# HIGH EFFICIENCY OF TRANSFECTION AGENTS USING BIO-COMPATABLE PH CLEAVABLE LINKERS

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## ABSTRACT

Transfection agents were synthesis using solid phase chemistry and utilising a pH cleavable linker. These compounds are based on 3-Amino-1, 2-propanediol and polyamine conjugate (spermine) to a steroid or bile acids. To optimize the transfection efficiency a series of structure-activity relationship studies were carried out by altering the steroid or fatty acid by systematic variation of the acid chain length. We demonstrate that transfection activity of our compound formulated with DOPE as a co lipid is comparable with commercial available, DC-Chol, transfectam, DOTAP and ESCORT™ respectively. This result suggests our bile acid based cationic lipid is more effective than those commercially available. The most potent compound was $N^1$-(3α,7α-dihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadecane.

**KEYWORDS**: Transfection agents, gene therapy, solid phase chemistry, safety-catch linker pH cleavable linker, liposome, pEGFPLuc plasmid DNA.

## 1. INTRODUCTION

Polyamines play important roles not only as lead compounds for a variety of therapeutic agents but also as templates for the synthesis of various compounds such as transfection agents [1]. These compounds have provided efficient transfer of genetic information into the target cells in a process with potential known as gene therapy. Gene therapy is generally considered as a promising approach for the treatment of diseases with genetic defects. These methods use either viral or non-viral systems for transfection. Although some viral systems show remarkable levels of transfection, they are limited for general use *in vivo* due to their low capacity for large-sized pieces of DNA and associated with immunological problems as function of high dose [2]. Consequently, there has been increasing attention focused on the development of efficient nonviral transfection agents. The most common used vectors are based on cationic lipid//DNA complexes or cationic polymer/DNA complexes. The most well-known synthetic transfection agents are cationic liposomes which are now recognized as potent agents for delivery of genes or other nucleic acids. Cationic liposomes form heterogeneous lipid vesicles, typically formed either a single cationic amphiphiles or more commonly from a combination of a cationic amphiphile and neutral lipids. They mediate gene delivery by interacting electrostatically with negatively charged DNA sequences to form complexes which due to the liposome may enter cells by endocytosis. Until now, many different organic compounds have been tested as synthetic vectors *in vitro* and *in vivo*.

However, the transfection efficiency of synthetic agents is displayed as low compared to viral-mediated. Previous studies using fluorescence cationic lipids /DNA complexes suggest that pentration into the cell cytoplasm is not the limiting barrier for gene expression. Indeed, physicochemical methods [4], suggest that although the complexes were generally observed in the cytoplasm of all cells, while gene expression was observed in only 5-12% of the cells. The result suggests that the important limiting step in none viral gene expression is the release of DNA from cationic lipid complexes. One method for obtaining increased gene expression is the DNA control-release approach [5]. This method is based on the use of a disulfide bridge as a reduction-sensitive group in lipopolyamines.

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As a part of our ongoing study into polyamine conjugates chemistry using solid phase chemistry [6] and bio-compatible pH cleavable linker [7], we were interested in the synthesis of various transfection agents. The bio-compatible pH cleavable linker offers not only the method of compound cleavage and attachment but also direct cleavage within the biological assay. In this paper we demonstrate the using bio-compatible pH cleavable linker to solid phase support for synthesizing and optimizing the effective transfection efficiency *in vitro*.

## 2. MATERIALS AND METHODS

### General

Solvents and reagents were obtained from commercial suppliers and used without further purification. Solid phase synthesis were performed in glass peptide vessels or polypropylene syringes. NMR spectra were recorded on a Bruker MHz. All chemical shifts are quoted in ppm on the δ scale using the residual protonated solvent as the internal standard. Coupling constants (*J* values) were measured in Hz. Mass spectra were obtained on a VG Platform single quadrupole mass spectrometer in electrospray ionisation (ES+ or ES-) mode. Analytical RP-HPLC was performed on a HP1100 system equipped with a Phenomenex Prodigy $C_{18}$ reverse phase column (150 x 4.6 mm i.d.) with a flow rate of 1 mL/min, monitoring at the wavelength of 220 and eluting with (A) 0.1% TFA in $H_2O$ and (B) 0.042% TFA in acetonitrile, gradient 0% (B) to 100% (B) in 20 minutes. Semi-preparative RP-HPLC were performed on a HP1100 system equipped with a Phenomenex Prodigy $C_{18}$ reverse phase column (250 × 10.0 mm i.d., flow rate 2.5 mL/min), monitoring at the wavelength of 220 nm and eluiting with (A) 0.1% TFA in $H_2O$ and (B) 0.042% TFA in acetonitrile, gradient 0% (B) to 100% (B) over 40 minutes. IR spectra were obtained on a BioRad FTS 135 spectrometer with a Goldengate ATR accessor with neat compounds. UV-VIS spectra were recorded using a 8452A Diode Array Spectrophotometer.

