Synthesis and Characteristics of Biodegradable Pyridinium Amphiphiles Used for in vitro DNA Delivery

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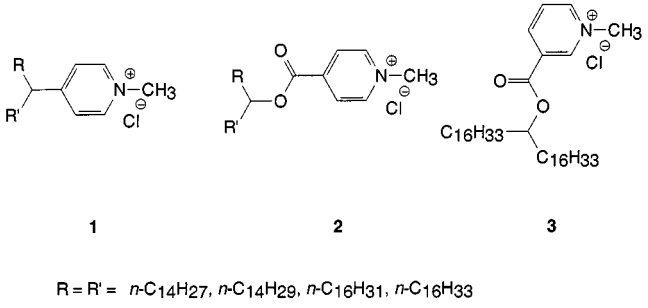
Pyridinium amphiphiles have found practical application for the delivery of DNA into eukaryotic cells. A general synthetic method starting from (iso)nicotinoyl chloride has been devised for the preparation of pyridinium amphiphiles based on (bio)degradable esters, allowing structural variation both in the hydrophobic part and in the headgroup area. By means of differential scanning calorimetry, transmission electron microscopy and UV measurements, some characteristics, including hydrolytic behaviour, have been determined. In vitro transfection ability and toxicity have been determined using the eukaryotic COS-7 cell line.

Introduction

Gene therapy has established itself a promising position as a principal method in the treatment of disorders with a genetic bias (such as cystic fibrosis) or in the development of therapeutic strategies for a wide range of diseases such as cancer and transmissible (e.g. AIDS, hepatitis) and acquired (such as Parkinson's disease, rheumatic arthritis, and others) diseases.[1,2] The concept of gene therapy is relatively straightforward, and involves the introduction of engineered or foreign genetic material into the target cells or organs to induce protein expression. Various introduction/delivery methods based upon either *viral* or *non-viral* carrier systems (*vectors*) have been developed. At present, the most efficient methods for the transfer of genetic material (*transfection*) involve the use of *viral* vectors[3,4] (e.g., retrovirus, adenovirus), although there are strong arguments relating to the risks with regard to immunogenicity and propagation.[5,6] Synthetic cationic amphiphiles (*cytofectins*), mostly in combination with neutral helper lipids such as dioleoylphosphatidylethanolamine (DOPE) partially circumvent these problems and have also proved efficient non-viral carrier systems both for in vitro and for in vivo delivery of DNA,[7–11] although improvements in transfection efficiency and toxicity are still necessary. We have previously reported on a class of cationic pyridinium-based amphiphiles (SAINTs) with good transfection potential towards eukaryotic cells[12–14] (Scheme 1).



1 **2** **3**

R = R' = *n*-$C_{14}H_{27}$, *n*-$C_{14}H_{29}$, *n*-$C_{16}H_{31}$, *n*-$C_{16}H_{33}$

Scheme 1. General structure of a SAINT amphiphile (**1**) and the derived ester amphiphiles (**2** and **3**)

The synthetic procedure permits easy structural modification of the "basic" SAINT concept (for example by **1**), yielding novel amphiphiles with different/improved characteristics both in terms of activity on a certain stage in the transfection pathway as well as on the overall transfection potential and toxicity.

One simple modification involves the introduction of ester functionalities between the cationic headgroup and the apolar lipid tails. The (effects of) incorporation of (bio)degradable ester moieties into cationic amphiphiles has previously been reported by, for example, Leventis and coworkers.[15] The introduction of ester groups ensured efficient intracellular metabolism after incorporation into animal cells, thus avoiding long-term persistence of unnatural cat-

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ionic lipid species in cellular membranes and its possible undesirable consequences for cellular functions.[16−20] Hydrolysis of the esters was activated by an environment with a low(ered) pH, conditions present in, for example, the endosome, increasing the overall transfection efficiency.

In this paper we report on the synthesis and application of several new pyridinium amphiphiles possessing (bio)degradable ester linkages between the cationic headgroups and the apolar lipid tails. We envisaged that the ester linkage would be activated towards hydrolytic processes promoting hydrolysis of the amphiphiles, such as during endocytosis. The lipoplex would consequently degrade and release the contents more readily, thereby increasing the transfection efficiency. Some of the physiochemical properties of the new amphiphiles relevant for influencing DNA delivering efficiency as well as their hydrolytic behaviour are also reported.

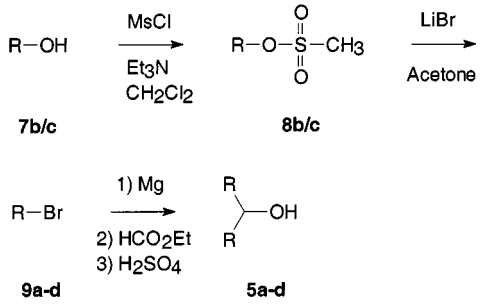
Results and Discussion

Synthesis

The amphiphiles **2a**−**d** were synthesised by treatment of isonicotinoyl chloride hydrochloride (**4**) with 1 equiv. of the appropriate alcohol **5a**−**d** and 2.1 equiv. of triethylamine as base, to yield the pyridine esters **6a**−**d** in moderate to good yields (Scheme 2). Quaternisation of the pyridinium nitrogen atom with a fivefold excess of methyl iodide in acetone afforded the methylated products in quantitative yield; subsequent ion exchange yielded the desired pyridinium amphiphiles **2a**−**d** as the more stable chloride salts. For **3**, the same procedure was followed, but starting from *meta*-substituted nicotinoyl chloride hydrochloride and alcohol **5d**.

