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Bile Acid-Oligopeptide Conjugates Interact with DNA and Facilitate Transfection

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Abstract

Bile acids conjugated to oligoarginine-containing peptides (BACs) form complexes with DNA based on the electrostatic interactions between negatively charged phosphate groups of the nucleic acid and the positively charged side chain guanidinium groups of the oligoarginine in the BACs. Charge neutralization of both components and subsequent increases of the net positive charge of the complex combined with the water-soluble lipophilic nature of the bile acid results in changes in the physicochemistry and biological properties of the complexes. We have examined the relationship of a series of 13 BACs on their interaction with circular plasmid DNA (pDNA). The formation of soluble, low-density and insoluble, high-density complexes was analyzed using several methods. The formation of high-density complexes was dependent on the DNA concentration, and was enhanced by increasing the BAC to pDNA charge ratio. Several of the BAC:pDNA complexes demonstrated exclusion of the DNA-intercalator Hoechst 33258 from pDNA, and were also protected from DNase activity. Several BAC conjugates interacted with pDNA to form nanometer-sized particles suitable for cell transfection in vitro. Five of the 13 BACs were transfection competent as single agents, and 11 of the 13 BACs showed enhancement of transfection in combination with DOPE containing liposomes or silica nanoparticles.

Keywords

Bile acid; cholesterol; gene therapy; polyarginine; plasmid; nanoparticles; silica articles

Introduction

Naked plasmid DNA has been shown to transfect cells both in vitro and in vivo; however, the transfection efficiency is generally low. In an effort to increase transfection capability, one area of research has focused on the use of chemical DNA condensing agents. A number of cationic chemical condensing agents have been used to augment transfection in vitro and in vivo, including cationic polyamino acids and cationic lipids.[1–4] The multimolecular complexes formed between these agents and DNA show a range of sizes and physicochemical properties in solution.[5] Polyamino acid polyplexes are usually unstable in isotonic electrolyte solutions, tending to undergo aggregation or flocculation with time. Positively charged polyplexes can enhance DNA uptake into cells, but often require the use of an endosomolytic

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agent, such as chloroquine, to facilitate endosomal escape and successful cellular transfection.[6] One of the main advantages offered by cationic condensing agents is the protection provided to plasmid DNA from DNases in the tissue or serum. For example, Godbey et al. have shown that poly-(ethylenimine) (PEI) DNA polyplexes were not degraded when exposed to either DNase I or II over the course of 24 h.[7]

Lipopolyamines (cationic lipids) have also been used to facilitate transfection and show significantly lower toxicity than many viral vectors, although they are less efficient transfection vectors than viruses.[8] These are generally composed of a hydrophobic lipid anchor, a linker or spacer, and a cationic headgroup with chemical proton accepting groups covalently linked to the lipid moiety. Cationic lipids when combined with other lipids, and with the addition of DNA, can form a variety of liposomal or micellar structures that are capable of enhancing cellular transfection. Several lipopolyamines based on the cholesterol structure have been used successfully as a scaffold for developing transfection reagents (e.g., 3-*β*-[*N*-(*N*′, *N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol)[9] and 3-*β*-(N4-spermine-carbamoyl) cholesterol).[10]

Bile salts are naturally synthesized cholesterol derivatives that possess surfactant properties that facilitate the formation of micellar and liposomal structures. In addition bile salts are chemically stable but readily derivatized at the 3, 7, or 12 positions of the bile acid steroid backbone or the 24-carboxyl group of the bile acid. We hypothesized that bile salts can be derivatized using cationic peptides and that the novel bile acid small peptide conjugates would function as transfection enhancing reagents.

Experimental Section

Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, Lipofectamine, and DNase free water were purchased from Invitrogen/Gibco (Carlsbad, CA). Chenodeoxycholic acid and other reagents for the BAC synthesis were from Sigma (St. Louis, MO) or Bachem (Torrance, CA). The pCF1 expression plasmid containing firefly luciferase cDNA driven by a CMV promoter (pCF1-Luc) was manufactured and quality controlled by the University of Michigan Vector Core (Ann Arbor, MI). Bisbenzimide (Hoechst 33258) was purchased from Sigma, and LE agarose gel and Bright-Glo luciferase lysis buffer and assay kit were from Promega (Madison, WI). DNase (Benzonase) was from Novagen (Novagen, USA). Silica nanoparticles (IPAST) were provided by Nissan Chemical Corporation America.

Synthesis of BACs

We synthesized the BAC analogues listed in Table 1 using solid-phase chemistry (see Figure 1 for structure). The peptides are first synthesized on the resin bed using modified solid-phase peptide chemistry.[11,12] Briefly, the resin and all amino acids used are protected (usually Pbf groups (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)). To attach the bile acid salt an excess of the desired bile acid was added to the resin after peptide synthesis and allowed to react with the immobilized peptide. After conjugation, the BAC was deprotected and cleaved from the resin (~6 h, reagent B, trifluoroacetic acid:phenol:water:acidic acid (88:5:5:2)) and purified to greater than 95% purity by HPLC. The final materials underwent analysis by mass spectrometry to ensure that the proper synthesis was completed.

Determination of Critical Micellar Concentration

The critical micellar concentration (CMC) of the BACs was determined using a dye solubilization method, which monitored the partitioning of dye into the micelle as a function of the BAC concentration.[13] Briefly, serial dilutions of a 7 mg/mL solution of the BACs were

made in 50 mM Tris buffer (pH 8.0). Ten microliters of the dye 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma) (0.4 mM in methanol) was added to each milliliter. Samples were incubated for at least 12 h in the dark, after which the absorbance of the samples at 356 nm was recorded. Linear regression of the data points above baseline was performed and the calculated CMC derived from the *x* intercept (concentration).

Preparation of BAC/pDNA Complexes

The general scheme for formation of BAC/DNA complexes was as follows. All solutions were maintained at room temperature. Equal volumes of the BAC solution at a known concentration (0 to 5 mg/mL) in water were triturated to a pDNA solution (50 mM Tris buffer pH 8.0) (<1 mg/mL) and then briefly vortexed. The concentrations of the two constituents were varied to adjust the mole-to-mole ratio between BAC analogue and DNA molecule. The use of higher concentrations of either the BAC or pDNA led to flocculation and aggregation of the complexes (data not shown).

