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QUARTERLY

Gemini (dimeric) surfactant perturbation of the human erythrocyte★✪

Martina Dubničková[1], Malgorzata Bobrowska-Hägerstrand[2], Thomas Söderström[2, 3] Aleš Iglič[4] and Henry Hägerstrand[2]✉

[1]*Department of Cell and Molecular Biology of Drugs, Faculty of Pharmacy, Comenius University, Bratislava, Slovakia;* [2]*Department of Biology, Åbo Akademi University, Biocity, Åbo/Turku, Finland;* [3]*Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Biocity, Åbo/Turku, Finland and* [4]*Laboratory of Applied Physics, Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, Slovenia*

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We studied the ability of di-cationic gemini surfactantsdi (amphiphiles), i.e. 1,4-butanediammonium-*N,N*-dialkyl-*N,N,N′,N′*-tetramethyl bromides (Di-C_m-di-QAS (s = 4), where m = 8,11,13,16 and s = the number of alkyl groups in the spacer) to induce shape alteration, vesiculation, haemolysis and phosphatidylserine exposure in human erythrocytes, and to protect erythrocytes against hypotonic haemolysis. At high sublytic concentrations the Di-C_m-di-QAS (s = 4) amphiphiles rapidly induced echinocytic (spiculated) shapes and a release of exovesicles, mainly in the form of tubes, from the cell surface. Following 60 min incubation erythrocytes were sphero-echinocytic and a few cells with invaginations/endovesicles were observed. No phosphatidylserine exposure was detected. The haemolytic potency increased with an increase of the alkyl chain length. At sublytic concentrations the Di-C_m-di-QAS (s = 4) amphiphiles protected erythrocytes against hypotonic haemolysis. It is suggested that the Di-C_m-di-QAS (s = 4) amphiphiles perturb the membrane in a similar way as sin-

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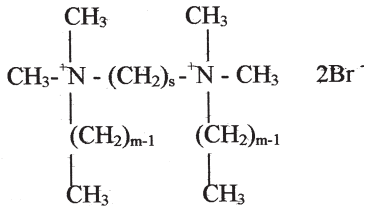
✉Corresponding author: Henry Hägerstrand, Department of Biology, Åbo Akademi University, Biocity, FIN-20520 Åbo-Turku; Finland; Tel. (35 82) 215 4089; Fax: (35 82) 215 4748; e-mail: Henry.Hagerstrand@aton.abo.fi

Abbreviations: cAH_{50} and cAH_{max}, the concentration giving 50% and maximal protection (respectively) against hypotonic haemolysis; cH_{50}, the concentration of the amphiphile which induces a 50% release of haemoglobin from the erythrocytes; DiC_m-di-QAS, 1,4-butanediammonium-*N,N*-dialkyl-*N,N,N′,N′*-tetramethyl bromides; TEM, transmission electron microscopy.

gle-chain cationic amphiphiles, but that they do not easily translocate to the inner membrane leaflet.

A strong antimicrobial effect of cationic amphiphiles has been taken advantage of in a variety of applications. In order to examine the antimicrobial and membrane disrupting effect of a relatively new group of amphiphiles, the so called gemini amphiphiles, the di-cationic gemini amphiphiles were synthesized and examined. Gemini amphiphiles can be considered as dimers of conventional single-chain (monomeric) amphiphiles (Zana *et al.*, 1998). Gemini amphiphiles are double-chained and their two polar head groups are connected by a hydrocarbon spacer, which can be of variable length.

The 1,4-butanediammonium-*N,N*-dialkyl-*N,N,N′,N′*-tetramethyl bromides (Di-C_m-di-QAS (s = 4); m is the number of the alkyl groups in the aliphatic alkyl chain and s is the number of alkyl groups in the spacer chain) are representatives of gemini amphiphiles. Their general formula is:



