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Interfacial indazolization: novel chemical evidence for remarkably high exo-surface pH of cationic liposomes used in gene transfection

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Abstract

Cationic liposomes are used as the carriers of polyanionic genes for combating against hereditary diseases in gene therapy. Studies directed to careful biophysical characterizations of the cationic liposomes commonly used in gene delivery have just begun. Herein, we report on a novel liposomal exo-surface bound indazolization reaction of an amphiphilic arenediazonium salt as evidence for the existence of remarkably alkaline exo-surface of cationic liposomes commonly used in gene transfection. Our results demonstrate that formation of 5-hexadecyl-7-methylindazole in thermal indazolization of 2,6-dimethyl-4-hexadecylbenzenediazonium tetrafluoroborate bound to liposome surface is a strong indication for the existence of significantly high exo-surface pH for cationic liposomes commonly used in gene delivery. The present method can be used in determining the relative exo-surface basicities of various cationic liposomes used in gene transfection and subsequently to find any possible correlation between the transfection efficiencies of these liposomes and their exo-surface basicities. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Gene transfection; Cationic liposome; Surface pH; Interfacial indazolization

1. Introduction

Transfection vectors including retrovirus [1,2], adenovirus [3], cationic liposomes [4–13], etc., are used as the carriers of polyanionic genes for combating against hereditary diseases in gene therapy. Reproducibility, high degree of targetability and low cellular toxicity associated with cationic liposomal transfection vectors have generated an unprecedented upsurge of multidisciplinary interests in developing efficient liposomal gene delivery vehicles. Since the first report of cationic lipid mediated gene delivery by Felgner et al. [4], an impressive number of efficient liposomal transfection vectors have been synthesized [14–23]. The key factors governing DNA–lipid interactions are still poorly understood. Reports on the structural details of the complexes between the cationic liposomes and DNA are appearing almost in every issue of the relevant scientific journals [6,7,15,24–41]. On the contrary, studies directed to careful biophysical characterizations of the cationic liposomes commonly used in gene delivery have just

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Abbreviations: DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; DOTMA, *N*-[1-(2,3-dioleoyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium chloride; DHDAB, dihexadecyl-dimethylammonium bromide; 16-Ind, 5-hexadecyl-7-methylindazole; 1-Ind, 5,7-dimethylindazole

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begun. Nantz and coworkers [15] recently studied the correlation of lipid hydration with in vivo transfection activity, and demonstrated that cationic lipids possessing the greatest imbalance between the effective cross-sectional areas occupied by their polar and hydrophobic domains are the most active transfection agents. They have also studied the counterion influence on the cationic lipid mediated transfection of plasmid-DNA [32]. The first step in the liposomal transfection process, namely, the formation of complexes between the negatively charged DNA and the cationic liposomes, is dictated by electrostatic interactions. The surface charge of a cationic liposome and the DNA–lipid interactions are most likely to be dependent on the interfacial pH. Using a pH-sensitive fluorophore, Zuidam and Barenholz [26] have recently studied the electrostatic parameters of the cationic liposomes commonly used in gene delivery, and have demonstrated the presence of a large positive surface potential and a high pH at the cationic liposomal surface. In their most recent work [42], they have also demonstrated that the electrostatic parameters play a key role in the formation of DNA–lipid complexes. Herein, we report on a novel liposomal exo-surface bound indazolization reaction of an amphiphilic arenediazonium salt as evidence for the existence of remarkably alkaline exo-surface of cationic liposomes commonly used in gene transfection. Our results demonstrate that formation of 5-hexadecyl-7-methylindazole in thermal indazolization of 2,6-dimethyl-4-hexadecylbenzenediazonium tetrafluoroborate bound to liposome surface is a strong indication for the existence of significantly high exo-surface pH for cationic liposomes commonly used in gene delivery.

2. Materials and methods

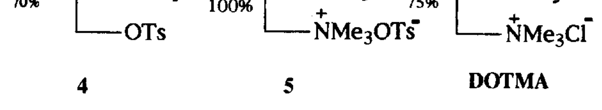
2.1. Materials

2,6-Dimethyl-4-hexadecyl benzene diazonium tetrafluoroborate (16-ArN$_2^+$BF$_4^-$) and 1-ArN$_2^+$BF$_4^-$ were synthesized following the procedure described in Refs. [43,44]. 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), dihexadecyl-dimethylammonium bromide (DHDAB), benzyl bromide, trimethyl silyl iodide and *rac*-1,2-isopropylidene-*sn*-glycerol were purchased from Fluka (Switzerland) and used without further purification. Amberlyst A-26 chloride ion exchange resin and *rac*-3-(dimethylamino)-1,2-propanediol were purchased from Lancaster, UK and Aldrich, USA. Cholesterol was purchased from Loba Chemicals (India) and was crystallized two times from methanol before use. All the other reagents used in the synthesis were procured from Spectrochem (India) and were of highest analytical grade. All the solvents used were of HPLC grade and were purchased from Qualigens (India).

