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Biologically active bisquaternary ammonium chlorides: Physico–chemical properties of long chain amphiphiles and their evaluation as non-viral vectors for gene delivery

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Abstract

The biological properties of bisquaternary ammonium salts, which are derivatives of *N*,*N*-bisdimethyl-1,2-ethanediamine (bis-C_nBEC), of general formula /$C_nH_{2n+1}OOCCH_2(CH_3)_2N^+CH_2CH_2N^+(CH_3)_2CH_2COOC_nH_{2n+1}/2Cl^-$, were investigated ($n$=10, 12, 14). The interaction with model membrane was studied by differential scanning calorimetry experiments, and the apparent adiabatic molar compressibility of their solution as a function of concentration was obtained by sound velocity measurements. Their biological activities were assayed by Electrophoresis Mobility Shift, MTT proliferation, and transient transfection. All the investigated compounds interact with the DNA and are able to transfect DNA, when they are coformulated with DOPE, with an efficiency significantly greater than that of a standard commercial transfection reagent. Bis-C_{14}BEC is the only molecule able to deliver DNA inside the cells without a helper lipid, as shown by EGFP expression, albeit with a low efficiency in comparison with a standard commercial transfection reagent. This may be due to a slightly different interaction of bis-C_{14}BEC from bis-C_{10}BEC and bis-C_{12}BEC with phospholipid bilayers. Bis-C_{10}BEC and bis-C_{12}BEC show a slight fluidising effect, while bis-C_{14}BEC increases stability of both the gel and the rippled gel phases.

Keywords: Bisquaternary ammonium salt; DPPC liposome; Non-viral vector for gene delivery; Differential scanning calorimetry; Sound velocity measurement; Apparent adiabatic molar compressibility; Electrophoresis mobility shift assay; Transfection; Green fluorescent protein

1. Introduction

Research in the field of *gemini* surfactants — i.e. surfactants in which at least two identical moieties are bound together by a spacer at the polar head level — has developed very quickly, because of their advantages over the monomeric ones owing to their increased surface activity, lower critical micelle concentration (CMC), and useful viscoelastic properties [1]. Of the *gemini* surfactants, the most studied structures under the profile of biological activity and of chemico–physical properties are the bisquaternary ammonium salts (bisQUATS), and, among these, derivatives of *N*,*N*-bisdimethyl-1,2-ethanediamine of general formula /$C_nH_{2n+1}OOCCH_2(CH_3)_2N^+CH_2CH_2N^+(CH_3)_2CH_2COOC_nH_{2n+1}/2Cl^-$ (bis-C_nBEC), where the subscript n stands for the number of carbon atoms of the alkyl chain bound to the carboxyl group, which, at least formally, can be treated as double amphiphilic betaine ester derivatives (C_nBEC) belonging to the class of soft surfactants, i.e. surfactants able to disintegrate into inactive products, avoiding unwanted side-reactions, and hence biodegradable. Cationic bisquaternary ammonium surfac-

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tants show a stronger biological activity than the corresponding monomers, and result as being more active both on a molar and on a weight scale as far as germicidal activity and protein binding ability are concerned [2,3]. Quite recently, the use of *gemini* surfactants as non-viral vectors in gene therapy has been proposed [4–7], on account of the possibility of taking advantage of their cationic character necessary for binding and compacting DNA and of their superior surface activity. Gene therapy is one of the major goals pursued by post-genomic research. It is based on the principle of curing a disease caused by a known defective gene by delivering a correct copy of the gene to the diseased cells, by means of a specially designed vector. Most of the vectors fall into two classes, namely viral or non-viral. Although viral vectors are generally very efficient in delivering genes into a targeted cell, their use is not without the risk of adverse or immunogenic reaction, or replication, depending on the virus being used. As a result, non-viral vectors have in many cases become a preferred means of gene delivery into eukaryotic cells, although their transfection efficiency is still low and needs to be optimised.