It appeared to be troublesome to exchange the iodide for the chloride counterion under the conditions routinely applied (Dowex 1×8, 200−400 mesh), due to transesterification of the long-tailed ester products with the methanol eluent. An alternative method using a large excess of AgCl in acetone did result in counterion exchange without side-reactions, although residual traces of Ag^+ were hard to remove due to excessive complexation, probably with the carbonyl oxygen atom. By use of the very mild ion exchange material Sephadex (DEAE A25, chloride form), however, transesterification was prevented despite the use of methanol as eluent and, moreover, resulted in quantitative ion exchange. In some cases, purification proved troublesome because of the tendency of the products to complex residual solvent, resulting in elemental analyses that deviated slightly from the theoretical values.

The double-tailed starting alcohols **5a**−**d** were obtained from the singly tailed precursor alcohols **7b/d** either by the three-step procedure outlined in Scheme 3, or from the starting bromides **9a** and **9c**.[21]

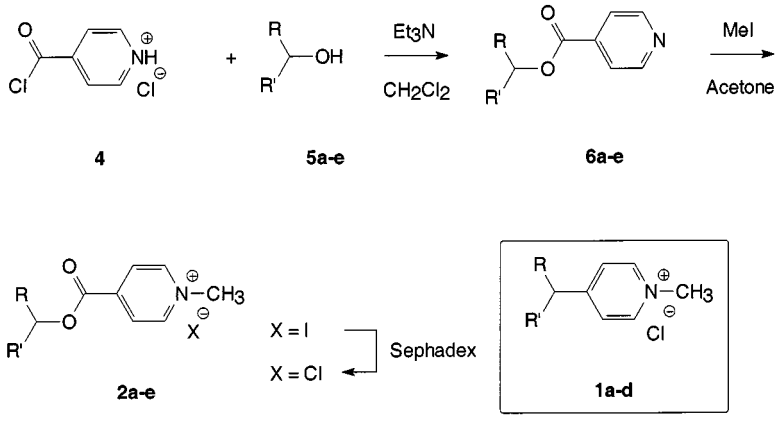


R = **a** *n*-$C_{14}H_{27}$, **b** *n*-$C_{14}H_{29}$, **c** *n*-$C_{16}H_{31}$, **d** *n*-$C_{16}H_{33}$

Scheme 3. Synthesis of double-tailed alcohols **5a**−**d**

The precursor alcohols **5b/d** were transformed into the corresponding mesylates **8b/d** by treatment with methanesulfonyl chloride in the presence of a slight excess of triethylamine as base. Treatment with LiBr in dry acetone afforded the bromides **9**, which were subsequently converted into the double-tailed alcohols **5a**−**e** by means of a mixed Grignard reaction with 1 equiv. of Mg and 1 equiv. of ethyl formate in ether, in moderate overall yields. This methodology also allows double-tailed alcohols with two distinct tails to be synthesised.

Besides the long-tailed pyridinium amphiphiles **2a**−**d**, designed to be studied in terms of both hydrolysis and trans-



R = R' = **a** *n*-$C_{14}H_{27}$, **b** *n*-$C_{14}H_{29}$, **c** *n*-$C_{16}H_{31}$, **d** *n*-$C_{16}H_{33}$, **e** CH_3

Scheme 2. Synthesis of the ester amphiphiles **2** and **3**

fection potential, **2e** was also synthesised as a model compound solely to optimise the hydrolysis assay.[22]

Some Characteristics of the Amphiphilic Aggregates

The amphiphiles used in this study, except for model compound **2e**, form bilayer vesicles when mixed with DOPE, as evidenced by transmission electron microscopy.[23,24] The sizes of the aggregates typically varied between 50 and 250 nm. Remarkably, the different connectivities of **2d** and **3** (*para* and *meta* substitution, respectively) did not result in significant differences in the bilayer packing and overall morphology. Figure 1 shows a transmission electron micrograph of **3**, negatively stained with phosphotungstic acid (PTA); the vesicle size generally varied between 20 and 200 nm.

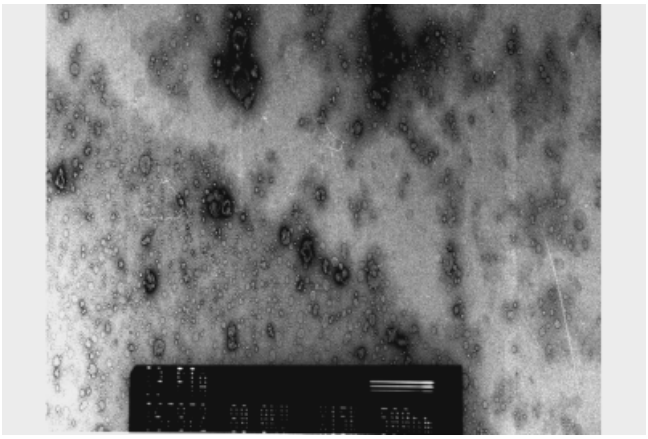


Figure 1. Transmission electron micrograph of vesicles of **3** and DOPE negatively stained with PTA; vesicle size varies between 20 nm and 200 nm

Earlier results with *non*-ester SAINT amphiphiles did suggest that the overall morphology has a distinct influence on the transfection efficiency and can be directed by changing several parameters, among which the molecular structure is the most evident.[25] Apparently, vesicle formation is overwhelmingly dominated solely by the orientation and dynamical behaviour of the apolar lipid tails, these being identical for **2d** and **3**, while the actual orientation of the cationic headgroup exerts little or no influence.