The importance of the spacer and alkyl chain length for the properties of gemini amphiphiles has attained attention and has been considered in many of the studies mentioned below. E.g. concerning: the critical micellar concentration of different gemini amphiles (Rozycka-Roszak *et al.,* 1989; Zana *et al.*, 1991; Alami *et al.,* 1993; Frindi *et al.,* 1994), their antimicrobial activity (Devinsky *et al.,* 1985; Dubničková *et al.,* 1997), aggregation (Alami *et al.*, 1993), formation of lyotropic mesophases (Danino *et al.,* 1995), surface activity (Diamant & Andelman, 1994), micelles (Hirata *et al.,* 1995, Rozycka-Roszak *et al.,* 1996, Danino *et al.,* 1997) and effect on model phospholipid membranes (Dubničková *et al.,* 1996; 1997). The physico-chemical and biological properties of gemini amphiphiles have been reviewed by Fisicaro (1997).

We have previously found that conventional single-chain amphiphiles have the ability to induce shape alterations, vesiculation and haemolysis in human erythrocytes, as well as to protect erythrocytes against hypotonic haemolysis (Isomaa *et al.*, 1986; Isomaa *et al.*, 1987; Hägerstrand & Isomaa, 1989; 1991; 1992; Hägerstrand *et al.*, 1998).

In this study we wanted to examine whether also gemini amphiphiles, (i.e. Di-C_m-di-QAS (s = 4), where m = 8,11,13,16) can induce shape alterations, vesiculation, and haemolysis in human erythrocytes, as well as protect them against hypotonic haemolysis, and to compare the membrane effects of gemini amphiphiles with those of other amphiphiles, particularly the single-chained cationic alkyltrimethylammonium bromides. This study was aimed at increasing the knowledge of effects of gemini amphiphiles on biological membranes.

METHODS

Chemicals. The 1,4-butanediammonium-*N,N*-dialkyl-*N,N,N′,N′*-tetramethyl dibromides, previously also called CmBAS, were synthesized at the Faculty of Pharmacy, Comenius University, Bratislava, Slovak Republic (Imam *et al.*,1983). Sample identity was confirmed by NMR and elemental analysis.

Erythrocytes. Blood was drawn from the authors by vein puncture into heparinized tubes. The erythrocytes were washed three times in a buffer containing 10 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 10 mM glucose (pH 7.4). The erythrocytes were then suspended at a concentration of 16×10^8 cells per ml in the medium and kept cold until used. All experiments were carried out within 48 h after the blood was drawn.

Incubation. Incubation was started by adding erythrocytes to tubes containing buffer and amphiphile. The final erythrocyte concentration was 1.6×10^8 cells per ml and incubation was carried out at 37°C.

Haemolysis. Erythrocytes were added to the buffer containing various concentrations of the amphiphiles. After incubation (60 min) the samples were centrifuged (1000 × ***g*** for 40 s) and the percentage of haemolysis was determined in supernatants by comparing with the supernatants of a standard curve (known degree of haemolysis). cH_{50} is the concentration which induces a 50% release of haemoglobin from the erythrocytes in the sample.

Phase contrast and interference contrast microscopy (LM). The morphology of amphiphile-treated (10 min) erythrocytes, fixed with 1% glutaraldehyde (30 min, room temperature) was studied by phase contrast microscopy at 400× magnification. Shapes were named according to Bessis (1973).

Transmission electron microscopy (TEM). Amphiphile-treated (30 min) erythrocytes were suspension-fixed in 1% glutaraldehyde in the buffer for 30 min at room temperature, postfixed in 1% OsO_4 in 0.9% NaCl for 30 min at room temperature, dehydrated in a graded series of acetone/water (50–100% w/w) and finally embedded in Epon. Thin sections were prepared as previously described in Hägerstrand & Isomaa (1989) and were studied with a JOEL 100SX electron microscope.

Flow cytometry. Flow cytometry was largely performed as previously described (Hägerstrand *et al.,* 1998). In short, following pre-treatment of erythrocytes with the aminophospholipid translocase inhibitor *N*-ethylmaleimide (10 mM, room temperature, 30 min, two washes) and amphiphile-treatment (60 min, 37°C), 25 μl of cell suspension was added to 200 μl of a 1 : 200 solution of FITC-annexin-V (209-250-T300, Alexis) in the buffer (containing 3.8 mM Ca^{2+}). The samples were then incubated on ice for about 20 min in the dark. Ten thousand cells/sample were measured for fluorescence intensity and size with a FACScan flow cytometer (Becton Dickinson).