With the aim of achieving a better insight into the interaction of *gemini* surfactants with membrane, we collected chemico–physical data, useful for correlating the structure of surfactants with their transfection ability.

2. Experimental

2.1. Materials

Both amphiphilic betaine ester derivatives and *N*,*N*-bisdimethyl-1,2-ethanediamine derivatives were synthesised at the Technical University of Wrocław [8]. The solutions were prepared by using freshly boiled doubly distilled water, stored under nitrogen. Solution concentrations are expressed as molality, m (mol kg^{-1}).

1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids, Birmingham, Alabama, and L-α-phosphatidylethanolamine dioleoyl (C18:1,[*cis*]-9) (DOPE) from Sigma-Aldrich.

2.2. Sound velocity measurements

Density and sound velocity of the solutions were measured by a Paar DSA 5000, oscillating U-tube density ($\pm$0.000001 g cm^{-3}) and sound velocity ($\pm$0.1 m s^{-1}) meter which measures to the highest accuracy in wide viscosity and temperature ranges. Based on an additional measuring cell made of stainless steel and high resolution electronics, the sound velocity of the filled in sample can be determined accurately. Both measuring cells are temperature controlled using a built-in solid state thermostat and two integrated Pt 100 platinum thermometers ($\pm$0.001 °C). The instrument was calibrated by water and dry air.

2.3. Sample preparation for differential scanning calorimetry (DSC)

Samples for DSC were performed, as before [9,10], on multilamellar vesicles (MLVs). We were interested in studying the influence of bisQUATS on both pre and main transitions. We therefore chose the multilamellar vesicles as model membranes because DSC curves of MLV show clear pre and main transitions. DPPC, with appropriate amounts of bis-C_nBEC compounds, was dissolved in chloroform. Chloroform was evaporated and a thin mixed film formed on the flask wall. After this, distilled water was added and the mixed film was intensively shaken at 60 °C until a milky suspension of liposomes was obtained. Final phosphatidylcholine concentration was 25 mg/cm^3. The lipid suspension was then loaded into the sample cell of a DSC microcalorimeter (Mettler Toledo Thermal Analysis System D.S.C. 821^e). Scan rate was 2 °C/min, and incubation (performed at 4 °C) lasted 5 days.

2.4. Electrophoresis mobility shift assay (EMSA)

Binding reactions were performed in a final volume of 14 μl with 10 μl of 20 mM Tris/HCl pH 8, 1 μl of plasmid (1 μg of pEGFP-C1) and 3 μl of bis-C_{10}BEC, bis-C_{12}BEC and bis-C_{14}BEC at different final concentrations, ranging from 25 to 200 μM. Binding reaction was left to take place at room temperature for 1 h; 5 μl of 1 g/ml in H_2O of glycerol was added to each reaction mixture and loaded on a TA (40 mM Tris–Acetate) 1% agarose gel. The gel was run for 2.5 h in TA buffer at 10 V/cm. EDTA was omitted from the buffers because it competes with DNA in the reaction.

2.5. Cell culture

The human rhabdomyosarcoma cell line RD-4, obtained from David Derse, National Cancer Institute, Frederick, Maryland, was maintained as a monolayer using growth medium containing 90% DMEM, 10% FBS, 2 mM L-glutamine, and 100 IU/ml penicillin, 10 μg/ml streptomycin. Cells were subcultured to a fresh culture vessel when growth reached 70–90% confluence (i.e. every 3–5 days) and incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2.

2.6. MTT proliferation assay

Briefly, after 48 h of treatments, 20 μl of MTT (5 mg/ml) was added to the culture for 4 h. Then, after the addition of 100 μl of solubilisation solution (10% SDS in HCl 0.01 M) cells were incubated at 37 °C overnight. Specific optical density was measured at 540 nm, using 690 nm as reference wavelength in an SLT-Lab microreader (Salzburg, Austria). For the proliferation studies, each experiment was done three times and each treatment was