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RESEARCH ARTICLE

Gene Delivery to Triple-Negative Breast Cancer Cells by Folic Acid-Polyethyleneimine Polyplexes

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SUMMARY

Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer. It lacks hormonal and growth factor receptors commonly expressed by other types of breast cancer, making it difficult to treat by conventional treatments. Although gene therapy might be a therapeutic option, delivery of genes into TNBC cells is still an obstacle. In this study, it was aimed to overcome this obstacle by folic acid (FA) conjugated polyplex formulations to target the folate receptor, which has been reported to be overexpressed in TNBC cells. Non-covalent complexes of FA and linear polyethyleneimine (LPEI) polyplexes (FA-LPEI polyplexes) were prepared at six different ratios. After characterization studies, cytotoxicity and transfection ability were evaluated. Conjugation of FA by increasing amounts of LPEI polyplexes increased the size from 204.1 to 469.8 nm. Their PDI values were between 0.31-0.51, and zeta potentials were positive. After treatment with polyplex formulations, cell viability decreased significantly, starting from 3:1(w/w) LPEI:pDNA ratio and from 3:3:1 (w/w/w) FA:LPEI:pDNA ratio. Cell viability decreased below 70%, only above the 5:1 (w/w) LPEI:pDNA ratio. Adding of FA to polyplex formulations reversed the cytotoxicity of P3, P4, and P5 formulations. Although LV-RFP pDNA was delivered successfully into 4T1 cells by all formulations, fluorescent microscope images showed that the optimal formulations were FA-P3 and FA-P4. This gene delivery system, generated by the non-covalent conjugation of FA to polyplexes, increased the uptake and decreased the cytotoxicity of LPEI polyplexes. Non-covalent complexes of FA-PEI polyplexes represent promising delivery systems in gene therapy, directed against cancer cells expressing folate receptors.

Key Words: *Triple-negative breast cancer, gene therapy, delivery system, folic acid, polyethyleneimine, polyplex*

Üçlü-Negatif Meme Kanseri Hücrelerine Folik Asit-Polietilenimin Poliplekslerı ile Gen Aktarımı

ÖZ

Üçlü-negatif meme kanseri (TNBC), meme kanserlerinin en agresif alt tipidir ve diğer meme kanseri türleri tarafından yaygın olarak eksprese edilen hormonal ve büyüme faktörü reseptörlerinden yoksundur, bu durum mevcut tedavilerle tedavi edilmesini zorlaştırmaktadır. Gen tedavisi terapötik bir seçenek olabilse de, genlerin TNBC hücrelerine aktarılması hala bir engeldir. Bu çalışmada, TNBC hücrelerinde aşırı eksprese edildiği bildirilen folat reseptörünü hedefleyen, folik asit (FA) konjuge edilmiş polipleks formülasyonları ile bu engelin aşılması amaçlandı. FA ve doğrusal polietilenimin (LPEI) poliplekslerinin (FA-poliplekslerı) kovalent olmayan kompleksleri altı farklı oranda hazırlandı. Karakterizasyon çalışmalarından sonra, hücre toksisitesi ve transfeksiyon yeteneği değerlendirildi. Folik asitin artan miktarlarda LPEI poliplekslerı ile konjugasyonu, boyutu 204.1 nm'den 469.8 nm'ye yükseltti. PDI değerleri 0.31-0.51 arasında olup, zeta potansiyelleri pozitifti. Hücre canlılığı, polipleks formülasyonları uygulandıktan sonra, 3:1 (a/a) LPEI:pDNA oranından ve 3:3:1 (a/a/a) FA:LPEI:pDNA oranından başlayarak anlamlı oranda azaldı. 5:1 (a/a) LPEI:pDNA oranının üzerinde, hücre canlılığı %70'in altına düştü. Polipleks formülasyonlarına FA eklenmesi, P3, P4 ve P5 formülasyonlarının sitotoksisitesini tersine çevirdi. LV-RFP pDNA, tüm formülasyonlar tarafından 4T1 hücrelerine başarılı bir şekilde aktarıldığı halde, floresan mikroskop görüntüleri optimum formülasyonların FA-P3 ve FA-P4 olduğunu gösterdi. Folik asidin poliplekslere kovalent olmayan konjugasyonu ile hazırlanan bu gen taşıyıcı sistem, LPEI poliplekslerinin hücre içine alımını arttırdı ve sitotoksisitesini azalttı. FA-PEI poliplekslerinin kovalent olmayan kompleksleri, folat reseptörünü eksprese eden kanser hücrelerine gen aktarımında kullanılabilecek gelecek vaadeden taşıyıcı sistemlerdir.