Protection against hypotonic haemolysis. These experiments were carried out in the buffer diluted to a tonicity where about 60% of untreated erythrocytes were haemolysed. Erythrocytes were added to the diluted medium containing various concentrations of the amphiphiles. After incubation (30 min) the samples were centrifuged (1000 × ***g*** for 40 s) and the percentage of haemolysis was determined in the supernatants as above. cAH_{50} and cAH_{max} are the concentration giving 50% and maximal protection against hypotonic haemolysis, respectively (see Isomaa *et al.,* 1986).

RESULTS

Haemolysis

The haemolytic activities of Di-C_m-di-QAS (s = 4) (m = 8,11,13,16) at a fixed erythrocytes concentration (1.65×10^8 cells/ml) are shown in Table 1. The concentrations which induced a 50% release of haemoglobin (cH_{50}) was about 100 μM for Di-C_{11}-di-QAS (s = 4), about 80 μM for Di-C_{13}-di-QAS (s = 4) and about 50 μM for Di-C_{16}-di-QAS (s = 4). With Di-C_8-di-QAS (s = 4) 50% haemolysis was not reached, although concentrations up to 50 mM were tested. The haemolytic concentrations (cH_{50}) for some other cationic amphiphiles are included for comparison.

Protection against hypotonic haemolysis

All Di-C_m-di-QAS (s = 4) amphiphiles protected erythrocytes against hypotonic haemolysis. They reduced the degree of haemolysis from about 60% in the control sample to 30–35%. The concentrations giving maximum protection against hypotonic haemolysis (cAH_{max}) are indicated in Table 2. cAH_{max} and the concentrations where

Table 1. Haemolytic concentrations (cH_{50}) of cationic amphiphiles in human erythrocytes in relation to alkyl chain length

Compound/chain length	C_8	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{16}
Di-C_m-di-QAS (s = 4)	Not achieved		100 μM		80 μM		50 μM
Di-C_8-di-QAS (s = 2)	330 μM						
Di-C_{12}-QAS				40 μM			
Di-C_{14}-amidine						~80 μM	
C_mTAB		7000 μM		650 μM		80 μM	40 μM

cH_{50}, concentration inducing a 50% release of haemoglobin (haemolysis); Di-C_m-di-QAS (s = 4), 1,4-butanediammonium-*N,N*-dialkyl-*N,N,N′,N′*-tetramethyl bromides, spacer = $4CH_2$ (Imam *et al.*, 1983) (double-tailed double-headed); Di-C_8-di-QAS (s = 2), *N,N′*-bisdimethyl-1,2-ethanediamine dichloride, spacer = $2CH_2$ (Fogt *et al.*, 1995) (double-tailed double-headed); Di-C_{12}-QAS, didodecyldimethylammonium bromide (Kodak 1357102) (double-tailed single-headed); Di-C_{14}-amidine, *N*-t-butyl-*N′*-tetradecyl-3-tetradecylaminopropionamidine (Vectamidine, BiotechTools) (Hägerstrand *et al.*, 1999) (double-tailed with a complex head group); C_mTAB, alkyltrimethylammonium bromides (Sigma) (single-tailed single-headed)

haemolysis starts at isotonic conditions largely coincide.

Shape change

Control erythrocytes were discoid or slightly echinocytic (D-E_3). Slightly echinocytic shapes are normal in erythrocytes isolated into buffer. The discoid resting shape of control erythrocytes can be restored by the addition of bovine serum albumin (not shown). All

Table 2. Protection against hypotonic haemolysis

Compound	cAH_{max} [μM]
Di-C_8-di-QAS (s = 4)	182
Di-C_{11}-di-QAS (s = 4)	11.2
Di-C_{13}-di-QAS (s = 4)	15
Di-C_{16}-di-QAS (s = 4)	11.8

cAH_{max} is the concentration giving maximal protection against hypotonic haemolysis. Full names of the amphiphiles are given in Table 1.

