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Increased Target Specificity of Anti-HER2 Genospheres by Modification of Surface Charge and Degree of PEGylation

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Abstract: Genospheres are cationic lipid−nucleic acid nanoparticles prepared by the assembly of the lipids and nucleic acids from an aqueous/organic liquid monophase that independently dissolves the components, where the resultant particles are homogeneously sized (70−110 nm), with efficiently incorporated and protected DNA. In the present study, we demonstrate pH-dependent modulation of the Genosphere surface charge using pH-titratable lipids. By incorporation of the lipids with titratable anionic or imidazole headgroups, Genospheres with neutral or anionic surface charge at neutral pH were produced and compared for cellular uptake and transfection of a reporter gene (luciferase) in culture of breast cancer cells. The extent of particle−cell association was also studied by fluorescent microscopy and quantified by cytofluorometery. The effects of Genosphere surface modification with poly(ethylene glycol) (molecular weight 2000) at low (0.5 mol %) and high (5 mol %) grafting densities, as well as the effects of HER2-receptor-directed targeting by an internalizable anti-HER2 scFv F5, linked via PEG spacer, were also studied. Inclusion in the Genosphere formulation of pH-titratable lipids CHEMS (cholesteryl hemisuccinate), CHIM (1-(3-(cholesteryloxycarbonylamino)propyl)imidazole), or DSGG (1,2-distearoyl-*sn*-glycero-3-hemiglutarate) rendered the particles surface-charge neutral or slightly anionic at neutral pH, and cationic at mildly acidic pH, as shown by ζ-potential measurements. In HER2-targeted systems, transfection activity and target specificity with HER2-overexpressing SKBR-3 breast cancer cells were dependent on Genosphere surface charge and PEGylation. The highest target specificity correlated with low cationic charge at neutral pH, while incorporation of 5 mol % PEG-lipid had only minor effects on Genosphere-cell association, internalization, and transfection activity. The implications of this work for potential in vivo applications are discussed.

Keywords: Nonviral gene delivery; plasmid encapsulation; targeted gene delivery; HER2 receptor; ζ-potential; surface charge; PEGylation

Introduction

The success of gene therapy relies on delivering nucleic acid efficiently to its cellular target in a functionally intact state. The advantages of using nonviral delivery strategies include lower toxicity, inexpensive components, and the ease

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of attaching cell surface targeting ligands. Variations of complexes formed from the electrostatic interactions between nucleic acid and cationic liposomes (lipoplex), usually made with an excess of cationic charge, have been used as delivery vehicles in vitro[1] and in vivo.[2]

However, poor pharmacokinetic characteristics with the majority of sample typically accumulating in the first-pass organs (lung, liver, and spleen) shortly after injection,[3] in conjunction with high inflammatory toxicity, serologic toxicity, and hepatotoxicity may limit their utility in vivo.[2] It is known that the increased efficacy of many anticancer liposomal formulations over free drug is in part due to their longer circulation times and natural accumulation in diseased tumor sites, leading to high drug concentrations in close proximity to tumor cells. Recent DNA−lipid complex preparation methods have been devised that attempt to modify the properties of the complexes to enhance the prospects of tumor localization, and therefore produce more suitable candidates for in vivo use.[4−7] To further increase the efficacy of lipid based gene delivery, researchers have often adopted some of the approaches that have been used successfully to enhance liposomal drug delivery, including PEGylation.[8] The reduction of excess cationic surface charge has also been shown to contribute to longer circulation times in a variety of these DNA−lipid particle assemblies.[8,9] Recently a technique involving the selective reduction of thiocholesterol-based cationic lipids on the outer surface of particles has been used to obtain charge-neutral particles.[10]

Targeted delivery of nucleic acids is an important consideration when designing a delivery vehicle. Targeting promotes increased delivery to the cells of interest with decreased nonspecific delivery to other cells. As release and subsequent intracellular delivery is more difficult to control for nucleic acids compared to small molecular weight drugs, molecular targeting provides a promising strategy for specific and efficient intracellular nucleic acid delivery.

Previously, we have shown that DNA−lipid particles (Genospheres) could be formed under conditions where lipid and DNA are each soluble, either in molecular or micellar form, *prior* to their combination.[11,12] Such conditions promote more favorable interactions between DNA and cationic lipids, and eliminate the bilayer structural rearrangements that occur when DNA interacts with preformed cationic liposomes.[13] The Genosphere nanoparticles were small in size (80−110 nm) and afforded excellent protection to the entrapped DNA in the presence of human plasma. In addition, they were capable of being immunotargeted to selectively transfect HER2 overexpressing cells by insertion of an anti-HER2 human single-chain monoclonal antibody (scFv)−PEG conjugate.

In the present work, we describe how altering the surface properties of these particles affects their interactions with cells and the efficiency of gene expression in vitro. Various lipid components were utilized to modulate surface charge, which affected cell binding, internalization, and transfection efficiency. A neutral PEG-diacylglycerol analogue was used to enhance the steric stability of the particles without affecting surface charge. We used an anti-HER2 human single-chain monoclonal antibody (scFv)−PEG conjugate to facilitate intracellular delivery of the Genospheres and selectively enhance gene transfection efficiency. Finally, we discuss the potential of Genospheres for various in vivo applications

Results

Construction and Characterization of Genospheres. The mixing of the nucleic acid solution to the lipid solution in 50% (v/v) aqueous ethanol medium at 55−60 °C, and subsequent removal of the ethanol by vacuum evaporation or dialysis at room temperature, effected the self-assembly of Genospheres.[12] The resulting particles had size distributions with a volume-weighted average of 80−150 nm and a standard deviation of 35−65 nm as determined by dynamic light scattering. The extent of DNA and lipid encapsulation in the particles under these conditions is typically over 90%;[11]

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Table 1. ζ-Potentials (ζ) of Genospheres at Different pH[a,e]

sample formulation	composition (nmol/μg of DNA)	Genosphere size (nm)	ζ (mV)	
			pH 5.5	pH 7.0
DOTAP/POPC/Chol	6/15/10 (A)	93.8 ± 41.7	nd[b]	+116.0[c](3)
DOTAP/POPC/Chol/CHEMS/CHIM	6/25/7/6/3.67 (B)	88.9 ± 48.9	+95.1(4)	∼0(1)
	6/18/6/5/1	81.1 ± 43.6	+70.9(3)	−6.1(1)
	5/25/7/6/3.67	88.2 ± 51.1	+70.1(3)	−3.9(3)
	5/15/3/5/2	96.8 ± 39.6	+55.3(5)	−6.0(1)
	5/15/5/5/1	82.7 ± 40.9	+55.2(3)	−1.2(1)
	6/25/6/9/1.67	100.5 ± 39.5	+37.6(3)	−30.8(3)
	5/25/6/8/2.67	89.0 ± 45.7	+24.1[d](3)	−24.9[d](3)
	5/25/6/9/1.67	98.6± 47.0	+24.0(3)	−39.3(3)
	6/25/5/10/1.67	115.7 ± 43.1	+16.7(3)	−32.8(3)
DOTAP/POPC/Chol/DSGG	6/25/16.67/5	78.5 ± 45.6	+82.6[d](2)	+46.3[d](4)
	5/25/16.67/6 (C)	149.2 ± 60.6	+23.6[d](3)	−18.1[d](3)
DOTAP/POPC/Chol/DSGG/CHIM	6/25/14.67/4/2	124.4 ± 63.8	+59.2[d](3)	+25.5[d](4)