2.2. Synthesis

The reported synthesis of DOTMA (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride) [4] consists of dioleoylation of *rac*-3-(dimethylamino)-1,2-propanediol with oleoyl *p*-toluene sulfonate in xylene at high temperature followed by quaternization of the resulting tertiary amine using condensed methyl chloride at 70°C in a sealed Paar apparatus. There are neither any yield reports at any step nor any spectral characterization of the final

Scheme I. Synthesis of DOTMA



Reagents : (a) Oleyl Mesylate(2.2eqv)/KOH (30 eqv)/ Xylene/6 hrs; (b) TMSI (6 eqv)/DCM/14 hrs;
(c) TsCl (3 eqv)/ DMAP (3 eqv)/DCM/0°C-R.T. 43 hrs;
(d) Me$_3$N-MeOH/60°C/sealed tube/48 hrs; (e) Amberlyst A-26 chloride ion exchange resin.

Scheme 1.

product in this published synthesis of DOTMA, and for this reason we felt the need to develop a well characterized chemical synthesis of DOTMA, the details of which are outlined below.

2.2.1. Synthesis of DOTMA

DOTMA was synthesized from *rac*-3-*O*-benzyl-*sn*-glycerol **1** (prepared by conventional benzylation of *rac*-1,2-isopropylidene-*sn*-glycerol using sodium hydride and benzyl bromide in dry dimethylformamide under nitrogen followed by quantitative deprotection of isopropylidene group by refluxing it in 80:20 (v/v) methanol/water containing few drops of added concentrated hydrochloric acid) in five steps as outlined in Scheme 1 with an overall yield of 41%. Dioleoylation [45] of **1** (1.22 g, 6.7 mmol) with 30 eq. of dried and powdered potassium hydroxide and 4.9 g (14 mmol) of oleoyl mesylate (prepared in three steps from commercially available oleic acid in overall 77% yield: (a) MeOH/2%H_2SO_4/6 h reflux; (b) LAH (3 eq.)/THF/0°C 3 h; and (c) CH_3SO_2Cl (1.5 eq.)/Py (1.5 eq.)/DMAP (cat)/DCM/0°C R.T./6 h) was carried out by refluxing in xylene for 6 h using a Dean–Stark apparatus. After removing xylene by distillation, the residue was extracted with 150 ml diethyl ether. The ether extract was washed thoroughly with water, dried with anhydrous sodium sulfate, filtered, and ether was rotavapored from the filtrate. Pure dioleoyl product **2** (3.6 g, 78.6% yield from **1**) was isolated from the residue by silica gel column chromatography using 8–10% ethyl acetate in pet-ether as the eluent. The benzyl group of **2** was then removed with trimethylsilyl iodide (7.35 g, 36 mmol) in dry dichloromethane following the protocol described in Ref. [46]. Tosylation of the resulting debenzylated compound **3** (3.2 g, 100% yield from **2**) was carried by stirring with *p*-tosyl chloride (3 g, 15.7 mmol) and DMAP (2.1 g, 17.2 mmol) in dry dichloromethane at room temperature for 40 h. The dichloromethane was removed by rotatory evaporation, and pure tosylated product **4** (2.7 g, 70% yield from **3**) was isolated from the residue by silica gel column chromatography using 2–5% ethyl acetate in pet-ether as the eluent. Quaternization of **4** with methanolic trimethylamine in a sealed tube for 20 h at 60–65°C afforded *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium tosylate (2.9g, 3.6 mmol) **5**, in 100% crude yield from **4**. The crude quaternary salt **5** was not purified. It was dissolved in chloroform, loaded over a freshly generated Amberlyst A-26 chloride ion exchange column, and eluted with chloroform. After evaporating the eluent chloroform, pure DOTMA (1.8 g, 75% yield from **5**) was finally obtained by recrystallization of the residue from acetone at −18°C.

^{1}H-NMR of DOTMA (400 MHz, $CDCl_3$): δ/ppm = 0.88 (t, 6H, $C\underline{H}_3–C_{17}H_{32}–$); 1.20–1.34 (m, 44H, $–(C\underline{H}_2)_n–$); 1.58 (m, 4H, $–C\underline{H}_2–CH_2–O–$); 1.90–2.08 (m, 8H, $–C\underline{H}_2–CH=CH–C\underline{H}_2–$); 3.34–3.48 (2t, 4H, $–CH_2–C\underline{H}_2–O–$); 3.54 (s, 9H, $(C\underline{H}_3)_3N^+–$); 3.60 (dd, 1H, $\underline{H}^1CH^2OR–CHOR–CH_2–N^+(C\underline{H}_3)_3$); 3.68 (m, 2H, $CH_2OR–CHOR–C\underline{H}_2–N^+(CH_3)_3$); 4.03 (dd, 1H, $H^1C\underline{H}^2OR–CHOR–CH_2–N^+(CH_3)_3$); 4.09 (m, 1H, $H^1CH^2OR–C\underline{H}OR–CH_2–N^+(CH_3)_3$); 5.32 (m, 4H, $–CH_2–C\underline{H}=C\underline{H}–CH_2–$).

