We describe a novel gene delivery system that specifically targets human epidermal growth factor receptor 2 (Her2)-overexpressing breast cancer cells. The targeting complexes consist of a PEGylated polylysine core that is bound to DNA molecules coding for either green fluorescent protein or shrimp luciferase. The complex is disulfide linked to the monoclonal antibody trastuzumab and to a pore-forming protein, Listeriolysin O (LLO). Trastuzumab is responsible for specific targeting of Her2 receptors and uptake of the gene delivery complex into endosomes of recipient cells, whereas LLO ensures that the DNA molecules are capable of transit from the endosomes into the cytoplasm. Omission of either trastuzumab or LLO from the nanocomplexes results in minimal gene product in targeted cells. Treatment of isogenic MCF7 and MCF7/Her18 cell lines, differing only in number of Her2 receptors, with the complete gene delivery system results in a 30-fold greater expression of luciferase activity in the Her2-overexpressing MCF7/Her18 cells. Our nanocomplexes are small (150–250 nm), stable to storage, nontoxic and generic in make-up such that any plasmid DNA or antibody specific for cell-surface receptors can be coupled to the PEGylated polylysine core.

INTRODUCTION
The goal of cancer gene therapy is to deliver therapeutic genes and achieve their expression in tumor tissue. Candidate genes include interleukin-12, which could provoke an antitumor immune response, and tumor necrosis factor-α, which could induce cancer cell apoptosis. However, these genes must be delivered specifically to avoid toxic side effects. Targeted delivery of genes to cancer cells has been achieved in a limited number of laboratories using liposomal delivery systems with antibody to human epidermal growth factor receptor 2 (Her2) receptors, 1,2 with an RGD peptide specific for integrin, 3 or with antibody to prostate-specific membrane antigen. 4

Alternative gene delivery systems have been based on polymers such as polyethylenimines (PEIs) or dendrimers, rather than on liposomes. Such polymeric targeting systems have been reported using epidermal growth factor (EGF) specific for EGF receptors, 5 anti-Her2 antibody (trastuzumab) specific for Her2 receptors, 6 transferrin specific for transferrin receptors, 7,8 a fibroblast growth factor (FGF)-11-mer peptide specific for FGF receptors 9 and lactoferrin or lactoferricin specific for transferrin receptors. 10

There are, however, problems associated with the use of polymeric-based targeting systems. PEI is highly cytotoxic, causing immediate disruption of the cell membrane and consequent necrotic cell death, or eventual disruption of the mitochondrial membrane leading to apoptosis. 11 Toxicity has been decreased somewhat by using lower molecular weight PEIs 12 or by shielding of PEI/DNA complexes via covalent modification with polyethylene glycol (PEG) to prevent nonspecific interactions with components in the plasma or with erythrocytes. 12

In contrast to gene targeting systems based on liposomes or on PEI- or dendrimer- polymers, the targeting complex we have developed is based on polylysine (PL), a nontoxic polymer, coupled to a N-hydroxysuccinimide and orthopyridyl disulfide hetero-functionalized PEG ester (OPSS-PEG-NHS). By including bound trastuzumab (Herceptin), our targeting complexes selectively deliver reporter DNAs to breast cancer cells overexpressing Her2 receptors. The test reporter genes used in our system code for either green fluorescent protein (GFP) or luciferase (Luc). Expression of these DNAs is dependent on the inclusion of a pore-forming protein, Listeriolysin O (LLO), in the targeting complexes to enable passage of the DNAs from the endosomal compartment to the interior of the targeted cells.

The practical advantage of these complexes is that they are generic in nature and are designed to carry any antibody recognizing a specific membrane receptor overexpressed in the targeted tumor cells, and similarly to carry any DNA molecule coding for a specific gene product in the targeted cells. Our nanocomplexes result in gene expression in specifically targeted cells and appear to be nontoxic to the recipient cells.