[a] Genosphere samples were prepared as described in Materials and Methods. Ionizable headgroups: DOTAP, a quaternary amine; CHEMS, a weak acid, pK_a = 5.1; CHIM, a weak base, pK_a = 6.65; DSGG, a weak acid, pK_a = 5.1. The samples were divided into two groups (approximately 50 μg of DNA per sample) and dialyzed against either 5 mM Na-HEPES, 5% (w/w) sucrose, pH 7.0, or 5 mM Na-MES, 5% (w/w) sucrose, pH 5.5. [b] Not determined. [c] Measured in 5 mM Na-HEPES, pH 7.4 with 5% (w/w) sucrose). [d] Solutions contain 5 mM NaCl in addition to the indicated buffers and sucrose. Electrophoretic mobilities were measured with a Beckman-Coulter Delsa 440-SX, and ζ-potentials were calculated as previously described. Uncertainties of the ζ-potentials are 5% or less, and the number of electrophoretic runs for each data point is given in parentheses. [e] In line 1, the ζ-potential reported for Genospheres prepared with DOTAP/POPC/Chol 6/15/10 (nmol of lipid/μg of DNA) at pH 7.0 is an estimate. Due to an uncertainty in ionic strength, the measured mobility was greater than the predicted O'Brien−White maximum value. The actual ζ-potential is estimated to lie between +58 mV (Smoluchowski value) and +116 mV (O'Brien−White value at the mobility maximum).

therefore the lipid and DNA compositon of the particles closely follows the composition of the initial lipid−DNA mixture. The proportion of DNA accessible to the environment as determined by a DNA-binding dye (PicoGreen) accessibility assay[11] of the Genospheres in the present study was ∼20%. In contrast, lipoplex particles, prepared by complexation of plasmid DNA with premade cationic liposomes, tend to encapsulate DNA rather incompletely, with higher proportions of the DNA "accessible" to the dye. Previously, we found that lipoplexes of identical lipid and nucleic acid compositon had considerably higher dye accessibility for DNA than the particles produced by the Genosphere method.[11]

Engineering the Charge on Genosphere Particles. The surface ionic charge of Genospheres was characterized by electrophoretic mobility and expressed in terms of ζ-potential (ζ). ζ-Potentials were calculated from the mobilities by the O'Brien−White algorithm,[14] using particle sizes determined by dynamic light scattering and ionic strengths determined by conductometry. The O'Brien−White calculations are necessary when the Debye screening length (κ^{-1}) is nonnegligible compared to the particle size, which was the case in our study. In the case of nontitratable cationic lipids, Genosphere constructs were prepared with approximately two ionic equivalents of cationic lipid to one ionic equivalent of DNA. At near-neutral pH (5 mM HEPES, 5% (w/w) sucrose at pH 7.4), these particles had positive ζ (Table 1). However, the positive surface charge is unfavorable for iv targeted gene delivery using lipid-based carriers,[15] while favorable for cytoplasmic delivery of the DNA once in the cell. Therefore, in order to construct Genospheres whose positive surface charge would decrease at neutral pH but increase upon acidification typical for endosomes, pH-titratable lipids (Figure 1) were included in the formulations.

Titratable lipids can be cationic or anionic and their net charge can be altered in a physiologically relevant pH range (4.5−7.5). Cationic titratable lipids increase their positive charge at low pH and diminish it at neutral or slightly basic pH. The lipophilic imidazole derivatives behave as cationic titratable lipids,[16] cholesteryl imidazole (CHIM) being an example. Anionic titratable lipids reduce their negative charge at low pH, and increase it at neutral or slightly basic pH. Examples of anionic titratable lipids are fatty acids, succinyl and glutaric esters of lipids, such as cholesteryl hemisuccinate (CHEMS), dioleoyglyceride hemisuccinate (DOGHEMS), and distearoylglyceride hemiglutarate (DSGG) (see Figure 1A−D). Genospheres with incorporated titratable lipids were prepared, mobilities measured, and ζ-potentials calculated at acidic and neutral pH (Table 1). While the inclusion of titratable lipids did not significantly affect the average particle size (range, 78−146 nm), the particle surface charge changed noticeably between pH 5.5 and pH 7.4. At pH 5.5, typical of endosomes or lysosomes, all the Genosphere constructs had a positive ζ, indicating the presence of