### Expression plasmids

The pEGFPLuc plasmid DNA contained a fusion of enhanced green fluorescent protein and luciferase was obtained from Clontech Laboratories Inc. Protein expression is under the control of the human cytomegatovirus (CMV) promoter. pEGFPLuc is a cotransfection marker that allows determination of transfection efficiencies in mammalian cells by fluorescence microscopy or a standard luciferase assay.

Plasmid DNA was isolated by the method of Laura Machesky [9]. Briefly, overnight culture of bacteria was centrifuged and the pellet resuspended in 50mM glucose, 25 mM Tris.HCl, pH 7.5, 10 mM EDTA with lysozyme (40 mg/mL) and incubated at room temperature for 10 min. The solution was then lysed by alkaline lysis and plasmid DNA was isolated by using diatomaceous earth.

### Formulation of cationic liposomes

Liposomes were prepared by adding 10 μM of transfection agents to 10 μM of dioleolyl L-α-phosphatidylethanolamine (DOPE) (prepared at 2 mM in $CHCl_3$). The solvent was removed under stream of nitrogen gas and the result lipid films were dried under vacuum for 2 h to remove trace amounts of organic solvent. The lipid films were hydrated in 0.2 μm filtered water and vortexing 2-3min at room temperature. Liposomes preparations were stored at 4°C under nitrogen gas before use.

### Cells culture and Transfections

ND7 cells (Mouse neuroblastoma x rat neurone hybrid cell line) were kindly donated by Dr. K Lilycrop (Univ. Southamp, Div. Biochem & Mol. Biol). Cells were maintained in (DMEM) + 2 mM Glutamine + 10% Foetal Bovine serun (FBS). Split confluent cultures 1:3 to 1:6 i.e. seeding at 1-3x$10^4$ cells/$cm^2$ by aspirating; 5% $CO_2$; 37°C. Cells are lightly attached therefore use of trysin is unnecessary. Twenty-four hours before transfection, 500 μl of ND7 cells was seeded into 24 well culture plates at a concentration of 5x $10^4$cells/mL. This dilution showed approximately 80% confluency after 24 hours. The cells were washed with medium without serum just before transfection and incubated with 200 μl of serum free medium. Plasmid DNA/liposome complex mixtures (100μl) were added to each well. Two hours affter tranfection, gently overlay the cells with 0.8 mL complete medium with serum. It is not necessary to remove the transfection medium containing the liposome/DNA complexes. Return the cell to the incubator and continue the incubation for 24-48 hours incubation at 37°C in 5% $CO_2$. The transfection efficiency was assayed by using microscopes and the cytotoxicity was also assessed using the fluorescent exclusion dye propidium iodide (PI). Briefly, PI (5μg/mL) was presented in the medium throughout the experiment period. PI is highly polar and is excluded from live cells but may enter damaged cells and becomes highly fluorescent upon binding to DNA. Fluorescence was exited at

450-490 nm or 515-560 nm using a standard Leica inverted microscope with FITC and rhodamine filter block N2 respectively (Leica UK, Milton Keynes, Bucks, UK). Fluorescent images were captures using a COUH CCD monochrome camera (Cohu Inc., Sandiago, CA, USA), stored on optical disk prior to analysis on an Apple Performa 5200 computer.