DNase Protection Assays

To examine the ability of BAC pDNA complexes to protect DNA from endonuclease activity, BACs were serially diluted from a 5 mg/mL stock by 1:1 dilution with 25 μL aliquots of buffer A (50 mM Tris pH 8.0, 1 mM $MgCl_2$). This was mixed with 25 μL of pCF1-Luc pDNA (80 ug/mL in buffer A) in the well of a 96-well plate. For DNase protection assays, 50 μL of DNase solution (1μL of Benzonase (Novagen, USA) in 15 mL of buffer A) was added to the BAC:pDNA complex and incubated at room temperature for 2 min. The DNA digestion was stopped by the addition of 5 μL of 500 mM EDTA (pH 8.0). Ten microliters of 10× loading buffer (20% Ficoll, 0.25% bromophenol blue) was added to the reaction mixture, and 10 μL was loaded per lane (0.2 μg DNA/lane) on a 0.9% agarose gel. Gels containing ethidium bromide (EB) were subjected to electrophoresis (30 min at 300 V), photographed, and interpreted by visual observation of EB staining. The values indicated are the BAC:pDNA molar ratios. DNase protection was defined as the molar ratio of BAC that prevents the DNA laddering due to endonuclease digestion.

Hoechst Dye Exclusion Assay

The dye exclusion assay was modified from the methods used by Labarca[14] and Held.[15] Hoechst dye stock (1 mg/mL in distilled water) was prepared in advance and stored at 4 °C in a light tight container. Fresh working assay solutions were prepared from stock prior to the start of each assay. A final concentration of 1 μg/mL of Hoechst 33258 in 10 mM Tris buffer pH 7.4 was used in the assay (Hoechst/buffer). pDNA was prestained overnight at 4 °C at a concentration of 5 μg/mL in the Hoechst/buffer. Fifty microliters of the prestained pDNA was mixed with 50 μL of the individual BAC analogues over a range of BAC concentrations (0.24–125 μg) and incubated at room temperature for 15 min. Ten microliters of 10× loading buffer (20% Ficoll, 0.25% bromophenol blue) was added to the reaction mixture, and 10 μL was loaded per lane (0.125 μg DNA/lane) on a 0.9% agarose gel without ethidium bromide. The gels were electrophoresed at 300 V for 30 min and photographed. Fluorescence quenching was defined as the BAC:pDNA molar ratio that completely prevented pDNA fluorescence in the gel. Quenching was determined by visual inspection of the photograph, and the results were reported as the BAC's effect on fluorescence and migration rate.

Particle Size Analysis

The size distribution of the BAC: pDNA complexes was determined using a NICOMP 380/DLS instrument following the manufacturer's recommended procedures. The final pDNA concentration was 3.75 μg/mL in 50 mM Tris (pH 8). A range of BAC concentrations were prepared by serial dilution and tested at varying molar ratios for the BAC:pDNA complexes

of 5339 to 170 837. All particle size measurements were performed after 15 min after mixing. Results are presented as the NiComp number distribution (>90% of the particles) because the formation of larger aggregates did not allow a Gaussian distribution analysis to be utilized.

Transmission Electron Microscopy

BAC(A6):pDNA complexes prepared at a 26 000 molar ratio were visualized by transmission electron microscopy (TEM) using 300-mesh carbon coated Formvar-film copper (Cu) grids, at 60 kV and magnifications ranging from 40 000- to 300 000-fold. Complexes were prepared at a 0.01 μg/μL pDNA concentration. Ten microliters of complexes were loaded on the Cu grid for 2 min followed by blotting of the excess liquid and incubation for another 2 min with 1% uranyl acetate followed by blotting. The grid was air-dried for another 2 min and visualized under the electron microscope.

Turbidity Assay

Serial dilutions of pDNA (from 30 to 0.234 μg/mL in 100 mM Tris pH 8) and BAC (3.75 to 0.117 mM in H_2O) were prepared separately. Equal volumes of the pDNA and BAC preparations were mixed in clear bottom 96-well plates and incubated for 40 min at room temperature. The absorbance of the solution at 630 nm was measured using a Biotek EL311 plate reader. The absorbance was plotted versus the pDNA concentration for a given molar ratio of BAC:pDNA for each BAC compound, and the rise in absorbance at 630 nm was attributed to the increasing turbidity.

Cell Culture

DAOY cells, a human medulloblastoma cell line, and NIH 3T3 cells, a mouse fibroblast cell line, were maintained at 37 °C, 5% CO_2, and 100% humidity in Dulbecco's modified Eagle medium supplemented with 1% antibiotics of penicillin–streptomycin–amphotericin and 10% fetal bovine serum. Cells were seeded onto 96-well tissue culture plates at ~15 000 cells/well 16 h before transfection.

In Vitro Transfection Assays

Transfection efficiency for the BAC/pDNA conjugates was determined by measuring the expression of firefly luciferase after transfection with pCF1-luciferase plasmid. To test the effect of changes in the BAC ratio at different concentrations of plasmid on transfection, 25 μL of BACs was mixed with 25 μL of diluted pDNA followed by a 30 min incubation. To each 50 μL mixture, 150 μL of serum free (SF) DMEM was added, and 50 μL (triplicate wells) containing 0.5, 1, 2, and 3 μg of luciferase plasmid per well was added to DAOY cells, confluent (1:10 split previous day, ~10^5 cells/well). The cells were transfected for 3 h, then the mixture was aspirated and 200 μL of DMEM/10% FBS was added, and the mixture was incubated for 48 h. The cultures were assayed using the Bright-Glo lysis buffer and Glo-Luciferase assay kit (Promega). Average light counts per second (LCPS) were plotted. When testing for stimulation of transfection by liposomes or silica nanoparticles, aliquots of Lipofectamine or IPAST silica nanoparticles were added to the transfection mixture after the BAC/pDNA 30 min incubation, and incubated a further 15 min.

Mixtures of the BAC and pCF1 and aliquots (100 μL) containing 0.5 μg of pDNA in serum free DMEM were added to wells of a 96-well plate containing DAOY cells. After 3 h the medium was aspirated and replaced with 200 μL of DMEM with 10% serum. Transfection efficiency was measured 48 h following transfection using Promega's luciferase assay kit. Luminescence was measured using a Perkin-Elmer 1450 Microbeta Trilux plate reader in the luminometer mode. Invitrogen's Lipofectamine and Plus reagents were used as a positive controls.