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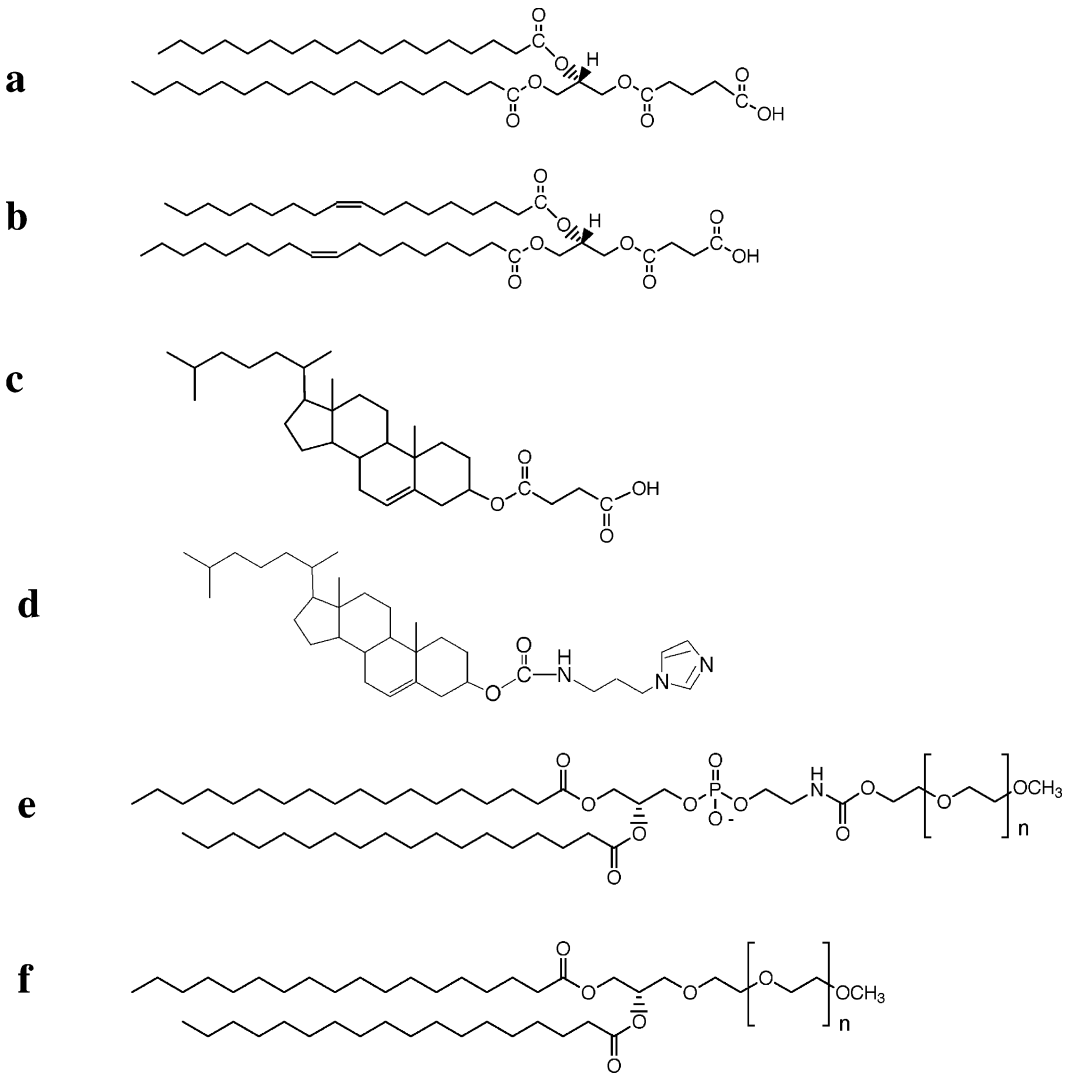

Figure 1. The chemical structures of Genosphere-modifying lipids used in this work: (a) 1,2-distearoyl-*sn*-glycero-3-hemiglutarate (DSGG); (b) 1,2-dioleoyl-*sn*-glycero-3-hemisuccinate (DOGHEMS); (c) cholesteryl hemisuccinate (CHEMS); (d) 1-(3-(cholesteryloxycarbonylamino)propyl)imidazole (CHIM); (e) *N*-[ω-methoxy-(poly(oxyethylene)-α-oxycarbonyl)]-DSPE (PEG_{2000}) (PEG-DSPE); (f) 2,3-distearoylglycerol 1-monomethoxypolyethyleneglycol ether (PEG_{2000}) (PEG-DSG).

un-neutralized cationic lipid charges. Addition of the anionic titratable lipid derivatives caused a reduction in ζ, detectable at low pH and much more prominent at neutral pH. In some instances this caused the charge to change sign, indicating a functioning pH-dependent "switch" of surface charge. Five of the formulations studied had ζ values close to 0 (i.e., deviating less than 10 mV) at neutral pH and were, therefore, designated as "neutral". Formulations containing CHEMS and CHIM showed the largest amplitude of surface charge switch, in the range of 50−96 mV.

The Effect of Charge, Degree of PEGylation, and Anti-HER2-Antibody Targeting on Cell Association and Transfection Activity of Genospheres. On the basis of ζ measurements, we selected for further studies three Genosphere formulations, having cationic, neutral, or anionic surface charge at neutral pH (Table 1, formulations A, B, and C, respectively). To effect target-specific internalization of Genospheres into cells, a highly internalizable anti-HER2 scFv′ fragment (F5)[17,18] was attached to the surface of Genospheres by coincubation with the F5−lipopolymer conjugate (F5-scFv−PEG-DSPE).[17−19] Grafting of PEG on the Genosphere surface at various densities (0.5 mol % and 5 mol % of the total lipid) was achieved by inclusion of a charge-neutral PEG-lipid derivative, PEG-DSG, in the lipid mixture prior to complexation of DNA.

The effect of PEGylation, charge, and anti-HER2-antibody targeting on the degree of Genosphere association with HER2-overexpressing cells was characterized qualitatively

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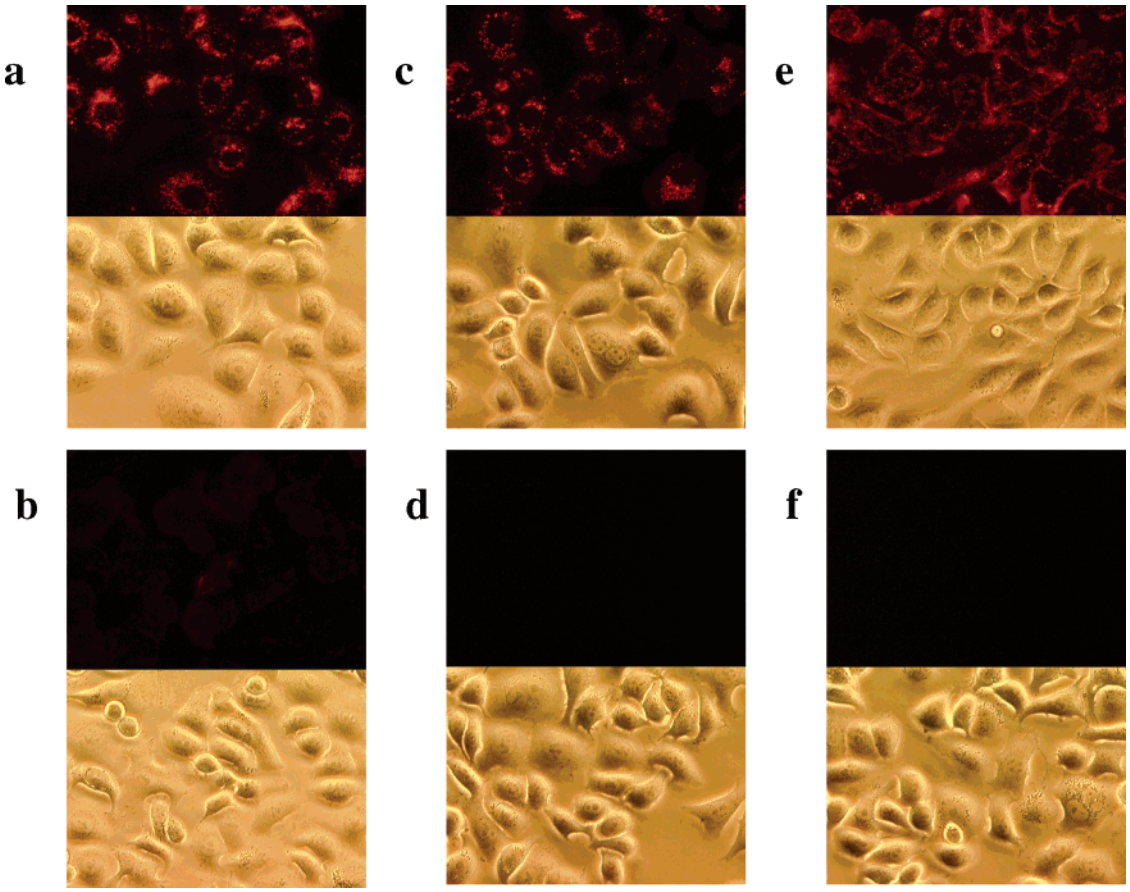

