pure peptide standard graph, the amount of peptide remaining in the conjugated peptide solution before dialysis was obtained to calculate the percentage of reaction efficiency.

One of the substantial factors in non-viral gene carrier is the ability to condense DNA into nano-sized particles. The capacity of the designed peptides to neutralize the negative charge of nucleic acid and condense DNA was investigated through gel retardation method. Our previous study confirmed that, both VV32 and VV45 peptides were able to condense the plasmid DNA ratio from C/P=1. This phenomenon prevents the movement of the plasmid in the electrophoresis gel by neutralizing its charge. Therefore, ratios more than 1 is suitable for other assessments due to its DNA protection against nucleases and gene delivery. For this reason, three C/P= 1, 3, 6 were used to prepare LPD polyplex and lipopolyplex.

After conducting toxicity and transfection tests on PD and LPD carriers, since the highest amount of transfection was observed at C/P=6, therefore, for the preparation of MEND and T-MEND formulations, only this ratio of peptide to plasmid (C/P=6) exploited [23].

For cellular uptake of nanocarriers, the size and charge of the manufactured particles determine the path of their penetrance into the cell. Particles with a size less than 200 nm enter the cell through clathrin-mediated endocytosis, particles with a size of 200-500 nm through caveolae-mediated endocytosis, and particles larger than 500 nm enter the cell through a non-phagocytic pathway [30, 31]. Additionally, by enhancing the interaction of positively charged complexes with negatively charged ones, the presence of net positive charge in the nanocarrier/pDNA formulation can ease cell entrance [32].

Table 1. The size and zeta potential of polyplex (PD) and lipopolyplex (MEND, T-MEND) carriers of VV45 and VV32 peptide at C/P=6. The polyethyleneimine (PEI 25 kDa) at C/P=0.8 is a control sample. Evaluation was performed in HEPES buffer medium and culture medium (without serum) and data was represented as (mean ± SD), (n= 3)

Sample name	Samples in HEPES buffer			Samples in serum-free medium		
	Z-Average (nm)	PDI	Zeta Potential (mV)	Z-Average (nm)	PDI	Zeta Potential (mV)
PD VV45 (C/P= 6)	174.8± 42.8	0.270± 0.016	-5.99± 1.73	191.2± 1.38	0.673± 0.005	0.97± 0.995
PD VV32(C/P= 6)	186.9± 27.6	0.272± 0.033	2.95± 1.09	187.9± 1.96	0.658± 0.011	0.11 ± 2.31
MEND (VV45)	304.33±45.7	0.54±0.08	-14.5±0.7	329.1±67.59	0.560±0.028	-3.28±1.48
MEND (VV32)	228.56±17.01	0.320±0.052	12.9±1	245.8±32.47	0.543±0.135	-0.15±2.54
T-MEND (VV45)	391.9±41.3	0.676±0.063	-13±2.77	403.3±54.49	0.569±0.051	-6.03±3.23
T-MEND (VV32)	353.1±75.5	0.607±0.055	-5.57±1.63	348.1±48.2	0.549±0.068	-5.89±1.50
PEI 25kDa (C/P= 0.8)	158.3± 1.67	0.251± 0.115	10.8± 0.53	199.3± 1.43	0.342± 0.122	6.41± 3.50

The size of polyplex carrier (PD) formulations in both peptides was below 200 nm and MEND formulations were between 200-300 nm. As can be seen in Table 1, the zeta potential of PD formulations in the deionized water medium was positive and in serum-free culture medium, their zeta potential was negative. The zeta potential of MEND (VV32) and PD VV32 formulations was positive, and MEND (VV45) and PD VV45 formulations were negative, and in T-MEND formulations, the zeta potential of both peptides decreased and became negative. The size and zeta potential data of MEND and T-MEND formulations showed that the presence of DSPE-PEG-GE11 on the surface of T-MEND formulations caused an approximate increase in particle size by 70-120 nm compared to the MEND formulations. It may signify the ligand's penetration into the liposomal bilayer of the MEND formulation, which is consistent with the increased particle size brought about by the surface presence of PEG-GE11. Also, the addition of DSPE-PEG-GE11 ligand into the liposomal bilayer in the T-MEND formulation created a negative charge compared to the MEND formulation, which could be attributed to the presence of PEG on the surface of the T-MEND liposome or the negative charge of GE11 at pH=7.4 [33].

Polyplex (PD) and lipopolyplex formulations of MEND and T-MEND were prepared with two peptides VV45 and VV32 at C/P=6, which had the highest transfection in LPD formulation [23], and were evaluated in terms of transfection efficiency. For qualitative and quantitative assessment of GFP protein expression resulting from the GFP gene transfer to the cells by the manufactured carriers, flowcytometry assay was carried out and microscopic imaging were checked. PEI 25 kDa was used as a gold standard for transfection. The transfection effectiveness of MEND and T-MEND formulations was much higher than that of pGFP (P<0.0001), as shown in Fig. 1. However, the expression of this level was not statistically significant when compared to PEI 25 kDa. The schematic of GFP transfection of the prepared polyplexes and lipopolyplexes were summarized in Fig. 2.