MATERIALS AND METHODS
Materials
OPSS-PEG-NHS (molecular weight 5000) was purchased from Nanocs (New York, NY, USA). Trastuzumab (Herceptin) was a generous gift from Dr Virginia Borges (UC Denver, Denver, CO, USA). LLO from Escherichia coli transfected with the LLO-pET29-DP-E3570 plasmid, kindly provided by Dr Dan Portnoy (UC Berkeley, Berkeley, CA, USA), was purified by the method described previously 13,14 and stored in storage buffer (50 mM phosphate buffer, pH 6.0, 1 M NaCl and 1 mM EDTA) without dithiothreitol to preserve its activity. Polylysine hydrobromide (molecular weight 37 000; degree of polymerization: 177) and 2-iminothiolane-HCl (Traut’s reagent) were purchased from Sigma Life Science (St Louis, MO, USA). CL-4B Sepharose...
used for the purification of the one-component complexes was purchased from Amersham Biosciences (Uppsala, Sweden). All other reagents, unless otherwise specified, were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA).

Cells and growth medium

The cell line ce2, derived from human mammary epithelial cell line MTSV1-27 that had been stably transfected with Her2 DNA, was kindly provided by Dr Joyce Taylor-Papadimitriou (King's College, London School of Medicine, London, UK). The overexpressing Her2 ce2 cells were grown in Dulbecco's modified Eagle's medium (Sigma Life Science) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The cell line ce2, derived from human mammary epithelial cell line MTSV1-27 that had been stably transfected with Her2 DNA, was kindly provided by Dr Joyce Taylor-Papadimitriou (King's College, London School of Medicine, London, UK). The overexpressing Her2 ce2 cells were grown in Dulbecco's modified Eagle's medium (Sigma Life Science) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The cell line ce2, derived from human mammary epithelial cell line MTSV1-27 that had been stably transfected with Her2 DNA, was kindly provided by Dr Joyce Taylor-Papadimitriou (King's College, London School of Medicine, London, UK). The overexpressing Her2 ce2 cells were grown in Dulbecco's modified Eagle's medium (Sigma Life Science) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The cell line ce2, derived from human mammary epithelial cell line MTSV1-27 that had been stably transfected with Her2 DNA, was kindly provided by Dr Joyce Taylor-Papadimitriou (King's College, London School of Medicine, London, UK). The overexpressing Her2 ce2 cells were grown in Dulbecco's modified Eagle's medium (Sigma Life Science) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The cell line ce2, derived from human mammary epithelial cell line MTSV1-27 that had been stably transfected with Her2 DNA, was kindly provided by Dr Joyce Taylor-Papadimitriou (King's College, London School of Medicine, London, UK). The overexpressing Her2 ce2 cells were grown in Dulbecco's modified Eagle's medium (Sigma Life Science) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The cell line ce2, derived from human mammary epithelial cell line MTSV1-27 that had been stably transfected with Her2 DNA, was kindly provided by Dr Joyce Taylor-Papadimitriou (King's College, London School of Medicine, London, UK). The overexpressing Her2 ce2 cells were grown in Dulbecco's modified Eagle's medium (Sigma Life Science) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA).

DNA purification

DS5a bacteria transfected with the pEFGP-N3 plasmid were kindly provided by Dr Jason Burkhed (University of Alaska, Anchorage, AK, USA). The growth of the bacteria in LB broth with kanamycin at a final concentration of 30 μg ml⁻¹, endotoxin-free DNA. The following day, a red blood cell (RBC) lysate assay was carried out to determine whether LLO had completely reacted with the OPPL-DNA to yield the Tmab/DNA complex. A 5 μl aliquot of the reaction mixture was placed in each of two tubes, with 2.5 μl 300 mm diethiothreitol added to one tube and 2.5 μl H₂O added to the other tube, mixed and incubated at room temperature for 15 min to allow for reduction of the disulfide bond. Sheep RBCs were prepared by centrifugation and resuspension of the pellet in 1 x DPBS, pH 7.45, in a 1:2.5 dilution. At the end of the 15-min incubation, 50 μl of the diluted RBCs were added to each of the two tubes and the time until lysis was noted. If LLO had fully reacted with the OPPL group, there was no lysis of the RBCs in the presence of diethiothreitol there was complete lysis of the RBCs within 2–3 min.