In the complex interactions between the vesicles and the DNA, the fluidity of the formed bilayers plays an important role. The main phase-transition temperatures for bilayer vesicles formed by **2d**/DOPE and **3**/DOPE were therefore determined by differential scanning calorimetry (DSC) and found to be comparable, despite the different connectivity of the ester moiety (*para* versus *meta*). In addition to the main phase transition at 30 °C, a second transition was found at 42 °C, with increasing intensity in successive experiments. The appearance of this second signal is attributable to secondary effects caused by hydrolysis of the amphiphiles due to the elevated temperatures during the prolonged time interval between measurements. These findings were also corroborated by ^{1}H NMR studies (not shown), which showed both starting material and hydrolysis products with increasing concentrations of the latter (vide infra). At transfection conditions (37 °C) all SAINT/DOPE bilayers exist in a liquid crystalline phase, which is apparently a prerequisite for efficient transfection under physiological conditions.[26]

Hydrolysis

In order to obtain information on the hydrolytic characteristics, the hydrolysis of the non-bilayer-forming model compound **2e** was studied by monitoring the changes in UV absorption as a function of time and of pH (Figure 2).

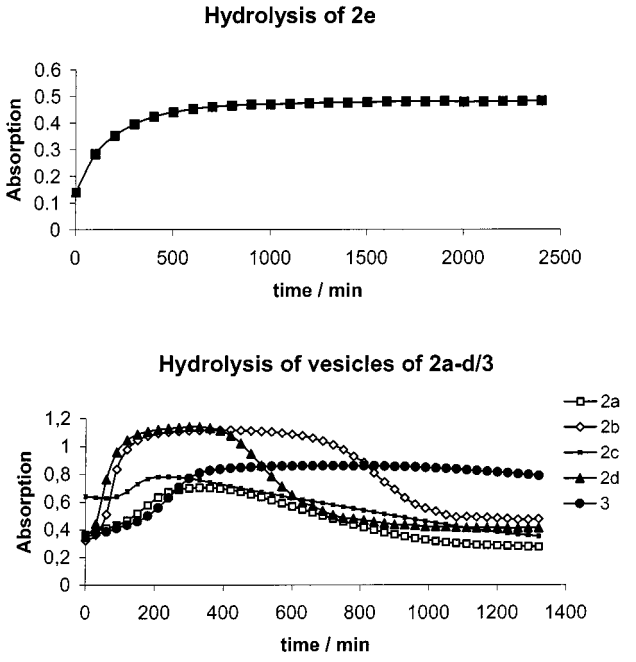


Figure 2. UV absorption profile for the hydrolysis of model compound **2e** (*i*) and vesicles formed from **2a**−**d**/**3** (*ii*); absorptions at λ = 250 nm, pH = 7.4 and at 37 °C in Hepes buffer (10 mM); see text for explanation

At pH = 7.4 (physiological conditions), the half-life for the hydrolysis of **2e** is 3 h 20 min, whereas at pH = 3.0 hydrolysis is strongly retarded. At pH = 10.6 and pH = 11.0 the half lives are reduced to 6.4 and 3.8 s, respectively,

Table 1. Pseudo-first-order (k_{obs}) rate constants and half-lifes for hydrolysis of **2a**−**e** and **3**; half lifes for **2a**−**d** were determined from the time at which half of the maximum absorption value was reached; see text for explanation

Compound	k_{obs} [min^{-1}]	$T_{1/2}$ [s]
2e (pH = 11.0)	10.85	3.8
2e (pH = 10.6)	6.48	6.4
2e (pH = 7.4)	3.45×10^{-3}	1.2×10^{4}
2e (pH = 3.0)	ca. 0	ca. ∞
2a		10.8×10^{3}
2b		4.8×10^{3}
2c		9.6×10^{3}
2d		3.6×10^{3}
3		1.5×10^{4}

indicating that the observed rate constant k_{obs} is mainly determined by the hydroxide ion catalysed reaction.[27] At pH = 3.0, the rate constant is almost zero, confirming that k_{H_2O} and k_{H^+} are only minor contributors to k_{obs}. The value of k_{OH^-} ($1.12 \cdot 10^4$ L·mol^{-1}·min^{-1}) was determined by plotting k_{obs} against the hydroxide ion concentration, and this allowed determination of the k_{obs} and half-life under endosomal conditions (pH ≈ 6.0);[28] they amounted to $1.12 \cdot 10^{-4}$ min^{-1} and ca. 103 h, respectively. The pseudo-first-order rate constants and half-lifes for the hydrolysis of **2e** determined at different pH values are listed in Table 1.

The hydrolytic characteristics of the bilayer vesicles formed from **2a**−**d** or **3** and DOPE were also studied. As a general trend, relatively stable absorptions were observed initially, followed by steep increases in absorption, levelling off by more or less linear and faint decreases. These rather sigmoidal curves were also observed for micelles of betaine ester derivatives and were presumably caused by aggregation of the amphiphiles.[29] The final decreases in absorption presumably originated from turbidity and/or precipitation of the resulting relatively insoluble alcohols, partly together with some of the UV-active material (either ester or acid). Because of the complex absorption curves and disturbing secondary effects, accurate rate constants could not be obtained, and so half-life values were approximated from the time at which the absorption reached half the maximum level (Table 1).