Di-C_m-di-QAS (s = 4) amphiphiles induced an echinocytogenic shape transformation in human erythrocytes (Table 3, Fig. 1A and B). At high sublytic concentrations of the surfactants erythrocytes became sphero-echinocytic. The spheroidal echinocytic shapes persisted even during extended incubations (5 h). For comparison, the type of erythrocyte shape transformations induced by some other cationic amphiphiles tested in our laboratory are included in Table 3. Cationic amphiphiles with a single head group, but not gemini amphiphiles, may induce a shape recovery, from initially echinocytic shapes to discoid or stomatocytic shapes (Table 3). Di-C_{14}-amidine, a double-chained amphiphile with a complex head group containing two charged moieties, induces stomatocytic shapes only. It should be noted that Di-C_{14}-amidine is not a gemini amphiphile, since it is not made up of two identical amphiphilic moieties (Zana *et al.*, 1998).

Vesiculation

At concentrations where sphero-echinocytic cell shapes occurred (Fig. 2A), exovesicles were released from the cell surface (Fig. 2C). Long tubular exovesicles, but also shorter prolate dumbbell shaped and spheroidal exovesicles occurred (Fig. 2C). It appeared as if the isolated microvesicles seen in the cross sections were (possibly due to different sedimentation rates) partly arranged in domains where tubular exovesicles were oriented parallel to each other. It should be noted that tubular exovesicles inclined with respect to

Table 3. Shape alterations and vesicles induced by cationic amphiphiles in human erythrocytes

Compound	Number of tails/number of head groups	Shape after 1 min/60 min	Shape of exovesicles	Presence of invaginations/endovesicles
Di-C_8-di-QAS (s = 4)	2/2	Ech./Ech.	Tubular and small fragments, incl. spheroidal	++
Di-C_{11}-di-QAS (s = 4)	2/2	Ech./Ech.	"	+
Di-C_{13}-di-QAS (s = 4)	2/2	Ech./Ech.	"	+
Di-C_{16}-di-QAS (s = 4)	2/2	Ech./Ech.	"	+
Di-C_8-di-QAS (s = 2)	2/2	Ech./Ech.	" (some branched)	
Di-C_{12}-QAS	2/1	Ech./Disc. and Stom.	Spheroidal mainly	++
Di-C_{14}-amidine	2/complex	Stom./Stom.	No exovesiculation detected	++
C_mTAB	1/1	Ech./Disc. and Stom.	Spheroidal mainly	++

Ech., echinocytic; Disc., discocytic; Stom., stomatocytic; ++, frequently; +, sparsely. Full names of amphiphiles are given in Table 1.

the plane of the cross section may be mistaken for spheroidal ones, and the number of tubular exovesicles may therefore be underestimated. Some erythrocytes, especially in samples treated with Di-C_8-di-QAS (s = 4), contained invaginations/endovesicles following 60 min incubation (Fig. 2B).

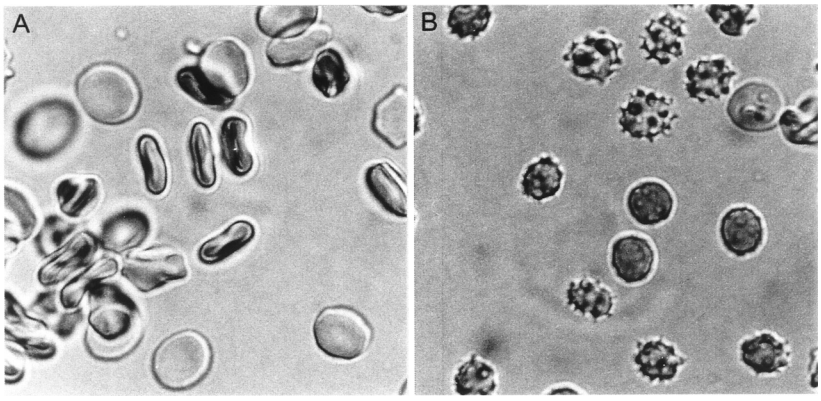


Figure 1. Interference contrast micrographs showing (A) untreated human erythrocytes and (B) erythrocytes incubated with Di-C_{11}-di-QAS (s = 4) (11.2 μM).