If the reaction of LLO with the Tmab/DNA complex had gone to completion, the reaction mixture was put over a CL-4B column pre-equilibrated with 1 x DPBS, pH 7.45, and fractions were collected. To identify the early-eluting DNA-nanocomplex fractions, aliquots of the fractions were placed in a mini-spectrophotometer (Eppendorf BioPhotometer, Hauppauge, NY, USA), and A260, A280 and A320 readings of each fraction were recorded. The peak nanocomplex fractions, characterized by a peak in absorption at all three wavelengths, were stored at 4 °C until use in the cell treatment experiments.

Sizing of the nanocomplex preparations was determined by dynamic light scattering analysis using a Nicomp 380 zeta potential/particle sizer (Particle Sizing Systems, Santa Barbara, CA, USA) or using a Malvern zetasizer Nano-S (Malvern Instruments, Malvern, UK). When the effect of presence versus absence of trastuzumab on DNA expression was tested, the size of the complete complex with both t-Tmab and LLO was 278 ± 94 nm, whereas that of the complex with LLO but minus t-Tmab was 267 ± 107 nm. When the effect of LLO on DNA expression, the size of the complete complex with both t-Tmab and LLO was 147 ± 31 nm, whereas that of the complex with Tmab but minus LLO was 148 ± 37 nm. For unknown reasons, some variability in the size of the nanocomplex preparations was noted, although within a given experiment the relative sizes of the complexes were well matched. In general, it can be seen that the range in nanocomplex size is between 150 and 250 nm approximately.

Treatment of cells with nanocomplexes

Actively growing ce2, HCC1954, MCF7 and MCF7/Her18 cells were plated out in Falcon microtiter 96-well assay plates, black/clear bottom (Becton Dickinson Labware, Franklin Lakes, NJ, USA), either in duplicate for fluorescence analysis or in triplicate for luciferase analysis. After 48 h of growth, when the cells were between 50 and 70% confluent, they were treated with the nanocomplex preparations. Before treatment, the CL-48 purified nanocomplex preparations were equalized with respect to the amount of DNA bound using the Qubit dsDNA high-sensitivity assay kit (Invitrogen Life Technologies, Thermo Fisher Scientific, Pittsburgh, PA, USA).

For the purposes of binding trastuzumab (Tmab) to the OPSS groups in OPPL-DNA, thiol groups were introduced into the trastuzumab molecules. The thiolated trastuzumab (t-Tmab) was prepared just before addition to OPPL-DNA, thiol groups were introduced into the trastuzumab molecules. The thiolated trastuzumab (t-Tmab) was prepared just before addition to OPPL-DNA, thiol groups were introduced into the trastuzumab molecules. The thiolated trastuzumab (t-Tmab) was prepared just before addition to OPPL-DNA, thiol groups were introduced into the trastuzumab molecules. The thiolated trastuzumab (t-Tmab) was prepared just before addition to OPPL-DNA, thiol groups were introduced into the trastuzumab molecules. The thiolated trastuzumab (t-Tmab) was prepared just before addition to OPPL-DNA, thiol groups were introduced into the trastuzumab molecules. The thiolated trastuzumab (t-Tmab) was prepared just before addition to OPPL-DNA, thiol groups were introduced into the trastuzumab molecules. The thiolated trastuzumab (t-Tmab) was prepared just before addition to OPPL-DNA, thiol groups were introduced into the trastuzumab molecules.
Analysis of gene expression

For examination of GFP fluorescence, the growth medium was removed from the ce2 cells, and 100 μl 1 × DPBS, pH 7.45, was added to each well. The cells were viewed with a Leica DMI6000B inverted fluorescence microscope (Leica Microsystems, Buffalo Grove, IL, USA), and photos were taken with a 5 × objective using Leica Application Suite, version 3.7.0 software. For the purposes of quantitation, NIH ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to compare the relative amounts of fluorescence in each field of cells subjected to different conditions of treatment with the nanocomplexes.

