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RESEARCH ARTICLE

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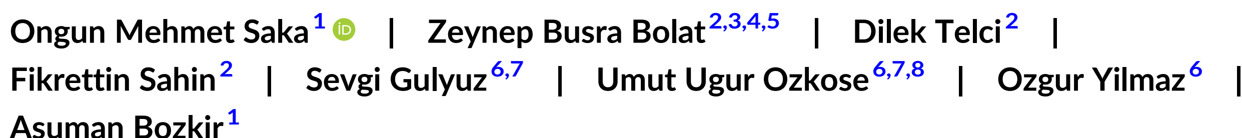
Development of peptide-18-targeted nanoliposome formulations with an alternative stealth coating copolymer for targeting breast cancer AU565 cell line


Ongun Mehmet Saka[1] | Zeynep Busra Bolat[2,3,4,5] | Dilek Telci[2] | Fikrettin Sahin[2] | Sevgi Gulyuz[6,7] | Umut Ugur Ozkose[6,7,8] | Ozgur Yilmaz[6] | Asuman Bozkir[1]

[1]Department of Pharmaceutical Technology, Faculty of Pharmacy, Ankara University, Ankara, Turkey

[2]Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, Istanbul, Turkey

[3]Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Istanbul Sabahattin Zaim University, Istanbul, Turkey

[4]Molecular Biology and Genetics Department, Hamidiye Institute of Health Sciences, University of Health Sciences-Turkey, Istanbul, Turkey

[5]Experimental Medicine Research and Application Center, University of Health Sciences-Turkey, Istanbul, Turkey

[6]Material Technologies, Marmara Research Center, TUBITAK, Gebze, Turkey

[7]Department of Chemistry, Faculty of Science and Letters, Istanbul Technical University, Istanbul, Turkey

[8]Department of Chemistry, Faculty of Science and Letters, Piri Reis University, Istanbul, Turkey

Correspondence
Asuman Bozkir, Department of Pharmaceutical Technology, Faculty of Pharmacy, Ankara University, Ankara, Turkey.
Email: bozkir@pharmacy.ankara.edu.tr

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Abstract

The incidence of breast cancer has increased considerably in recent years. Many efforts have been made to develop various nano-drug delivery systems with specific properties to achieve effective and specific therapy. The stealthy nano-liposomes are the most successful and promising candidates for providing targeted tumor therapy. In this paper, a synthesized PEtOx-DOPE (poly(2-Ethyl 2-Oxazoline)-Dioleoyl Phosphatidylethanolamine) copolymer was equipped with a breast cancer-recognizing tumor-homing peptide to achieve cell-specific delivery. The prepared liposomes provided stability for 6 months, and their hydrodynamic diameters are around 100 nm. Targeted liposomes remarkably exhibited 8 times higher cellular internalization in comparison with the nontargeted cells in flow cytometry and confocal microscopy. Furthermore, liposome constructs displayed slight toxicity on HaCaT cells when treated with high doses. Hence, Peptide 18-conjugated PEtOx-DOPE liposome systems can serve as favorable candidates in breast cancer-targeted therapy.

KEYWORDS

cancer targeting, drug targeting, gene delivery vehicles, liposomes, polymer

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1 | INTRODUCTION

Lipids and fatty acids, which are inherently present in cell membranes, are the primary components of liposomes. Due to their primary components, liposomes are considered biocompatible and biodegradable carriers.[1] Liposomes are structurally defined by the self-assembly of the amphipathic molecules to form a bilayer sphere. Also, the amphiphilicity of the liposomes makes them an ideal drug carrier for active molecules, which have varying polarities. The encapsulation of these active molecules into the liposomes reduces toxicity and improves tolerable dose regimens for cancer treatment. In addition, high loading capacity, excellent biocompatibility, and convenient modifications to preparations are other advantages of liposomes.[1,2]

According to the Turkish Cancer Society, breast cancer ranks first among the cancer types seen in women. One out of every eight women has a lifetime risk of developing cancer, and one out of every 30 women dies from breast cancer.[3] On the other hand, worldwide breast carcinomas are expected to increase to 2.50 million, and the mortality of breast cancer will be 768,646 by 2025.[2] The standard care approach is removing primary tumors by surgical operation. But the secondary foci of cancer can be left in the operation area. Therefore, radiotherapy or chemotherapy are commonly used treatments for metastatic breast cancer. The key to effective cancer therapy is targeting the unique microenvironment of pre-metastatic niches.[2,4]

Researchers have worked on delivery systems for effective breast cancer treatment in the last decade. One of the major strategies is to minimize the side effects of the anticancer drugs by using targeted liposomes.[2,5] The surface of liposomes must be modified by attaching appropriate ligands to achieve selective delivery. For instance, poly (ethylene glycol) modified liposomal doxorubicin was the first liposomal drug used for breast cancer approved by FDA.[5] In our early research, we have highlighted the synthesis of poly(2-ethyl-2-oxazoline) (PEtOx) for targeting liposomes. PEtOx shows the same stealth properties as poly (ethylene glycol). A well-defined PEtOx-liposome offers low toxicity, good hydrophilicity, flexibility, and biocompatibility.[6] These superior properties make liposomes promising in cancer therapy. Although liposomes exhibit superior properties compared with other nanocarriers, structural instability is the major issue for liposomal formulations.[1,2,4]

In this study, we first developed liposomal delivery systems for plasmid DNA by using peptide 18 (WXEAAYQRFL) for targeting breast cancer AU565 cell lines. Physico-chemical properties and the stability of the liposomes were analyzed. The cytotoxicity of the liposomes was investigated in HaCaT cell line, followed by targeting specificity and cellular uptake assessment of peptide-18 nanoliposome in breast cancer AU565 cell lines.