Erythrocytes were fixed in 1% glutaraldehyde.

Phosphatidylserine exposure

The absence of FITC-annexin V binding to erythrocytes incubated at sublytic concentrations of Di-C_m-di-QAS (s = 4) (m = 8,11,13,16), as monitored by flow cytometry, indicates that no phosphatidylserine exposure had occurred (Fig. 3). It should be noted that pre-treatment of erythrocytes with *N*-ethylmaleimide increased their haemolytic sensitivity to amphiphile treatment, why low amphiphile concentrations were used.

DISCUSSION

According to the bilayer couple hypothesis (Sheetz & Singer, 1974) amphiphiles induce shape alterations in human erythrocytes by being asymmetrically distributed between the

bilayer leaflets, thereby expanding one leaflet relative to the other. The difference in location of differently charged membrane-permeable amphiphiles within the bilayer is mainly attributed to an attraction or a repulsion of amphiphiles with acidic phospholipids, mainly phosphatidylserine, in the inner leaflet. At equilibrium anionic amphiphiles are regarded to preferentially stay in the outer leaflet, thereby being echinocytogenic, while cationic ones are trapped in the inner membrane leaflet, thereby being stomatocytogenic.

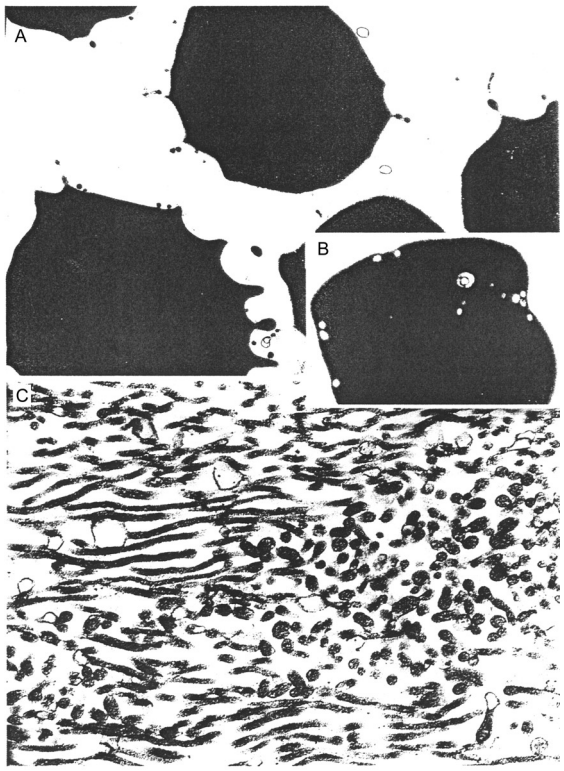


Figure 2. TEM micrographs showing (A) human erythrocytes incubated with Di-C_{16}-di-QAS (s = 4) (23.6 μM). (B) Following 60 min incubation some erythrocytes with invaginations/ endovesicles occurred. (C) Released exovesicles.

Our results showed that the studied di-cationic Di-C_m-di-QAS (s = 4) gemini amphiphiles rapidly induced echinocytic shapes, which persisted through incubation. Apparently, Di-C_m-di-QAS (s = 4) amphiphiles are readily intercalated into the outer membrane leaflet, but can not translocate, probably due to the head group properties, to the inner membrane leaflet at a high rate. Largely persistent echinocytic shapes were previously reported to be induced also by the di-cationic gemini amphiphile Di-C_8-di-QAS (s = 2), differing from Di-C_m-di-QAS (s = 4) mainly by having only two hydrocarbon groups in the spacer (Fogt *et al.*, 1995). On the other hand a slow shape recovery, from echinocytic to discocytic or stomatocytic shapes, was observed with the cationic double-chained but single-headed Di-C_{12}-QAS (see legend to Table 1) and cationic single-chain and -headed alkyltrimethyl ammonium bromides (Table 3). A similar very slow shape transformation from echinocytes to stomatocytes has been observed with some phosphatidylcholines (Tamura *et al.,* 1987). Interestingly, Di-C_{14}-amidine, having two alkyl chains and a complex cationic head group, induced a stomatocytogenic shape transformation only.