HRMS (FABS) *m/z*: Calcd (for $C_{42}H_{84}NO_2$, the 4°-ammonium ion) 634.6502, found 634.6484.

2.2.2. Synthesis of 5-hexadecyl-7-methylindazole (16-Ind)

2.6 g (7.13 mmol) hexadecyl trimethyl ammonium bromide (CTAB) was dissolved in 500 ml 20 mM aqueous phosphate buffer (pH 7.0) to prepare a cationic micellar solution. 110 mg (0.25 mmol) of 16-$ArN_2^+BF_4^-$ dissolved in 10 ml HPLC grade acetonitrile was added to the micellar solution and the solution was kept stirred overnight (12 h). The solution turned light yellow, and 1.5 g (10.7 mmol) of sodium perchlorate monohydrate was added to this yellow solution. Immediately, a heavy white precipitate appeared. The precipitate was filtered and washed with a copious amount of water. The washed precipitate was air-dried using a water aspirator for 4 h and finally the air-dried precipitate was vacuum-dried for 2 h. The dried precipitate was then extracted with ether (3×200 ml). The combined ether extract was dried with anhydrous sodium sulfate and filtered. The ether was removed from the filtrate using a rotatory evaporator and 16-Ind was isolated from the residue by silica gel column chromatography, changing the eluent from pet-ether to pet-ether/ethyl acetate (88:12, v/v). The fractions with $R_f = 0.6$ (using 60:40 pet-ether/ethyl acetate, the TLC developing solvent) were combined and the solvent was evaporated. The white residue left after evaporation of the solvent afforded 50 mg of pure 16-Ind (57% yield) upon crystallization from acetone.

^{1}H-NMR of 16-Ind (200 MHz, $CDCl_3$): δ/ppm = 0.88 (t, 3H, $C\underline{H}_3$–$C_{15}H_{31}$–); 1.20–1.34 (m, 26H, –$(C\underline{H}_2)_n$–); 1.68 (m, 2H, –$C\underline{H}_2$–CH_2–Ar); 2.50 (s, 3H, $C\underline{H}_3$–Ar); 2.68 (t, 2H, –CH_2–$C\underline{H}_2$–Ar); 7.0 (s, 1H, Ar$\underline{H}$ *para* to –CH = N–); 7.37 (s, 1H, Ar$\underline{H}$ *ortho* to –CH = N–); 8.0 (s, 1H, Ar–C$\underline{H}$ = N–NH–Ar).

HRMS *m/z*: Calcd (for $C_{24}H_{40}N_2$) 356.3192, found 356.3196.

2.2.3. Synthesis of 5,7-dimethylindazole (1-Ind)

1.0 g (4.27 mmol) of 1-$ArN_2^+BF_4^-$ was added to 500 ml aqueous 0.1 M NaOH solution and the solution was kept stirred overnight (12 h). The reddish-orange solution was then extracted with ether (3×200 ml). The combined ether extract was dried with anhydrous sodium sulfate and filtered. The ether was removed from the filtrate using rotatory evaporator and 1-Ind was isolated from the red residue by silica gel column chromatography, changing the eluent from pet-ether to pet-ether/ethyl acetate (80:20, v/v). The fractions with $R_f = 0.4$ (using 50:50 pet-ether/ethyl acetate, the TLC developing solvent) were combined and the solvent was evaporated. The light yellow solid residue left after evaporation of the solvent afforded 70 mg of pure white 1-Ind (11.2% yield) upon activated norit treatment in hot methanol followed by crystallization from ethyl acetate containing a few drops of methanol.

^{1}H-NMR of 16-Ind (200 MHz, $CDCl_3$): δ/ppm = 2.38 (s, 3H, $C\underline{H}_3$–Ar *para* to –NH–); 2.50 (s, 3H, $C\underline{H}_3$–Ar *ortho* to –NH–); 6.9 (s, 1H, Ar$\underline{H}$ *para* to –CH = N–); 7.28 (s, 1H, Ar$\underline{H}$ *ortho* to –CH = N–); 7.9 (s, 1H, Ar–C$\underline{H}$ = N–NH–Ar).

HRMS *m/z*: Calcd (for $C_8H_{10}N_2$) 146.0844, found 146.0843.

2.2.4. Synthesis of DOTAP

DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride) was synthesized basically following the protocol reported by Leventis and Silvius [23]. The iodide counterion was exchanged with chloride ion by ion-exchange chromatography using an Amberlyst A-26 chloride ion exchange column.