Results

Synthesis of BAC Conjugates

We synthesized a series of BAC analogues using the variations shown in Table 1. In motif A, the size and charge were varied by increasing the arginine (R) chain length; in motifs B–D, the DNA binding moiety was altered by the addition of L-alanine (A) as a neutral spacer;[16] and in motif E, specific DNA binding peptides (RAWA) that form an α-helical structure were added.[17] The use of bile acids as the scaffolding for anchoring the DNA binding peptide has the advantage of allowing a variety of potential structural modifications to regulate the hydrophilic/hydrophobic properties of the BAC.

TEM of BAC/pDNA Complex

The BAC analogues form distinct particles when mixed with pDNA. As revealed with TEM, rod shaped or hollow cylinder particles were formed, indicative of DNA condensation. Aggregation of multiple structures was also apparent (Figure 2A). The exact physical structure of the BAC:pDNA complexes remains unknown, but we hypothesize that the peptide condenses the DNA with the bile acid projecting toward the solution interface. For several BAC compounds the formation of micellar structures was possible as the aggregation of the BAC:pDNA complexes was increased by association of the individual complexes (data not shown). The effect of the pDNA binding multiple numbers of BACs within a constrained space may also increase the interactions between individual bile acid moieties within the complexes.

Determination of the Critical Micelle Concentration for the BACs

Since bile acids naturally form micelles in solution, we determined the critical micelle concentration (CMC) of the BACs. As noted above, the formation of micelles has the potential to enhance the formation of micron scale BAC:pDNA particles and supramolecular aggregate mixtures, but might also facilitate transfection in the context of specific BAC formulations. The calculated CMC values for the BACs ranged from 0.8 to greater than 9 mM and are listed in Table 1. A possible limitation of this assay is the high concentration of BAC:pDNA complexes required to perform the assay.

Measurement of Particle Size and Onset of Turbidity

For particle size measurements the BAC:pDNA complexes were formed in a low salt (50 mM Tris pH 8) buffer at six different mole:mole ratios. The charge ratio for the BACs increased as the arginine content increased, and because of this, we expected that the condensation of pDNA and the formation of particles would occur at different molar concentrations for each specific BAC. Therefore we chose to express the data as molar ratios, rather than the more commonly used charge ratios, since it was unclear whether the bile salt component of the BACs could inhibit pDNA binding to the arginine groups via steric hindrance. Additionally, it was not clear if all of the guanidinium groups were available for DNA binding when incorporated into the BAC as polyarginine peptide since we observed that BACs with 3 or fewer arginines did not form particles. Eight of the 13 BACs formed measurable particles using a pDNA concentration of 3.75 μg/mL. Table 1 lists the average particle size formed and the minimum mole:mole ratio at which the particles were formed.

Since particle formation was measured at low pDNA concentrations with the NICOMP analysis, we also measured the development of turbidity in a separate assay to evaluate the effect of pDNA concentration on particle formation. Figure 3 shows representative results for an experiment with the “D” series of BACs. These results show that both the concentration of pDNA and the molar ratio of BAC affected the onset of particle formation and aggregation, and that the degree of variability was dependent on the peptide composition of an individual

BAC. Generally, the concentration required to produce turbidity decreased with increasing arginine content of the BAC. Table 1 shows the molar ratio required to induce turbidity as an indicator of particle formation and aggregation for all the BACs. While the results observed in the turbidity assay generally agree with the results of the particle sizing measurements, two unpredicted observations were made. First, A3 and A4, which did not show measurable particle formation at a 3.75 μg/mL pDNA concentration, both formed supramolecular aggregates at higher concentrations of pDNA. Second, B11, which has measurable particle formation at high molar ratios of the BAC, does not show increases in turbidity with increasing pDNA concentrations. The reasons for these observations are not presently known but may indicate intermolecular reorganization after initial particle formation. The stoichiometry and kinetic characterization of these phenomena remain under study.

Fluorescence Dye Exclusion and Protection from DNase Activity

We examined the effect of BACs on dye exclusion in intact pDNA using Hoechst 33258 prestained pDNA. Table 2 summarizes the results of the interaction of the BAC with Hoechst stained pDNA showing a rank ordering of weakest to strongest interactions. Interestingly BAC analogues provided a wide spectrum interactions from none detected (A3 and D3) to complete quenching of fluorescence (D11) at 2:1 w/w ratio for BAC:pDNA.

To determine the ability of BAC conjugates to protect DNA against DNase activity, we examined the migration shift of DNA fragments after incubation with DNase using agarose gel electrophoresis and ethidium bromide staining. Figure 4 shows an example of the agarose gel used in the DNase protection experiments using the BAC D9.

Figure 5 is a summary figure showing the BAC:pDNA molar ratios required to protect the DNA from degradation or to exclude the Hoechst dye from intercalating into the pDNA. Prevention of degradation was defined as the concentration that prevents the DNA laddering and was reported as the ratio that starts to shift the pDNA migration pattern to lower molecular weights. D11, A6, E9, and C8 all protected the plasmid within a 2-fold molar concentration range in two experiments; three of these, D11, A6, and C8, contain 6 arginines (50–100% amino acid content). Interestingly, the analogue E9 (3 arginines, 9 amino acids) protected pDNA from DNase better than B7 (6 arginines, 7 amino acids) by 2-fold, based upon molar ratios, but showed less effective Hoechst dye exclusion. B11 with 9 arginines ranked much lower at protection than the four best protecting BACs that contained half the total number of amino acids. From the protection results, the composition and order of amino acids has a greater effect than what would be predicted on the basis of total charge and chain length.

Measurement of In Vitro Gene Transfection

We determined the effect of pDNA concentration on the ability of BAC:pDNA complexes to mediate transfection. Figure 6 shows that with a decreasing amount of pDNA an increasing amount of BAC (A6) was required to form complexes capable of optimal transfection. As the plasmid concentration decreased from 2 to 0.5 μg/well, the molar ratio of BAC required for maximum transfection efficiency was increased. The BAC ratio for maximum transfection efficiency did not decrease when the plasmid concentration increased from 2 to 3 μg. This showed that BAC:pDNA ratios vary with plasmid concentration and that increases in luciferase expression were not linear with increasing concentrations of plasmid DNA. We hypothesize that a threshold concentration of BAC is required for transfection competence rather than just a particular molar ratio. At low concentrations of pDNA, increasing the plasmid concentration 2-fold decreased the molar ratio of BAC required 2-fold (i.e., a constant amount of BAC or threshold amount was needed). This lends support for our theory that the formation of micellar or other forms of complex structures are required for transfection.

