Anticancer Genes

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17β-Estradiol-Associated Stealth-Liposomal Delivery of Anticancer Gene to Breast Cancer Cells**

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Estrogen receptor (ER), a member of the nuclear hormone receptor superfamily, is classically known to express as intracellular steroid receptor. It shows up predominantly in estrogen-responsive organs, such as ovary, uterus, and mammary cells. ER possesses mainly genomic properties but nongenomic, steroidal functions are also known.[1–5] Interestingly, classical ERs are implicated in several cancer phenotypes in the female population and are expressed in 60–80% of breast cancer samples.[6] These receptors are mostly confined to the primary stage of the tumor, and therefore endocrine-based therapy is generally used in the initial stages of neoplastic transformation.

β-Estradiol or estrogen is the endogenous ligand of ER. Estrogen-induced ER acts as a ligand-dependent transcription factor during its genomic activity, and regulates cell proliferation in cancer cells such as that observed in breast carcinoma, thereby becoming the principal target for breast cancer therapy. There are several ER antagonists, aromatase inhibitors, and selective estrogen receptor modulators (SERMs) that alone, or in combination, provide several clinical options for breast cancer therapy with limited efficacy.[7–11]

Liposomes have long been utilized as viable options for targeting cancers.[12–14] Poly(ethylene glycol)(PEG)-associ-

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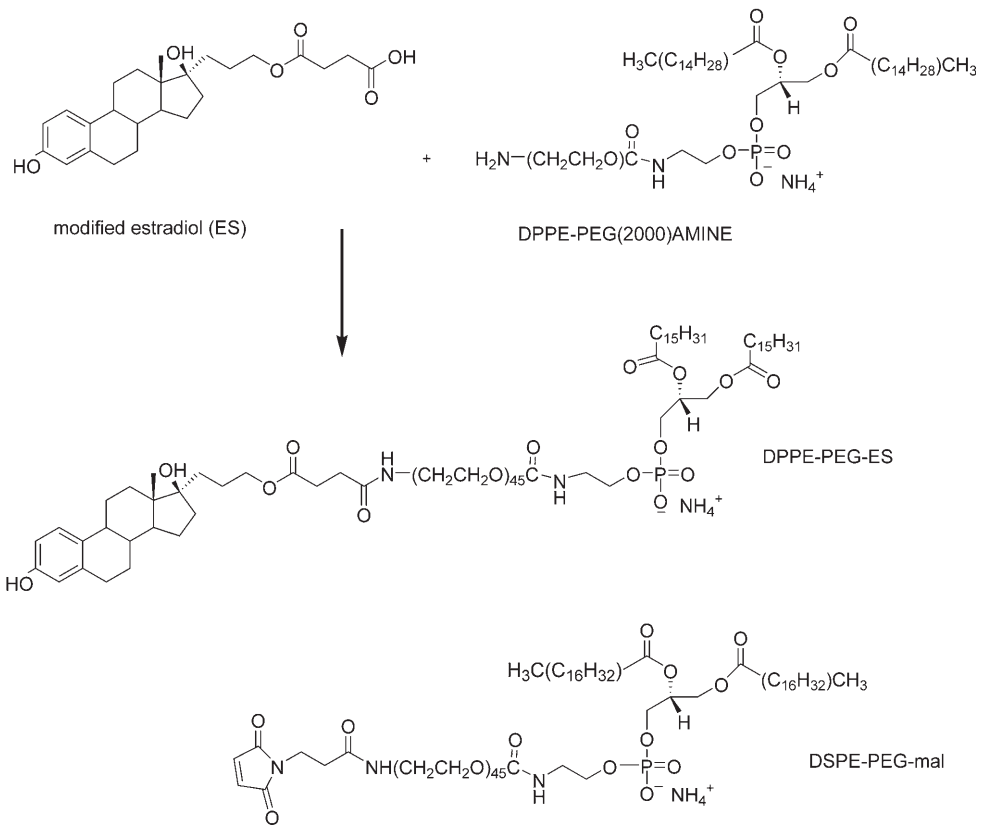
ated liposomes offer a stealth property to the payloads encapsulated in liposomal formulations with enhanced circulation half-lives.[14–16] Recently, a PEG- and cyclic RGD-associated (RGD: Arg-Gly-Asp) polymeric micelle has been exploited to deliver encapsulated doxorubicin to integrin-expressing specific tumorigenic cell types.[17] We have also shown that genetic payloads can be efficiently targeted to sigma-receptor-expressing breast cancer cells when haloperidol, a low-molecular-weight sigma-receptor-targeting drug, is covalently grafted to the headgroup region of a stealth cationic liposome.[18] This finding prompted us to investigate whether estrogen, the natural ER ligand, associating with a gene-carrying stealth liposomal system can act as a targeting ligand for delivering genes to breast cancer cells implicated with ER. This option of targeting genes at breast or estrogen-responsive cancer cells is expected to eliminate the chemotherapeutic drug-induced toxicity to normal cells.