For measuring luciferase activity, the growth medium was removed from the MCF7 and MCF7/Her18 cells, the cells were rinsed with 100 μl 1 × DPBS, pH 7.45, and then 100 μl 1 × reporter lysis buffer (Promega) was added to each well. Lysis of the cells was enhanced by one freeze/thaw cycle of the 96-well plate at −80 °C, followed by transfer of the extracts to microfuge tubes that were spun at 11 900 g for 3 min at 4 °C in an Eppendorf microfuge 5417R. A 5 μl aliquot of each extract was diluted with 45 μl 1 × DPBS, pH 7.45, in a white 96-well assay plate and then 50 μl of Nano-Glo luciferase assay reagent (Promega) was added to each well. The luminescence produced by each cell extract was measured in a Biotek luminometer using Gen5 software (BioTek, Winooski, VT, USA). Protein determinations were done on the same cell extracts using a modification of the bicinchoninic acid (BCA) protein assay (reagents A and B; Pierce, Rockford, IL, USA) for a 96-well plate (Corning, Corning, NY, USA). Protein determinations were done on the same cell extracts using a modification of the bicinchoninic acid (BCA) protein assay (reagents A and B; Pierce, Rockford, IL, USA) for a 96-well plate (Corning, Corning, NY, USA). Protein determinations were done on the same cell extracts using a modification of the bicinchoninic acid (BCA) protein assay (reagents A and B; Pierce, Rockford, IL, USA) for a 96-well plate (Corning, Corning, NY, USA). To each well, we added 100 μl BCA reagent (50 parts reagent A/1 part reagent B) plus 10 μl of each cell extract or 10 μl of each bovine serum albumin standard. The wells were mixed, covered with parafilm, heated at 60 °C in a dry incubator for 15 min to develop the color and then read at 562 nm after removing the parafilm.

Analysis of nanocomplex toxicity

To analyze whether the nanocomplex treatment of cells had any effect on cell viability or proliferation, the XTT cell proliferation assay kit (ATCC) was used. Equal numbers of MCF7/Her18 cells were plated out in the requisite number of wells in a 96-well plate at a concentration of 0.9 × 10⁴ cells per well and grown overnight at 37 °C in a CO₂ incubator. The following day, replicate wells were treated either with 100 μl of growth medium (plus 10% fetal bovine serum and penicillin–streptomycin) containing 10 μl of luciferase nanocomplex (complex-treated) or with 100 μl of medium containing 10 μl of 1 × DPBS, pH 7.45 (control-treated) for 3 h at 37 °C in 5% CO₂. At the end of a 3-h incubation, the treatment mix was pipetted off from each well, replaced with 100 μl of fresh growth medium and the treated cells were then grown for another 24, 48 or 72 h before adding 50 μl activated-XTT solution to each well for a further incubation of 3 h at 37 °C. The specific absorbance of the treated (complex- versus control-treated) cells in each well and in the blank medium-only wells was measured at 475 nm, followed by measurement of the nonspecific absorbance at 660 nm. The specific absorbance of each sample was expressed mathematically according to the manufacturer's instructions, as follows:

Specific Absorbance(S.A.) = A₄₇₅nm(Test)−A₄₇₅nm(Blank)−A₆₆₀nm(Test)

Parallel wells of complex-treated versus control-treated MCF7/Her18 cells were harvested simultaneously at 24, 48 and 72 h for analysis of luciferase expression, as specified above under ‘Analysis of gene expression’.
Tmab19 and LLO 14 were essential for speciﬁc delivery of DNA into the endosome via LLO-formed pores.14,17,18 Because of these data, we reasoned that ce2 cells would be a good choice for studying the targeting ability of our GFP nanocomplexes bearing Tmab and LLO.

Our initial experiments on gene targeting were done with ce2 cells, derived from a human mammary epithelial cell line transfected with Her2-DNA and known to overexpress Her2 receptors.19 Previous work in our laboratory had shown that both Tmab19 and LLO14 were essential for speciﬁc targeting and delivery of a payload (calcein) to the cytoplasm of these cells. Because of these data, we reasoned that ce2 cells would be a good choice for studying the targeting ability of our GFP complexes bearing Tmab and LLO.