^{1}H-NMR of DOTAP (200 MHz, $CDCl_3$): δ/ppm = 0.88 (t, 6H, $C\underline{H}_3$–CH_2–$C_{15}H_{28}$–); 1.20–1.40 (m, 40H, –$(C\underline{H}_2)_n$–); 1.50–1.68 (m, 4H, –$C\underline{H}_2$–CH_2–CO–O–); 1.90–2.08 (m, 8H, –$C\underline{H}_2$–CH = CH–$C\underline{H}_2$–); 2.21–2.40 (2t, 4H, –CH_2–$C\underline{H}_2$–CO–O–); 3.42 (s, 9H, $(C\underline{H}_3)_3N^+$–); 3.70–3.90 (m, 1H, CH_2OCOR–CHOCOR–$\underline{H}^1H^2C$–$N^+(C\underline{H}_3)_3$); 4.02–4.18 (m, 1H, CH_2OCOR–CHOCOR–$H^1\underline{H}^2C$–$N^+(C\underline{H}_3)_3$); 4.21–4.38 (m, 1H, $\underline{H}^1CH^2OCOR$–CHOCOR–CH_2–$N^+(CH_3)_3$); 4.45–4.59 (m, 1H, $H^1C\underline{H}^2OCOR$–CHOCOR–CH_2–$N^+(CH_3)_3$); 5.32 (m, 4H, –CH_2–$C\underline{H}$ = $C\underline{H}$–CH_2–); 5.61 (m, 1H, CH_2OCOR–$C\underline{H}OCOR$–CH_2–$N^+(CH_3)_3$).

HRMS (LSIMS) *m/z*: Calcd (for $C_{42}H_{80}NO_4$, the 4°-ammonium ion) 662.6087, found 662.6089.

2.3. Methods

2.3.1. High-performance liquid chromatography (HPLC)

The yields of both the short-chain and the long-chain indazole products (1-Ind and 16-Ind, respectively) were determined by quantitative HPLC. The LC system consisted of a Shimadzu Model LC10A and a Hewlett Packard HP series 1050 high-performance liquid chromatograph. A 20-μl sample loop was used for quantitative determination of both 1-Ind and 16-Ind. The wavelength of the UV detector was set at 212 nm in an HP series 1050 HPL chromatograph for detection of 1-Ind. The separation of the 1-Ind from other products was effected on a 4.6×250 mm Whatman reverse phase C18 column. The mobile phase used was acetonitrile/water (60:40, v/v) with a flow gradient where the initial flow rate (0.6 ml/min) was maintained up to 8.0 min and changed to 1.5 ml/min at 13.0 min. This final flow rate was maintained for 12 min before returning to the initial flow rate. 16-Ind was detected in a Shimadzu Model LC10A HPL chromatograph using a UV detector at the wavelength of 219 nm. 16-Ind was separated from the other products using a 3.5×150 mm Nova-Pak C18 (Waters) steel cartridge column. The initial flow rate (0.6 ml/min) was linearly increased to 1.0 ml/min at 25.0 min using a constant mobile phase acetonitrile/methanol/isopropanol/water (100:75:70:9.5, v/v).

2.3.2. Preparation of small unilamellar liposomes with bound 16-$ArN_2^+BF_4^-$

The lipid mixtures in the desired ratio were dissolved in chloroform in a glass vial. The chloroform was removed with a thin flow of moisture-free nitrogen and the dried film of lipid left in the vial was then kept under high vacuum for 8 h. Five ml aqueous buffer was added to the vacuum-dried lipid film and the mixture was allowed to swell for 15 h (overnight). The hydrated lipid mixture was then sonicated at 60–70°C in a ULTRAsonik 28X bath sonicator at maximum power for 10 min. The resulting multilamellar vesicles were sonicated at 100% duty cycle and 25 W output power in a Branson 450 Ti-probe sonicator for 3–5 min. The translucent solutions were finally extruded five times through 50-nm pore size polycarbonate membranes under a 200–300 p.s.i. nitrogen pressure using a Lipex Biomembrane Extruder system. To 1.5 ml of the resulting optically almost transparent small unilamellar liposomes were added 15 μl of an appropriate stock solution of the exo-surface basicity sensor, 16-$ArN_2^+BF_4^-$, so that in all the experiments the molar ratio of lipid to surface basicity sensor were 100 or 50 to 1.

