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Improved stability of black lipid membranes by coating with polysaccharide derivatives bearing hydrophobic anchor groups

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Black lipid membranes were coated with modified polysaccharides bearing hydrophobic palmitoyl and cholesteryl moieties. The changes in membrane structure were investigated using dipicrylamine, a lipophilic ion, as membrane probe. The kinetics of ion transport through the black lipid membranes were studied using the charge pulse relaxation technique. With this technique it was found that it is possible to detect the insertion of the hydrophobic anchor groups of the polysaccharides into the membrane bilayer. As a result of the surface coating, these membranes exhibit a drastically increased long-term stability.

Artificial models for cell walls

Bacterial and plant cell membranes are coated with cell walls composed mainly of polysaccharide derivatives [1,2]. These cell walls are required to maintain the shape of the cells and to protect the cell membrane against both chemical and physical forces (e.g., osmotic pressure). On the other hand the sugar moieties from glycolipids and glycoproteins play an important role in biological recognition processes including antigen-antibody interaction, toxin recognition and cell-cell adhesion [3,4].

In an attempt to mimic such stabilization processes of cell membranes in artificial, bilayer membranes, different approaches have been employed [5–8]. Regen et al. [5] and Aliev et al. [7] used ionic forces to achieve a coating of the surface of liposomes with polymers. In another attempt, polysaccharides bearing hydrophobic anchor groups were used to build up artificial cell-wall-like structures on the surface of liposomes [8]. After treatment of egg phosphatidylcholine liposomes with *O*-palmitoylpullulan or *O*-palmitoylamylopectin, these vesicles showed a reduced permeability for carboxyfluorescein and increased resistance against enzymatic lysis with phospholipase D.

The structural changes in the membrane of liposomes coated with partially hydrophobized polysaccharides have, however not yet been investigated. To gain some information about these structural changes, black lipid membranes were used for further model investigations.

First attempts to stabilize black lipid membranes via a polymerization of lipid molecules or by the addition of hydrophobic polymers to the membrane-forming solution have already been reported [9–11]. In the present study, black lipid membranes made from glycerolmonooleate and diphytanoylphosphatidylcholine were coated with pullulans and amylopectins carrying either palmi-

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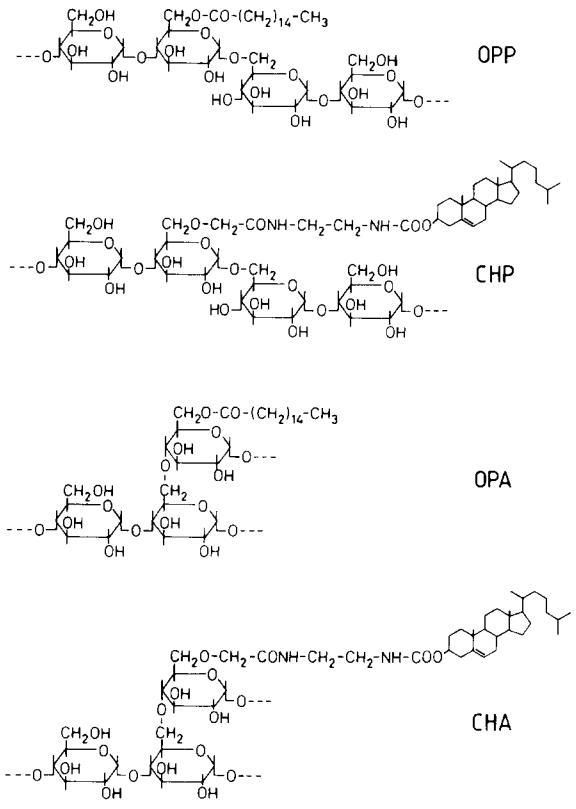

Fig. 1. Structures of the polysaccharide derivatives.

toyl or cholesteryl moieties as hydrophobic side groups (shown schematically in Fig. 1). Information about the structural changes in the membranes after this coating was obtained using the lipophilic anion dipicrylamine as membrane probe. Its transport kinetics were studied using charge pulse relaxation measurements as described by Benz et al. [12].