When ce2 cells were treated with Tmab/GFP nanocomplexes, GFP signal was detectable by 24 h and increased through 48 h. We chose the latter time point for our studies on the speciﬁcity of delivery. As the treatment dosage of Tmab/GFP targeting complexes was increased from 5 to 20 μl, ce2 cells showed an increase in GFP expression at 48 h after treatment (Figure 1). We noted a marked decrease in GFP expression when Tmab was omitted from the targeting complexes as well as when Tmab/GFP targeting complexes were competed by free Tmab during treatment of the cells (Figure 1). The proﬁle of GFP expression when the complexes were competed with free Tmab was in fact similar to that seen when the targeting antibody was omitted from the nanocomplex preparation (Figures 1 and 2). ImageJ analysis to quantitate the ﬂuorescence showed that the expression level of GFP in the presence of free Tmab as competitor was ~20% of that seen when competing free Tmab was not present (Figure 2).

Similarly, GFP expression in ce2 cells was used to evaluate the effect of LLO on targeted gene delivery. The expression of GFP from delivery complexes constructed without LLO was only 5% of the expression from complexes with LLO (Figure 2). Interestingly, this 5% residual expression was further reduced by free Tmab competition to ~1% of the expression achieved by complexes containing LLO (Figure 3). This result suggests that at least a portion of the GFP expression without LLO is due to Her2-speciﬁc uptake of the targeting complexes and leakage of GFP DNA out of the endosome in some other manner than through LLO-formed pores.

Fluorescence in MCF7/Her18 cells targeted with Tmab/GFP nanocomplexes and competition with free trastuzumab

To visualize the efﬁciency of DNA delivery to targeted cells, we treated Her2-overexpressing MCF7/Her18 (referred to as Her18) cells with Tmab/GFP complexes and compared bright-ﬁeld images with ﬂuorescence images of cells in the same microscopic ﬁeld (Figure 4). In this example, a manual count of all visible cells in the bright-ﬁeld and ﬂuorescent images using ImageJ indicated that 18% of the cells expressed detectable GFP (Figure 4, left-hand panels). It can be seen that the level of ﬂuorescence varied from cell to cell within the treated population. Variation in degree of GFP expression in targeted cells could result from variation in a number of different factors, such as variation in binding of complexes to receptors in the 3 h incubation period, successful uptake into endosomes, efﬁciency of transit out of the endosomes and timing of subsequent transcription and translation.
We also demonstrated that GFP expression was significantly reduced when cells were co-incubated with targeting complexes and free Tmab added at the time of treatment. The reduced expression is presumably because of competitive inhibition by free Tmab on Tmab/GFP complex binding to Her2 receptors (Figure 4, right-hand panels).

Effect of Her2 receptor overexpression and requirement of Tmab for targeted delivery of luciferase DNA
To evaluate the components essential to our nanocomplex delivery system, we compared the expression of targeted DNA in the isogeneic breast cancer cell lines MCF7 and MCF7/Her18. The Her18 cell line differs from the parent MCF7 cell line by virtue of a 45-fold increase in the number of Her2 receptors located in its plasma membranes. To test the versatility of our nanocomplex system and to simplify the quantitation of the gene product, we replaced the GFP DNA with shrimp luciferase DNA in the targeting complex preparation (Tmab/Luc complexes).

When both the MCF7 and Her18 cell lines were treated with varying doses of Tmab/Luc complexes, there was a 25- to 30-fold increase in the luciferase activity of Her18 cells as compared with that of MCF7 cells (Figure 5). When Tmab was omitted from the complexes (non-Tmab/Luc) used for treatment of the Her18 cells, the luciferase activity was reduced by > 90% (Figure 5), demonstrating that the specificity for Her18 cells is indeed based on Tmab binding to overexpressed Her2 receptors.

Requirement of LLO for expression of luciferase DNA in cells targeted with Tmab/Luc nanocomplexes
Studies done with Tmab/Luc complexes constructed with or without LLO present in the complex showed that LLO was essential for expression of the luciferase DNA in both Her18 and MCF7 cells (Figure 6). When LLO was present, 20- to 22-fold more luciferase activity was expressed in the Her18 cells than in the MCF7 cells (Figure 6). It seems likely that this difference is because of specific targeting of the Tmab-containing complexes to the overexpressed Her2 receptors that are much more plentiful in the Her18 cells than in the MCF7 cells.