Scheme 1. Representative synthesis and structure of the lipids. Modified ES, which is produced in five steps, is treated with DPPE-PEG-NH_2 to obtain the targeting lipid DPPE-PEG-ES. DSPE-PEG-mal is the control lipid (DSPE = distearoylphosphatidylethanolamine, mal = maleimide).

Recently, it was found that estrogen exhibits high affinity to GPR30, an orphan receptor ubiquitously present in most of the cell types. In association with estrogen, GPR30 executes several nongenomic functions.[1,19] We envisaged that an estrogen-associated reporter-gene-carrying liposomal delivery system would uniquely target ER-expressing cells irrespective of existing GPR30.

With such a rationale in mind, we conjugated 17β-estradiol (ES) to the distal end of a phospholipid with a PEG spacer in between. The conjugation was performed without potentially diminishing the estrogenic property of the ligand. 3-Hydroxy-protected estrone was subjected to a Grignard reaction by following a previously reported procedure[20] to obtain the 17α-allyl moiety. This moiety was converted to a primary alcohol which was reacted with succinic anhydride to obtain an estradiol-associated molecule with a carboxylic acid end group. After silyl deprotection, the carboxylic acid group was conveniently conjugated to the amine end group of the 1,2-dipalmitoyl-Sn-glycero-phosphatidylethanolamine (DPPE)-linked PEG moiety to obtain the targeting lipid DPPE-PEG-ES (Scheme 1). NMR peaks at $\delta = 6.6$–7.2 ppm ascertained the conjugation of the estrogen moiety in DPPE-PEG-ES (Figure 1B), whereas no aromatic peaks were obtained in the PEG-suppressed NMR spectrum of DPPE-PEG-NH_2 (Figure 1A). The MALDI mass spectrum of DPPE-PEG-ES showed an inverted U-shaped pattern with an increment of 44 mass units between molecular weights 2756 and 3410. In spite of the two PEG populations in the sample, the peak of the U-shaped spectral pattern was obtained at a molecular weight of 3018.8, which was about 132 (that is, about three CH_2CH_2O residues) less than the calculated molecular weight of the product of 3151.67 (comparable to the peak at 3151.7 in Figure 1C). The calculated molecular weight was obtained by summing the molecular weights of the contributing estrogen fragment (413.3) and the starting material (DPPE-PEG-NH_2; 2739.37), and then subtracting one (the hydrogen lost from the lipid fragment upon conjugation). The mass spectrum of the starting material also revealed two different populations of PEG (the spectrum contains a molecular ion peak at 2739; see the Supporting Information).

This ligand-conjugated PEG-lipid was included at 5 mol% with respect to the known cationic lipid *N*,*N*-di-*n*-octadecyl-*N*,*N*-di-hydroxyethylammonium chloride (DODEAC)[18] and the neutral colipid cholesterol (at 1:1 molar ratio), to form long-circulating and targeting cationic liposomes. We used 5 mol% DSPE-PEG-mal (Scheme 1) in association with a DODEAC/cholesterol (1:1) formulation as our control liposome. This nontargeting lipid was included in the study to show the effect of the estradiol moiety in targeting liposomes to ER-expressing MCF-7 cells.