Figure 2. Representative fluorescent microscopy pictures of SKBR-3 cells incubated with Genospheres of various surface charge and PEGylation. Panels: neutral anti-HER2 targeted and nontargeted Genospheres with 0.5% PEG-DSG (panels a and b, respectively); anionic targeted and nontargeted Genospheres 5% PEG-DSG (panels c and d, respectively). The lipoplex formulation with 5% PEG-DSG is shown in panel e, and cells with no treatment are shown in panel f. Fluorescent and phase contrast modes are displayed on the top and bottom respectively for each panel.

by fluorescence microscopy (Figure 2) and quantified by cytofluorometry (Figure 3) using Genospheres and lipoplexes containing a fluorescent lipid label. HER2-directed antibody targeting significantly increased the extent of cell association of the particles in a manner dependent both on particle charge and on degree of PEGylation. Reducing the cationic charge and increasing the PEG-DSG content to 5 mol % significantly increased the specificity of targeting neutral and anionic particles as judged by the magnitude of increase in the average fluorescence intensity. It was apparent that cationic Genospheres and lipoplexes bound to the cells in a relatively nonspecific manner. However, while higher PEGylation attenuated the cellular association of cationic Genospheres, it did not affect lipoplex binding.

In addition to increased association with the receptor-overexpressing cells, targeted preparations rapidly entered the cells forming a characteristic punctate perinuclear pattern, indicative of endosomal/lysosomal localization[20,21] (Figure 2, panels a and c). Nontargeted Genospheres displayed only weak binding and primarily localized at the cell surface (Figure 2, panels b and d), while the lipoplexes appeared to be located both intracellularly and surface bound in a pattern different from that of targeted Genospheres.

The transfection activity of anti-HER2 immunoGenospheres, as well as of nontargeted Genospheres and lipoplexes in HER2-overexpressing SKBR-3 cells, is shown in Figure 4. The activity for targeted Genospheres at low and high PEG-DSG content was comparable to that obtained for the lipoplex formulation with 0.5 mol % PEG-DSG. Lipoplexes with high PEG-DSG content had low transfection activities. Remarkably, the transfection efficiency of the plasmid delivered by the HER2-targeted Genospheres was *less* dependent on the degree of PEGylation. Targeted preparations showed enhanced transfection activities over their nontargeted counterparts, with neutral and anionic Genospheres exhibiting the largest increases in activity (167- and 850-fold, respectively). The lower transfection activities of the nontargeted preparations varied with surface charge and degree of PEGylation. Generally, the cationic particle exhibited higher nonspecific transfection at both PEG-DSG

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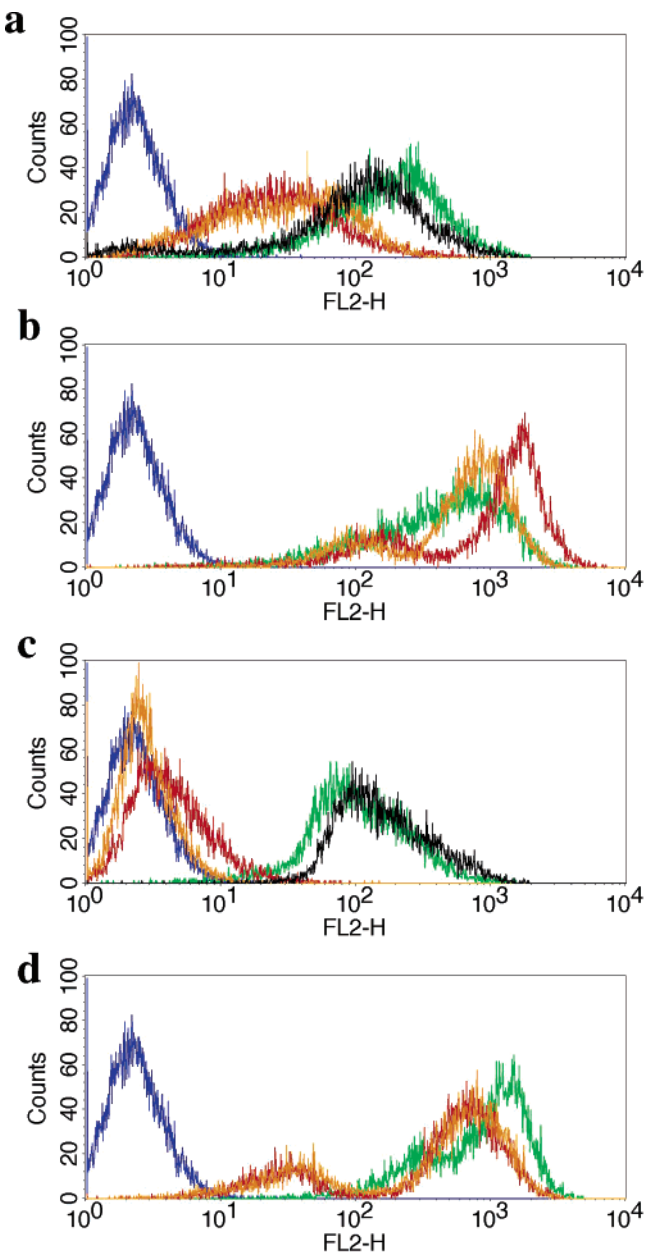