In the absence of LLO, there was effectively no luciferase activity in either cell line treated with Tmab/Luc complexes (Figure 6). We have previously shown that LLO is essential for the formation of pores in the endosomal membrane and subsequent transit of calcine payload to the cytoplasm when Tmab-containing liposomes are bound to Her2 receptors and taken up into endosomes. Similarly, our results with the Tmab/Luc complexes

Figure 4. Fluorescence in Her18 cells treated with trastuzumab/green fluorescent protein (Tmab/GFP) complexes. Her18 cells were treated for 3 h with a 10 μl per well dose of Tmab/GFP complexes either in the absence or in the presence of free Tmab competitor (2.5 μl per well) and visualized 48 h later. A bright-field image and a fluorescent image of the same field were taken for comparison, with the 20 x objective on the inverted fluorescence microscope. Scale bar, 100 μm.

Figure 5. Effect of trastuzumab (Tmab) on luciferase (Luc) activity. Luciferase activity (relative light unit (RLU) x 10^5 units per μg protein) is plotted against dose (5 to 15 μl) of DNA complexes used for treatment. Data are compared for Her18 versus MCF7 cells targeted with Luc complexes containing Listeriolysin O (LLO) and with or without Tmab present in the complexes. Each treatment was set up in triplicate, and cells were harvested 24 h after treatment for analysis of luciferase activity. Bicinchoninic acid (BCA) protein determinations were done on the same cell extracts used for the luciferase assay. The data are shown as mean ± s.e. (n = 3).
in the absence of LLO suggest that there is a lack of luciferase activity in either cell line because the Luc-DNA is also unable to cross the endosomal membrane into the cytoplasm of the targeted cells.

Viability of Her18 cells treated with Tmab/Luc nanocomplexes and luciferase expression over a 3-day time course
To determine whether treatment with Tmab/Luc complexes was toxic or nontoxic to Her2-overexpressing breast cancer cells, we simultaneously measured luciferase activity and cell viability of complex-treated versus control nontreated Her18 cells over a 72-h time period. When examined visually under the microscope, treated and nontreated cells appeared to be identical over time period. When examined visually under the microscope, treated and nontreated cells appeared to be identical over time with respect to morphology, viability and confluency, with ~ 40–50% confluency at 24 h, 75% confluency at 48 h and finally complete confluency by 72 h after treatment.

The apparent lack of effect of nanocomplex treatment on cell viability over 72 h was confirmed by an XTT viability assay (Figure 7, upper panel). Over the same time period, the complex-treated cells showed a marked increase in luciferase expression (Figure 7, lower panel), suggesting that the cells were viable and continued to synthesize luciferase. As expected, the control Her18 cells, which received only a medium change and no complex treatment, had luciferase readings at the level of background, and are therefore not visualized in the bar graph.

Targeting Tmab/Luc nanocomplexes to other breast cancer cell lines and competition with free Tmab
To examine the targeting of the Tmab/Luc nanocomplexes to Her2-overexpressing breast cancer cell lines other than MCF7 and MCF7/Her18, we treated ce2 cells and HCC1954 cells with Tmab/Luc nanocomplexes and measured the luciferase expression in the absence versus the presence of free Tmab as competitor. An increase in luciferase expression was noted in both the ce2 and HCC1954 cell lines in comparison with the low Her2-expressor MCF7 cell line (Figure 8), although both cell lines exhibited less luciferase end product than did the Her18 cells. It is noteworthy that the HCC1954 cell line is trastuzumab resistant but, in spite of this, specific gene therapy was accomplished with our targeting system. Competition with free Tmab was most effective with the Her18 and HCC1954 cells, less effective with the ce2 cells and with no competition of the basal luciferase expression in the MCF7 cells (Figure 8).