Generally, the half-lifes of vesicles of the *para*-substituted compounds **2a**−**d** were lower than those found for the *non*-bilayer-forming model compound **2e**, suggesting that hydrolysis was greatly facilitated by aggregation. Furthermore, hydrolysis of the saturated alkyl surfactants **2b** and **2d** was faster than that of the unsaturated analogues **2a** and **2c**.[30] The *meta*-substituted material **3** was less easily hydrolysed than the *para*-substituted analogue **2d**, which might be attributable to the differences in the directing resonance effects of the electron-withdrawing pyridinium headgroup. At this stage, we have to emphasise that, under *physiological* conditions, such as during transfection, hydrolytic characteristics are influenced not only by the pH, but also, and probably more strongly, by available enzymatic pathways. Results relating to hydrolysis both of the materials presented here and of closely similar ones under enzymatic conditions will be published in due course.[31]

Transfection Experiments

Transfections were carried out by means of a β-galactosidase assay.[32] Vesicles made of the cationic amphiphiles and DOPE (1:1) were mixed with plasmid DNA containing encoded reporter protein. The resulting aggregates were mixed with cultured cells (COS-7) and analysed for the presence of reporter protein after 2 d. The transfection results are listed in Table 2. The transfection efficiencies are given in arbitrary units and are compared to that of Lipofectin [a mixture of dioleyloxypropyltrimethylammonium chloride (DOTMA) and DOPE], one of the most commonly used commercially available transfection agents.[33]

Table 2. Transfection efficiencies and toxicity evaluation of SAINTs **1a**−**d** and SAINT esters **2a**−**d**/**3**; transfection efficiencies were evaluated by β-galactosidase assay of the vesicle/DOPE/DNA complexes in COS-7 cells, reported in arbitrary units

Compound	Transfection efficiency (%)[a]	Cell survival (%)[b]
Lipofectin	100	65
1a	230	70
2a	235	25
1b	95	85
2b	380	98
1c	180	75
2c	210	98
1d	100	85
2d	330	98
3	1290	98

[a] Lipofectin transfection efficiency arbitrary set to 100%. [b] Cell survival is expressed as percentage of surviving cells after the transfection event.

In order to access the influence of the ester linkage, a comparison was made with the transfection efficiencies found for the analogous *non*-ester-containing SAINT materials **1a**−**d**.[14] Within this group, a steady increase in transfection potential is found both for an increase in lipid tail length (**1a** > **1c**, **1b** > **1d**) and for the introduction of the double bond (**1b** > **1a**, **1d** > **1c**). These results are in agreement with previous work on SAINT amphiphiles.[14] The introduction of an ester moiety has an even more profound influence on the transfection efficiency, as becomes evident on comparing **1a**−**d** with the corresponding esters **2a**−**d**. Surprisingly, the introduction of a double bond appears not to be synergistic with the introduction of an ester-linking moiety, as the *non*-ester SAINTs **1a**−**d** show the highest efficiencies with *unsaturated* lipid tails (**1a** vs. **1b** and **1c** vs. **1d**), whereas the ester SAINTs **2a**−**d** display their highest transfection potentials with *saturated* lipid tails (**2b** versus **2a** and **2d** versus **2c**). An exceptionally large increase in transfection efficiency was shown by *meta*-substituted material **3** in comparison with **1d** and **2d**, increasing the efficiency by a factor of 12 and 6, respectively. A transfection efficiency a remarkable 12 to 13 times higher than that of lipofectin was found for **3**.

Except for **2a**, all the ester derivatives displayed either no or hardly any toxicity towards the cell cultures used (in terms of cell survival). This decrease in overall low toxicity upon the introduction of an ester moiety has also been reported by other groups.[15]

Conclusions

A number of cationic pyridinium amphiphiles have been synthesised, and have displayed increased transfection potential through influencing both the overall morphology

and the DNA-releasing ability of the formed lipoplexes. We had anticipated that increases in the hydrolytic rates of ester group containing amphiphiles at low(ered) pH values, a situation present in the endosome, should facilitate the escape of DNA, although the systems so far studied proved remarkably stable at pH = 6 in comparison to under physiological or more basic conditions. Although the initial idea proved incorrect, a sudden release of DNA in the endosome might bring about a fast degradation of the exposed DNA and/or problems in the transport of the DNA to the nucleus in the absence of (protecting) vector material. The novel ester derivatives **2a−d** and **3** displayed transfection potentials similar to, or much better than, that of lipofectin, with improvements of up to four/fivefold for **2a−d** and twelve/thirteenfold for **3**, accompanied by a further decrease in toxicity.