Figure 3. Cytofluorometric analysis of the uptake of fluorescently-labeled Genospheres and lipoplexes by SKBR-3 cells. Signal intensity histograms of cells incubated with fluorescently labeled Genospheres. Nontargeted (panels A, C) or HER2-targeted, scFv-conjugated Genospheres (panels B, D) containing either 0.5 mol % (panels A, B) or 5 mol % (panels C, D) PEG-DSG. Genosphere compositions are in nmol of respective lipids per μg of DNA: cationic (green lines), DOTAP/POPC/DOPE/Chol (6/12/3/10); neutral (red lines), DOTAP/POPC/DOPE/Chol/CHEMS/CHIM (6/20/5/7/6/3.67); anionic (orange lines), DOTAP/POPC/DOPE/Chol/DOGHEMS (5/20/5/16.67/6). DOTAP/DOPE (12/12) lipoplexes with "low-0.5% PEG-DSG" and "high-5% PEG-DSG" (black lines) are displayed alongside the nontargeted particles in panels a and c, respectively. Blue lines are nontreated cells (autofluorescence controls).

concentrations, and is in agreement with the results found previously using folate-targeted liposomal entrapped polylysine-condensed DNA.[22] In contrast, in the absence of a targeting ligand, the levels of transfection activity for both the neutral and anionic particles decreased with additional PEGylation and increasing anionic surface charge.

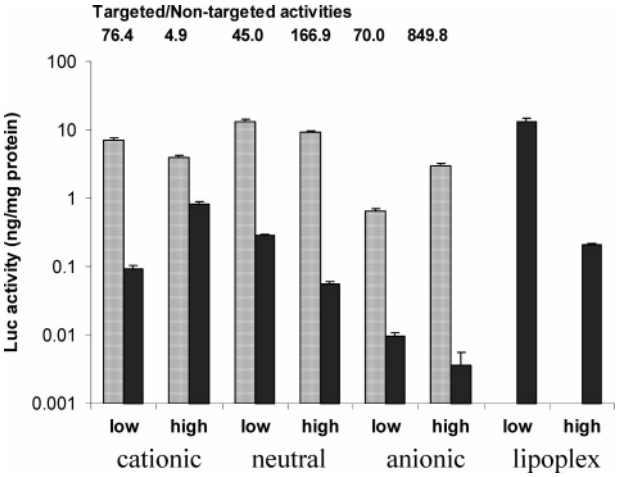

Figure 4. Luciferase expression in SKBR-3 cells following incubation with Genospheres of varying surface charge and degree of PEGylation. The Genosphere compositions are given in the Figure 2 legend. Hatched and filled bars represent targeted and nontargeted particles, respectively (n = 3). Labels "low" and "high" refer to complexes containing 0.5 and 5 mol % PEG-DSG (total lipid), respectively. Numbers above the bars indicate ratios of activities for targeted Genospheres/activities for nontargeted Genospheres.

However, the levels of gene expression of the targeted particles did not always correlate with the levels of cell association. For the targeted cationic and neutral Genospheres with low PEG-DSG content (0.5 mol %), cell association and transfection correlated well, with neutral particles having approximately twice the cell association and transfection levels of the cationic particles. In the case of anionic particles having lower positive charge attainable under endosomal/lysosomal pH levels, gene expression was considerably reduced even in the presence of the targeting ligand, although the target-dependent cell association was of the same magnitude as that of targeted cationic and neutral particles. On the other hand, lipoplexes with low PEGylation had transfection activity equal to that of the targeted neutral particles with 0.5% PEG-DSG, while having 8 times less cell association.

At higher PEG content (5 mol %), the targeted neutral and anionic Genospheres had similar cell association values, but the neutral Genosphere was approximately 3 times more active in the transfection assay. In agreement with our previous findings, higher PEGylated lipid content did not substantially inhibit the activity of the targeted cationic Genospheres, and moreover had very little effect on the activity of the neutrally charged targeted Genospheres. In

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contrast, increased levels of PEG-DSG (5 mol %) rendered lipoplexes substantially less active (65 times lower) than lipoplexes containing 0.5 mol % PEG-DSG, even though the cell association values were similar.

As a control, the transfection activity of the targeted and nontargeted neutral formulation (with 5% PEG-DSG) in a non-HER2 overexpressing cell line (MDAMB468) were similar at 0.03 ± 0.02 and 0.04 ± 0.02 ng luciferase/mg cell protein, respectively. Under identical conditions, a commercial transfection formulation (Lipofectamine 2000) complexed with the same plasmid gave luciferase values approximately 20,000 times higher.

Discussion

Previously, we described a methodology for producing small, stable, target-selective particles (Genospheres) from lipids and nucleic acids micellarly or molecularly dissolved in a mixture of water and infinitely water-miscible organic solvent that can be used for delivery of therapeutic nucleic acids.[11] Initially, Genospheres were constructed with excess cationic lipid. Genospheres formed with 5−6 nmol of a cationic lipid per μg of plasmid DNA, which gives a positive/negative charge ratio of 1.67−2, assuming the average nucleotide weight of 330. This was enough cationic lipid charge to completely neutralize the DNA phosphate charge and ensure stability, as well as highly efficient entrapment of the nucleic acid (plasmid DNA) during formation of the particle. However, the uptake of such particles by tumors may be impaired due the presence of persistent cationic charge that increases the particle clearance and accumulation in vascular sites other than tumors. Reducing the cationic charge on lipid carriers promotes longer circulation times, decreases interactions with opsonins and uptake by macrophages, and reduces nonspecific interactions with cell surfaces.[23,24] To avoid excessive positive surface charge of a DNA−lipid particle while in the bloodstream, and at the same time provide for sufficient cationic charge to effect intracellular transfection, we took a 2-fold approach. First, we partially substituted titratable anionic and/or cationic lipid for a strongly charged cationic lipid species (such as lipids with quaternary-amine headgroups, e.g., DOTAP) in the particle formulation (Table 1). As the entrapment of DNA was carried out at a reduced pH (pH 5.5), the particles containing anionic titratable lipids in an amount enough to bring the particle's surface charge at neutral pH (pH 7.0) to close to neutral, or even anionic, still entrapped the plasmid very effectively. Upon a change of pH from 7.0 to 5.5, the particles containing nontitratable cationic lipid components along with the titratable anionic lipids showed an amplitude of the charge change of about 40 mV. Addition of a titratable cationic lipid (positive-to-neutral), along with the charge-changing anionic lipid (neutral-to-negative), increased the amplitude of the charge change to 50−90 mV (Table 1), producing more effective transfecting particles (Figure 4). The strategy of modulating surface charge in DNA/lipid microparticles, using lipids with titratable amines as the cationic component at low pH, is known; however, the entrapment efficiencies of the nucleic acids in such particles are limited.[6] Supplementing a titratable aminolipid with a strongly cationic lipid component, counterbalanced at neutral pH with a titratable anionic lipid component, seems to improve the entrapment of DNA and particle recharging upon cytophysiologically relevant acidification.