Anahtar Kelimeler: *Üçlü-negatif meme kanseri, gen tedavisi, taşıyıcı sistem, folik asit, polietilenimin, polipleks*

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INTRODUCTION

Cancer is a complex disease, which is developed by the uncontrollable proliferation of cells. Various cancer treatment methods are chosen based on the type, stage, prognosis, and individual circumstances. Surgery, chemotherapy, biological therapy, and radiotherapy are among the available forms of treatment.

One of the most prevalent cancers affecting women worldwide is breast cancer (Siegel et al., 2021). Breast cancer can be divided into numerous subtypes based on histological and molecular properties (Zubair et al., 2021). The most aggressive and fatal subtype of breast tumors is triple-negative breast cancer (TNBC) (Sharma et al., 2010). In contrast to other subtypes, TNBC lacks estrogen, progesterone, and human epidermal growth factor receptor 2 (HER2). TNBC cells are resistant to available therapies, and there is currently no effective and targeted therapy therefore, alternative treatment methods are required (Zubair et al., 2021; Kumar & Aggarwal, 2016).

Gene therapy is a potentially beneficial cancer treatment approach. It consists of the delivery of genes such as "DNA, RNA, mRNA, siRNA, antisense oligonucleotide, CRISPR/Cas9 system, ZFNs, and TALENs" to patients to repair responsible damaged genes that cause disease (European Medicines Agency, 2018; Demir-Dora, 2021; Cesur-Ergün & Demir-Dora, 2023). The European Medicines Agency (EMA) defines gene therapy medicinal products as "those that contain genes that have therapeutic, prophylactic, or diagnostic effects and are applied to repair the tissue damage, replenish the deficiencies to maintain the functionality of the body, and prevent the expressions of unwanted genes" (European Medicines Agency, 2018).

By manipulating genetic material to repair or modify dysfunctional genes, suppress their expression, or introduce therapeutic genes to counteract the disease process, breast cancer gene therapy offers a promising way to increase the efficacy of conventional treatments and lengthen survival rates (Dastjerd et al., 2022). Targeted gene therapy aims to deliver related genes only to target cells or tissues (Esam et al., 2021).

For successful gene therapy, non-viral vectors must be designed specifically for the cell type to deliver the cargo into the target cells (Demir-Dora, 2021). Many diseases, including cancer, make cells express specific cell surface receptors at higher levels (Antignani et al., 2020). By attaching ligands or antibodies to vectors that match these receptors, gene therapy vectors can be designed to bind preferentially to the targeted cells (Patil et al., 2019; Krystofiak et al., 2012).

The folate receptor is a glycoprotein in the cell membrane that is primarily expressed in epithelial cancer cells (Prabhu et al., 2015). They are attractive targets for delivering therapeutic genes to cancer cells specifically because they are overexpressed in various cancers, including ovarian, lung, and breast cancer. Folic acid (FA) acts as a ligand that can bind to gene delivery systems by chemical modifications. FA is more important for the proliferation of cancer cells than it is for normal cells. Cancer cells use the folate receptor (FR) for external folic acid uptake because they cannot produce folic acid independently. This allows them to grow and maintain themselves. Higher levels of FR expression are observed on the surface of solid tumors (Jahan et al., 2021; Krystofiak et al., 2012).