Our experiments also showed that the spare occurrence of invaginations in Di-C_m-di-QAS (s = 4)-treated cells, following sphero-echinocytosis, occurred more frequently when m = 8 (Table 3). This may be taken to indicate that the transbilayer movement from the outer to the inner leaflet is easier for compounds with a shorter alkyl chain. A similar conclusion was drawn following studies on the shape recovery of human erythrocytes from echinocytes to discocytes following treatment with phosphatidylcholines having variable acyl chain lengths (C_8-C_{12}) (Tamura *et al.,* 1987).

To summarize, single- and double-chain amphiphiles with a single cationic head-group (possibly including Di-C_{14}-amidine) seem to flip from the outer to the inner membrane leaflet at a higher rate than gemini amphiphiles. In the case of gemini amphiphiles the role of the spacer and its length remains unknown.

The present study revealed that di-cationic gemini amphiphiles, like cationic double-chained amphiphiles with a single head group and conventional cationic single-chained single-headed amphiphiles (Hägerstrand & Isomaa, 1992), induce a release of exovesicles.

The ratio long tubular/small exovesicles (including spheroidal, prolate dumbbell shaped exovesicles, etc.) seemed to be high with gemini amphiphiles but very low with conventional alkyltrimethylammonium bromides. Theoretical analysis, starting from the single-molecule (inclusion) energy (Kralj-Iglič *et al.,* 1996), which takes into account the anisotropic effective shape (Bobrowska-Hägerstrand *et al.,* 1999; Kralj-Iglič *et al.,* 1999) of dimeric amphiphiles, indicates that the deviatoric properties of the membrane, induced by the orientation ordering of the anisotropic inclusions (molecules), may be a plausible explanation for the observed stable tubular microexovesicles released from the erythrocyte membrane upon incubation with dimeric amphiphile (Kralj-Iglič *et al.*, 2000). It should be noted that the single-chained amphiphile dodecylmaltoside, which has a large polar head group, induces a release of predominantly tubular microexovesicles (Hägerstrand & Isomaa, 1992), indicating the importance of the structure and shape of the amphiphile polar head (Israelachvili, 1992) for the exovesicle shape.

In samples treated with the previously studied gemini amphiphile Di-C_8-di-QAS (s = 2) branched tubular-exovesicles were observed (Hägerstrand & Isomaa, 1992). Such exovesicles were not observed in erythrocytes treated with Di-C_m-di-QAS (s = 4). In line with these observations, it has been reported that gemini amphiphiles with short spacers (s = 2) may form threadlike micelles at low concentrations (Zana & Talmon, 1993, Danino *et al.*, 1995).