T-MEND formulations contained VV32 and VV45 peptides show slightly higher transfection than similar MEND formulations. Although this value is lower than the expected amount due to the presence of the target ligand on the surface of T-MEND carriers, it may be attributed to the increase in the size of T-MEND formulations

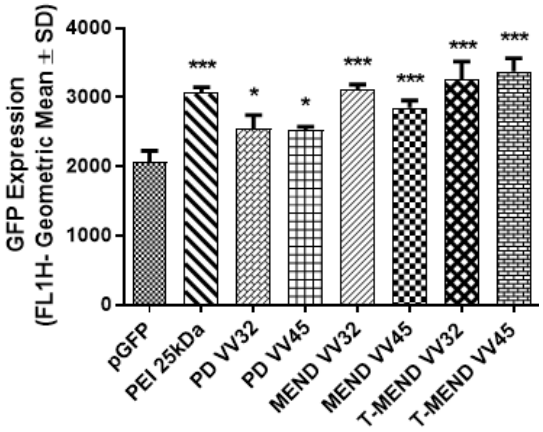


Fig. 1. Transfection efficiency of polyplex (PD) and lipopolyplex (MEND, T-MEND) samples of VV45 and VV32 peptides at C/P=6 in A549 cells. PEI 25kDa as a control group was compared with all of the vectors. (P<0.0001 = ***, P<0.001 = **, P<0.05 = *, compared mean with pGFP) (Mean ± SD, n= 3).

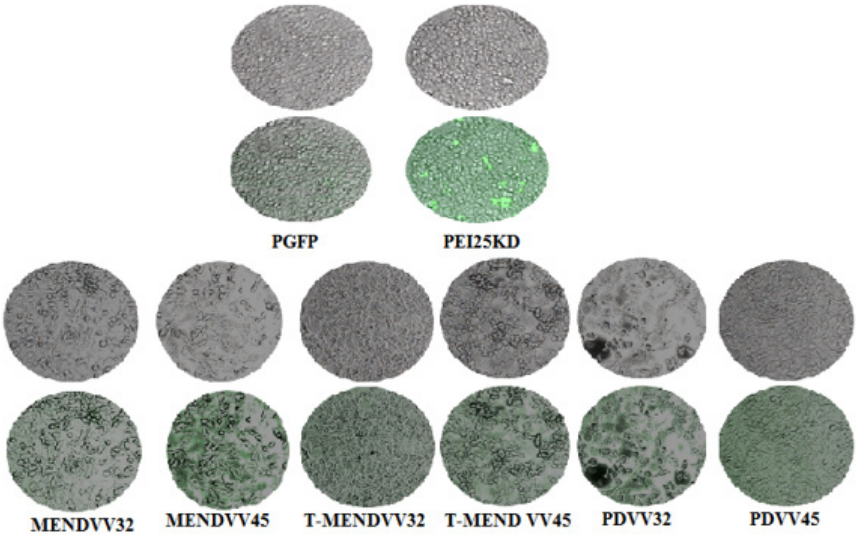


Fig. 2. GFP transfection of A549 cells by VV45 and VV32 polyplexes and lipopolyplexes in comparison with PEI 25 kDa (positive control) and PD (protamine) using fluorescent microscopy. The upper and lower images are dedicated to the bright-filed and merged fluorescent images, respectively

compared to MEND formulations that altered the cellular uptake pathway. It is essential to note that while NPs move through the biological environment, their sizes change [34]. Comparing identical PD complexes to the peptide-DNA complex inserted into two liposomal layers in the MEND formulation, however, boosted transfection efficiency, similarly to how LPD complexes did. This increase can be explained by lack of significant cytotoxicity and can be considered as a promising point for conducting more studies on these formulations.

Due to the result of metabolic activity test (Fig. 3), despite the use of peptide-DNA complex in the lipopolyplex core at C/P=6, MEND lipopolyplex did not represent significant cytotoxicity. Lack of cytotoxicity can be the result of the type of applied lipids in nanoparticle structure. DOPE and CHEMS are the fundamental component in the MEND structure which did not have the cationic properties of DOTAP, hence it did cause less damage to the cell membrane and cytotoxicity. Exceptionally in the case of MEND and T-MEND carriers corresponded with VV45 peptide, a little cytotoxicity is seen which can be related to the size of manufactured nanoparticle.

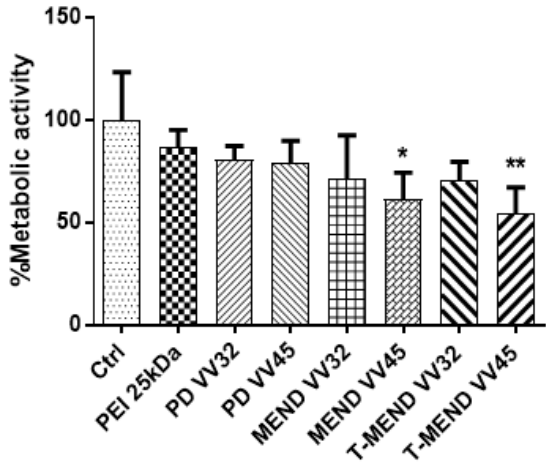


Fig. 3. Percentage of Metabolic activity of A549 cells incubated with MEND or PD complexes in comparison with PEI 25kDa as control. (P<0.001 = **, P<0.05 = *) (Mean ± SD, n=3).

CONCLUSION

To sum up, MEND can incorporate different functional moieties including a specific ligand to specific cells, intracellular sorting devices that accelerate endosomal escape, long circulation, etc. it can be concluded that multifunctional carriers designed based on LMWP are considered as the safe carrier for gene delivery. Presence of protamine in the nanoparticulate structure did not increase the risk of cytotoxicity of carriers, when included in lipopolyplex formulations, but also they showed high transfection efficiency. In MEND formulation, in spite of the fact that it did not increase transfection significantly like LPD, but it showed low cytotoxicity and acceptable

transfection efficiency at the level of PEI 25 kDa. Nevertheless, further studies are essential to explore the potential of these novel synthesized NPs to improve their efficiency in gene delivery.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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