Second, we used a ligand-directed "active targeting" approach to achieve specific uptake of the Genospheres into acidifying intracellular compartments via receptor-mediated endocytosis. While cationic particles have natural and often nonspecific tendency to bind to cell surfaces and become endocytosed,[1] neutral or anionic lipid particles are taken up much less, as we also observed in our cytofluorometric studies (Figure 3a,c). To make Genospheres targetable, they were incubated with the micelles of a lipopolymer (PEG-DSPE) conjugated to a ligand (scFv antibody) to the free terminus of the PEG chain, effecting "insertion" of the DSPE anchor in the outer lipid bilayer of the Genosphere particle and appending the ligand, via PEG spacer, to the particle surface, as we described previously.[12] HER2, a tyrosine kinase receptor of EGFR family, was chosen as a targeting epitope. HER2 is a validated target for anticancer therapeutic strategies, as it is a readily accessible surface antigen, is overexpressed in many cancer cell types such as breast, lung, and ovarian carcinomas,[25,26] and is essential for tumor progression. A single chain Fv antibody ligand, F5, is highly internalizable by HER2-overexpressing cells[27] and effectively internalizes appended lipid nanoparticles, such as liposomes, in vitro[17] and in vivo.[28] Conjugation of F5 scFv with maleimide-terminated PEG-DSPE via C-terminally engineered cysteine residue produced stable, water-soluble, micellar F5−PEG-DSPE conjugate, that, upon incubation with liposomes, self-inserted into the outer monolayer of the liposome membrane, forming anti-HER2 immunoliposomes.[17,18]

This targeting strategy leads to a more than 100-fold increase in luciferase reporter gene expression in HER2-overexpressing cells.[12] In the present report, we observe a

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5- to 850-fold enhancement of luciferase expression with targeted Genospheres compared to nontargeted Genospheres in SKBR-3 cells, while in human breast carcinoma cells with low expression of HER2 (MDA-MB-468), the expression was not increased by attachment of HER2-specific, internalizable scFv ligand. The HER2-directed targeting of Genospheres increased the level of reporter gene expression in absolute terms, and decreased the dependence of transfection efficiency on the particle charge and the density of particle surface coating with sterically stabilizing hydrophilic polymer (PEG) (Figure 4). The charge-changing Genospheres that were neutral at neutral pH and had an increased PEG coating (5 mol %) showed increased selectivity of transfection in the HER2 overexpressing cells compared to our previously described nontitratable cationic Genospheres.

Surface coating with a hydrophilic polymer terminally grafted to lipid residues is a recognized method for improvement of blood stability and longevity of lipidic microcarriers.[29,30] The effect of surface PEGylation on the association and transfection activity of Genospheres was studied using a charge-neutral PEG-lipid derivative, PEG-DSG (Figure 1), to avoid imparting additional negative charge carried by a more conventional PEG-lipid, PEG-DSPE. Reducing the magnitude of cationic surface charge alone diminished nonspecific Genosphere−cell interactions regardless of the PEG-DSG content. We previously observed that addition of PEG-DSPE to nontargeted lipoplexes inhibited transfection in a PEG-DSPE dose-dependent manner.[31] In this study we observed a similar effect, with transfection activity of 5 mol % PEG-DSG lipoplexes having only ∼1.5% of the activity of 0.5 mol % PEG-DSG lipoplexes. Increasing surface density of PEG chains further reduced nonspecific, i.e., targeting-independent, association of Genospheres with the cells. However, addition of a targeting ligand restored the transfection activity, leading to increased specificity of transfection. HER2-targeted Genospheres with 5 mol % PEG-DSG transfected HER2-overexpressing cells in vitro 15−45 times more efficiently than DOTAP/DOPE cationic lipoplexes with similar PEG-DSG content, and the neutrally charged, PEGylated, HER2-targeted Genospheres were the most active (Figure 4). Higher PEGylation of Genospheres (2−5 mol % PEG-DSPE compared to 0.5 mol % PEG-DSPE) also improves the particle size stability in the presence of human plasma.[12]

Anti-HER2 immunoliposomes are rapidly taken up by SKBR-3 cells into acidifying compartments (endosomes and lysosomes), consistent with coated pit receptor-mediated endocytosis, forming distinct, punctate perinuclear patterns revealed by epifluorescent microscopy of the liposomal fluorescent label.[20] In this study we observed a similar intracellular pattern of the fluorescently labeled HER2-targeted Genospheres (Figure 2) after incubation with the same cell line, suggesting that HER2-targeted Genospheres were similarly internalized via HER2-mediated endocytosis.

The steps subsequent to the receptor-mediated endocytosis of the targeted Genospheres and the mechanisms that govern the release of DNA with further entry into cytoplasm and to the nucleus are as yet unclear. An interesting observation is that charge-changing Genospheres which contained an imidazole-lipid derivative CHIM were more active than those that did not (formulations B and C, Table 1; Figure 4). This might be related to additional cationic charge from the protonation of an imidazole ring, by the "proton sponge" effect observed with imidazole-containing transfection reagents,[32] or both, leading to the rupture of endosomes and escape of the particles into cytoplasm. It was hypothesized that the anionic lipids normally found on the cytoplasmic facing side of an endosome interact with the cationic lipid and cause a destabilization of the cationic lipid−DNA complex.[33] The observation of lower transfection activity of "anionic" Genospheres that attain less positive surface potential upon contact with acidified endosomal interior (commonly reported pH for endosomal interior is 5.0[34]) seems to support this hypothesis.

Despite 8-times-lower levels of cellular association for lipoplexes, that is, complexes of plasmid DNA with premade, small cationic lipid vesicles, they showed the same transfection activity as targeted, neutral Genospheres. We believe that the combination of increased amounts of the polymorphic lipid DOPE, and the nonspecific mode of cellular uptake by highly cationic lipoplexes, may contribute to the higher transfection activity. However, the loss of specificity, aggregation stability, and DNA protection against degradation[11] are unacceptable tradeoffs in the choice of lipoplexes vs Genospheres for future in vivo systemic applications. Unlike lipoplexes, Genospheres stably entrapped DNA within the lipid matrix of the particle, shielding the DNA from nuclease degradation even in the presence of human plasma, as measured both by an intercalating dye assay and by a DNase enzymatic degradation assay.[11]

Comparing Genospheres with two other well-known methodologies for formulating DNA−lipid particles, we found that Genospheres exhibited the unexpected combination of small size, high degree of lipid and DNA incorporation, and narrow particle density distribution.[11] This indicated

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good particle homogeneity and leads us to suggest that the Genosphere methodology may serve as a good tool for producing uniform nucleic acid carrying particles that are stable enough to be used in vivo.