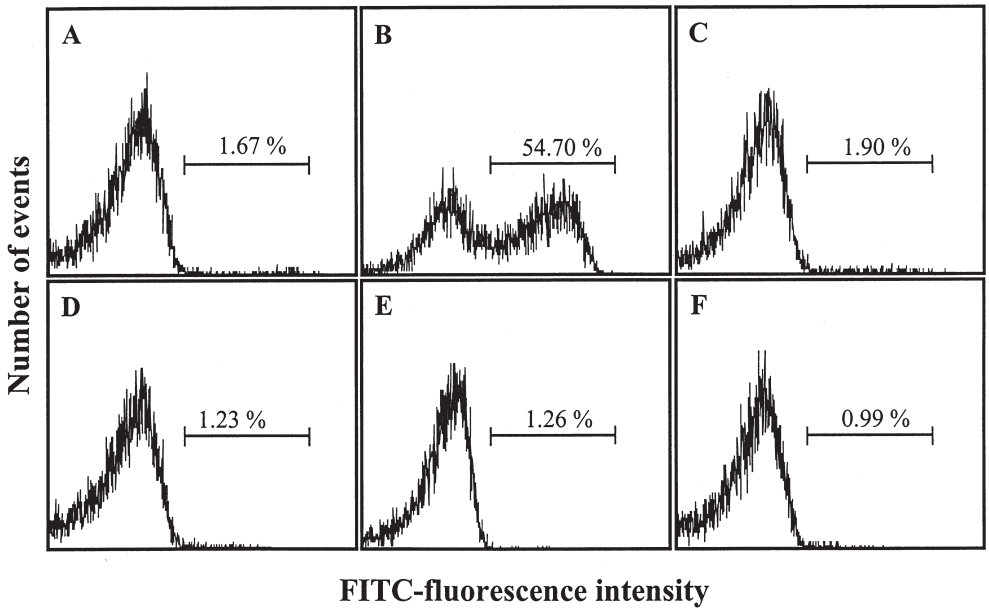


Figure 3. Flow cytometry analysis of FITC-annexin-V binding to human erythrocytes following selected treatments at 37°C.

Erythrocytes were (A) pretreated with *N*-ethylmaleimide (NEM; 10 mM, 30 min, room temperature, two washes), pretreated with NEM and incubated for 60 min with (B) octaethyleneglycol dodecyl ether (C12E8; 44 μM, positive control), (C) Di-C_8-di-QAS (s = 4) (182 μM), (D) Di-C_{11}-di-QAS (s = 4) (6 μM), (E) Di-C_m-di-QAS (s = 4) (7 μM) and (F) Di-C_{16}-di-QAS (s = 4) (6 μM). The values are the percentage of cells binding FITC-annexin V above a threshold.

Data given in Table 1 indicate that single-chained alkyltrimethylammonium bromides (C_mTAB) with shorter chain lengths (C_{10}, C_{12}) are much less haemolytically effective than the related cationic double-chained amphiphiles. A similar observation was reported by Kleszczynska *et al.* (1990) comparing Di-$C_{8\text{-}16}$-di-QAS (s = 2) with conventional alkyltrimethylammonium bromides. The dif-

ference may be due to the membrane perturbation of amphiphiles when intercalated into the membrane, or due to the membrane partition. A difference in the transbilayer distribution and mobility of gemini amphiphiles and alkyltrimethylammonium bromide may also affect the haemolytic potency of the compounds.

Di-C_m-di-QAS (s = 4) did not induce phosphatidylserine exposure at the sublytic concentrations tested. This is in line with our previous study showing that single-chain cationic amphiphiles, e.g. dodecyltrimethylammonium bromide, at sublytic concentrations induce none, or a very weak, phosphatidylserine exposure compared to anionic, zwitterionic and nonionic amphiphiles (Hägerstrand *et al.*, 1998). The reason for the differences in the phosphatidylserine exposing capacity of differently charged amphiphiles is not known.

Previous studies have also shown that Di-C_m-di-QAS (s = 4) gemini amphiphiles affect the thickness and area of model phosphatidylcholine bilayers (Dubničková *et al.*, 1996; 1997). The thickness of the bilayer decreased with the alkyl chain length from m = 7 to m = 9, but then increased again from m = 10 to m = 16. Simultaneously, the area of the bilayer increased with the chain length from m = 7 to m = 9 but then decreased from m = 10 to m = 16. Thus, there is a non-linear relation between the amphiphile chain length, and its effect on bilayer thickness and area. Also the antimicrobial activity shows a non-linear dependence on the alkyl chain length (Dubničková *et al.*, 1997). The antimicrobial activity increased with the chain length from m = 7 up to a maximum at m = 11–12, after which it decreased. In contrast to those studies showing a non-linear effect/alkyl chain length relationship of Di-C_m-di-QAS (s = 4), the present study showed that the relationship between the alkyl chain length (m = 8,11,13,16) and the concentration needed to induce haemolysis (cH_{50}), and to protect against hypotonic haemolysis (cAH_{50}), is almost linear. This linearity (the absence of a "cut-off" effect) indicates that the alkyl chain length of Di-C_m-di-QAS (s = 4) affects the erythrocyte membrane partition and/or the membrane perturbation by Di-C_m-di-QAS (s = 4) in a simple direct way.

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