Materials and Methods

Materials

Diphytanoylphosphatidylcholine was purchased from Avanti Biochemicals (Birmingham, AL). Glycerol monooleate was synthesized and purified as described by Buchnea [13]. Pullulane-51 (weight-average molecular weight 51 000) and amylopectin (weight-average molecular weight 112 000) were gifts from Hayashibara Biochemical Laboratories (Okayama, Japan). Dipicrylamine (puriss. grade) was obtained from Fluka (Buchs, Switzerland). *O*-Palmitoylpullulan and *O*-palmitoylamylopectin were prepared according to the procedure for the preparation of *O*-palmitoyldextran [14,15]. Cholesterylpullulan and Cholesterylamylopectin were prepared as described elsewhere [8,16]. The degree of substitution with hydrophobic side-groups per 100 glucose units in these polysaccharides was determined by ^{1}H-NMR and found to be 1.8 and 2.6 for *O*-palmitoylpullulan-51, 1.4 and 2.8 for *O*-palmitoylamylopectin-112, 0.9 and 1.3 for cholesterylpullulan-51 and 0.6 and 1.0 for cholesterylamylopectin-112. The terminology used for the investigated polysaccharides includes the molecular weight and the degree of hydrophobic substitution. They are listed below: *O*-palmitoylpullulan-51 (1.8), *O*-palmitoylpullulan-51 (2.6), *O*-palmitoylamylopectin-112 (1.4), *O*-palmitoylamylopectin-112 (2.8), cholesterylpullulan-51 (0.9), cholesterylpullulan-51 (1.3), cholesterylamylopectin-112 (0.6) and cholesterylpullulan-112 (1.0).

Black lipid membrane experiments

Black lipid membranes were formed from solutions of glycerol monooleate and diphytanoylphosphatidylcholine in *n*-decane (1–2% (w/v)).

The aqueous KCl solutions (1 M for all experiments) were prepared using distilled water that had been purified by a Millipore purification system (Milli Q, Millipore Corp., U.S.A.). The polysaccharides were dissolved in the electrolyte solution. Dipicrylamine was added to the aqueous phase from a concentrated solution in ethanol. Its concentration in the electrolyte solution was $1 \cdot 10^{-8}$ mol/l for all experiments.

Experiments were performed at 25°C in Teflon chambers with two compartments separated by a thin wall with an aperture of 1 mm in diameter as described earlier [9]. The stationary conductance was measured by applying a voltage of 10 mV to the membrane and measuring the current with a Keithley 610C solid-state electrometer. For the measurement of the electrical capacity, rectangular voltage pulses (approx. 25 mV) were applied to the membrane from a Hewlett Packard HP 8011A pulse generator through Ag/AgCl electrodes. The decay of the capacitive current was measured as the voltage drop across a resistor (100 KΩ) with a Gould OS 4040 digital storage oscilloscope. The

capacitance of the membrane, C, was determined from the exponential decay time, T, of the current according to the relation

$$C = I_0 T / V_0 \quad (1)$$

where I_0 is the current extrapolated to zero time and V_0 is the applied voltage. The result was normalized to unit area. The experimental apparatus was checked using equivalent circuits simulating the bilayer membrane system and the results agreed within 1%.

The charge pulse relaxation measurements were carried out with one Ag/AgCl electrode grounded and the other Ag/AgCl electrode connected to the pulse generator (HP 8011A) through a fast diode (reverse voltage resistance greater than $1 \cdot 10^{11}$ Ω). The voltage between these two electrodes was measured by a fast-voltage amplifier with a high input resistance (risetime 20 ns) and a digital storage oscilloscope (Gould OS 4040). The time resolution of the system was about 1 μs. A Commodore CBM 4032 microcomputer was used to analyse the relaxations and to calculate the kinetic constants. The voltage relaxations in the charge pulse experiments could be described in each case by a sum of two relaxation processes. The kinetic constants of dipicrylamine transport were calculated according to Benz et al. [12]. In order to reach equilibrium of ion concentration in the bilayer, all kinetic measurements were carried out at least 0.5 h after the membrane had completely turned black.