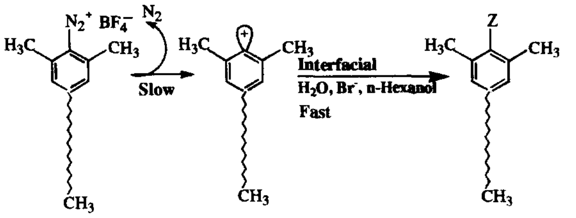
3. Results and discussion

3.1. Background

Romsted and his coworkers have demonstrated that the local molar concentrations of water, counterion and alcohol co-surfactant in the interfacial region of CTAB-based cationic microemulsions can be determined using the product distribution of the interface-bound dediazoniation reaction of (16-$ArN_2^+BF_4^-$), an amphiphilic arene diazonium salt [43,44]. In this method for determining the interfacial concentrations, the aggregate-bound probe molecule is allowed to undergo thermal dediazoniation reaction in presence of acid in the dark. The relative amounts of the three dediazoniation products namely, 16-ArOH, 16-ArBr and 16-ArOHex (determined quantitatively by HPLC analysis of the dediazoniation product mixtures) were shown to be dependent on the local molar concentrations of water, bromide ions and *n*-hexanol, respectively (Scheme 2). In our pursuit of deciphering any possible correlation existing between the transfection efficiencies of various cationic liposomes and the exo-surface concentrations of water and counterions, we decided to extend the use of the same interfacial probe (16-$ArN_2^+BF_4^-$) for determining the local molar concentrations of water and counterions present in the interfacial regions of various small unilamellar cationic liposomes prepared at pH 7.0. To our utter surprise, the HPLC chromatograms for the product mixtures formed from the cationic liposome-bound reaction of the probe molecule showed a significant new peak right after the 16-ArOH peak in all the chromatograms. The same product was also formed in significant yields when the probe molecule was dissolved in aqueous CTAB at pH 7 and the micellar solution was heated at 50°C. Isolation in pure form followed by NMR, high-resolution mass spectral characterization of the purified product (as described in Section 2.2.2) and HPLC spiking experiment conclusively revealed the identity of the peak that appeared after the 16-ArOH peak as 5-hexadecyl-7-methylindazole (16-Ind in Fig. 1). More than three decades back, in

Scheme II

(A) Interfacial dediazoniation of CTAB-based cationic microemulsion associated interfacial probe.

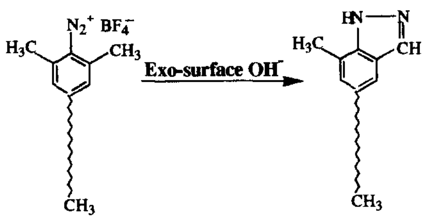


16-ArN$_2^+$ (Interfacial Probe)

16-Ar$^+$

16-ArOH when z = OH
16-ArBr when z = Br
16-ArOHex when z = OHex

(B) Indazolization of 16-ArN$_2^+$ associated with the exo-surface of cationic liposomes used in gene delivery.



16-ArN$_2^+$ (Interfacial Probe)

16-Ind

Scheme 2.

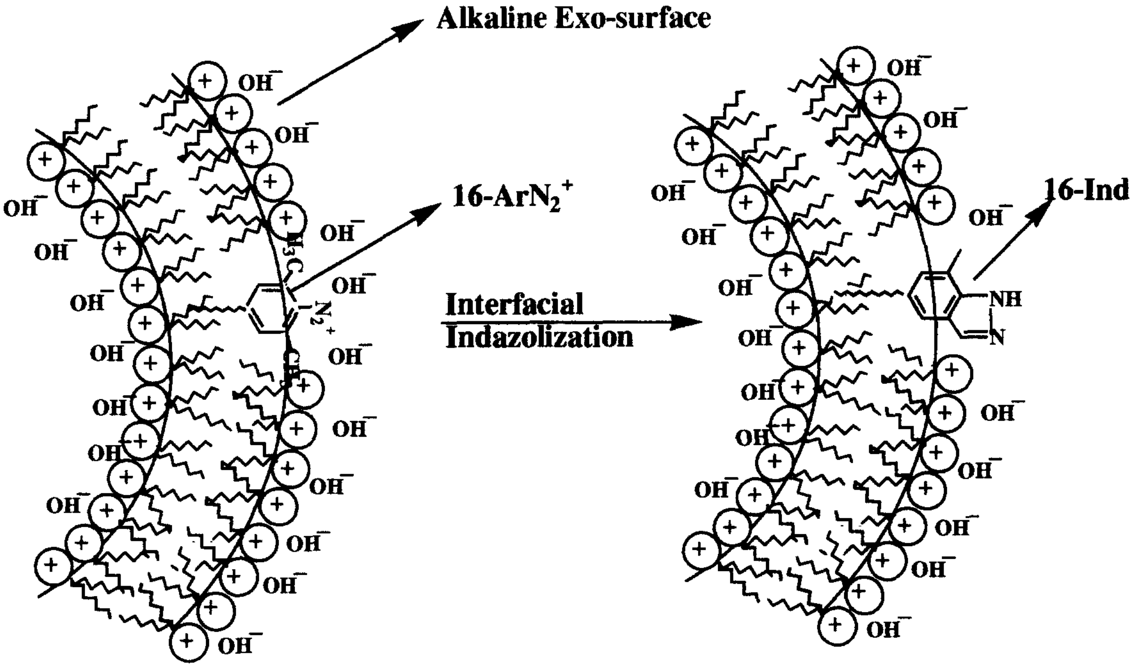


Fig. 1. Indazolization of 2,6-dimethyl-4-hexadecylbenzenediazonium salt (16-ArN$_2^+$) bound to the exo-surface of a cationic liposome.