Experimental Section

Synthesis

General: All reactions were carried out with dried solvents under nitrogen in oven-dried glassware. For column chromatography, Al_2O_3 (activity II−III), prepared by addition of the indicated amount of water to Merck aluminium oxide 90 active neutral (activity I) was used with the indicated eluent system(s). Melting points (uncorrected) were determined by a Kofler melting point microscope. Several compounds displayed liquid crystalline behaviour; in these cases no melting points are reported. NMR spectra were recorded with Varian Gemini 200 and Varian VXR 300 spectrometers operating at 200 and 300 MHz for the proton channels, respectively. Mass spectra were recorded with a Nermag R-3010 triple quadrupole mass spectrometer equipped with an in-house atmospheric pressure ionisation source and ion-spray interface. Elemental analyses were carried out in the Analytical Department of the University of Groningen. Accurate elemental analyses were difficult to obtain, due to the presence of longer and shorter carbon chain homologues in the alkyl-chain starting materials (technical grade), as evidenced by electron spray mass spectrometry. UV measurements were performed at λ = 250 nm and 37 °C ± 0.2 °C with a Perkin−Elmer Lambda-5 UV/Vis spectrophotometer. Vesicle solutions ($5 \cdot 10^{-4}$ M at pH = 7.4) were mixed 1:4 with 50 mM Hepes buffer (prior to measurement) to a final volume of ca. 2.5 mL and a concentration of ca. $1 \cdot 10^{-4}$ M. No substantial hydrolysis due to this treatment was observed, and no precipitation due to osmotic shock was seen prior to measurement. EM experiments (negative staining) were carried out with a Philips EM-300 microscope operating at 80 kV. Differential scanning calorimetry measurements were performed with a MicroCal MA-01060 apparatus. Samples used were 1.7 mM, and measurements were carried out with heating rates of 30 °C/h. (*Z*)-11-Tetradecen-1-ol, (*Z*)-11-hexadecen-1-ol, 1-bromotetradecane, cetyl bromide, ethyl formate, isonicotinoyl chloride hydrochloride, and nicotinoyl chloride hydrochloride were obtained from Aldrich (Minneapolis, MN).

General Procedure for the Synthesis of Mesylates 8a/c:[21] Triethylamine (42.5 mmol) was added slowly to a solution of alcohol (20.0 mmol) in dichloromethane (50 mL). The reaction mixture was cooled to 0 °C and mesyl chloride (22.5 mmol) in dichloromethane (5 mL) was added dropwise. The reaction mixture was then stirred at room temperature for approx. 90 min, with the progress monitored by TLC. After addition of dichloromethane to a final volume of approx. 100 mL, the organic layer was washed with water, diluted HCl, saturated $NaHCO_3$ solution, and brine, and dried with Na_2SO_4. Evaporation of the solvent yielded yellowish oils in 85−99% yields, typically. The mesylates were used without further purification; storing times were kept as brief as possible.

(*Z*)-11-Tetradecen-1-mesylate (8a): Slightly yellow oil; yield 5.74 g, 99%. ^{1}H NMR ($CDCl_3$): δ = 0.95 (t, 3 H), 1.27 m, br., 14 H), 1.74 (m, 2 H), 2.01 (m, 4 H), 3.00 (s, 3 H), 4.21 (t, 2 H), 5.33 (m, 2 H).

(*Z*)-11-Hexadecen-1-mesylate (8c): Slightly yellow oil; yield 5.41 g, 85%. ^{1}H NMR ($CDCl_3$): δ = 0.89 (t, 3 H), 1.27 (m, br., 18 H), 1.74 (m, 2 H), 2.01 (m, 4 H), 3.00 (s, 3 H), 4.21 (t, 2 H), 5.33 (m, 2 H).

General Procedure for the Synthesis of Bromides 9a/c: A solution of mesylate **8** (20 mmol) and an excess of pre-dried LiBr (60 mmol) in dry acetone (40 mL) was refluxed for 3 h while being vigorously stirred, the progress being monitored by TLC. After filtration, the mixture was taken to dryness and the resulting yellowish oil was purified on Al_2O_3 (act. II−III), with chloroform/hexane (1:1) as eluent system. The bromides were obtained as colourless, viscous oils, typically in 55−90% isolated yield.

(*Z*)-1-Bromo-11-tetradecene (9a): Colourless oil; yield 2.90 g, 53%. ^{1}H NMR ($CDCl_3$): δ = 0.95 (t, 3 H), 1.27 (m, 14 H), 1.85 (m, 2 H), 2.01 (m, 4 H), 3.41 (t, 2 H), 5.35 (m, 2 H). ^{13}C NMR ($CDCl_3$): δ = 14.4, 20.5, 28.0, 28.2, 28.8, 29.2, 29.4, 29.5, 29.8, 32.8, 34.1, 129.3, 131.5.

(*Z*)-1-Bromo-11-hexadecene (9c): Colourless oil; yield 3.86 g, 64%. ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 3 H), 1.27 (m, 18 H), 1.85 (m, 2 H), 2.01 (m, 4 H), 3.41 (t, 2 H), 5.35 (m, 2 H). ^{13}C NMR ($CDCl_3$): δ = 14.0, 22.3, 26.9, 27.2, 28.2, 28.8, 29.3, 29.4, 29.5, 29.7, 31.9, 32.7, 33.9, 129.8.

General Procedure for the Synthesis of Carbinols 5:[21] A solution of bromide **9** (40 mmol) in dry ether (10 mL) was slowly added under nitrogen to 40 mmol of magnesium turnings in dry ether (20 mL). To start the reaction, some crystals of iodine were added and the mixture was briefly heated. The bromide solution was subsequently added at such a rate that the reaction continued to reflux without additional heating. After completion, the mixture was refluxed for a further 30 min, and ethyl formate (20 mmol) was added dropwise, followed by an additional 1 h of refluxing. The mixture was cooled to 0 °C and water (5 mL) was carefully added, followed by a 1 M sulfuric acid solution (ca. 15 mL). The two phases slowly separated after addition of water (100 mL) and ether (200 mL). The organic layer was washed with water (3 × 100 mL) and brine and dried with Na_2SO_4. After removal of the solvents under reduced pressure, oils were purified on Al_2O_3 (act. II−III), with chloroform/hexane (1:1) as eluent, to yield wax-like material, whereas solid materials were crystallized from acetone to afford white solid materials. Yields typically ranged from 40−75%.