To determine the transfection activity for the BAC conjugates we tested them as single agents or in combination with liposomes added after BAC:pDNA complexation. The addition of dioleoylphosphatidylethanolamine (DOPE), which has been shown to increase fusion ability of a lipid membrane of lipoplexes, greatly enhanced the transfection efficiency of the BAC:pDNA complexes (Figure 7).[18,19]

Several of the BACs, most notably B7, C8, C11, and D11, were capable of mediating significant transfection activity as single agents, i.e., transfection competent. However, the peak efficiency of these BACs was approximately half that of the nonoptimized liposomal control (water, Figure 7, BAC + Lipofectamine panel). The addition of liposomes to preformed BAC:pDNA complexes enhanced the transfection efficiency of competent BACs. Interestingly, transfection incompetent BAC:pDNA complexes were rendered functional by the addition of liposomes to the preformed complexes. BACs A3, A4, and especially D9 each showed a dramatic increase in transfection efficiency with the addition of liposomes, compared to the liposome and liposome with Plus reagent controls.

Since several of these BACs were capable of forming micelles, a property of detergents in solution, this may be partially responsible for the formation of transfection competent nanoparticles without the addition of exogenous lipids. However, the CMC for compounds such as E13 (lowest measured CMC, 0.8 mM) showed no potential for particle formation or cell transfection. This supports the importance of the BAC peptide component in the formation of transfection competent particles.

To further clarify that BAC molecules directly participated in the enhancement of liposome transfection, we tested the ability of silica nanoparticles to enhance BAC transfection. Silica nanoparticles in the presences of condensing agents are thought to enhance transfection by physical concentration of DNA at the cell surface.[20,21] Additionally, stereotaxic injections of cationic-modified silica nanoparticles, complexed with plasmid DNA encoding for EGFP, into the mouse ventral midbrain and into lateral ventricle, allowed fluorescent visualization of extensive transfection of neuronal-like cells in substantia nigra and areas surrounding the lateral ventricle of the brain.[22] No toxicity was observed 4 weeks after transfection, and the efficiency of transfection equaled or exceeded that obtained in studies using a viral vector.

Figure 8 shows the results comparing silica nanoparticle to liposome enhancement of BAC (A6) transfection. These results support the idea that both liposomes and silica nanoparticles increase the concentration of the pDNA at the cell surface, and that BACs are transfection competent in the absence of exogenous lipids when exposed to the cell surface at sufficient concentrations.

Discussion

In this report we describe the synthesis and functional characteristics of a series of new cationic bile acid derivatives termed BACs. The data shows that BACs are able to mediate and modulate transfection of cells using expression plasmid DNA and enlarges the panel of amphiphilic compounds available for this application. We have combined short peptides (3–13 amino acids) composed of polyarginine and varying numbers of spacer amino acids coupled with a bile acid (incorporating the steroidal backbone found in many cholesterol containing transfection reagents) to increase the hydrophilicity and water solubility of the lipopolyamines. Bile salts are steroids with detergent properties that can form micellar structures when concentrations exceed a critical concentration, a property that is retained for several of the BACs. The conjugation of the DNA binding peptide can occur at the 3, 7, or 12 positions of the bile acid steroid backbone or for this series of BACs on the 24-carboxyl group of the bile acid. The short polycation peptides provide multiple functions: (a) affinity for nucleic acid; (b) act as a DNA

condensing agent; (c) protect the nucleic acid from nuclease activity; and (d) assist in the cellular internalization of the complex. Uptake of condensed DNA cationic polyplexes by target cells is generally thought to proceed via endocytosis, or possibly micropinocytosis.[23, 24] However, polymers containing arginine have been reported to increase the permeability of proteins and DNA to the cell interior by a nonendocytic mechanism.[24–27]

Cationic polymers such as polylysine and other protonable amino acid peptides have long been used as to produce plasmid DNA cationic polyplexes that are capable of transfecting cells. However, the length of the amino acid polymer chain requires a minimum length, and longer chains, although exhibiting enhanced transfection efficiencies, also exhibit higher toxicity, reducing cell viability in vitro and in vivo.[28,29] BACs, in contrast, showed no toxicity at concentrations lower than 100 μM (data not shown).

Other groups have investigated similar transfection systems composed of either the polyamine attached to a steroid nucleus,[30] or bile salts attached to chemical groups such as the guanidinium to provide the cationic charge.[31] Futaki et al. found that oligoarginines of 4–16 residues were found to be able to transfect cells, and N-terminal stearylation of the peptides increases the transfection efficiency by approximately 100 times to reach the same order of magnitude as that of Lipofectamine.

Unlike these previous reports we have used the COOH group of the bile acid scaffold for synthetic derivatives. Others have reported synthesized cationic structures utilizing bile acids coupling the cationic structure to either the hydroxyl groups of the steroidal backbone at the 3-OH group (spermine analogues),[32] or the carboxyl group using spermine, pentamine, or hexamine,[33] or diaminobutane and spermidine.[32] These authors found that the attached polyamine chains provided increased transfection efficiencies compared to commercially available reagents.

Yingyongnarongkul et al.[31] formed three libraries each that included four molecules with steroidal backbones with one or two guanidinium polar head groups attached at the 24-carboxyl position. The steroids bearing hydroxy groups in library 2, with two attached guanidinium groups, bound DNA more efficiently than the steroid bearing carbonyl groups that exhibited transfection activity at higher charge ratios (>20). These compound libraries did not show significant transfection activity when dioleoyl-l-α-phosphatidylethanolamine (DOPE) was omitted.[31] Only one of the steroidal compounds, the deoxycholate with two attached guanidinium groups, showed exclusion of ethidium bromide indicating condensation of the DNA, but this compound showed no transfection activity when formulated in DOPE containing liposomes. Our present work demonstrates that a varying range of molar ratios are capable of forming effective transfection complexes with BACs either as single agents, or when the complexes are added to a liposomal mixture containing DOPE or with uncharged silica nanoparticles.