Non-viral gene delivery vectors can be prepared by natural or synthetic polymers. In research studies, cationic polyethyleneimine (PEI), poly(L-lysine), chitosan, and cyclodextrin are mainly used polymers besides poly[2-(dimethylamino) ethyl methacrylate] (PDMAEM) and poly (amidoamine) (PAMAM) dendrimers (Fahira et al, 2022; Yuan & Li, 2017; Küçüktürkmen et al., 2017; Saka & Bozkır, 2012; Salva et al., 2013; Masimov & Büyükköroğlu, 2023, Demir-Dora & Öner, 2023). Because of its strong buffering ability at the acidic endosomal pH, PEI has endosomolytic activity (Fahira et al., 2022; Sabin et al., 2022). PEI/DNA complexes help DNA pass through the cell membrane

during endocytosis by adhering to negatively charged proteoglycans on the cell surface. Different PEI forms and molecular weights can affect a PEI's effectiveness and toxicity in the delivery of genes (Ahn et al., 2002). Although linear PEIs (LPEIs) have a low molecular weight and are not toxic, their effectiveness in gene delivery is limited. Branched PEIs (BPEIs) are toxic and have a high molecular weight, but they also have a high transfection efficiency (Zakeri et al., 2018).

In this study, a novel polymeric delivery system was developed for plasmid DNA (pDNA) delivery into triple-negative breast cancer cells, which are very difficult to transfect. Electrostatic interactions between negatively charged pDNA and positively charged LPEI were used to prepare PEI polyplexes. By non-covalent binding of FA to PEI polyplexes, a new gene delivery system was obtained (FA-PEI polyplexes) with various FA, LPEI, and pDNA amounts. The obtained polyplex formulations were characterized by their particle sizes, polydispersity indexes, and zeta potential values. Cytotoxicity and transfection ability of these formulations were evaluated on 4T1 cells.

MATERIALS AND METHODS

Materials

LPEI (Polysciences; Inc Cat. No. 23966, MW: 25 kDa) was used as a cationic polymer. Plasmid pLV-RFP (Beronja et al., 2010), which encodes red fluorescent protein, was from Addgene (Addgene plasmid #26001). The DNA isolation kit was QIAGEN (Cat No/12162). 4T1 cells were from ATCC. All other chemicals were from Sigma & Aldrich.

Isolation of Plasmid DNA

E. coli DH5α cells, which were made competent by the $CaCl_2$ method, were used for amplification of pLV-RFP pDNA. After the Ampicillin selection of transformants, pDNA was purified by QIAGEN maxi kit. The purity of pDNA was checked by either electrophoretic mobility on 1% (w/v) agarose gel or evaluating (A260 nm / A280nm) UV absorbance values.

Preparation of formulations

Polyplex formulations were prepared by using LPEI and LV-RFP pDNA. 1 μg/μL pDNA solution and 1mg/mL LPEI solution were prepared in ultra-pure (DNase/RNase-Free) distilled water (pH 7.0). LPEI:pDNA complexes (polyplexes) were freshly prepared at increasing amounts of LPEI to the constant amount of pDNA (1 μg) at 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 (w:w) ratios and labeled as P1, P2, P3, P4, P5, and P6 subsequently. pDNA was added to LPEI solution and, after mixing, incubated at 37°C for 30 minutes to allow complete binding via electrostatic interactions.

Non-covalent FA:LPEI:pDNA polyplex formulations were prepared at increasing amounts of FA and LPEI to the constant amount of pDNA (1 μg) at 1:1:1, 2:2:1, 3:3:1, 4:4:1, 5:5:1, 6:6:1 (w/w/w) ratios and labeled as FA-P1, FA-P2, FA-P3, FA-P4, FA-P5 and FA-P6 subsequently. FA was mixed with polyplex formulations and incubated at 37°C for 30 minutes to allow complete binding via electrostatic interactions. Non-covalent complex formation and pDNA binding ability were evaluated by gel retardation assay by 1% (w/v) agarose gel electrophoresis. Samples were run at 90 v for 60 min in 1% TAE buffer, pH 8.0. FA:LPEI:pDNA polyplex formulations were freshly prepared before use at all stages.