2 | MATERIALS AND METHODS

2.1 | Materials

Cholesterol and L-α-Phosphatidylcholine were purchased from Sigma. PEtOx-DOPE and P18-PEtOx-DOPE were synthesized at Tübitak MAM Laboratories, Turkey. pDNA (pEGFP-C3 plasmid; Addgene) was prepared at Yeditepe Laboratories, Turkey (DOI: 10.1080/21691401.2018.1491478). The HaCaT (CLS 300493) cell line was provided by DKFZ, Heidelberg. MCF10A (CRL-10317) and AU565 (CRL-2351) cell lines were obtained from ATCC. All other chemicals and reagents used were of analytical grade.

2.2 | Synthesis of PETOx-DOPE and P18-PEtOx-DOPE polymers

P18-PEtOx-DOPE (for chemical structure, see Figure 1) was prepared in several synthetic steps by following our earlier reported protocol.[6]

PEtOx-N_3 ($M_{n,GPC}$ = 5800 g/mol, Đ = 1.05) was prepared similar to the previous works by living cationic ring-opening polymerization of 2-ethyl-2-oxazoline.[7–9] Briefly, a solution of mesyl functional initiator (0.35 g, 1.25 mmol) and dry 2-ethyl 2-oxazoline (5 mL, 49.5 mmol) in 15 ml dry acetonitrile were placed in a dried Schlenk tube equipped with a

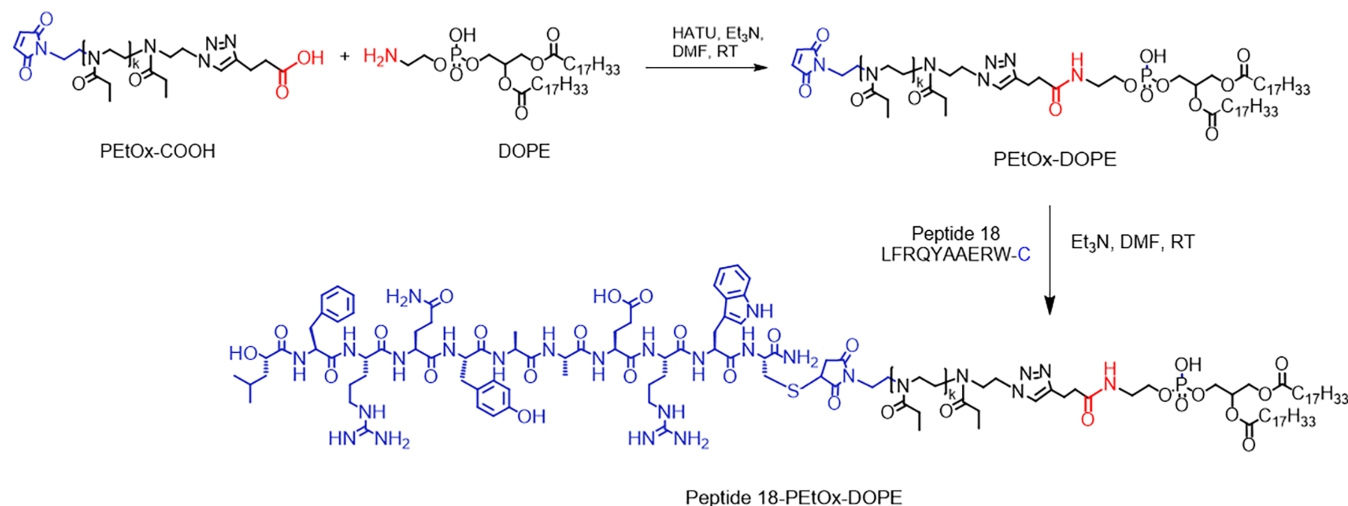

FIGURE 1 Synthesis of the Peptide 18-modified PEtOx-DOPE.

magnetic stir bar. The polymerization was continued overnight under nitrogen atmosphere at 130°C. The reaction mixture was arranged at 65°C, and a 5-fold excess of NaN_3 was added to terminate for 3 days. Upon completion of the termination, excess NaN_3 was removed by column chromatography. The recovered filtrate was concentrated in a vacuum. The final product was dissolved with a small amount of DCM, precipitated in cold diethyl ether and then dried under vacuum (Yield: 92%).

Subsequently, PEtOx-COOH was synthesized with click chemistry and protection from light. 4-pentynoic acid (183 mg, 1.86 mmol), PEtOx-N_3 (4.0 g, 0.69 mmol), sodium ascorbate (683 mg, 3.45 mmol), and $CuSO_4$ (275 mg, 1.73 mmol) in 1:2 DMF/H_2O (16, 32 mL) were placed in a tube with a stir bar. The tube was evacuated and backfilled with nitrogen. The reaction was vigorously stirred under nitrogen at ambient temperature for 1 day. The carboxyl-functional PEtOx was dialyzed with a membrane (cut off 2000 Dalton) against water for 5 h.