Results and Discussion

Influence of O-palmitoylamylopectin on membrane thickness and stability

Black lipid membranes were formed in presence of *O*-palmitoylamylopectin in the electrolyte solution. The first striking effect of the addition of this compound was a drastic increase in the long-term stability of the black lipid membranes. This effect was checked using black lipid membranes from glycerol monooleate and the amylopectin derivative *O*-palmitoylamylopectin-112 (2.8) at a concentration of 50 mg/l in the electrolyte solution. Under these conditions the glycerol monooleate membranes, which usually are stable only for about 1 h were stable for up to 2 days, sometimes longer. This striking stabilization of black lipid membranes is in our opinion a most interesting effect.

The conductivity and capacitance of the membranes thus stabilized were measured. The results were compared to the values obtained in the absence of the polysaccharide and are summarized in Table I.

The conductivity of the membranes remains

TABLE I

CONDUCTIVITY, CAPACITANCE AND THICKNESS OF BLACK LIPID MEMBRANES WITH AND WITHOUT *O*-PALMITOYLAMYLOPECTIN IN THE AQUEOUS PHASE

Electrolyte solution: 1 M KCl. Membrane thickness was calculated from the capacitance via $C = \varepsilon_M \varepsilon_0 A/d$, where ε_M = is the dielectric constant of the membrane ($\varepsilon_M = 2.0$); ε_0, permittivity of the vacuum ($\varepsilon_0 = 8.854 \cdot 10^{-12}$ F·m^{-1}); A, area of the membrane; d, thickness of the membrane, OPA, *O*-palmitoylamylopectin.

Lipid	Number of membranes	Polysaccharide added	Conductivity (S·cm^{-2})	Capacitance (μF·cm^{-2})	Membrane thickness (nm)
Glycerol monooleate	5	–	10^{-7}	0.39 ± 0.04	4.5 ± 0.5
Glycerol monooleate	6	OPA-112 (1.4) 0.1 g/l	10^{-7}	0.35 ± 0.03	5.1 ± 0.4
Diphytanoyl-phosphatidylcholine	5	–	10^{-8}	0.35 ± 0.03	5.1 ± 0.4
Diphytanoyl-phosphatidylcholine	5	OPA-112 (1.4) 0.1 g/l	10^{-8}	0.29 ± 0.03	6.1 ± 0.6

unchanged in the presence of *O*-palmitoylamylopectin, whereas the membrane capacitance is slightly decreased. For the glycerol monooleate membranes, this effect is still within the limits of the experimental error. However, for the black membranes formed from diphytanoylphosphatidylcholine, the decrease in capacitance is significantly more than the experimental error. These results point to the fact that the *O*-palmitoylamylopectin-112 (1.4) interacts with the black lipid membranes. This interaction slightly increases the thickness of the hydrophobic parts of the bilayer as shown in Table I.

To gain more information about the interaction of partially hydrophobized polysaccharides with black lipid membranes the lipophilic ion dipicrylamine was used as membrane probe and the transport kinetics of dipicrylamine through these bilayer membranes was studied.

Dipicrylamine transport through polymer coated black lipid membranes

Lipophilic ions such as tetraphenylborate and dipicrylamine are able to permeate through membranes and thus increase the membrane conductivity by several orders of magnitude [17–21]. A detailed mechanism for the transport of these hydrophobic ions through lipid membranes was proposed in 1971 [22]. This mechanism is based on the results of electrical relaxation studies and proposes a transport in three distinct steps, as demonstrated in Fig. 2. The first step is the adsorption of the ions from the aqueous phase to the membrane-solution interface (rate constant, k_{AM}). The second step is translocation through the hydrophobic part of the membrane to the opposite interface (rate constant, k_i). The final step is desorption into the aqueous solution (rate constant, k_{MA}).