DISCUSSION
Overexpression of specific receptors in the plasma membranes of various cancer cells provides a mechanism by which such cells can be targeted specifically in vitro and in vivo with antibody-containing nanocomplexes. In the case of human breast cancers, overexpression of the Her2 occurs in 25–30% of breast cancers and is associated with an aggressive phenotype. By conjugating trastuzumab (Herceptin), an antibody specific for the Her2 receptor, to our DNA-containing complexes, we have achieved specific delivery of DNA to Her2-overexpressing breast cancer cells. In the presence of competitor free Tmab, expression of end product in these cells is markedly decreased, demonstrating that targeted gene expression is indeed dependent on nanocomplexes binding to the Her2 receptors on the cancer cells.

To better conceptualize our DNA-targeting nanocomplex, we provide an interpretive illustration of a single complex (Figure 9). The targeting complexes in our model are based on a core of
OPSS-PEG-NHS (OPN), a PEG derivative capable of forming covalent amide linkages at its NHS end with amino groups of proteins and disulfide bonds at its OPSS end with thiol groups in proteins. After binding of PL to the NHS ends of OPN, supercoiled plasmid DNA is added and interacts with the bound PL via noncovalent electrostatic interactions. Finally, t-Tmab and LLO compete for the OPSS ends of OPN to form reversible disulfide bonds. The electrostatic bonds are responsible for the subsequent release of DNA and the reversible disulfide bonds for the release of LLO from the complex within the interior of the endosomes.14,24

The addition of the pore-forming protein, LLO, to our nanocomplexes enables the successful transit of the DNA from the endosomal compartments to the cytoplasm of the targeted cells. Under the reducing conditions found in endosomes, disulfide-bound LLO is released either from low molecular weight PEL19 or from liposomes23 such that it can form pores in the endosomal membrane and allow exit of the delivered endosomal contents to the cytoplasm. We have now extended these findings by showing that the disulfide bond between the LLO and the OPN core in our nanocomplexes also appears to be reduced in the endosome, and the released LLO then functions to allow passage of the DNA to the cytoplasm. In addition, the acidic environment of the endosome presumably affects the ionic bonds between the DNA and the PL, resulting in dissociation of the DNA from the polysine, although the PL itself would remain covalently bonded to the OPN core.

It is assumed that the DNA in our system is expressed only in targeted cells that are actively dividing because, once delivered to the cytoplasm, DNA molecules must relocate to the nucleus, during breakdown and reformation of the nuclear membrane, in order for transcription to take place.26 The transcripts subsequently exit the nucleus and are translated in the cytoplasm to result in detectable gene product. In our studies, we have successfully detected two different gene products, green fluorescent protein and luciferase, as a result of specificity of delivery of their respective DNAs to Her2-overexpressing cells by our Tmab-coupled nanocomplexes. Expression of GFP and luciferase in our system is dependent on the presence of both Tmab and LLO in the DNA-containing targeting complexes. Omission of either in construction of the nanocomplexes results in a marked decrease in expression of these gene products in cells grown in vitro.

The DNA-containing nanocomplexes that we have designed could be constructed using any plasmid DNA coding for a desired gene product such as interleukin-12, which can stimulate a T-cell response, or tumor necrosis factor-α, which can induce apoptosis in tumor cells. Nonspecific delivery of these therapeutic genes results in significant toxicity, necessitating a system that results in tumor-specific gene delivery.27–29 In addition to versatility of gene delivery, it should be straightforward to substitute Tmab with any other desired antibody or targeting peptide that binds to receptors uniquely expressed on the cells being targeted. Our complexes are stable to storage for a period of >6 weeks at 4 °C without loss of activity (data not shown), both with respect to targeting ability and expression of luciferase. Finally, our toxicity experiments show that our nanocomplexes are not toxic to Her18 cells for at least 72 h after Tmab targeting and in fact allow subsequent growth of Her2-overexpressing cells in vitro.

The specificity and other desirable characteristics demonstrated for our gene delivery system in vitro justify further study in an in vivo model. It must be determined whether our nanocomplexes...
are capable of reaching their intended target in vivo before being degraded or cleared from the circulatory system and whether LLO or any other component present in our targeting complexes is sequestered sufficiently so as not to trigger an immune response in recipient tumor-bearing animals.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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