Then, PEtOx-DOPE was prepared under a nitrogen atmosphere via carbodiimide activation chemistry. PEtOx-COOH (3.25 g, 0.56 mmol), DOPE (508 mg, 0.62 mmol), HATU (426 mg, 1.12 mmol), and Et_3N (156 mL, 1.12 mmol) were dissolved in DMF/DCM (1/1 v/v: 10 mL,10 mL) in a flask with a magnetic stir bar. The reaction was stirred at room temperature for 3 days. The product was purified by the dialysis membrane (MWCO 2000) against a mixture of DCM and methanol. The desired product was obtained with lyophilization (yield: 88,6%).

Peptide 18 modified PEtOx-DOPE was prepared via thiol maleimide Michael-addition. PEtOx-DOPE (320 mg, 0.048 mmol) was dissolved in 5 ml of DMF. Et_3N (8 ml, 0.11 mmol) and Peptide 18 (sequence Cys-WXEAAYQRFL, 85 mg, 0.059 mmol) were added. The reaction was sparged with nitrogen and stirred for 2 days at room temperature. The resulting product was dialyzed (MWCO 2000) for 24 h against DCM and deionized water, respectively and then freeze-dried (Yield: 86.25%).

2.3 | Preparation of liposomes

The most common method employed for cationic liposome preparation is thin film hydration.[1,10,11] pDNA-loaded liposomes constructed by PEtOx:DOPE with or without P-18 consists of homogenization and lyophilization steps for gene delivery systems. Briefly, phosphatidylcholine/cholesterol/polymer with a molar ratio of 7:2:1 was dissolved in chloroform (15 mL, total concentration of 13.25 mg/mL). This solution was immediately added to a rotavapor (Heidolph, GmbH, Germany) and rotated at 150 rpm for 1 h. The ethanol was eliminated by heating the solution at 50°C for 4 hours at room pressure, followed by the utilization of a vacuum (200 mbar) for all night. After the evaporation step, the formed lipid film was hydrated with 20 -mL pDNA solution (5 ng/μl) to achieve multilamellar vesicles. Nonencapsulated pDNA was removed by ultracentrifugation at 40,000 × g for 1 h (Beckman-Coulter Optima, USA). The supernatant, which contains free pDNA, was removed, and the pellet was resuspended with bidistilled water. In order to achieve nano-unilamellar vesicles, we used an ultra homogenizer (Ika HPH 2000/4-SH5, Germany) for 25 min at 250 ppm under 1000 mbar pressure. The nanoliposomes were lyophilized to avoid the stability problem. Before further characterization, the liposomes were reconstituted via dispersion in bi-distilled water.

2.4 | Particle size and surface charge of liposomes

In order to evaluate nano-vesicle formation, the mean particle size, size distribution, and zeta potential of the liposomes were assessed using a particle size analyzer (Malvern Instruments, Worcestershire, UK). All measurements were conducted in triplicate at room temperature using reconstituted liposomes in bi-distilled water.

2.5 | Morphology characterization of liposomes

The morphology of the reconstituted liposomes was observed using Transmission Electron Microscopy (Tecnai G2 F30, Netherlands). After resuspension with the bi-distilled water, one drop of suspension was poured on the TEM grid and negatively stained by a 2% phosphotungstic acid solution.

2.6 | Stability studies

pDNA-loaded lyophilized nanoliposomes were kept at refrigerated conditions (5 ± 3°C) for 6 months. Stability was investigated with the change in particle size, PDI, and zeta potential measurements of nanoliposomes.

2.7 | In vitro cytotoxicity

The cytotoxicity of the PEtOx-DOPE copolymer and liposome without pDNA was evaluated on the normal human keratinocyte cell line HaCaT using the cell proliferation reagent WST-1 solution (Roche, USA). HaCaT cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, USA) and 100 U/mL of penicillin and 100 μg/mL of streptomycin (1% PS, Gibco, Thermo Fisher Scientific, USA). All cells were seeded at a density of 5×10^3 cells/well in 96-well plates and incubated at 37°C in a humidified atmosphere (5% CO_2). After 24 h, HaCaT cells were treated with 25, 50, 75, 100, and 150 μg/mL of PEtOx-DOPE copolymer and liposome. At each 24 -h time interval, cells were subjected to a WST-1 assay according to the manufacturer's protocol, and the absorbance values were measured at 450 nm in a microplate reader (Bio-tek ELx800, USA). Cell viability percentage was calculated by assigning nontreated cells absorbance value as 100%.