Physicochemical characterization

For the physicochemical characterization of polyplex and FA-LPEI polyplex formulations, measurements of particle size (z-average), polydispersity index (PDI), and zeta potential (surface charge) were carried out. Dynamic light scattering (DLS) (Malvern Zetasizer ZEN3600, UK) was used to measure the mean particle diameter (Z-average) and the PDI of non-covalent polyplexes. At least, three copies of each measurement were made at room temperature. The zeta potential was calculated using the pure water's viscosity and dielectric constant and measured using laser Doppler velocimetry at 25°C on the same instrument.

Evaluation of cytotoxicity

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the cytotoxicity of formulations on the 4T1 cell line. RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and penicillin-streptomycin (100 IU/mL penicillin, 100 µg/mL streptomycin) was used to culture 4T1 triple negative mouse breast cancer cells in a 96-well plate at a density of $15x10^3$ cells/well. The cells were then incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO_2. After incubation, the growth medium was removed, and PBS (pH 7.4) was used to wash the cells. Following the addition of 100 µL of fresh medium (control group), formulations of polyplex or FA-polyplex, as well as fresh medium, were added. 20 µL of MTT (AppliChem, A2231) solution (5 mg/mL in PBS) was added to each well after the initial incubation period, and the cells were then incubated at 37°C for an additional 4 h. To dissolve formazan crystals, which are produced by live cells, 100 µL of DMSO (Sigma D8418) was added to the culture medium after the incubation period.

Using a microplate reader, the optical densities of each well were measured at 570 and 630 nm. To eliminate the background absorption, blank samples were used. The percentage of absorbance that was used to measure cell viability in comparison to the control group of cells that had been given the culture medium was calculated. For each sample, three replicates were carried out.

Evaluation of transfection ability

Transfection studies were done in three groups to evaluate the effect of folic acid. In the first group (Group A), freshly prepared PEI polyplexes by increasing amounts of LPEI (P1 to P6) were used for transfection studies. In the second group (Group B), non-covalently bound FA–LPEI polyplex formulations (FA-P1 to FA-P6) were used. In the third group (Group C), 1 mM FA was added to the RPMI 1640 medium to test whether FA added into the media could lead to a similar transfection for LPEI polyplexes.

RPMI 1640 medium supplemented with 10% FBS, L-glutamine, and penicillin-streptomycin was used to maintain 4T1 cells. Before transfection experiments, 4T1 cells ($15x10^3$ cells/well) were cultured for 18 hours in a humidified incubator (5% CO_2) at 37°C. Cells were then incubated with formulations of LPEI polyplexes and folic acid-polyplexes for 4 hours at 37°C and washed with PBS after the incubation period. For groups A and B, fresh growth medium and for group C, growth medium containing 1mM folic acid was then added to the wells. After 24, 48, and 72 hours, the fluorescence signal was evaluated under the fluorescence microscope.

Statistical analysis

All experiments' results were presented as means ± standard deviation. The SPSS® statistical package, S®version 26.0 (SPSS, Inc., Chicago, IL, USA), and the GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA) for Windows® were used to conduct the statistical analyses. One-way ANOVA was used to analyze the data, and Tukey's post-hoc test was used to determine its statistical significance. Significant results were defined as p-values < 0.05.

RESULTS AND DISCUSSION

TNBC is the most aggressive subtype of breast cancer. It lacks hormonal and growth factor receptors commonly expressed by other types of breast cancer, making it difficult to treat by conventional treatments used in breast cancer. Although gene therapy might be a therapeutic option, delivery of genes into TNBC cells is still an obstacle. The folate receptor, which has been reported to be overexpressed in 4T1 mouse TNBC cell lines, offers a good choice for targeting TNBC cells (Jahan et al., 2021; Krystofiak et al., 2012).

A new non-viral gene delivery system was created in this study by non-covalently conjugating FA. It was characterized and tested on the 4T1 mouse TNBC cell line for pDNA condensation ability, cytotoxicity, and transfection ability.

Preparation of formulations

Formulation of effective delivery systems that can accurately deliver genes into target cells is a complex process, and optimizing this process is essential for success. Nonviral gene delivery systems must be specially created for the genetic material and cell type to have a therapeutic effect on target cells (Mohammadi et al., 2023; Ren et al., 2019). To improve the stability, cellular uptake, and intracellular trafficking of pDNA, viral or non-viral gene delivery systems are used. TNBC is one of the cancer cells that express folate receptors, and FA-conjugated polyplexes represent promising vectors in gene therapy targeted against these cells (Jiang et al., 2011; Laemon & Low, 2001; Lu & Low, 2012; Cao et al., 2020; Jahan et al., 2021).