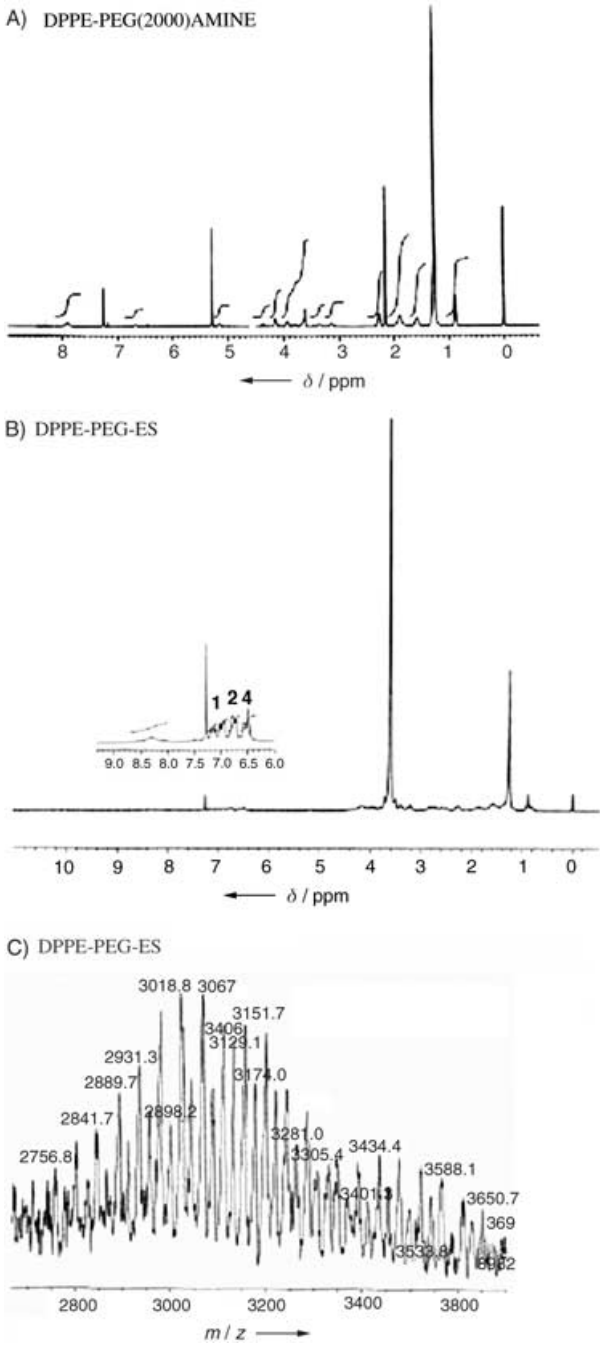


Figure 1. ^{1}H NMR spectra of A) the starting material, DPPE-PEG-NH_2, and B) the final product, DPPE-PEG-ES. The inset in (B) shows the aromatic proton signals from the lipid-associated estradiol. C) MALDI mass spectrum of DPPE-PEG-ES.

The DNA-binding characteristics of both the targeting and the nontargeting liposomes were confirmed by a simple gel retardation assay, and the DNase I sensitivity of the lipoplex-associated DNA was evaluated by a conventional DNase I protection experiment (see the Supporting Information). The sizes and surface charges of the lipoplexes under transfection conditions were also measured (see the Supporting Information). The targeting and nontargeting lipoplexes showed a comparable stability. Except for lipoplex sizes with a charge ratio of 8:1, the surface charges and sizes of lipoplexes in the presence of serum were comparable.