Successful in vivo gene delivery may require not just a stable carrier system but also selective targeting. In the present work we have demonstrated that Genospheres can selectively target and transfect HER2-overexpressing cells in vitro and can be tailored by formulating with a judicious choice of surface modifying lipids for reduced nonspecific cell interactions. The nonreactive nature of the carrier and its ease of combination with a targeting ligand make the use of Genospheres an attractive methodology for efficient nucleic acid delivery. Future studies will concentrate on the evaluation of Genospheres as a targetable gene delivery vehicle in vivo.

Materials and Methods

Materials. 1,2-Dioleoyl-3-(trimethylammonio)propane (DOTAP), 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC), 1,2-dioleoyl-3-*sn*-phosphatidylethanolamine (DOPE), and 1,2-dioleoyl-*sn*-glycero-3-hemisuccinate (DOGHEMS) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Dioctadecyldimethylammonium bromide (DDAB), cholesteryl hemisuccinate (CHEMS), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and 2-morpholinoethanesulfonic acid (MES) were obtained from Sigma (St. Louis, MO). 1-Monomethoxypoly(ethylene glycol)-2,3-distearoylglycerol PEG_{2000} (distearoylglycerol-PEG_{2000}, Sunbright DSG-20H, PEG-DSG) was acquired from NOF Corporation (Tokyo). Cholesterol was purchased from Calbiochem (San Diego, CA). Stock solutions of lipids dissolved in ethanol were stored at −40 °C. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid ($DiIC_{18}$(3)-DS) and the DNA intercalating dye Picogreen were purchased from Molecular Probes (Eugene, OR). A pH-titratable lipid 1-(3-(cholesteryloxycarbonylamino)propyl)imidazole (CHIM) was synthesized as described previously.[16] Donor human plasma was obtained from the local blood bank.

Purified firefly luciferase and D-(−)luciferin were obtained from Roche (Indianapolis, IN). Other reagents of the highest possible grade were purchased and used without further purification. Cell culture media were obtained from the UCSF cell culture facility (UCSF, San Francisco), and cells were obtained from ATCC (Rockland, MD). F5−PEG-DSPE conjugate was prepared from the purified anti-HER2 scFv, F5, by conjugation to the maleimide-activated PEG terminus of the PEG-DSPE lipid anchor through an engineered C-terminal cysteine as described previously.[18,19]

A bacterial plasmid containing firefly luciferase gene under the control of an early cytomegalovirus promoter (pCMV/luc$^+$) and a penicillin resistance gene was constructed, amplified, and purified as described previously.[31] The plasmid concentration was determined by absorbance at 260 nm ($\epsilon = 6600$ L mol^{-1} cm^{-1}), and purity was determined from the ratio A_{260nm}/A_{280nm} (Shimadzu, UV160U). DNA was considered to be sufficiently protein-free, with an A_{260nm}/A_{280nm} ratio of at least 1.85.

Synthesis of 1,2-Distearoyl-*sn*-Glycero-3-Hemiglutarate (DSGG). 1,2-*O*-Dioctadecyl-*sn*-glycerol (0.2 g, 0.335 mmol), glutaric anhydride (76.4 mg, 0.76 mmol), and 4-(dimethylamino)pyridine (81.8 mg, 0.76 mmol) were stirred in anhydrous chloroform under argon at room temperature for 12 h. The solvent was removed by rotary evaporation and the product purified on a silica gel column by isocratic elution with hexane/ethyl acetate (2:1). The product-containing fractions were combined, and the solvent was removed by rotary evaporation, yielding 115 mg (0.162 mmol, 48.4%) of product. The purity and identity were confirmed by TLC and ^{1}H NMR. Analytical data: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.866 (t, 3H, CH_2-CH_3), 1. 26 (s, 60H, CH_2), 1.55 (q, 4H, $O-CH_2-CH_2-CH_2$), 1.95 (q, 2H, $CO-CH_2-CH_2-CH_2-CH_2-CO$), 2. 40 (s, 4H, $CO-CH_2-CH_2-CH_2-CO$), 3.47 (q, 4H, $O-CH_2-CH_2$), 3.57 (t, 2H, $CHO-CH_2-O$), 3.64 (q, 1H, $O-CH(CH_2)-O$), 7.39 (s, 1H, COO*H*).

Genosphere Preparation. Genospheres were prepared as described previously.[12] Briefly, the plasmid DNA and the mixture of lipids, as indicated in the text, were separately dissolved in 5 mM aqueous HEPES−Na, pH 7.4, containing 50 vol % ethanol, and combined at 60 °C at a DNA concentration of 0.2−0.5 mg/mL. The mixtures were allowed to attain ambient temperature, and dialyzed overnight against unbuffered saline to remove ethanol. The dialyzed samples containing Genosphere-formulated DNA at about 0.1 mg/mL were used without further purification. If titratable lipids were used in the formulation, 5 mM MES, pH 5.5, was used instead of 5 mM HEPES, pH 7.4, in the aqueous-ethanolic solutions and then dialyzed against 5 mM Mes, 144 mM NaCl in order to increase the positively charged lipid species and reduce anionic charges if present, thus maximizing interactions of the lipids with DNA. HER2-targeted Genospheres were prepared by incubation of Genospheres with F5-cys-mal−PEG(2000)-DSPE antibody conjugate in saline overnight at 37 °C.[12] Typically the amount of conjugate equal to 15 μg of antibody was added per μmol of POPC in the sample. The antibody−PEG-DSPE conjugate is presented in a micellar solution containing a proportion of cysteine-quenched maleimide-PEG-DSPE,[17] and therefore, to account for the effects of PEG introduced in the course of antibody conjugation, nontargeted control samples were incubated with an equivalent amount of PEG-DSPE (i.e., 0.22 mol % of the PC content). Genosphere samples were stored at 4 °C until use.

In Vitro Delivery and Gene Expression. HER2-overexpressing human breast adenocarcinoma cells (SKBR-3 cells) were cultured in McCoy's 5A medium supplemented with 10% (v/v) fetal bovine serum, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin G at 37 °C, 5% CO_2. The cells were plated at a density of 250 000 cells per well in a 12-well plate (Corning) and acclimated overnight. The final medium volume in each well was 1 mL, and each well received 1 μg of pCMV/luc$^+$ in the form of (i) free DNA, (ii) simple plasmid/liposome complexes, or (iii) Genosphere

preparations. Genospheres were sterilized by passing through a 0.2 μm polyethersulfone (PES) filter (Corning) and the DNA concentration was measured as described previously using the Picogreen assay. The control lipoplexes were aseptically prepared by the addition of pCMV/luc$^+$ to a sonicated dispersion of DOTAP/DOPE (1:1) liposomes for a final ratio of 12 nmol of DOTAP/μg of DNA in 5 mM HEPES, pH 7.4. Both plasmid and liposome solutions were filter-sterilized prior to mixing, and the resulting suspension was added to the cells 20 min following complexation.