This study aimed to develop a non-viral polymeric gene delivery system due to LPEI's low toxicity and cost-effectiveness. By non-covalently conjugating FA to LPEI:pDNA complexes, a new gene delivery system was developed. Electrostatic interactions between the negatively charged LV-RFP pDNA phosphate groups and protonated nitrogen atoms of the LPEI resulted in the formation of polycation/pDNA complexes. The purity range for pDNA was 1.8 to 2. Our previous research demonstrated that LPEI complexes can condense pDNA starting at a polymer:pDNA ratio of 1:1 (w/w), which corresponds to an N/P of 3 (Demir-Dora & Öner, 2023).

Non-covalent conjugated FA:LPEI:pDNA polyplex formulations were prepared by complexation of an increased amount of FA and LPEI with the constant amount of pDNA (1 μg) at 1:1:1, 2:2:1, 3:3:1, 4:4:1, 5:5:1, 6:6:1 (w/w/w) ratios. A gel retardation assay was performed to evaluate the pDNA condensation ability of FA-LPEI polyplex formulations. In all FA-LPEI polyplex formulations, pDNA migration was wholly inhibited at all ratios while the free pDNA moved from the well (Figure 1).

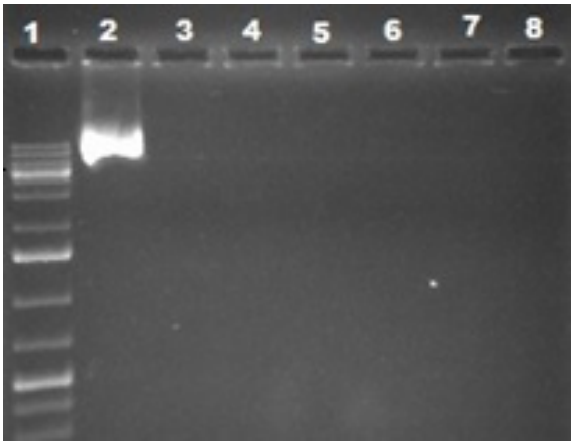

Figure 1. Gel retardation assay photograph of FA-LPEI polyplexes containing increasing amounts of FA and LPEI polymers and constant amount of LV-RFP pDNA between 1:1:1 and 6:6:1 (w/w/w) FA:LPEI:pDNA ratios. Lane 1: DNA mw marker (1 kb plus DNA ladder, Thermo), Lane 2: Naked LV-RFP pDNA (7539 bp), Lane 3: FA-P1, Lane 4: FA-P2, Lane 5: FA-P3, Lane 6: FA-P4, Lane7: FA-P5, Lane 8: FA-P6

Due to its effectiveness, affordability, ability to form electrostatic complexes, and interaction with cell membranes, PEI is widely utilized in developing gene and drug delivery systems. The condensation of pDNA by PEI polyplexes is a critical step in gene delivery, as it enhances the stability, cellular uptake, endosomal escape, nuclear localization, and expression of the delivered gene (Mohammadi et al., 2023; Zakeri et al., 2018).

Characterization of gene delivery systems

The physical characteristics of the non-viral delivery systems, such as size, surface charge, and the presence of various functional groups, can impact their effectiveness, cell toxicity, and cellular uptake (Honary et al., 2013). By measuring particle size, PDI, and zeta potential, LPEI polyplexes and FA-LPEI polyplexes were characterized. Table 1 presents the findings for all formulations prepared at different amounts and ratios of FA, LPEI, and pDNA.