We measured the β-gal gene expression in ER-expressing breast adenocarcinoma MCF-7 cells pretreated or untreated with 100 μM tamoxifen, an ER antagonist. Cells were transfected with cationic lipoplex containing either the targeting lipid DPPE-PEG-ES or the nontargeting lipid DSPE-PEG-mal. To mimic in vivo conditions, the transfection was carried out in the presence of a serum-containing medium. Figure 2

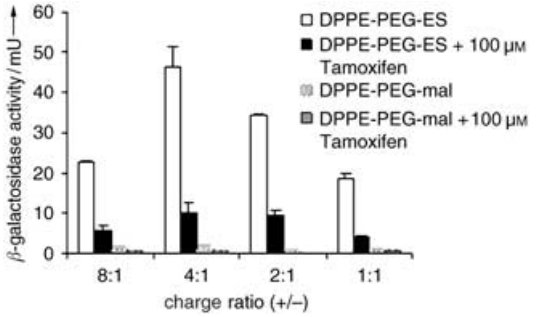


Figure 2. β-Gal gene expression in MCF-7 cells. MCF-7 cells, with or without pretreatment by tamoxifen (100 μM), were transfected with 0.3 μg of pCMV-β-gal DNA complexed in the estradiol-associated targeting cationic liposome (DPPE-PEG-ES) or in the nontargeting control cationic liposome (DSPE-PEG-mal). All cells were treated in the presence of 7% serum. Transgene expression is represented as β-galactosidase activity. The targeting and nontargeting lipoplexes were treated with the cells in cationic lipid/DNA charge ratios (+/−) of 8:1, 4:1, 2:1, and 1:1. The transgene expression obtained for each data point was acquired from triplicate treatments performed in a single day. The difference between the data obtained for DPPE-PEG-ES and DSPE-PEG-mal is statistically significant at all charge ratios (the probability of getting more extreme results than the observed value (p) < 0.0001), whereas that for DPPE-PEG-ES and DPPE-PEG-ES + 100 μM tamoxifen is statistically significant at all charge ratios ($p < 0.0001$) except for a charge ratio of 4:1, when $p = 0.00029$.

shows that the cells treated with targeting lipoplex express significantly more reporter gene than cells treated with nontargeting lipoplex at all the charge ratios (20- to 100-fold). Moreover, the presence of tamoxifen significantly inhibits targeting-lipoplex-mediated gene expression at all the charge ratios. However, in the case of the nontargeting-lipoplex-mediated transfection, the presence of tamoxifen has almost no effect ($p > 0.1$). This result demonstrates the ability of the DPPE-PEG-ES containing targeting lipoplex to transfect MCF-7 cells in a target-specific manner. The number of ligands is higher at a charge ratio of 8:1, but the highest transfection was obtained at a ratio of 4:1. This is probably because the lipoplex size at a charge ratio of 8:1 is almost three times that at a charge ratio of 4:1 (see the Supporting Information), and also because the larger lipoplex size may not be favorable for efficient cellular uptake, particularly in this system. However, the size and surface charge of lipoplexes with other charge ratios are comparable. Therefore, in comparison to lipoplexes obtained at charge ratios of 2:1 or 1:1, the lipoplex obtained at a charge ratio of 4:1 tends to have a greater targeting ability as a result of the availability of more

ligands on the surface, and hence a higher transfection efficiency.

MDA-MB-231 is an advanced-stage breast cancer cell with drug-resistant properties. This cell does not possess ERs but expresses GPR30 protein[19] and therefore it is a natural, ER-knockout cell line. This cell line was chosen for transfection with targeting lipoplex, and the extent of transgene expression was compared with that obtained from MCF-7 cells. Figure 3 shows that there was almost negligible β-gal

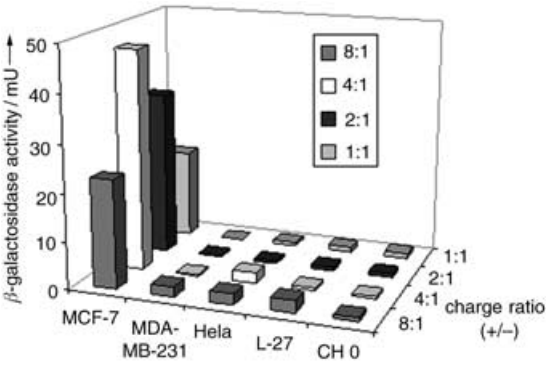


Figure 3. β-Gal transfection in various cell lines. MDA-MB-231 (ER-knockout cell), Hela, CHO, and L-27 cells were transfected with targeting cationic lipoplex containing DPPE-PEG-ES and the results were compared with those for MCF-7 (ER-positive cell) for lipid/DNA charge ratios (+/−) of 8:1, 4:1, 2:1, and 1:1. The transgene expression obtained for each data point was acquired from triplicate experiments performed in a single day. The transgene expressions obtained in MCF-7 cells were all statistically significant ($p < 0.0001$) when compared to those obtained from other cell lines.