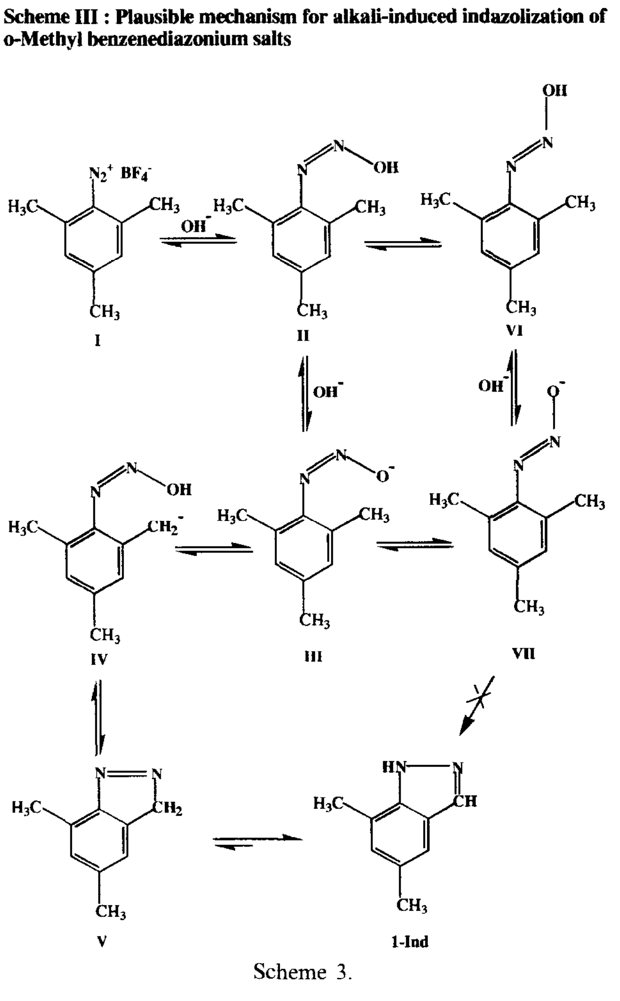
their investigations on the rearrangement of benzeneazotribenzoylmethane and its derivatives, Curtin and Poutsma [47] reported the formation of 5,7-dimethylindazole (1-Ind in Scheme 3) in low yield (9%) when 2,4,6-trimethyl benzene diazonium salt was treated with strong alkali. This report suggested to us that the origin of formation of 16-Ind (Scheme 2) in the liposome-associated conversion of 16-ArN$_2^+$BF$_4^-$ is probably the remarkable alkaline exo-surface of the cationic liposomes (Fig. 1).

3.2. *Correlation between %1-Ind and pH in the non-liposomal indazolization*

The yields of indazole not being the main focus of their investigations, Curtin and Poutsma, in their report [47], did not mention anything about the dependence of 1-Ind yield on the alkali concentrations. We decided to carry out a detailed HPLC-based product distribution study for establishing the correlation between the yields of 1-Ind and the concentrations of alkali. The results shown in Table 1 demonstrate that within the pH range 9–12.5, the yields of indazole product increase with increasing solution pH, and when the pH of the solution exceeds 12.5 the yields of 1-Ind decrease again. As to the origin of formation of the indazole product from short-chain diazonium salt in alkaline pH, we propose the mechanistic route shown in Scheme 3. The indazole product is likely to result via the formation of the *syn*-hydroxy azoate intermediate **III** formed in the alkaline media. Intramolecular abstraction of a proton from the *ortho-methyl* group results in the formation of carbanion **IV** which then expels the hydroxide ion, intramolecularly yielding intermediate **V**. The intermediate **V** finally undergoes aromatization to form 1-Ind. Obviously, the intramolecular proton abstraction from the *ortho-methyl* group is feasible only with *syn*-hydroxy azoate intermediate **III** and not with its *trans*-isomer **VII** (Scheme 3). The formation of such *syn*-hydroxy azoate from diazonium salts in the presence of alkali and the isomerization of *syn*-azoate to its *trans*-isomer in strongly alkaline medium has already been reported [48–50]. Thus, most likely the isomerization of the *syn*-hydroxy azoate intermediate **III** to the corresponding *trans*-isomer **VII** (Scheme 3) results in the observed low yields of 1-Ind when the pH of the solution exceeds 12.5 (Table 1).