Bis[(*Z*)-11-tetradecenyl]carbinol (5a): White powder obtained after crystallisation from acetone; yield 3.44 g, 41%, m.p. 57−58 °C. ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.27 (m, br., 36 H), 2.03 (m, 8 H), 3.54 (m, 1 H), 5.36 (m, 4 H). ^{13}C NMR ($CDCl_3$): δ = 14.4, 20.5, 25.6, 27.1, 29.3, 29.5, 29.6, 29.7, 29.8, 37.5, 72.0, 129.3, 131.5.

Bis(tetradecyl)carbinol (5b): White powder obtained after crystallisation from acetone; yield 4.66 g, 55%, m.p. 85−87 °C. ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.26 (m, br., 52 H,), 3.58 (m, 1 H). ^{13}C NMR ($CDCl_3$): δ = 14.1, 22.7, 25.6, 29.4, 29.7, 31.9, 37.5, 72.0.

Bis[(*Z*)-11-hexadecenyl]carbinol (5c): Chromatography on Al_2O_3 (act. II−III) with chloroform/hexane (1:1) followed by crystallis-

ation from acetone yielded **5c** as a white powder; 5.05 g, 53%, m.p. 47−48 °C. ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.27 (m, 44 H), 2.01 (m, 8 H), 3.56 (m, 2 H), 5.35 (m, 4 H). ^{13}C NMR ($CDCl_3$): δ = 14.0, 22.3, 25.6, 26.9, 27.2, 29.3, 29.5, 29.6, 29.7, 29.8, 31.9, 37.5, 72.0, 129.8, 129.9.

Bis(hexadecyl)carbinol (5d): The compound was obtained after Soxhlet extraction with chloroform. Subsequent crystallisation from chloroform afforded a white powder; yield 4.51 g, 47%, m.p. 90−92 °C. ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.26 (m, 60 H), 3.59 (m, 1 H). ^{13}C NMR ($CDCl_3$): δ = 12.6, 21.2, 24.2, 27.9, 28.1−28.3 (several), 30.4, 36.0, 70.5.

General Procedure for the Synthesis of Pyridinium Esters 6: Isonicotinoyl chloride hydrochloride (**4**; 3.0 mmol) was added in small amounts to a refluxing solution of carbinol **5** (2.5 mmol) in toluene (20 mL). Triethylamine (7.0 mmol) was then added dropwise, and the mixture was refluxed overnight. After filtration, the solvents were removed and the resulting yellow-white solid was purified on Al_2O_3 (act. II−III) with a dichloromethane/hexane (1:1) to pure dichloromethane gradient followed by crystallization from acetonitrile to afford esters **6** as white solids in yields ranging from 60−70%.

Bis[(*Z*)-11′′-tetradecenyl]methyl Pyridine-4-carboxylate (6a): Chromatography on Al_2O_3 (act. II−III) with a dichloromethane/hexane (1:1) to pure dichloromethane gradient afforded **6a** as a colourless oil; yield 0.90 g, 69%. ^{1}H NMR ($CDCl_3$): δ = 0.93 (t, 6 H), 1.24 (m, br., 44 H), 1.66 (m, 4 H), 1.98 (m, 8 H), 5.15 (q, 1 H), 5.32 (m, 4 H), 7.88 (d, $^3J_{AB}$ = 5.3 Hz, 2 H), 8.78 (d, $^3J_{AB}$ = 5.3 Hz, 2 H). ^{13}C NMR ($CDCl_3$): δ = 20.3, 22.9, 25.5, 29.5, 29.7, 29.7, 29.8, 32.1, 34.2, 123.1, 138.2, 150.7, 165.0.

Bis(tetradecyl)methyl Pyridine-4-carboxylate (6b): Chromatography on Al_2O_3 (act. II−III) with a dichloromethane/hexane (1:1) to pure dichloromethane gradient followed by low temperature crystallisation from acetonitrile afforded **6b** as a white solid; yield 0.87 g, 66%, m.p. 38.0−39.6 °C. ^{1}H NMR ($CDCl_3$): δ = 0.87 (t, 6 H), 1.26 (m, br., 48 H), 1.68 (m, 4 H), 5.15 (q, 1 H), 7.88 (d, $^3J_{AB}$ = 5.3 Hz, 2 H), 8.78 (d, $^3J_{AB}$ = 5.3 Hz, 2 H). ^{13}C NMR ($CDCl_3$): δ = 20.3, 22.9, 25.5, 29.5, 29.7, 29.7, 29.8, 32.1, 34.2, 123.1, 138.2, 150.7, 165.0.

Bis[(*Z*)-11′′-hexadecenyl]methyl Pyridine-4-carboxylate (6c): Chromatography on Al_2O_3 (act. II−III) with a dichloromethane/hexane (1:1) to pure dichloromethane gradient afforded **6c** as a colourless oil; yield 0.93 g, 64%. ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.30 (m, br., 52 H), 1.67 (m, 4 H), 2.01 (m, 8 H), 5.14 (q, 1 H), 5.34 (m, 4 H), 7.85 (d, $^3J_{AB}$ = 8.9 Hz, 2 H), 8.76 (d, $^3J_{AB}$ = 8.9 Hz, 2 H). ^{13}C NMR ($CDCl_3$): δ = 17.2, 22.3, 25.3, 29.7, 27.1, 29.3, 29.5, 29.7, 31.9, 34.0, 76.3, 122.8, 129.8, 138.0, 150.5, 164.8.