2.8 | Flow cytometry

Human normal epithelial breast cell line MCF10A and breast cancer AU565 cells were cultured with complete MEGM bullet kit medium (Lonza, CC3150) and RPMI 1640 medium supplemented with 10% FBS and 1% PS, respectively. AU565 and MCF10A cells were seeded with a density of 3×10^5 cells/well and 2.5×10^5 cells/well in 6-well plates, respectively, due to cell size differences, and maintained

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in a 5% CO_2 atmosphere at 37°C in a humidified condition. For cellular uptake, flow cytometry was used to detect the targeted specificity of P18-PEtOx-DOPE liposomes. P18-PEtOx-DOPE liposomes were loaded with fluorescein dye, and MCF10A and AU565 cells were treated with 0.25 μg/mL of P18-PEtOx-DOPE liposomes for 1 h at 37°C. Both cells were washed with PBS after being treated with P18-PEtOx-DOPE liposomes and collected in a 0.5% formaldehyde solution. The fluorescence intensity was analyzed at FL1-H (excitation/emission, 488:525 nm) by a Becton Dickinson FACSCalibur™ flow cytometer using CellQuestPro software (Becton Dickinson, USA).

2.9 | Confocal microscopy

Confocal microscopy was used to visualize the targeted efficiency of cells treated with fluorescein-loaded liposome constructs. AU565 and MCF10A cells were seeded in 8-well chamber slides with a density of 2.4×10^4 cells/well and 2.0×10^4 cells/well, respectively. Following 1 h incubation of the P18-PEtOx-DOPE liposome, AU565 and MCF10A cells were fixed with 2% paraformaldehyde for 20 min, washed with PBS, and stained with 5 μg/mL DAPI for 10 min. The fluorescently labeled cells were then visualized by Zeiss LSM 700 confocal microscopy.

2.10 | Statistical analysis

Data were analyzed by Microsoft Excel 2016 software (Microsoft, Office Professional Plus 2016). A two-tailed Student's *t*-test was used for comparing the results between the groups. A student *t*-test was performed on data reported as mean ± SD ($n = 3$), and statistically significant was defined as $p < 0.05$ (*).

3 | RESULTS

3.1 | Synthesis of P18-PEtOx-DOPE

The P18-PEtOx-DOPE bioconjugate (for chemical structure, see Figure 1) was achieved in several synthetic steps by following our earlier reported protocol.[6] The peptide 18 (P18) (Cys-WXEAAYQRFL) was prepared via the solid-phase peptide synthesis (SPPS) method. The synthesis and modification of PEtOx were synthesized similar to the previous works by living cationic ring-opening polymerization and "CuAAC" click chemistry, respectively. Conjugation of DOPE to PEtOx-COOH polymer was performed using TEA/HATU by carbodiimide activation chemistry. Finally, P18 was attached to maleimide functional PEtOx-DOPE via thiol maleimide Michael-addition. The successful synthesis of P18-PEtOx-DOPE was confirmed using GPC, ^{1}H NMR, and FT-IR spectroscopies.

3.2 | Characterization of the liposomes constructed by P18-PEtOx-DOPE

The physicochemical properties of the nanoliposomes prepared with molar ratio of 7:2:1 are given in Table 1. The mean diameter size of pDNA-loaded nanoliposomes was around 100 nm over 6 months. Before the lyophilization process, the size of the liposomes was 87.26 nm. Reconstituted liposomes, which the freeze dry process applied, show a little increase in size. However, this increase shows no significant change statistically. The polydispersity (PDI) values of all samples were below 0.5 and no relevant differences were found between formulations. Regarding zeta potential, formulations showed negative values around −25 mV.

One of the best methods to visualize liposomes close to their native structure is using electron microscopy. The morphology and size of the freshly prepared nanoliposomes without lyophilization (Figure 2A) and lyophilized nanoliposomes (Figure 2B) were also evaluated using transmission electron microscopy.

3.3 | Cytotoxicity of PEtOx-DOPE copolymer and liposomes

In order to determine the nontoxic concentration for PEtOx-DOPE copolymer and liposomes without pDNA, the WST-1 assay was used to measure the cell viability of HaCaT treated with increasing concentrations of the copolymer and liposomes. PEtOx-DOPE copolymer did not cause any significant cytotoxicity in HaCaT cells at 24 h. After 48 h incubation, PEtOx-DOPE copolymer increased cell proliferation slightly ($p > 0.05$) in HaCaT cells with a percentage of 10, 13, and 9 at concentrations of 25, 75, and 100 μg/mL,

TABLE 1 Characterization results of pDNA loaded nanoliposomes throughout stability studies. Each value represents the mean ± standard deviation of three measurements.

Time (month)	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
Initial time (before lyophilization)	87.26 ± 1.38	0.191 ± 0.006	−25.2 ± 1.11
Initial time (after lyophilization)	95.69 ± 0.63	0.247 ± 0.017	−27.0 ± 1.44
2nd months	93.34 ± 2.60	0.234 ± 0.004	−25.0 ± 0.46
4th months	97.45 ± 0.78	0.252 ± 0.019	−24.9 ± 1.35
6th months	106.20 ± 0.15	0.130 ± 0.009	−22.6 ± 2.21