Other groups have found that gene transfection was influenced by the polyamine chain length and steroid structure;[32] however, these compounds again were always formulated into DOPE containing liposomes so their effectiveness as single agent transfectants is not known. Our results show that molar ratios for binding of the BAC to the plasmid are important determinants of transfection competence especially in the absence of added cationic or fusogenic lipids.

In conclusion, BACs allow for the design and preparation of more efficient lipopolyamine-based vectors and molecular probes for DNA binding. Because the transfection efficiencies for several BACs were greatly enhanced with the addition of silica nanoparticles or preformed liposomes, these very water-soluble BACs offer the possibility of creating sophisticated modular gene-delivery systems capable of self-assembly via hydrophobic interactions between their components. The roles of the different functional moieties are to overcome the distinct

extracellular and then intracellular barriers to efficient in vivo gene transfection of specific target somatic tissues.

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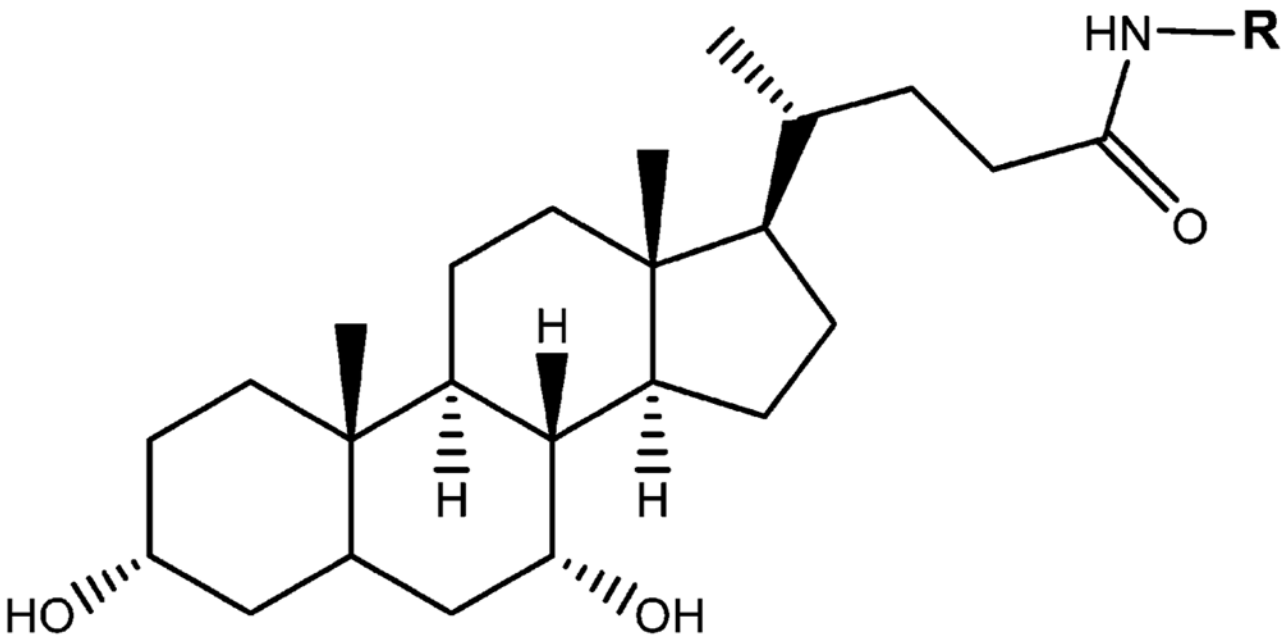

Figure 1.
Structure of BAC analogues with DNA-binding peptides (–R).

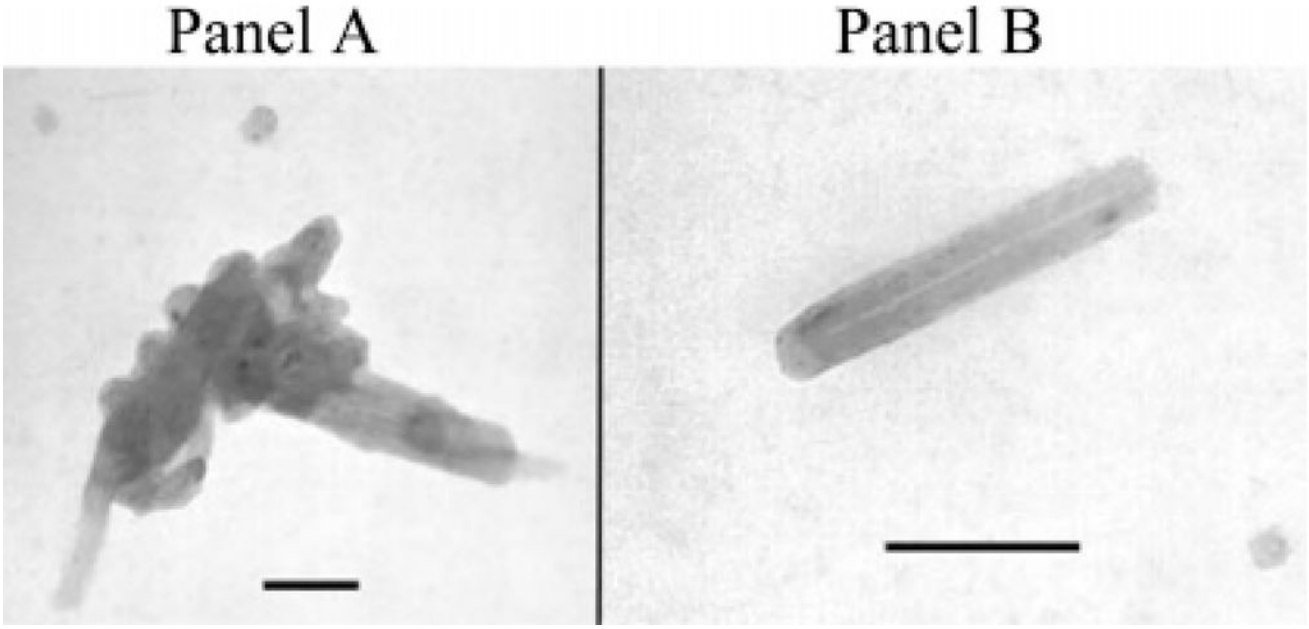

Figure 2.
TEM of BAC/pDNA complex. A negatively charged mixture of BAC(A6)/pDNA was applied to a carbon-coated Formvar-film grid, dried, and stained with uranyl acetate. In panel A the shapes of the representative complexes include spherical and rod in a supramolecular complex. In panel B a 200 nm rod or hollow cylinder is present in isolation. Bars = 100 nm.