The kinetics of lipophilic ion transport through black lipid membranes were studied using the charge pulse relaxation technique as described by Benz et al. [12]. Two kinetic constants are obtained by this method: the translocation rate constant, k_i, and the partition coefficient, β, of the lipophilic ions between membrane and electrolyte solution. This partition coefficient is given by:

$$\beta = k_{MA}/k_{AM} \qquad (2)$$

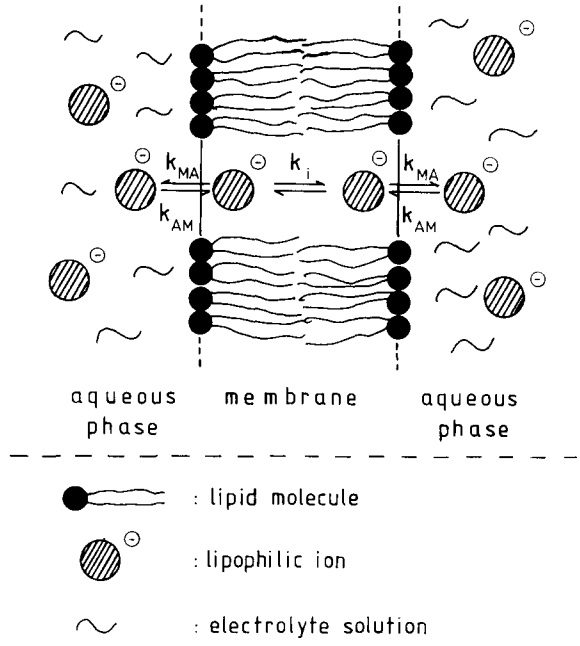

Fig. 2. Model for the transport of lipophilic ions through lipid membranes (according to Ref. 22). k_{MA}, rate constant for the adsorption to the membrane interface; k_{AM}, rate constant for the desorption into the aqueous phase; k_i, translocation rate constants.

β is strongly dependent on the surface potential of the membrane, whereas k_i depends mainly on the microviscosity and the thickness of the hydrophobic part of the membrane.

Measurements were taken from black lipid membranes made from glycerolmonooleate in the presence of various amounts of polysaccharide derivatives in the electrolyte solution. *O*-Palmitoylpullulan was soluble only in very small amounts (less than 10 mg/l) in 1 M KCl. There were used saturated solutions of *O*-palmitoylpullulan-51 (1.8) and *O*-palmitoylpullulan-51 (2.5) in 1 M KCl containing less than 10 mg/l.

The two cholesteryl-containing pullulans (51 (0.9) and 51 (1.3)) showed a very strong interaction with the black lipid membranes resulting in a destruction of the membranes a few minutes after their formation already at a concentration of 10 mg/l. Approx. 30 min equilibrium after formation of the black lipid membrane is required to reach an equilibrium in ion concentration between the membrane and the aqueous phase. Consequently, the kinetics of the lipophilic ion transport in pres-

TABLE II

INFLUENCE OF HYDROPHOBIZED PULLULAN AND AMYLOPECTIN DERIVATIVES OF DIPICRYLAMINE TRANSPORT THROUGH BLACK LIPID MEMBRANES MADE FROM GLYCEROL MONOOLEATE

k_i, translocation rate constant; β, partition coefficient. OP, *O*-palmitoyl; CH, cholesteryl; P, pullulan; A, amylopectin.