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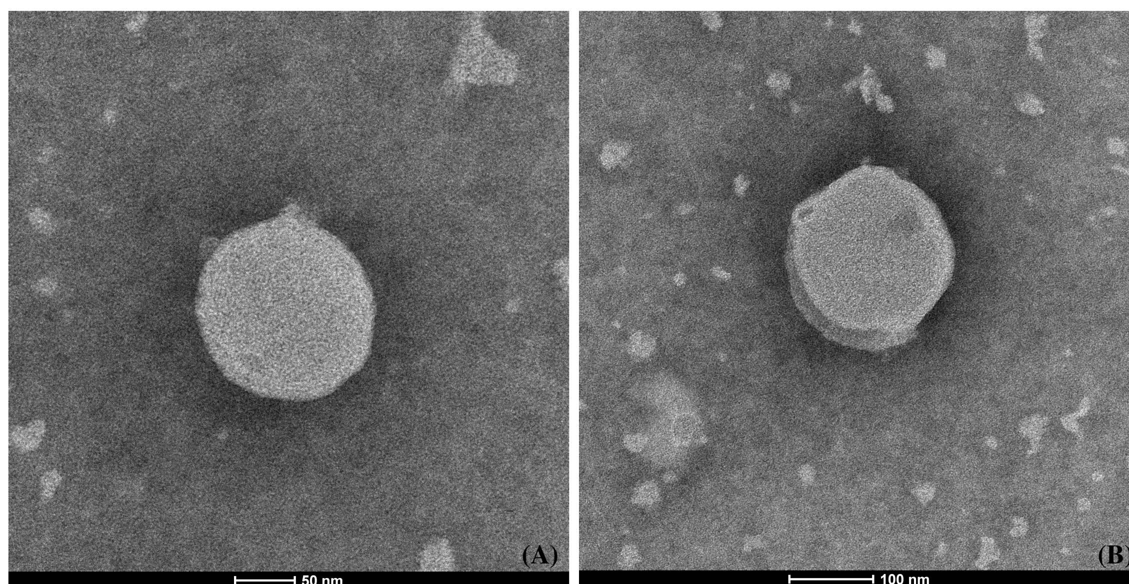

FIGURE 2 TEM images of (A) nonlyophilized liposomes and (B) lyophilized liposomes.

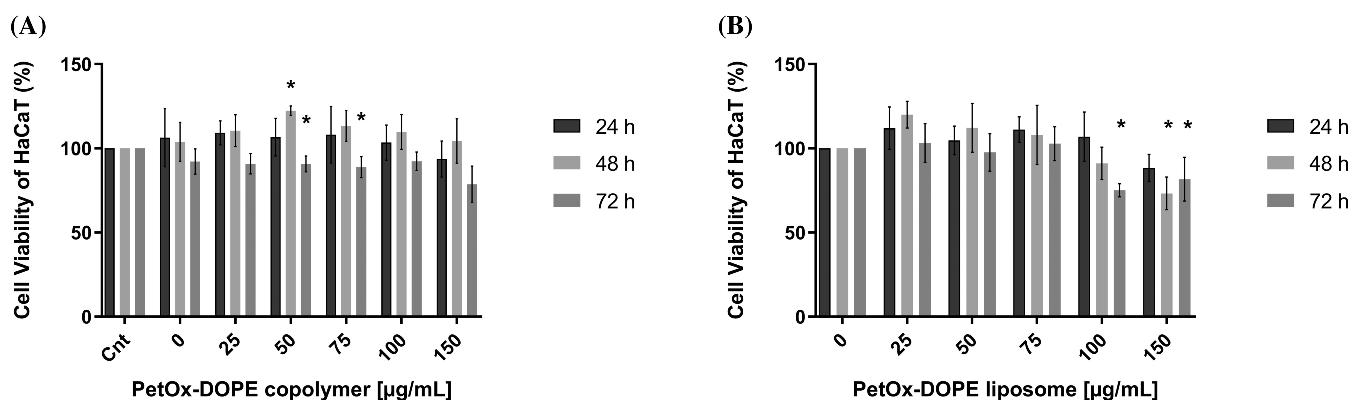

FIGURE 3 Effect of PEtOx-DOPE (A) copolymer and (B) liposome on HaCaT cell viability. Cells were treated with 25, 50, 75, 100, and 150 μg/mL of PEtOx-DOPE copolymer and liposome. Cell viability was assessed at each 24 h interval by measuring the absorbance change using a microplate reader at 450 nm.

respectively. A significant increase in the proliferation rate was detected in HaCaT cells when treated with 50 μg/mL of PEtOx-DOPE copolymer at 48 h. At the 72 -h mark, however, the cell viability of HaCaT cells was pulled back to almost control levels. There was a significant decrease ($p < 0.05$) in cell viability of HaCaT cells when treated with 50 and 75 μg/mL of PEtOx-DOPE copolymer (Figure 3A). A slight increase in cell viability ($p > 0.05$) of HaCaT cells was detected when treated with 25–75 μg/mL concentrations of PEtOx-DOPE liposomes at all time points. On the contrary, significant toxicity of 20% and 27% in HaCaT cells was recorded when treated with 150 μg/mL concentrations of PEtOx-DOPE liposomes after 48 and 72 h post treatment (Figure 3B). Overall, results revealed that PEtOx-DOPE copolymers and liposomes have no significant toxicity toward HaCaT cells unless used at 150 μg/mL concentrations.