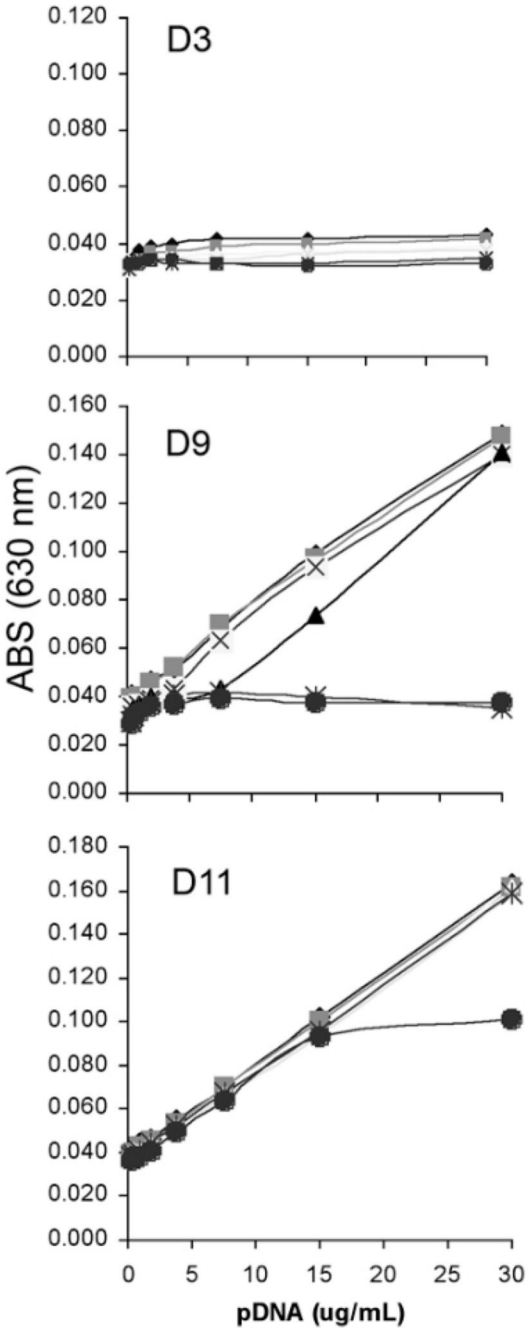

Figure 3.

Turbidity measurements of the D-series of BACs. Serial dilutions of both the BAC and plasmid were mixed in clear polystyrene tissue culture plates and incubated at room temperature for 40 min before the absorbance was read at 630 nM. BAC:pDNA ratios: ◆ 85 418, ■ 42 709, × 21 355, ▲ 10 677, boldface × 5339, ● 2669. The data are presented as representative experiments. See Table 1 for a summary.

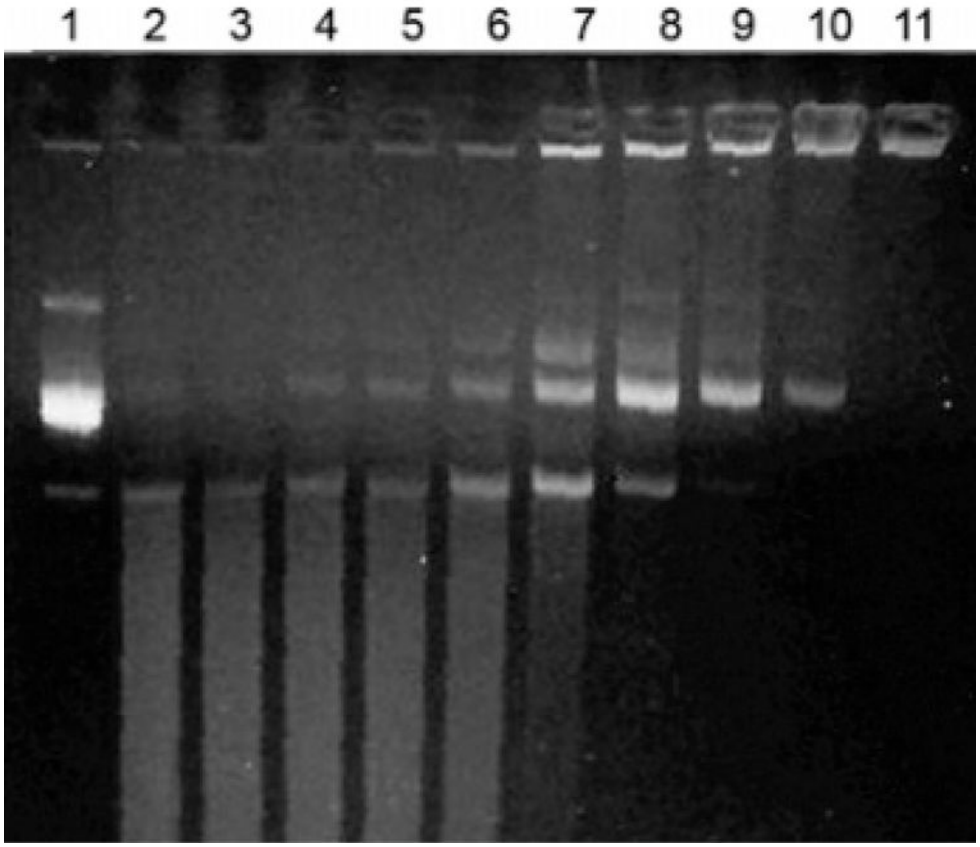

Figure 4.

Example of protection of pDNA from nuclease degradation. The protection assay was performed as described in the Experimental Section. Lane 1: pDNA. Lanes 2–11, DNase treated pDNA with increasing concentrations of BAC mixed with pDNA in the following BAC:pDNA molar ratios: (lane 2) 411, (3) 821, (4) 1643, (5) 3286, (6) 6571, (7) 13 143, (8) 26 286, (9) 52 572, (10) 105 143, (11) 210 287. DNase activity was visualized for BAC D9 at BAC:pDNA molar ratios of 13 143 (lane 7) or less, by an increase of smaller DNA fragments (DNA laddering).