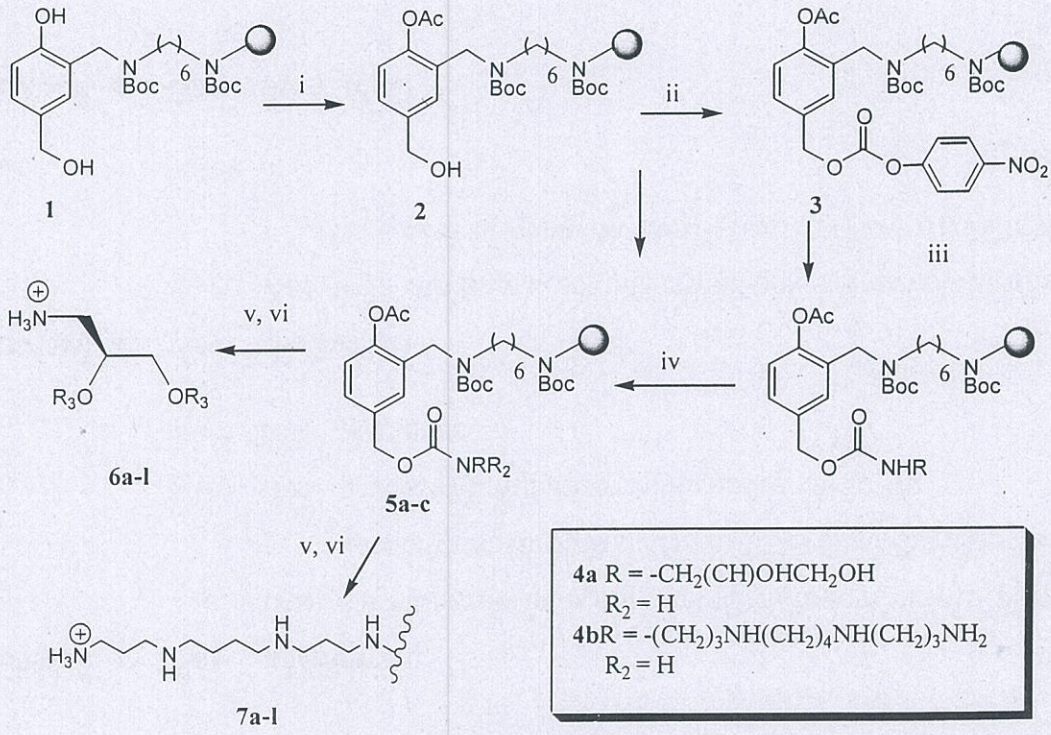
### MTT Assay

MTT assays [10] on samples were performed under the same conditions as transfection. The MTT was added to each well containing 90 µl of phenol red-free media. Following 3h incubation at 37 $^0$C, the resulting formazan crystals were dissolved in 100 µL, 10 % Triton X-plus o.1 N HCl in anhydrous isopropanol. Absorbance was measured at 570 nm and coverted to % viability relative to control (untranfected) cells.

## 3. RESULTS AND DISCUSSION

### Synthesis of Transfection compounds on polystyrene beads

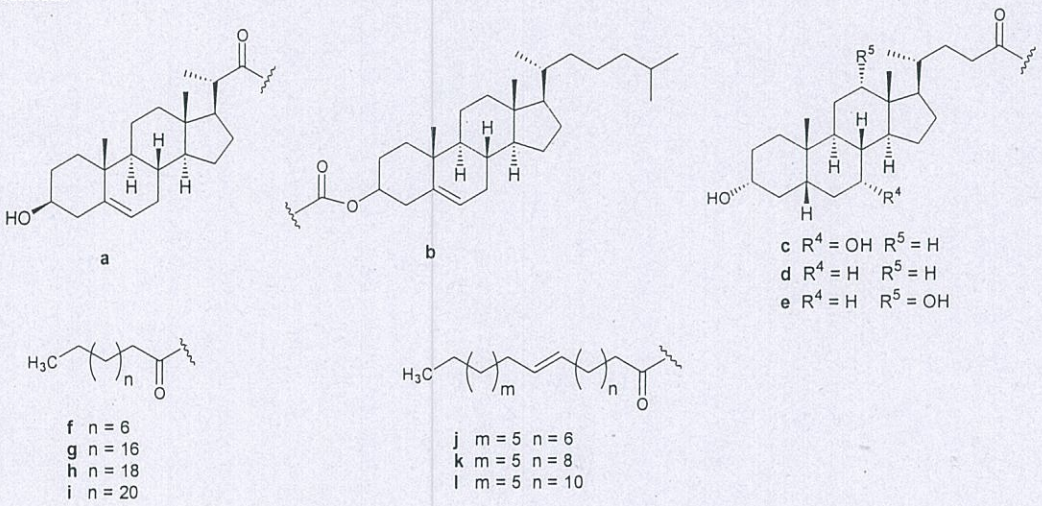
The synthesis of transfection agents was realized in a straightforward strategy as shown in scheme1. The selective acetylation of phenol on solid phase 2 was achieved modified from our method [7] based on the solution mode study. The *p*-nitrophenol activated linker 3 was achieved by using *p*-nitrophenol choroformate at room temperature. This linker was then reacted with 3-Amino-1, 2-propanediol (4a) and spermine (4b) to give the product 5a and 5b respectively. The loading of the resin was calculated using the ninhydrin test. The 3-Amino-1, 2-propanediol linker (5a) was first coupled to 3β-acetoxybisnor-5-chlolenic acid to give a desired product on the solid phase. These compounds were cleaved from the resin using 50% TFA in DCM followed by treatment with phosphate buffer (pH 8. These results suggest that the linker can be used for screening.