The polyplex sizes ranged between 181.6 nm and 416.1 nm for LPEI polyplexes and 204.1 nm and 469.8 nm for FA-LPEI polyplexes. Smaller nanocarriers were obtained for P1 and FA-P1 formulations, which represented 1:1 LPEI:pDNA (w/w) and 1:1:1 FA:LPEI:pDNA (w/w/w) ratios. As the polymer amount increased, the particle size tended to increase as expected. FA-LPEI polyplexes had a bigger particle size compared to the LPEI polyplexes. If the delivery vector has a small size of <200 nm, entry into the intracellular compartment will be easy (Valente et al., 2021). Although the FA-LPEI polyplexes have a bigger size, they have FA ligands to target folate receptors on TNBC cells.

The polyplexes' polydispersity index (PDI) is a crucial measure for determining particle dispersion. A low PDI value signifies that the polyplex formulations' particle sizes are distributed quite uniformly. Polyplexes with a narrow size distribution are more stable over time and are less prone to aggregation or dissociation. PDI values of less than 0.3 are considered suitable for polyplexes (Elsana et al., 2019). According to our results, binary polyplex formulations P3 and P4 had 0.35 and 0.33 PDI values, respectively, and FA-LPEI polyplex formulations FA-P3 and FA-P4 had 0.32 and 0.31 PDI values, respectively.

The stability and behavior of polyplexes in a solution are revealed by zeta potential. Due to electrostatic repulsion between particles, zeta potential levels between +16 and +55mV are high enough to guarantee colloidal stability (Elsana et al., 2019). Zeta potential can be affected by several variables, including PEI concentration, PEI molecular weight, pDNA size, and charge density (Valente et al., 2021).

The zeta potential results showed that PEI polyplex formulations were cationic, and the zeta potential value was increased by increasing the amount of polymer. After the forming of ternary polyplexes by folic acid, zeta potential values were decreased because of the carboxylic acid group of folic acid but remained cationic between +20.2 mv and +28.6 mv, allowing colloidal stability. The results of the gel retardation assay were also validated by zeta potential values.

Since negatively charged components of the inner layer of the cell membrane tend to translocate, cell surfaces, particularly cancer cell surfaces, are typically negatively charged. When the delivery system was positively charged and could bind to anionic cell surface proteoglycans, gene transfer was most effective. Positively charged nanocarriers are more easily taken by cancer cells than negatively charged or neutral carriers (Honary & Zahir, 2013). In addition to improving pDNA loading efficiency, a positive zeta-potential also improves the effective accumulation in the target cells (Elsana et al., 2019).

Table 1. Characterization results of LPEI polyplexes and FA-LPEI polyplexes (n=3)

Formulation	FA:LPEI:pDNA (w/w/w)	Size (nm±SD)	PDI ±SD	Zeta Potential (mv±SD)
P1	0:1:1	181.6±14	0.59±0.07	+20.9±1.2
P2	0:2:1	362.0±18	0.57±0.05	+24.3±1.1
P3	0:3:1	398.2±12	0.35±0.05	+27.2±1.1
P4	0:4:1	406.4±11	0.33±0.06	+31.7±0.9
P5	0:5:1	418.0±16	0.45±0.08	+32.9±0.9
P6	0:6:1	416.1±13	0.61±0.09	+35.8±1.2
FA-P1	1:1:1	204.1±11	0.50±0.05	+20.2±0.9
FA-P2	2:2:1	397.4±14	0.51±0.07	+22.1±1.1
FA-P3	3:3:1	415.8±10	0.32±0.09	+25.7±0.8
FA-P4	4:4:1	430.2±12	0.31±0.08	+26.2±1.1
FA-P5	5:5:1	441.0±13	0.40±0.06	+27.9±1.2
FA-P6	6:6:1	469.8±11	0.49±0.04	+28.6±0.9

Evaluation of Cytotoxicity

The effectiveness of *in vivo* and *in vitro* gene transfer is constrained by PEI's high toxicity (Costa et al., 2018). Cytotoxicity of LPEI polyplexes and FA-LPEI polyplexes on 4T1 cells was assessed by the MTT assay. Cytotoxicity studies were done to observe the effect of folic acid on PEI polyplex toxicity and explore the safety of polyplexes used for pDNA delivery to 4T1 cells.