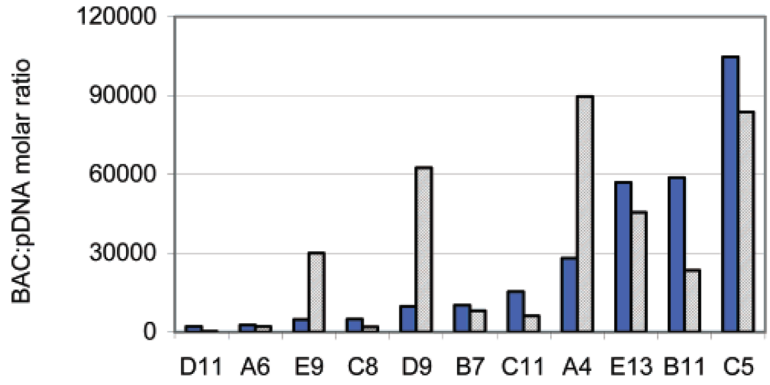

Figure 5.

BAC:pDNA resistance to DNase activity and Hoechst fluorescence quenching. Prevention of pDNA degradation (blue bars) is defined as the concentration that prevents the pDNA laddering (see Figure 4). Hoechst 33258 quenching (cross-hatched bars) is the ratio that completely prevents Hoechst fluorescence in the gel. Values are the BAC:pDNA molar ratios. Compounds A3 and D3 did not inhibit DNase activity or Hoechst quenching at the highest concentration tested (data not shown). Compounds D9 and A4 and protected pDNA in the DNAse protection assays, but failed to quench Hoechst fluorescence at the highest concentration tested (See Table 2).

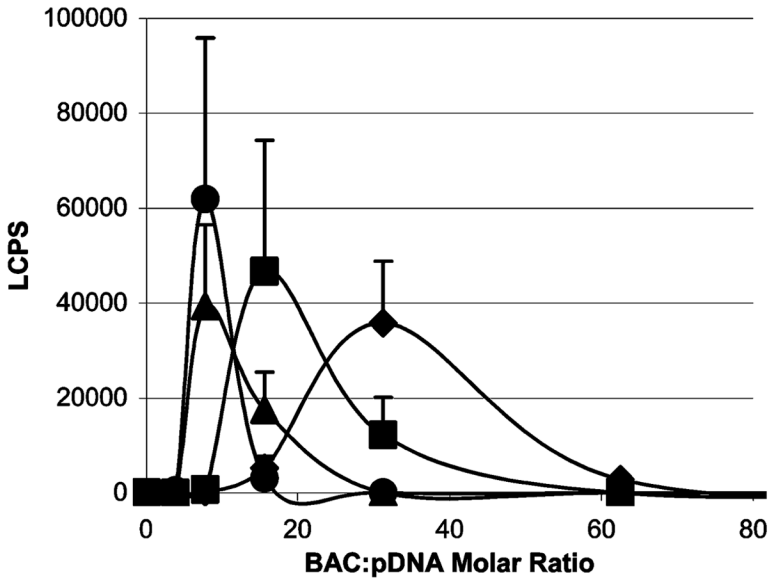

Figure 6.

BAC:pDNA molar ratios at specific pDNA concentrations modulate transfection efficiency. Twenty-five microliters of a BAC (A6) solution was mixed with 25 μL of pDNA solution containing a variant amount of total pDNA followed by a 30 min incubation. To each 50 μL mixture, 150 μL of serum free (SF) DMEM was added, and 50 μL (triplicate wells) containing ◆ 0.5, □ 1, ▲ 2, and ● 3 μg of luciferase plasmid per well was added to subconfluent DAOY cells (1:10 split previous day, ~10^5 cells/well). The cells were exposed to the BAC:pDNA complexes for 3 h, and then the mixture was aspirated, 200 μL of DMEM/10% FBS was added, and cells were outgrown for an additional 48 h. Wells were assayed using the Bright-Glo lysis buffer and Glo-Luciferase assay kit (Promega) as described in the Experimental Section. Average LCPS were plotted along the *y*-axis.

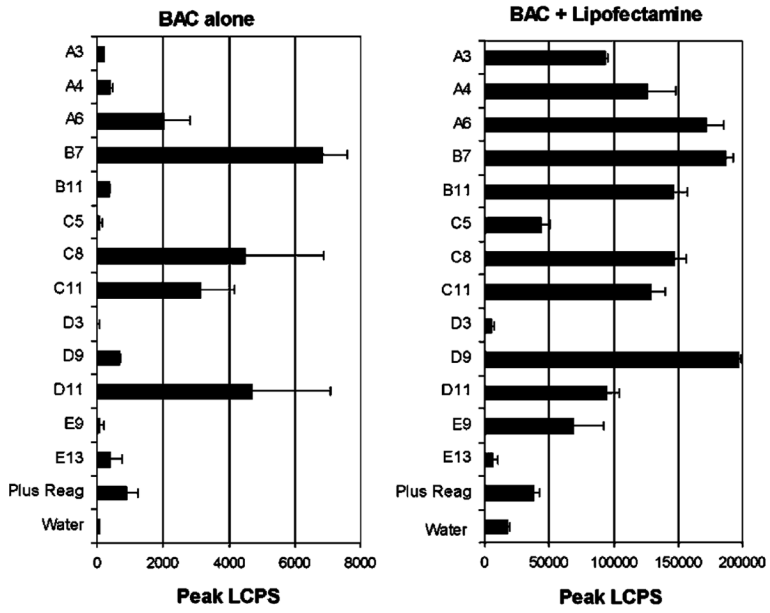

Figure 7.

Liposomes enhance the transfection efficiency of BAC:pDNA complexes. Transfection efficiencies defined as the peak light counts per second (LCPS) for BAC molar ratios in the ranges 5000–170000 were measured with and without the addition of liposomes (Lipofectamine) as a lipid donor. Plus reagent (Invitrogen) was used as a positive control for condensing agent. Lipofectamine was used as a lipid donor at a suboptimal concentration compared to that required for maximal transfection (0.5 μL of lipofectamine/μg of pDNA/100 μL). Peak LCPS values are presented as the mean ± SD.