expression in MDA-MB-231 cells compared to that obtained in MCF-7 cells ($p < 0.0001$). This result shows that the targeting lipoplex can deliver and hence express genes only in cells possessing ER. GPR30 has a high affinity for estrogen,[19] and perhaps upon interacting with targeting lipoplex it can deliver gene to the cytoplasm. However, the lipoplex is probably unable to mediate delivery of genes to the nucleus because of the absence of ER. It is not clear how ER mediates selective delivery of genes to the MCF-7 nucleus in association with or in the absence of a lipid shell. This interesting observation in itself warrants further investigation, especially in the light of the finding that PEG blocks the intracellular trafficking of DNA associated with the liposomal system.[21]

Next, we evaluated the gene-targeting efficiencies of the DPPE-PEG-ES-associated lipoplex in three more cell lines not known to express ER, namely human cervical carcinoma (Hela), Chinese hamster ovary (CHO), and murine sarcoma cells (L-27, which is genetically modified to overexpress integrin receptors), by using β-gal plasmid. The transgene expression from these cell lines was compared with that obtained from MCF-7 cells. Figure 3 shows that gene expression in Hela, CHO, and L-27 cells is statistically insignificant in comparison to that obtained in MCF-7 cells. In general, the CHO and Hela cell lines are highly transfectable with cationic lipids and the present targeting, stealth, cationic transfection lipid/DNA complex mediates negligible transfection in these cells, probably because the gene targeting is inefficient in the absence of ER.

Finally, we tested the targeting delivery system and estimated the relative anticancer efficacies of the apoptosis-inducing p53 gene delivered to MCF-7 cells with both the targeting and nontargeting lipoplexes. Figure 4 shows that the

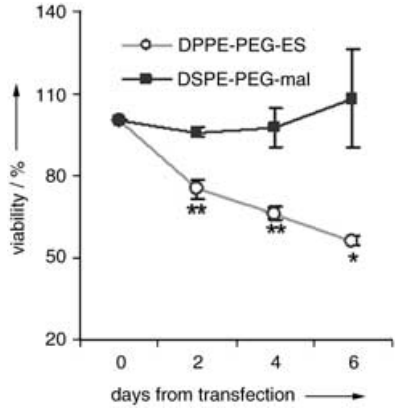


Figure 4. Anticancer-gene-mediated targeted killing of MCF-7 cells. The cells were treated with 0.3 μg of pCMV-p53 DNA complexed with cationic liposomes containing targeting lipid, DPPE-PEG-ES (○), and nontargeting lipid, DSPE-PEG-mal (■), in a cationic lipid/DNA charge ratio (+/−) of 2:1. The treatment was similar to that described for the β-gal transfection experiments. The cells underwent MTT assay on days 2, 4, and 6 after transfection. The *y* axis shows the percentage viability, and each data point was acquired from triplicate treatments; $p < 0.005$ (**), $p = 0.007$ (*).

targeting lipoplex can indeed induce cell death, possibly through apoptosis, in a target-specific manner. From the second day onwards, the cell viability of the group treated with DPPE-PEG-ES-associated targeting lipoplex showed significant toxicity ($p < 0.005$ and $p = 0.007$), which persisted during the subsequent six days. Furthermore, we examined the stability of the targeting lipoplex at various serum concentrations. No perceptible change in the targeting efficiency, even at the biologically relevant in vivo serum concentration of 53%, was observed (see the Supporting Information). This result proves that the targeting gene delivery system will remain undeterred in its targeting efficiency in vivo.

In summary, our findings demonstrate a highly efficient, novel route for targeting anticancer genes at breast cancer cells through the use of 17β-estradiol-associated stealth liposomes. The chemistry involved is simple and inexpensive. Evaluation of the in vivo potential of the technique for eradicating breast cancer is currently in progress.

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