**Scheme 1**. Synthesis of pH cleavable linker **5** (i) 1N NaON (aq), $Ac_2O$/THF, RT, (ii) p-$NO_2$PhCOCl, pyridine, DCM, RT (iii) polyamine ($NH_2CH_2CHOHCH_2OH$ and spermine (iv) $R_3CO_2H$/DIC/HOBt (DCM: DMF (3:1)) (v) 50%TFA/DCM, 1h, (vi) Phosphate buffer pH 8, 50 mM

The synthesis of transfection agents was carried out in the manner described above. As expected, the products were released from the solid support and used directly for screening biological

activity. The various transfection agents based on bile acid and long chain fatty acid are shown in scheme2.




Scheme 2 Structure of cationic lipids based on bile acid and long chain fatty acid

**Transfection screening by using ND7 cells line**

ND7 cells (Mouse neuroblastoma x rat neurone hybrid cell line) were used for the transfection assay. After several passages and subsequent growth, the cells were plated out at a concentration of $5x10^4$ cells/well in one mL of medium. They were allowed to grow for 24 hours to 60-80% confluence. The degree of confluency on the day of transfection is a parameter that needs to be optimized for each individual cell line. The transfection "vector" consisted of transfection agent/DOPE vesicles (2 mM) prepared in filtered water by vortexing the mixture 2-3 min at room temperature. Plasmid DNA (pEGFPLuc, Clontech Laboratories) contained the gene for green fluorescent protein (GFP) and luciferase as a co-transfection marker. Liposome complexes were prepared by mixing 1 μg of plasmid DNA and 5-10 μl of transfection agent (2 mM) in 200 μl of serum free medium. These complexes were incubated for 15-20 minutes at room temperature. The cells were then rinsed with 1 mL of serum free medium followed by gentle application of the liposome complexes. After 1 hour incubation at 37°C in 5% $CO_2$, 0.8 mL of complete medium was added to the cells. After 24 hours incubation, the cells were assayed for transfection efficiency. The fraction of fluorescent cells was determined under a microscope by cell counting and the results are shown in Figure 1. The percentage transfection was calculated by comparison with non-transfected cells and the results are shown in Figure 1.

The first compounds to be tested were the 3-Amino-1,2-propanediol analogues (**6a-l**). Disappointingly, results indicated no transfection at all. However the structurally related analogue (**7b**) showed modest activity. The bile acid derivatives (**7c–e**) proved more successful (Figure 2). Of this series of compounds, (**7c**) was found to be the most potent mediator of gene delivery, compound (**7d**) showing only modest activity, but comparable to (**7b**) and commercially available agents such as Transfectam, DOTAP and Lipid-67. Liposome complexes derived from the long chain fatty acids showed little activity, only (**7f**) yielding any appreciable transfection. The results of cells transfected with pEGFP DNA using the bile acid derivative (**7c**) is shown in Figure 2.

Analysis of our results indicates that conjugate (**7c**) is the most potent transfection agent, as evidenced by the greater population of GFP positive cells. This result differs from the structurally related steroidal analogues (**7d** and **7e**). A possible explanation for this dissimilarity between the series may be partly attributable to the ability of the respective compounds to bind DNA. It has been suggested that DNA binding is affected not only by the polyamine moiety (in our case spermine, which is constant in all the conjugates) but also the lipid anchor group[8] compound (**7c**). It has a charge ratio ($CR_{50}$) of 2.3 whilst the $CR_{50}$ values for (**7d**) and (**7e**) are 0.7 and 1.6, respectively, indicating that DNA is most tightly bound by (**1d**). (The binding affinities for DNA are expressed as the charge ratio at which 50% ($CR_{50}$) of ethidium bromide was quenched.) (Their relative binding affinity for calf thymas DNA were determined using an ethidium bromide displacement assay). The proposed mechanism of DNA delivery into cells is via endocytosis[11], however effective transfection also requires the release of

the DNA inside the cell. The lack of activity of compounds (**7d**) and (**7e**) may be related to the reduced propensity of DNA escape from the endosomal compartment as compared to (**7c**) due to the greater affinity for DNA.