The results showed that treatment of PEI polyplexes at 1:1 and 2:1 (w/w) polymer:pDNA ratios did not affect cell viability, with cell viability being 94% and 91%, respectively. After the addition of FA to P1 and P2 formulations, there was no meaningful change ($p>0,05$), and cell viability was 96 % and 92 %, respectively. After treatment with polyplex formulations, cell viability was reduced compared to the naked pDNA group and demonstrated considerable toxicity starting at a 3:1 (w/w) LPEI:pDNA ratio. After treatment with FA-LPEI polyplex formulations, cell viability was decreased starting from 3:3:1 (w/w/w) FA:polymer:pDNA ratio (Figure 2).

The addition of FA to the PEI polyplex formulation reversed the cytotoxicity of PEI polyplex for P3, P4, and P5 formulations. After the addition of FA to PEI polyplex formulations, cell viability was changed from 80% to 84%, 77% to 80%, and 65% to 69% for P3, P4, and P5 formulations, respectively ($p<0,05$) (Figure 2).

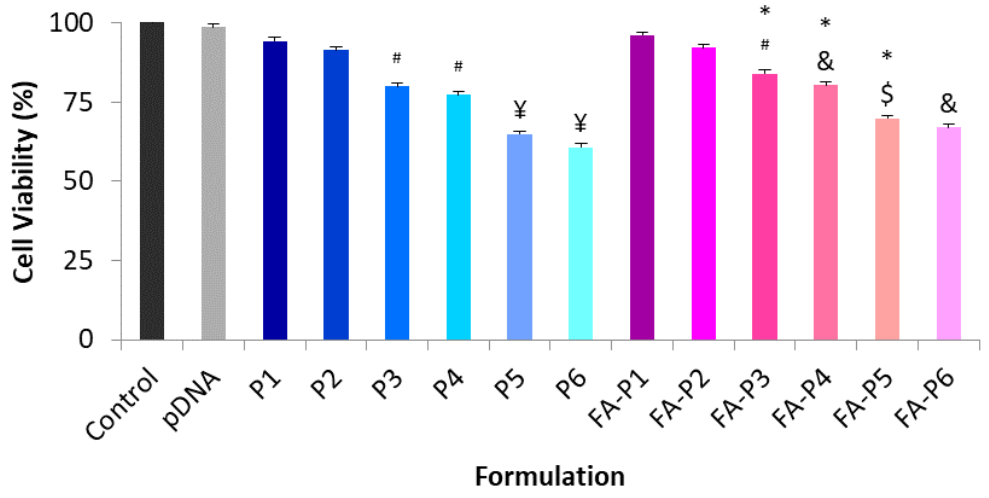

Figure 2. Cell viability of 4T1 cells treated with LPEI polyplex and FA-LPEI polyplex formulations. Comparisons were made using one-way ANOVA and Tukey's post-hoc testing, and they were as follows:*p<0.05: LPEI Polyplex vs. FA-LPEI Polyplex; $^{\#}$p<0,05: pDNA vs. LPEI Polyplex or pDNA vs. FA-LPEI Polyplex; $^{\&}$p<0,01: pDNA vs. LPEI Polyplex or pDNA vs. FA-LPEI Polyplex; $^{\$}$p<0,001: pDNA vs. LPEI Polyplex or pDNA vs. FA-LPEI Polyplex; $^{¥}$p<0,0001: pDNA vs. LPEI Polyplex or pDNA vs. FA-LPEI Polyplex.

Transfection Studies

Transfection ability of either polyplex or non-covalent FA conjugated polyplex formulations was evaluated qualitatively in 4T1 mouse triple-negative breast cancer cells by using red fluorescent protein-encoding LV-RFP pDNA with fluorescence microscopy.

All polyplex formulations successfully transfected cells despite the large particle sizes of FA-LPEI polyplexes, and they all maintained cell viability for up to 72 hours. The number of transfectants was higher after treatment of P3, P4, and P5 PEI polyplex formulations compared to P1, P2, and P6 PEI polyplex formulations. The number of transfected cells was increased by FA-LPEI polyplexes and FA in the growth medium (Figure 3). FA considerably improved polyplex transfection efficiency in various cell lines in a previous study in the presence of serum (Guo & Lee, 2001).