Compound added	Number of membranes	Concentration ($mg \cdot l^{-1}$)	k_i (s^{-1})	β (10^{-3} cm)
Reference	9	–	700 ± 200	3.5 ± 1.5
Pullulan	5	10	660 ± 130	2.0 ± 0.4
Amylopectin	5	50	670 ± 90	1.5 ± 0.3
	5	500	800 ± 200	2.8 ± 0.3
OPP-51 (1.8)	5	< 10	380 ± 70	1.8 ± 0.4
OPP-51 (2.5)	6	< 10	390 ± 40	2.5 ± 0.3
OPA-112 (1.4)	7	10	720 ± 150	2.3 ± 0.4
	6	50	610 ± 90	2.4 ± 0.5
	5	100	320 ± 40	1.8 ± 0.3
OPA-112 (2.8)	5	50	350 ± 50	2.9 ± 0.4
CHA-112 (0.6)	5	50	580 ± 80	1.9 ± 0.2
CHA-112 (1.0)	5	50	740 ± 130	2.1 ± 0.3
	5	100	330 ± 70	2.7 ± 0.4
	5	500	150 ± 30	2.0 ± 0.5

ence of cholesteryl-containing pullulans could not be measured.

The results of the measurements that have been carried out with the other pullulan and amylopectin derivatives are summarized in Table II. For comparison, this table also contains results from measurements in the presence and absence of polysaccharides without hydrophobic anchor groups.

The upper part of Table II shows that, within the limits of the experimental error, the unsubstituted polysaccharides pullulan-51 and amylopectin-112 do not show any detectable interaction with the glycerolmonooleate membranes. Conversely, with all hydrophobized polysaccharides a concentration dependent interaction was found. The translocation rate constant k_i is decreased upon this interaction, whereas the partition coefficient, β, remains unaffected. The decrease in k_i is due to an insertion of the palmitoyl and cholesteryl side-groups into the membranes. These hydrophobic moieties are linked together via the polysaccharide chains and so their movement within the membrane is hindered, resulting in a slight increase in the viscosity of the hydrophobic part of the bilayer. This and the effect that the thickness of the membranes increases slightly in presence of the hydrophobized polysaccharides allows one to describe the interaction of these derivatives with membranes as schematically drawn in Fig. 3. The palmitoyl or cholesteryl moieties act as hydro-

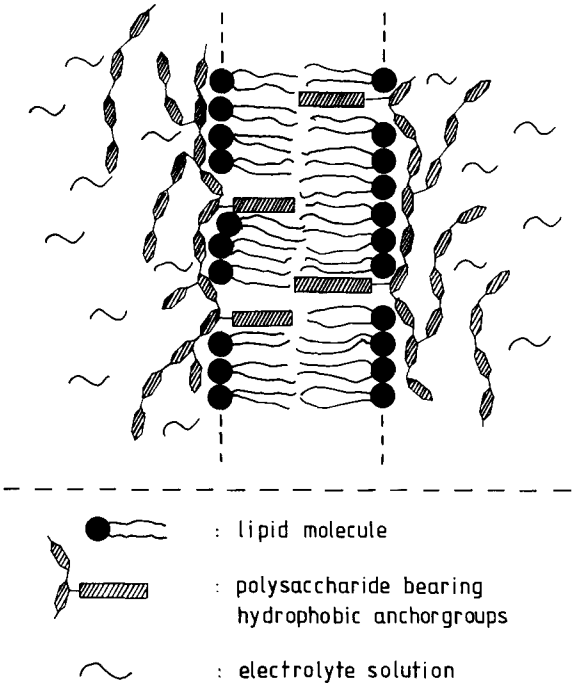

Fig. 3. Model for the structure of black lipid membranes after the adsorption of the hydrophobized polysaccharides.

phobic anchor groups for the polymer in the membrane. The membrane is thus covered by a polysaccharide layer in an aqueous phase comparable to the bacterial cell wall.

A similar way to stabilize black lipid membranes by adding hydrophobic polymers to the membrane forming solution has been published by Hider and co-workers [10,11]. In their experiments, a strong increase in membrane thickness and an increased stability against the lytic action of melittin were found.

The stabilization of black lipid membranes by coating them with partially hydrophobized polysaccharides from the aqueous phase is very similar to nature's way of stabilizing biomembranes by coating them with cell walls. In this case, the membrane properties are influenced only slightly by the stabilization reaction. Thus, this type of membrane stabilization shows promise for reconstitution experiments with proteins which usually destroy black lipid membranes, e.g., melittin or halorhodopsin [10,23].

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