Bis(hexadecyl)methyl Pyridine-4-carboxylate (6d): Chromatography on Al_2O_3 (act. II−III) with a dichloromethane/hexane (1:1) to pure dichloromethane gradient followed by low temperature crystallisation from acetonitrile afforded **6d** as a white solid: yield 0.98 g, 67%, m.p. 47.8−49.8 °C. ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.24 (m, br., 56 H), 1.66 (m, 4 H), 2.01 (m, 8 H), 5.14 (q, 1 H), 5.32 (m, 4 H), 7.83 (d, $^3J_{AB}$ = 5.9 Hz, 2 H), 8.76 (d, $^3J_{AB}$ = 5.9 Hz, 2 H). ^{13}C NMR ($CDCl_3$): δ = 14.3, 20.4, 25.2, 27.0, 29.2, 29.4, 29.7, 34.0, 76.2, 122.8, 129.2, 131.4, 131.7, 137.9, 150.5, 164.7.

General Procedure for the Synthesis of Methylated Pyridinium Esters 2: A solution of pyridine ester **6** (1 mmol) and methyl iodide (9 mmol) in dry acetone (20 mL) was refluxed for 5−6 h under nitrogen, with the progress being monitored by TLC. The mixture was taken to dryness and the crude iodide salts were used in ion exchange without additional purification. Ion exchange chromatography on a Sephadex column (chloride form, DEAE, A25) with methanol as eluent afforded white (waxy) solids that were recrystallized from acetone/acetonitrile mixtures. All compounds showed liquid crystalline behaviour, depending upon the amount of water present. Yields were 60−90%.

4-({Bis[(*Z*)-11′-tetradecenyl]methyl}oxycarbonyl)-1-methylpyridinium Chloride (2a): Off-white, gel-like solid; yield 0.34 g, 60%.

Iodide Salt: ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.25 (m, br., 44 H), 1.71 (m, 4 H), 2.06 (m, 8 H), 4.86 (br. s, 3 H), 5.19 (q, 1 H), 5.34 (m, 4 H), 8.51 (d, $^3J_{AB}$ = 5.9 Hz, 2 H), 9.50 (d, $^3J_{AB}$ = 5.9 Hz, 2 H).

Chloride Salt: ^{1}H NMR ($CDCl_3$): δ = 0.92 (t, 6 H), 1.25 (m, br., 44 H), 1.68 (m, 4 H), 2.05 (m, 8 H), 4.94 (br. s, 3 H), 5.17 (q, 1 H), 5.35 (m, 4 H), 8.45 (d, $^3J_{AB}$ = 6.1 Hz, 2 H), 9.79 (d, $^3J_{AB}$ = 6.1 Hz, 2 H). ^{13}C NMR (CDCl3): δ = 14.4, 20.5, 25.3, 27.1, 29.3, 29.5, 29.6, 29.7, 33.8, 49.7, 79.1, 127.3, 129.3, 131.5, 147.3, 161.1. $C_{36}H_{62}ClNO_2$ (576.35 + 1.0 H_2O): calcd. C 72.75, H 10.85, Cl 5.96, N 2.36; found C 72.51, H 10.97, Cl 5.83, N 2.41.

4-{[Bis(tetradecyl)methyl]oxycarbonyl}-1-methylpyridinium Chloride (2b): White solid, crystallised from acetone; yield 0.43 g, 73%, m.p. 155 °C.

Iodide Salt: ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.25 (m, br., 48 H), 1.71 (m, 4 H), 4.83 (br. s, 3 H), 5.19 (q, 1 H), 8.51 (d, $^3J_{AB}$ = 6.3 Hz, 2 H), 9.50 (d, $^3J_{AB}$ = 6.3 Hz, 2 H).

Chloride Salt: ^{1}H NMR ($CDCl_3$): δ = 0.87 (t, 6 H), 1.24 (m, br., 48 H), 1.67 (m, 4 H), 4.93 (s, 3 H), 5.18 (q, 1 H), 8.45 (d, $^3J_{AB}$ = 6.4 Hz, 2 H), 9.77 (d, $^3J_{AB}$ = 6.4 Hz). ^{13}C NMR ($CDCl_3$): δ = 14.1, 22.7, 25.3, 29.3, 29.5, 29.6, 31.9, 33.8, 49.8, 79.4, 127.5, 145.1, 161.3. $C_{36}H_{66}ClNO_2$ (580.38): calcd. C 74.50, H 11.46, Cl 6.11, N 2.41; found C 74.47, H 12.12, Cl 6.07, N 2.61.

4-({Bis[(*Z*)-11′-hexadecenyl]methyl}oxycarbonyl)-1-methylpyridinium Chloride (2c): Off-white, gel-like solid; yield 0.38 g, 60%.

Iodide Salt: ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.25 (m, br., 52 H), 1.69 (m, 4 H), 2.03 (m, 8 H), 4.86 (br. s, 3 H), 5.19 (q, 1 H), 5.34 (m, 4 H), 8.47 (d, $^3J_{AB}$ = 6.2 Hz, 1 H), 9.55 (d, $^3J_{AB}$ = 6.2 Hz, 2 H).