3.4 | P18-PEtOx-DOPE liposomes targeting specificity on breast cancer

The target specificity of Peptide 18-conjugated PEtOx-DOPE liposomes on breast cancer was studied by flow cytometry. For this purpose, the human normal breast epithelial MCF10A cell line and the breast cancer AU565 cell line were used. Fluorescein-loaded P18-PEtOx-DOPE liposome binding affinity was confirmed by flow cytometry in the FL-1 filter, the results of which showed that the cellular uptake of P18-PEtOx-DOPE liposomes by MCF10A cells was 10.2% (Figure 4A), while AU565 cells demonstrated a high uptake of 81.5% (Figure 4B). Confocal microscopy imaging was used to confirm the flow cytometry results, where P18-PEtOx-DOPE liposomes showed a stronger binding for AU565 cells in comparison to that of MCF10A cells (Figure 5).

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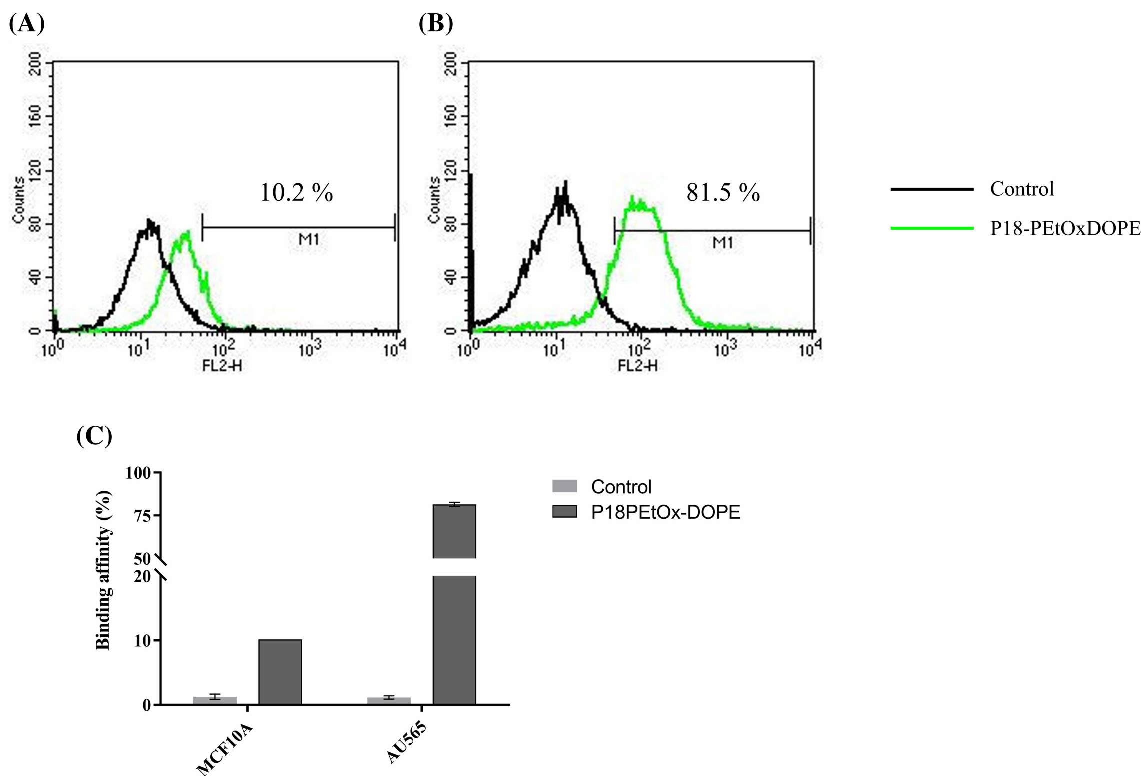

FIGURE 4 P18-PEtOx-DOPE liposomes uptake studies by MCF10A (A) and AU565 (B) cells using flow cytometry. Cells were incubated at 37°C for 1 h followed by measurement of fluorescence (excitation/emission, 488:525 nm).