All plasmid preparations were incubated with the cells for 6 h and removed by aspiration following washing with Hanks buffered saline (2 × 1 mL), and the incubation continued for another 18 h following the addition of fresh media (1 mL). The cells were washed with PBS (2 × 1 mL), detached from the plate by incubation with 1 mL of PBS (containing 3 mM EDTA) at 37 °C for 10 min, pelleted by centrifugation at 3500*g* for 3 min, and resuspended in cold PBS. The cells were lysed by two cycles of freezing (−80 °C) and thawing (37 °C). The lysates were centrifuged (7200*g*, 30−40 s) for removal of cellular debris and assayed for luciferase activity as described previously.[12] Luciferase activity was normalized to the protein concentration in the lysates, determined using the Micro-BCA assay (Pierce, Rockford, IL). A HER2-negative human breast adenocarcinoma cell line MDA-MB468 was transfected with the F5-targeted and nontargeted variants of the neutral Genosphere formulation in the same fashion.

Genosphere Size Measurements. Particle size was measured by dynamic light scattering (DLS) using a Nicomp C370 particle size analyzer (Nicomp Particle Sizing Systems, Santa Barbara, CA). For measurements, the samples were diluted in 0.2 μm filtered HBS, pH 6.5, chosen for its biocompatibility, isotonicity, and pH reported to be optimal for preventing phospholipid hydrolysis.[35] The particle-size distribution (mean and standard deviation) were calculated using the volume weighted Gaussian method and either the solid-particle mode (Genospheres) or vesicle mode (liposomes).

ζ-Potential Measurements. Electrophoretic mobility measurements were made using a Coulter Delsa 440-SX (Beckman Coulter, Fullerton, CA). Each value quoted is the average of a number of runs and is indicated in the table. Measurements were made in either 5 mM Na-HEPES, 5% (w/w) sucrose, pH 7.0, or 5 mM Na-MES, 5% (w/w) sucrose, pH 5.5. All buffers were degassed prior to use. The ζ-potentials were calculated from the measured electrophoretic mobilities using the O'Brien−White algorithm[14] as described in the Results section. Electrical conductivities were measured with the Delsa 440-SX and used to calculate the ionic strengths, required for determination of the Debye−Hückel constants (κ) of the electrolytes. The particle sizes of Genospheres measured at pH 5.5 or pH 7.0 were identical regardless of the presence of titratable lipids (data not shown). The particle radii (a) were then used to determine the parameters κa required in the O'Brien−White calculations.

Cell Association and Transfection Activity of Genospheres of Varying Surface Charge. For transfection studies, formulations of Genospheres having at neutral pH cationic (A), neutral (B),or anionic (C) charges were prepared as described above. The lipid compositions A, B, and C of Table 1 were used, where, instead of a saturated fatty acid derivative DSGG, we used an unsaturated, anionically titratable lipid DOGHEMS, so that the acyl chain composition was consistent with the most abundant dialkyl-lipid in the formulation, unsaturated POPC. Also, up to 20 mol % of the POPC content of the Genospheres in these formulations was replaced with the zwitterionic fusogenic lipid DOPE, which is known to improve transfection of lipoplexes in vitro.[36] Each formulation was prepared with a low and a high PEG-DSG content (0.5 and 5 mol % of total lipid, respectively). The fluorescent membrane probe $DiIC_{18}$(3)-DS was added to all samples (0.2 mol % total lipid) to facilitate quantification of cell association by FACS and visualization by fluorescent microscopy. The DOTAP/DOPE/DNA lipoplex samples were prepared as described above, adding either 0.5 or 5 mol % PEG-DSG. Genospheres were sterilized by passing through a 0.2 μm polyethersulfone filter (Nalgene), and lipoplexes were prepared aseptically as described previously.

SKBR-3 cells were plated in a 12-well plate (Corning) as described above, and the following day the cells were washed with Hanks BSS (2 × 1 mL). Individual samples (1 μg of DNA) were added to each of 6 wells in McCoy's 5A medium supplemented with 10% (v/v) FBS and 1% (v/v) Pen-Strep in a final volume of 1 mL. After 6 h, the wells were washed with Hanks BSS (3 × 1 mL), and 1 mL of medium was replaced in 3 of the wells and incubated further at 37 °C. The cells in these wells were analyzed for luciferase activity and protein content 24 h post-transfection using the freeze−thaw lysis method outlined above. The remaining cells were photographed through a fluorescence microscope (Eclipse TE 3000, Nikon, Japan) using a constant exposure time for all phase contrast (1/30 s) and for all fluorescence (1/15 s) photographs. The cells were harvested by incubation with 3 mM EDTA in PBS fixed with 2% paraformaldehyde in PBS for 15 min**,** and collected by centrifugation at 3500*g* for 5 min. The cells were resuspended in 1 mL of cold PBS and analyzed by cytofluorometry (FACSCalibur 2, Becton Dickinson) using the FL2 channel with (λ_{Ex} 488 nm and λ_{Em} 585 nm). The values reported are the mean and standard deviation of three experiments with 1 × 10^4 events counted per run.

Abbreviations Used

CHEMS, cholesteryl hemisuccinate; CHIM, 1-(3-(cholesteryloxycarbonylamino)propyl)imidazole; Chol, cholesterol;

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DDAB, dioctadecyldimethylammonium bromide; $DiIC_{18}(3)$-DS, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid; DOGHEMS, 1, 2-dioleoyl-*sn*-glycero-3-hemisuccinate; DOPE, 1,2-dioleoyl-3-*sn*-phosphatidylethanolamine; DOTAP, 1,2-dioleoyl-3-(trimethylammonio)propane; DSGG, 1,2-distearoyl-*sn*-glycero-3-hemiglutarate; DSPE, 1,2-disteroyl-3-*sn*-phosphatidylethanolamine; HBS, HEPES buffered saline (5 mM HEPES, 144 mM NaCl, pH 6.5); HEPES, 2-(4-(2-hydroxyethyl)piperazino)ethanesulfonic acid; MES, 2(*N*-morpholino)ethanesulfonic acid; PBS, phosphate buffered saline; PEG-DSG, 2,3-distearoylglycerol 1-monomethoxypolyethyleneglycol ether (PEG_{2000}); PEG-DSPE, *N*-[ω-methoxy-(poly(oxyethylene)-α-oxycarbonyl)]-DSPE (PEG_{2000}); POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine.

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