3.3. Liposomal indazolization of 16-$ArN_2^+BF_4^-$

The indazolization pathway shown in Scheme 3 is not likely to be mechanistically different when the interfacial probe (16-$ArN_2^+BF_4^-$) undergoes indazolization at the exo-surface of cationic liposomes. Because of its surfactant-like structure, the interfacial probe molecule (16-$ArN_2^+BF_4^-$), when added to cationic liposomes, is likely to position its polar diazonium group in the hydrated and polar exo-surface region of the various cationic liposomes (Fig. 1). Thus, the reactive diazonium group of the interfacial probe experiences an essentially aqueous environment of the liposomal exo-surface and being used in 1–2 mol% with respect to the liposome-forming cationic lipid, the probe causes minimum perturbation of the liposome structure. However, caution should be exercised at this point. The dielectric constants of the hydrated interfacial regions of liposomes are generally less than that of bulk water [51]. Therefore, possible medium effects on the various steps involved in the formation of indazole (Scheme 3) during the liposomal exo-surface associated indazolization cannot be ruled out. In other words, the correlation between the 16-Ind yield and the liposomal exo-surface pH can never be identical to that between the 1-Ind yield and the pH of the aqueous buffer solutions observed in the non-liposomal aqueous indazolization of 1-$ArN_2^+BF_4^-$ (Table 1). The short-chain indazolization results (Table 1) demonstrate that the onset of non-liposomal indazolization is around pH 9, and higher indazole yields



Scheme 3.

Table 1
Observed yields[a] of 1-Ind for non-liposomal indazolization of 1-ArN_2^+ within the pH range 9–14

pH[b]	% Yields of 1-Ind
9.0	3.2
9.2	4.5
9.4	5.8
9.6	7.7
9.8	10.8
10.0	14.4
10.2	17.6
10.4	22.6
10.6	27.4
10.8	30.9
11.0	35.8
11.2	39.2
11.4	40.6
11.6	43.5
11.8	46.0
12.0	50.8
12.5	50.6
13.0	47.6
13.5	45.5
14.0	34.5

[a] The yields of 1-Ind were determined through quantitative HPLC analysis of the product mixtures using the HPLC calibration graph for independently synthesized pure 1-Ind. The details of the HPLC conditions are described in Section 2. The non-liposomal indazolization was carried out by heating 1×10^{-4} M aqueous solution of the short-chain diazonium salt at 40°C for 6 h. The yields are reproducible within ±1% for duplicate experiments.

[b] The aqueous solutions within the 9.0–12.0 pH range were prepared using 20 mM $Na_2CO_3/NaHCO_3$ buffer solutions. The pH range 12.5–14.0 was maintained using aqueous NaOH solutions.

are obtained in the pH range 10.4–12.5. Given this high pH requirement for the formation of significant indazole and assuming minimal medium effects, the observed significant yields (23–32%, Table 2) of 16-Ind in most of the liposomal exo-surface bound indazolizations indicate that the exo-surface of the cationic liposomes commonly used in gene delivery are remarkably alkaline. The most remarkable feature of the results shown in Table 2 is the fact that the pH of the aqueous Hepes buffer solutions used to prepare the cationic liposomes were all near 7.0 and yet the yields of 16-Ind are in the range of 23–32%. In the non-liposomal indazolization, comparable yields (23–32%) of 1-Ind are obtained at solution pH 10.5–11.0 (Table 1). Thus, assuming minimal medium effects in the alkali-induced indazolization, the observed yields of 16-Ind in liposomal exo-surface bound indazolization (Table 2) strongly indicate that the local exo-surface pH for cationic liposomes used in gene transfection are in the range of 10.5–11.0. In other words, the molar concentrations of hydroxide ions in the exo-surface of the cationic liposomes commonly used in gene delivery are orders of magnitude higher than the hydroxide ion concentrations in the bulk aqueous Hepes buffer solutions (pH 7.0) used to prepare these liposomes. That the medium effects on the alkali-induced indazolization reactions (our basic assumption) are indeed minimal is strongly supported by the recent measurement of surface pH for cationic liposomes used in gene delivery by Zuidam and Barenholz [26] through a completely independent method. Using a pH-sensitive fluorophore 4-heptadecyl-7-hydroxycoumarin, Zuidam and Barenholz have measured the liposomal surface pH in 20 mM Hepes buffer (pH 7.4) for various cationic liposomes commonly used in gene transfection, and the value reported by them for cationic liposomes made from DOTAP is 11.6 [26]. If there is zero medium effect, the observed average percentage yield of 16-Ind (32.3%, Table 2) for indazolization in liposomes made from DOTAP when compared with non-liposomal data (Table 1) indicates that the exo-surface pH of cationic liposomes made from DOTAP is about 11. Given that there has to be some medium effect in the alkali-induced indazolization, our result for the DOTAP-based liposomes is consistent with the recently reported results of Zuidam and Barenholz [26]. The yields of 16-Ind observed for DOTAP/cholesterol-based liposome, a liposomal vector that has recently been demonstrated [7] to have significantly improved gene transfection efficiency, is lower than the observed yield of 16-Ind for liposomes made from only DOTAP (Table 2). This is expected because the auxiliary lipid cholesterol is going to reduce the positive charge density at the liposomal surface. The exo-surface, because of its reduced positive charge density, concentrates fewer hydroxide ions at the liposome surface, resulting in a lower percentage yield of 16-Ind for DOTAP/cholesterol-based liposome. The fact that we could not detect any 16-Ind product (Table 2) for both DOTAP/DOPE and DOTMA/DOPE liposomes indicates that the exo-surface pHs of the liposomes with DOPE as the auxiliary lipid are probably not as high as the exo-surface pH of cholesterol-containing liposomes. Such a