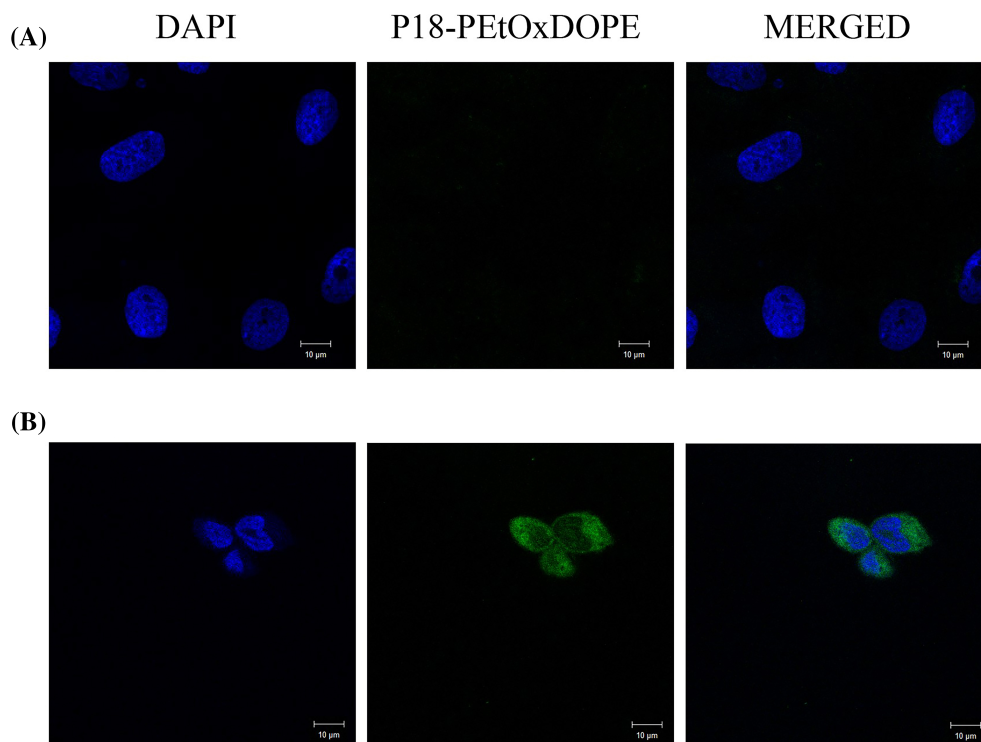

FIGURE 5 Confocal microscopy images after incubation of P18-PEtOx-DOPE liposomes by MCF10A (A) and AU565 (B) cells at 37°C for 1 h. Images were taken using 63× objectives and bars scaled to 10 μm.

4 | DISCUSSION

The major challenge that remains for developing efficient drug delivery liposomes is the binding of unprotected formulations to opsonin proteins for phagocytic clearance. Therefore, it is very important to understand the interaction between the surface of the nanoliposomes and the biological environment that affects the recognition and clearance by the reticuloendothelial system. Researchers made some

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optimizations to reduce the rapid clearance of the liposomes, such as preparing carriers containing glycolipids, albumin, or derivatives of polyethylene glycol. The camouflage technique is a common technique used on liposomes. The surface of the carrier grafted a stealth coating layer to restrict the interactions with opsonin proteins for improving the blood circulation half-life.[6,11,12] Preparing the gold standard polyethylene glycol (PEG) derivatives to improve the stability of liposomes and the increase in circulation time is already widely studied. However, there are some problems in the cellular uptake; the PEGylated nanoliposomes is more difficult according to the non-PEGylated ones.[13] For this reason, the effects of the addition of this polymer to the liposomal surface must be carefully evaluated to achieve the expected action.[7,9,13] Nunes et al. prepared PEGylated and non-PEGylated liposomes with similar mean diameters and zeta potentials, but indicated a critical amount of polymer to ensure maximum uptake/delivery to target tissues.

2-ethyl-2-oxazoline (PEtOx) is a promising polymer that has gained increasing interest for its highly functionalized while exhibiting a stealth property. Increasing concern about the increased use of PEG and the associated problem of possible immunogenicity has led to renewed interest in alternative polymers. In particular, PEtOx-conjugated liposomes can resist protein adsorption and uptake by macrophages with similar stealth behaviors as PEGylated liposomes.[14,15] We have shown the synthesis of PEtOx-DOPE as detailed in our early research.[6] Furthermore, Viegas et al. showed that PEtOx is a nonimmunogenic polymer even through repeated subcutaneous and intravenous injections. Also, Bludau et al. concluded that PEtOxylated nanoparticles show better stealth behaviors from antibody recognition compared to PEGylated nanoparticles.[16] Additionally, it was reported that when PEtOx is compared to PEG, it has a more stable main chain that facilitates the introduction of various active groups. Also, PEtOx can provide a chemical basis for further linking to increase uptake in targeted cells.[14] According to all of PEtOx's high functionalization possibilities, it can be used in drug targeting as a PEG alternative.

Stegh et al. showed that P-18 (large) and P-10 (small) catalytic subunits have higher binding affinity for the breast carcinoma cell line.[17] To achieve active targeting, we prepared P-18-conjugated PEtOx-DOPE-based nanoliposomes for pDNA delivery in breast cancer therapy. pDNA-loaded nanoliposomes were successfully prepared by using a common method known as thin-film hydration. Prepared and lyophilized liposomes were nanoscale (Table 1) and Figure 2 shows evidence that pDNA-loaded nanoliposomes showed an unilamellar spherical shape.

Different types and compositions of lipidic nanocarriers have been studied as novel drug delivery systems to achieve the maximum therapeutic effect and cellular uptake. Researchers prepared PEGylated liposomes around 250 nm by using the thin-film hydration method.[17,18] Usually nanocarriers are prepared in 50–200 nm size so that they are not able to escape from normal blood vessels. Nevertheless, when extravasated from blood capillaries, phagocytes can be retained nano liposomes (larger than 100–150 nm) for an extended period of time. So, most therapeutic nano-liposomes are designed to possess 50–100 nm diameters to avoid phagocyte uptake and to achieve prolonged blood circulation time.[18,19] We used an ultra-homogenizer to achieve smaller particles (95.69 nm) with narrow size distribution (Table 1).

The term "polydispersity index (PDI)" is used to describe the uniformity degree of a size distribution of nanoliposomes. If the values of PDI are bigger than 0.7, it indicates that the sample has a very wide particle size distribution.[18] Frequently, measurements lower than 0.25 refer to a narrow size distribution.[7] We successfully prepared nanoliposome formulations with a constricted size distribution (<0.25 PDI). Danaei et al. indicate that PDI with the average particle diameter is an indication of formulation quality with respect to the size distribution. The suitability of nanoliposomal formulations for any route of drug administration depends on their average size, PDI, and size stability, among other parameters.[13,18]

The lyophilization process is routinely used to increase stability by removing all the solvents of formulations. Consistency between the formulations before and after the lyophilization step was validated (Table 1). Specifically, nonlyophilized and lyophilized formulations showed a similar mean diameter with an average particle size (87.26 and 95.69, respectively). Liposome lyophilization did not affect the mean size statistically ($p > 0.05$). Both formulations showed lower PDI values (0.191 and 0.247) indicates that the liposomes are evenly distributed in the system.