Chloride Salt: ^{1}H NMR ($CDCl_3$): δ = 0.89 (t, 6 H), 1.28 (m, br., 52 H), 1.69 (m, 4 H), 2.01 (m, 8 H), 4.94 (br. s, 3 H), 5.17 (q, 1 H), 5.35 (m, 4 H), 8.45 (d, $^3J_{AB}$ = 6.4 Hz, 2 H), 9.78 (d, $^3J_{AB}$ = 6.4 Hz, 2 H). ^{13}C NMR ($CDCl_3$): δ = 14.0, 22.3, 25.3, 26.9, 27.2, 29.3, 29.5, 29.6, 29.7, 31.9, 33.8, 49.8, 79.0, 127.3, 129.8, 144.7, 127.3, 161.1. $C_{40}H_{70}ClNO_2$ (632.46 + 1.0 H_2O): calcd. C 73.86, H 11.16, Cl 5.45, N 2.15; found C 73.77, H 11.22, Cl 5.35, N 2.18.

4-{[Bis(hexadecyl)methyl]oxycarbonyl}-1-methylpyridinium Chloride (2d): Off-white solid; yield 0.48 g, 75%.

Iodide Salt: ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.24 (m, br., 56 H), 1.69 (m, 4 H), 4.80 (br. s, 3 H), 5.19 (q, 1 H), 8.49 (d, $^3J_{AB}$ = 5.4 Hz, 2 H), 9.43 (d, $^3J_{AB}$ = 5.4 Hz, 2 H);

Chloride Salt: ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 6 H), 1.24 (m, br., 56 H), 1.68 (m, 4 H), 4.93 (br. s, 3 H), 5.16 (q, 1 H), 8.45 (d, $^3J_{AB}$ = 5.5 Hz, 2 H), 9.79 (d, $^3J_{AB}$ = 5.5 Hz, 2 H). ^{13}C NMR ($CDCl_3$): δ = 14.4, 22.9, 25.6, 29.6, 29.7, 29.9, 32.2, 34.1, 49.8, 79.4, 127.5, 145.1, 161.3. $C_{40}H_{74}ClNO_2$ (636.49 + 1.0 H_2O): calcd. C 73.41, H 11.70, Cl 5.42, N 2.14; found C 73.31, H 11.83, Cl 5.26, N 2.25.

4-Isopropyloxycarbonyl-1-methylpyridinium Chloride (2e): Low-melting, off-white solid; yield 0.19 g, 89%.

Iodide Salt: ^{1}H NMR ($CDCl_3$): δ = 1.40 (t, 6 H), 4.84 (br. s, 3 H), 5.43 (m, 1 H), 8.67 (d, $^3J_{AB}$ = 6.6 Hz, 2 H), 9.76 (d, $^3J_{AB}$ = 6.6 Hz, 2 H).

Chloride Salt: ^{1}H NMR ($CDCl_3$): δ = 1.42 (t, 6 H), 4.82 (br. s, 3 H), 5.38 (m, 1 H), 8.48 (d, $^3J_{AB}$ = 6.6 Hz, 2 H), 9.53 (d, $^3J_{AB}$ = 6.6 Hz, 2 H). ^{13}C NMR ($CDCl_3$): δ = 20.2, 48.7, 70.7, 125.8, 143.8, 145.5, 159.2. $C_{10}H_{14}ClNO_2$ (215.68): calcd. C 55.69, H 6.54, Cl 16.44, N 6.49; found C 55.60, H 6.61, Cl 16.26, N 6.54.

Transfection

Preparation of Vesicles: A solution of amphiphile, alone or in a 1:1 molar ratio with DOPE and in a minimum amount of chloroform or methanol, was concentrated under a stream of nitrogen. Residual solvent was removed under vacuum and the resulting lipid film was hydrated in water to a total lipid concentration of 1 mM, sonicated to clarity at 45 °C with a Branson Sonifier Cell Disrupter B15 and used immediately.

Transfection Experiments: A 7.1 kb plasmid containing the E. coli β-galactosidase gene under control of the cytomegalo virus immediate early gene promoter/enhancer (pCMV β-gal Clontech, Palo Alto, CA, USA) was used as the reporter gene. DNA was isolated from E. coli with a Qiagen Plasmid Kit (QIAGEN® Inc., USA). The plasmid concentration was determined by measuring the absorption at λ = 260 nm, using the relation 1.0 OD = 50 μg/mL. Typically, the OD_{260}/OD_{280} ratio was 1.95. COS-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM® Gibo®, The Netherlands) containing 7% of fetal calf serum, 2 mM of L-glutamine, 100 units/mL of penicillin and 100 mg/mL of streptomycin at 37 °C in CO_2/air (1:19). Cells ($1\cdot10^5$ cells/well) were seeded in 12-well plates and allowed to grow overnight. The complex of SAINT/DOPE (1:1) with pCMV β-gal was prepared at a charge ratio of 2.5:1 (15 nm of lipid and 1 μg of DNA), in 100 μL of 10 mM HEPES buffer (pH = 7.4, 150 mM NaCl). After 10−15 min of incubation at room temperature, the lipoplex was diluted in 1 mL of DMEM medium, and 0.5 mL of the mixture was added to the cells and incubated for 4 h at 37 °C. The β-gal assay was performed 48 h later on lysate (5 or 15 μL), by using the CPRG substrate (Boehringer, Mannheim).

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