Nucleic acids need to get to the desired cell compartment to have a therapeutic impact. The plasmid DNA has to be delivered to the cell nucleus. The proton sponge effect, brought on by the PEI's ability to act as a proton buffer and cause the endosomal membrane to rupture, is thought to be the mechanism by which PEI polyplexes escape from endosomes. For the endosomal escape of the gene payload, PEI's high buffer capacity is also advantageous. Although PEI is a widely used transfection agent, it is inexpensive and can be used to successfully transfer plasmid DNA to the nucleus of cells which are very difficult to transfect (Costa, et al., 2018; Sabin, et al., 2022; Zakeri, et al., 2018).

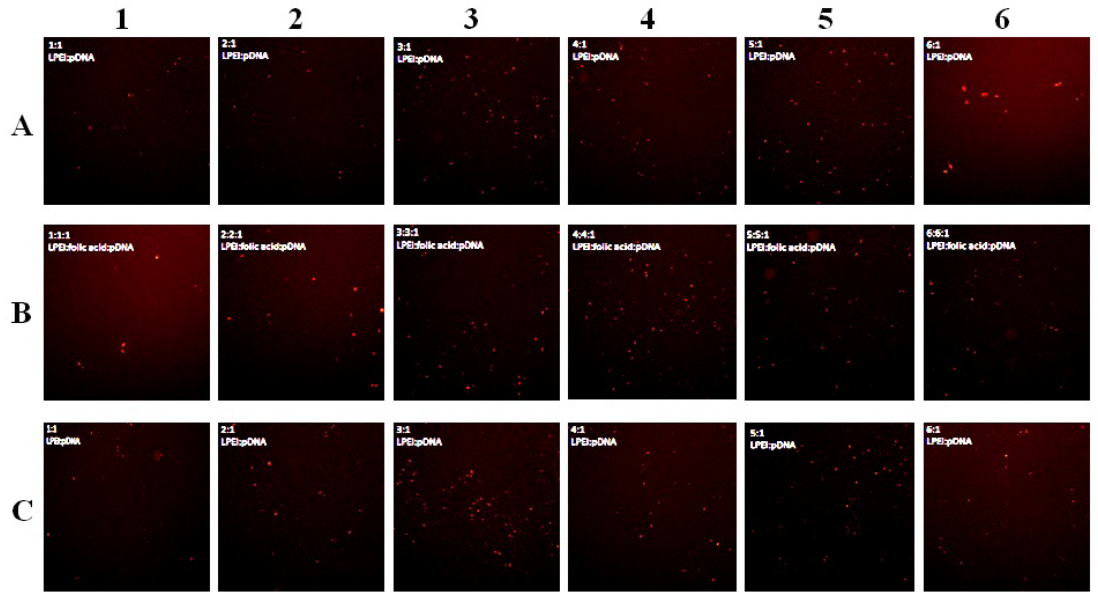

Figure 3. Qualitative transfection ability of LPEI polyplexes and FA-LPEI polyplexes. Images of red fluorescent protein (RFP) positive cells under a fluorescence microscope. A (1-6): Cells transfected by P1 to P6 polyplex formulations. B (1-6): Cells transfected by FA-P1 to FA-P6 polyplex formulations. C (1-6): Cells transfected by P1 to P6 polyplex formulations, which were incubated in the 1 mM folic acid containing medium during the transfection process.

CONCLUSION

Although gene therapy might be a therapeutic option for TNBC, which is difficult to treat, delivery of genes into TNBC cells is still an obstacle. A new gene delivery system prepared by non-covalent conjugation of FA to PEI polyplexes using FA as a ligand increased the uptake of polymeric pDNA delivery systems due to the increased overexpression of FA receptors in TNBC cells and was associated with less cytotoxicity. To treat cancer cells that express folate receptors, we suggest that FA-conjugated polyplexes are promising delivery systems. Future *in vivo* experimental investigations for breast cancer gene therapy will benefit from these findings.

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AUTHOR CONTRIBUTION STATEMENT

Design, experimental studies, data interpretation and manuscript writing (DDD)

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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