We prepared P-18-conjugated PEtOx-DOPE-based nanoliposomes for pDNA delivery with negatively charged zeta potential values around −25 mV (Table 1). It was indicated that highly negative charges residing on the surface inhibit the formation big liposomes by agglomeration because they create repulsive forces between vesicles membranes.[1,11,18] The liposomal formulations prepared with negatively charged lipids also enhance the delivery and transfection of pDNA.[1,11,20] Further, nonspecific cell uptake is reduced because of electrostatic repulsion between negatively charged liposomes and cell membranes. This situation also enables our liposomes to circulate in the bloodstream longer than neutral or positively charged liposomes.[1,21] Liposomal blood clearance rate is greatly affected by liposomal composition. We successfully composed PEtOx-conjugated negatively charged liposomes to increase blood circulation time and avoid from liposomal opsonization. Furthermore, Alavi et. al[5] demonstrate that the liposomal formulations, which prolong blood circulation, enable higher accumulation and deposition of the therapeutic pDNA within target cells. Physical and chemical stability describe the ability of the liposomal formulation to maintain its properties over time.[10,12,20] Stability is one of the most important indicators of liposomal formulations' potential efficacy. We evaluated the stability studies by performing physical assessments of the nanoliposomes for 6 months at 5 ± 3°C. When the particle size and zeta potential values were compared between timepoints (initial, 2,4, and 6 months), no significant difference ($p > 0.05$) was obtained. Although there was a very small increase calculated between the initial time and the 6th month's data, this increase was not statistically significant. This small calculated increase also does not reflect the degradation of the lipid membrane or the aggregation of the particles. Similarly, observed PDI

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values have not got any significant change (Table 1). According to these results, we can conclude that lyophilized nanoliposomes were stable for at least 6 months period.

PEtOx-based copolymers showed no significant toxicity on human epithelial kidney (HEK293), endothelial cells (HUVEC), or mesenchymal stem cells (MSC).[8,9] In our previous study, we synthesized poly(2-ethyl-2-oxazoline)-based copolymers by click reactions and reported that these copolymers showed no toxicity on human epithelial breast cell (MCF10A) and two different breast cancer (AU565 and MDA-MB-231) cell lines.[6] Furthermore, PEtOx-DOPE liposome constructs displayed a low toxicity profile on HEK293, HUVEC, MSCs, and MCF10A, suggesting that they may serve as nanocarriers for drugs.[7,22] In this study, the cytotoxic activity of PEtOx-*b*-DOPE copolymers and liposomes on HaCaT cells was evaluated. While PEtOx-*b*-DOPE copolymers had no significant toxicity in HaCaT cell viability, liposome constructs displayed slight toxicity on HaCaT cells when treated with high doses (150 μg/mL) of PEtOx-DOPE liposomes at 48 and 72 h, suggesting that PEtOx-*b*-DOPE liposomes may also serve as nanocarriers for drugs in breast cancer therapy. In order to enhance the tumor-homing ability of PEtOx-*b*-DOPE liposomes, a suitable targeting peptide specific for breast cancer was needed. P18 is a tumor-homing peptide that shows targeting specificity for breast cancer cell lines.[12] We have previously demonstrated that there was a high binding profile of P18 to breast cancer AU565 cell lines in comparison to other tumor homing peptides such as peptide 11 and MC-10 peptide.[7] Twenty-five micrograms of microliters of fluorescein-loaded P18-conjugated PEtOx-*b*-DOPE liposomes revealed a strong selectivity for AU565 cells with an 8-fold higher signal intensity compared to MCF10A cells. In agreement with flow cytometry results, confocal microscopy confirmed a strong binding affinity of the P18-conjugated PEtOx-*b*-DOPE liposomes to AU565 cells. Our results are in parallel with the literature, where P18 conjugated to nanocarriers is suggested as a potential agent in breast cancer targeted therapy [23]. Thus, success in the P18-PEtOx-*b*-DOPE liposome shows a potential candidate for delivery of cargo to breast cancer in targeted therapy.

5 | CONCLUSION

We successfully prepared pDNA-loaded nanoliposomes by using the P18-PEtOx-DOPE copolymer. The hydrophilic PEtOx was used as an alternative stealth coating for a pDNA-loaded nano-liposomal delivery system. Results indicate that PEtOx-DOPE copolymer and liposomes treatment with HaCaT cells showed no significant toxicity except at very high concentrations of PEtOx-DOPE liposomes for the late time point of 72 h. P18-conjugated to PEtOx-DOPE liposomes revealed specific and high binding affinity to AU565 cells compared to MCF10A cells. Hence, P18 conjugated PEtOx-DOPE liposome systems can serve as favorable candidates in breast cancer targeted therapy.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Ongun Mehmet Saka https://orcid.org/0000-0002-8726-6391

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