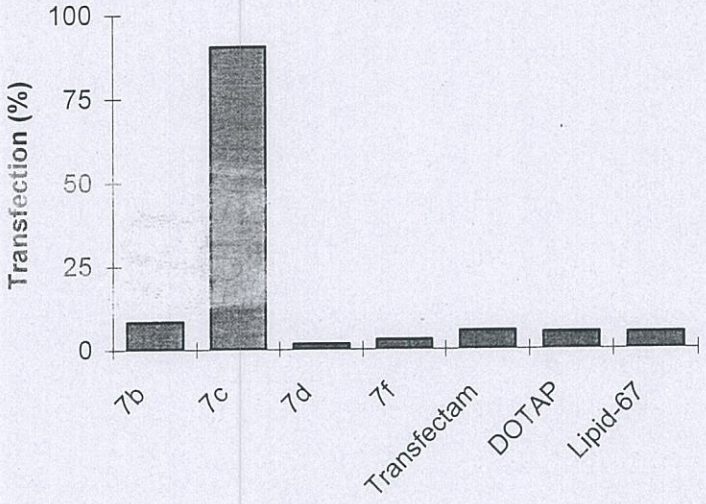
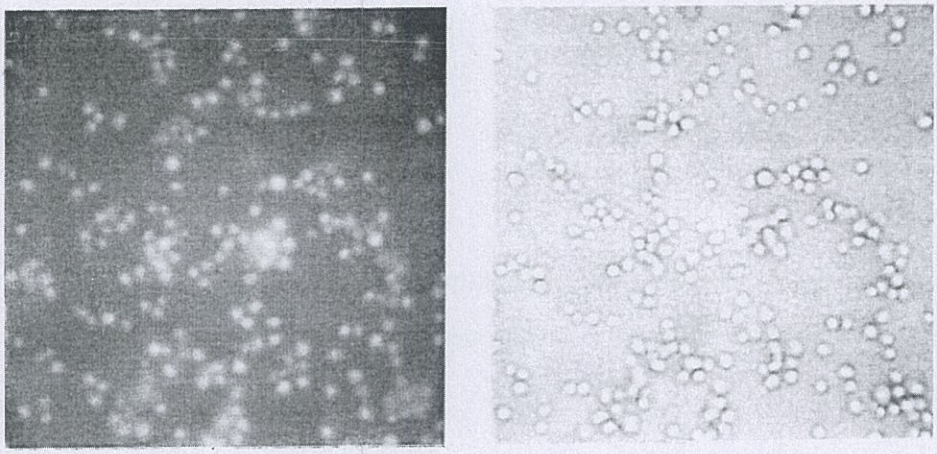



Figure 1 **Initial screen of transfection library 7a-l**



**A** **B**

Figure 2 ND7 cells were treated with transfection agent (7c) and green fluorescent protein (GFP) reporter gene. (A) cells analysed under a fluorescein filter and (B) represents cells under normal light ratio 1:1 comparation of transfection.

Finally, none of the conjugates alone (in the absence of DOPE) showed any significant degree of transfection, indicating that the polyamine conjugates alone do not facilitate the uptake of DNA and the neutral co-lipid DOPE[11] is required. This supports the argument that DOPE may help to promote endosome escape resulting in increased transfection.[11]

## Optimisation parameters

To achieve maximum transfection efficiency of ND7 cells, it was essential to optimise the lipid DNA ratio. The optimal amount of DNA per transfection was the first critical parameter to determine, although this will vary among cell types. It can be seen that increasing the amount of DNA may not necessarily result in higher transfection levels of the GFP reporter gene. For ND7 cells, the ratio of DNA/charge ratio (3:4) per well was the optimal amount of pEGFPLuc.

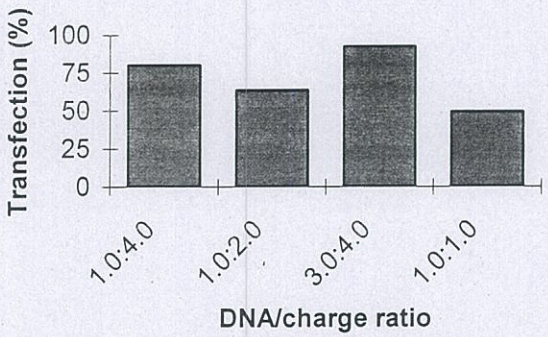



**Figure 3**. Optimisation of the amount of DNA required using compound (**7c**)

Toxicity

Generally, exposure of the cells to complexes did not cause significant toxicity. Most samples exhibited survival above 90 % of controls (cells exposed to the reagent free media) as determined by an MTT assay. However, toxicity was observed with compound 7b (70 %).

## 4. CONCLUSIONS

In conclusion, we have described the development of safety-catch linker for solid phase synthesis of some novel cholesterol based amphipiles. These cationic liposomes displayed high efficient capability to transfect rat neural cells *in vitro*. This method is simple and quick for searching lead compound. Their transfection efficiency with relatively low toxicity and ease of preparation would make them further promising candidates for general gene therapy.

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