Table 2
Observed yields of 16-Ind[a] for liposomal exo-surface bound indazolization of 16–ArN_2^+

Liposome[b]	% Yields of 16-Ind
DOTAP[c]	31.8
	32.7
DOTAP/cholesterol[d] (molar ratio 1:1)	23.9
	24.4
DOTAP/DOPE[d] (molar ratio 1:1)	Nil
DOTMA[c]	23.1
	22.4
DOTMA/cholesterol[d] (molar ratio 1:1)	31.7
	32.5
DOTMA/DOPE[d] (molar ratio 1:1)	Nil
DHDAB[c]	28.6
	29.9
DHDAB/cholesterol[d] (molar ratio 1:1)	41.4
	40.1

[a] The final concentrations of 16–ArN_2^+ were maintained in the range 1×10^{-4} to 5×10^{-5} M. The yields of 16-Ind (data for duplicate experiments are shown) were determined through quantitative HPLC analysis of the product mixtures using the HPLC calibration graph for independently synthesized pure 16-Ind. The details of the HPLC conditions are described in Section 2.

[b] All the liposome solutions were prepared in 10 mM aqueous Hepes buffer (pH 7.0). The details for liposomal indazolization are described in the text.

[c] The concentrations of lipid used in preparing pure cationic liposomes were 5 mM.

[d] The concentration of each lipid was 2.5 mM when the liposomes were prepared using 1:1 molar ratio of cationic lipid and the auxiliary lipid (either DOPE or cholesterol).

decrease in exo-surface pH is not unlikely when the auxiliary lipid is DOPE. The exo-surface alkalinity depends upon the net positive charge of the surface. Unlike cholesterol, a substantial portion of DOPE is likely to change their head-group nature from normal zwitterionic form to negatively charged form, even if the surface pH is moderately high (it need not be as high as that of cholesterol-containing liposomes). Such negatively charged DOPE will neutralize a significant portion of the exo-surface positive charge, thereby reducing the exo-surface positive charge density. However, the observed 16-Ind yields in liposomal indazolization for DOTMA-based liposomes is less than that for DOTMA/cholesterol-based liposomes (Table 2). The same trend is also observed for DHDAB-based liposomes. Based on our non-liposomal indazolization results (Table 1), we believe that the observed higher yields of 16-Ind for DOTMA/cholesterol and DHDAB/cholesterol liposomes compared with liposomes made from only DOTMA and DHDAB (Table 2) originate from the significantly high exo-surface pH (compared to DOTAP-based liposomes). Addition of cholesterol to DOTMA- and DHDAB-based liposomes reduces the very high exo-surface pH (as in case of DOTAP/cholesterol liposome) to a moderately high value, thereby favouring the formation of more 16-Ind. Such an increase of indazole yield with decrease of pH was observed at the high pH end (pH 14.0 to 12.5) of the %1-Ind vs. pH correlation (Table 1) during non-liposomal indazolization.

Clearly, further investigations need to be carried out to extract the exact value of the exo-surface pH of various cationic liposomes used in gene transfection from liposomal exo-surface associated indazolization. Our results demonstrate that 16-Ind yields, the indicator for the exo-surface basicities of the cationic liposomes commonly used in gene transfection, depend significantly on the compositions of the liposomes. The exo-surfaces of liposomes with cholesterol as the auxiliary lipid are more basic than the liposomes prepared using DOPE as the auxiliary lipid (Table 2). In their recently published work, Templeton et al. [7] have demonstrated that the efficacy of cationic liposome-mediated intravenous DNA delivery improves 75-fold to 150-fold when cholesterol is used as the neutral lipid (compared with using DOPE). Thus, there could be possible correlations between the efficacy of liposomal gene transfections and the exo-surface basicities of cationic liposomes used for transfection. The present method can be used in determining the relative exo-surface basicities of various cationic liposomes used in gene transfection and subsequently, to find any such possible correlation between the transfection efficiencies of these liposomes and their exo-surface basicities. The present method can also be used in determining the relative interfacial basicities of various cationic reversed micelles which, in turn, can throw new insight into the correlation between the activities of interfacially associated enzymes, e.g., lipases in reversed micellar biotechnology across a wide range of solution compositions. Studies towards these ends are in progress.

In conclusion, the present work shows that formation of a significant amount of 16-Ind in liposome-bound indazolization provides evidence for the presence of a remarkable alkaline exo-surface in cationic liposomes used in gene transfection.

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