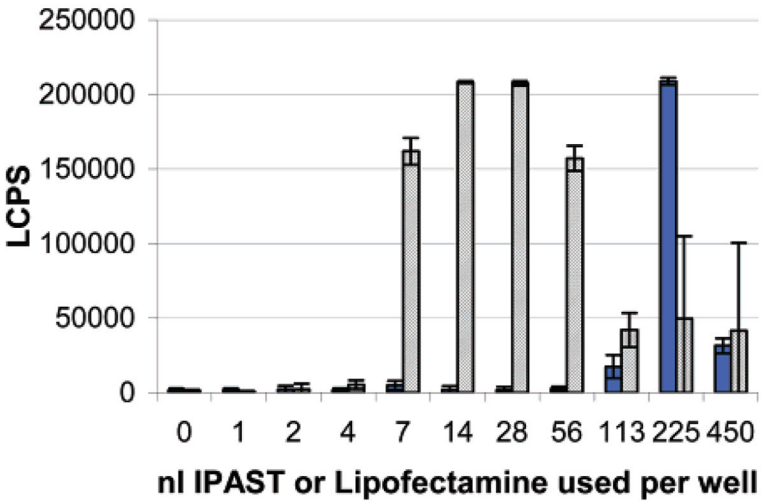

Figure 8.

BAC transfection efficiencies are enhanced by liposomes or silica nanoparticles. Subconfluent 293T cells (20–25%) were maintained in 96-well plates. BAC:pDNA complexes were made in serum free medium using 225 ng of pCF1 and 3.375 μg of BAC (A6) (final concentration per well) incubated for 15 min at room temperature. Serially diluted aliquots of Lipofectamine (blue bars) or IPAST silica nanoparticles (cross-hatched bars) were added to the BAC pDNA mixture and transferred immediately to the tissue culture wells. Cells were exposed to transfection reagents for 2 h, and then an equal volume of complete medium (10% FBS) was added. Luciferase activity was measured after an additional 18 h of static culture. Error bars are SD, $N = 4$.

Table 1

BAC Properties

BAC abbreviation	DNA binding peptide	BAC MW	calcd CMC concn (mM) [a]	av particle size formed (nm) [b]	min molar ratio for particle formation [c]	min molar ratio for turbidity onset [d]
motif A (R–)						
A3	–RRR	861.14	3.5 ± 0.0	none	NP	85 418
A4	–RRRR	1017.3	1.8 ± 0.0	none	NP	21 355
A6	–RRRRRR	1329.7	2.6 ± 0.4	103 ± 24	5339 [f]	2669 [f]
motif B (R–R–R–A–)						
B7	–RRRARRR	1400.8	>5.0 [e]	294 ± 289	21 355	21 355
B11	–RRRARRRARRR	1940.4	1.9 ± 0.0	550 ± 545	85 418	none
motif C (R–R–A–)						
C5	–RRARR	1088.4	>6.4 [e]	none	NP	none
C8	–RRARRARR	1471.8	4.4 ± 0.1	220 ± 191	10 677	5339
C11	–RRARRARRARR	1855.3	1.2 ± 0.3	319 ± 174	42 709	10 677
motif D (R–A–)						
D3	–RAR	776.03	>9.0 [e]	none	NP	none
D9	–RARARARAR	1457.8	1.7 ± 0.3	180 ± 161	42 709	10 677
D11	–RARARARARAR	1685	2.5 ± 1.1	242 ± 199	5339 [f]	2669 [f]
motif E (R–A–W–A–)						
E9	–RAWARAWAR	1517.8	1.2 ± 0.0	850 ± 203	10 677	42 709
E13	–RAWARAWARAWAR	2002.3	0.8 ± 0.0	none	NP	none

[a] CMCs were determined by the DPH dye method described in the Experimental Section.

[b] Particle size measurements were performed as described in the Experimental Section. Average particle size was calculated by averaging all conditions that formed measurable particles. Particle formation was defined as requiring more than 1000 counting events. Error is the standard deviation at all molar ratios for the particle sizes formed.

[c] Particles formed at all concentrations tested above the minimum molar ratio (BAC:pDNA). NP: no particles formed.

[d] Turbidity was used to measure the formation of light absorbing structures at higher plasmid concentrations. Serial dilutions of both the BAC and plasmid were mixed in clear polystyrene tissue culture plates and incubated at room temperature for 40 min before the absorbance was read at 630 nm. The results are reported as the lowest BAC molar ratio required to increase the OD 50% above the background value with a concentration of 15 μg/mL of plasmid. None: Turbidity did not exceed 150% of background.

[e] The CMCs for three BACs D3, C5, and B7 could not be determined since they were greater than 7 mg/mL (5–9 mM), the highest concentration tested. The experimental range reflects the range of CMCs calculated by varying the range of data points included in the linear regression. Error indicated is the SD.

[f] Lowest molar ratio tested.

Table 2

Increasing Rank Order of the Alteration in Gel Migration and Fluorescent Quenching of pDNA by BACs[a]

	μg of BAC used									
	0.24	**0.5**	**1.0**	**2.0**	**3.9**	**7.8**	**15.6**	**31.3**	**62.5**	**125.0**
A3	–	–	–	–	–	–	–	–	–	–
D3	–	–	–	–	–	–	–	–	–	–
D9	–	–	–	–	FM	FM	FM	FM	FM	FM
A4	ND	–	–	–	–	SM	SM	SM	SM	SM
E9	–	–	–	–	–	–	–	–	SM	Q
C5	–	–	–	–	–	–	SM	SM	SM	Q
E13	–	–	–	–	–	–	–	–	Q	Q
B11	–	–	–	–	FM	FM	FM	FM	Q	Q
B7	ND	–	–	–	–	–	Q	Q	Q	Q
C11	–	–	–	–	–	SM	Q	Q	Q	Q
A6	–	–	SM	SM	Q	Q	Q	Q	Q	Q
C8	ND	–	SM	SM	Q	Q	Q	Q	Q	Q
D11	–	Q	Q	Q	Q	Q	Q	Q	Q	Q

[a] pDNA was predyed with Hoechst 33258 overnight 1 μg/mL in 10 mM Tris buffer pH 7.4. 0.25 μg of pDNA was mixed with BAC, incubated for 30 min, and then photographed after agarose gel separation. "–" = visible fluorescence, FM = visible fluorescence, faster migration, SM = visible fluorescence, slower migration, Q = no visible fluorescence, ND = not determined.