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BILAYER-GEL MEMBRANES

FORMATION AND SOME PROPERTIES

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Summary

We developed a new procedure which induces multifunctional reagents to crosslink at one interface between a black bilayer and the adjacent water phase. This procedure yields 'bilayer-gel' membranes, i.e. membranes consisting of a bilayer and a polymer layer. The bilayer-gel membrane may tentatively be considered to be a new membrane system, because the formation of the polymer layer changes some bilayer properties. We studied bilayer-gel membranes composed of a bilayer of oxidized cholesterol and of a polymer layer of poly-L-lysine crosslinked by glutardialdehyde. Compared to unmodified bilayers, this membrane system has an electrical conductance of the same magnitude, the same electrical capacity and similar shapes of current-voltage dependences. However, this system is asymmetrical and differs in ion selectivity and increased stability from an unmodified bilayer.

Introduction

Artificial bimolecular bilayers between aqueous solutions, which are formed according to the method of Mueller and Rudin [1] are relatively unstable. The intention to stabilize these membranes motivated us to develop the procedure described here. We started with information given by King and Steinrauf [2] who increased the life time of lipid bilayers by adding poly-lysine and glutaraldehyde to the aqueous solutions. They assumed their procedure produced a two-dimensional network of poly-lysine crosslinked in the bilayer. We modified this procedure as follows: (1) We locally restricted the crosslinking reaction to one interface between bilayer and adjacent water phase, and (2) we controlled the reaction time. Thus we obtained a three-dimensional network, i.e., a polymer layer attached to the bilayer.

Materials and Methods

Procedure. The lipid membranes are formed at the aperture of a septum by means of a spatula. The diameter of the aperture is 6 mm. The septum separates the two compartments of a measuring double cell (as described below) filled with a buffered aqueous solution. The long blackening time of the membrane caused by the relatively large aperture is shortened to 10—20 min by heavily stirring the solution of one compartment for a few seconds.

The cross-linking reaction is started at room temperature, after the membrane has turned black, i.e. has more or less become a bilayer. In order to start the reaction we add, by means of an Eppendorff 100-μl pipette and stirring, an aqueous solution of glutardialdehyde to the *trans*-side * and an aqueous solution of poly-L-lysine to the *cis*-side * of the bilayer. The final concentrations in the compartments were 450 μg/ml glutardialdehyde and 100 μg/ml poly-L-lysine, respectively. Glutardialdehyde, not poly-L-lysine, must be added first, otherwise the black bilayer will burst. The bilayer is permeable to glutardialdehyde but almost impermeable to poly-L-lysine. After the addition of the reagents, the molecules of glutardialdehyde diffuse from the *trans*-side through the bilayer and crosslink the molecules of poly-L-lysine at the *cis*-side of the bilayer. By this technique the formation of a crosslinked polymer layer of poly-L-lysine is restricted to the *cis*-sided interface between bilayer and water. The crosslinking reaction is stopped after 1 h by exchanging the solutions of both compartments for solutions without glutardialdehyde and poly-L-lysine. For optimal formation of the gel layer aqueous solutions with a pH of 10 (at pH = 10 poly-L-lysine changes its conformation from random coil (pH < 10) to α-helix (pH > 10); see ref. 4) and an ionic strength of $c < 0.1$ M are required. Changes of the pH and an ionic strength of $c > 0.1$ M after the crosslinking reaction decrease the electrical stability of the bilayer-gel membrane (see Results). Thus, the pH of the solutions in which the gel layer is formed and the pH of the substituting solutions that fill both compartments after the crosslinking reaction, are kept at a value of 10. The ionic strength of all solutions is adjusted to $c < 0.1$ M. In this paper the polymer layer is called 'gel layer' and the combination of bilayer and polymer layer is called 'bilayer-gel membrane'.

Materials. The solution used for forming the bilayers consisted of 50 mg oxidized cholesterol dissolved in 1 ml decane. The oxidized cholesterol (kindly supplied by B. Dobias, Department of Chemistry, University of Regensburg. The oxidation is described in ref. 3.) was produced by oxidizing cholesten-(6)-ol-(3), $C_{27}H_{46}O$ cod. France, Merck-Schuchard. The polymer layer is formed from poly-L-lysine, (20 000 dalton, Miles-Yeda) and glutardialdehyde (No. 6L043, Merck-Schuchard). The pH of the aqueous solutions was adjusted by $NaOH/Na_3BO_3$ buffer (5 mM).

Measuring double cell. In order to stop the crosslinking reaction, the solutions of both compartments must be replaced by solutions without glutardialdehyde and poly-L-lysine. For establishing a concentration gradient through the membrane, the content of at least one compartment also has to be

* *cis*-side being that side of the bilayer where the gel layer is formed, and *trans*-side the opposite side.

exchanged. Additionally, the formation of the gel layer was rather susceptible to mechanical concussions or vibrations. Thereby, parts of the membrane area are liable to disappear into the torus or to be drawn out of it. Hence these parts prevent an optimal formation of the gel layer near the torus. In order to avoid this, the membrane has to be kept motionless with regard to the aperture. Compartments that can be sealed keep the membrane motionless and they allow a quick exchange of their solutions.

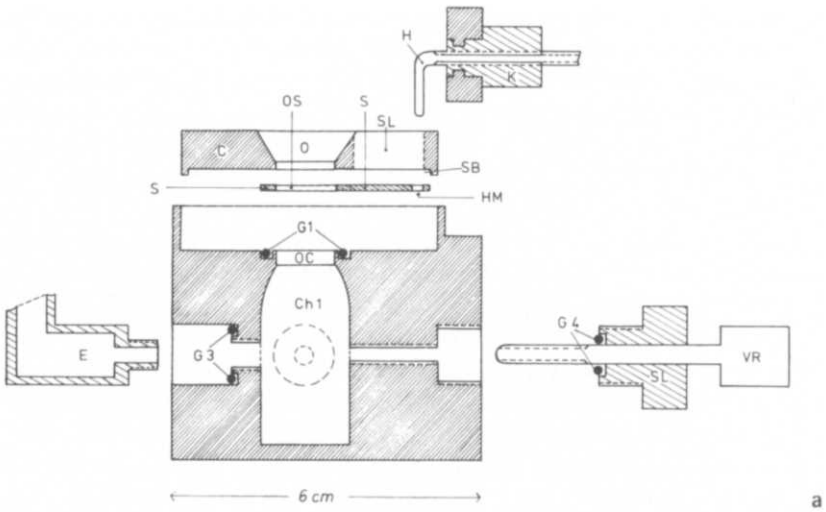
These reasons caused us to develop a measuring double cell as shown in Fig. 1. Both compartments of the double cell can be sealed or opened during an experiment without breaking the membrane. Each single cell is made out of a black perspex block. A replacable teflon sheet with an aperture is inserted between the two single cells which are linked together by screws. The circular opening in the top of each compartment can be opened or closed by adjusting a thin sliding sheet which is inserted between the top and the cover of the compartment. When closed the sliding sheet is sealed by an O-ring gasket. A special screw (volume regulation) in each perspex block allows curved membranes to level out. The compartments are linked to electrical devices via agar bridges and calomel electrodes. The membrane can be optically observed through the window of one cell. Before the solution of one compartment is exchanged, the other compartment must be closed. The substituting solution flows through a pipe put above the bottom of the compartment while on top the substituted solution is withdrawn by a suction pipe. For electrical measurements both pipes are removed in order to avoid electrical interference.

Measuring devices. The electrical resistance R and the electrical membrane potential U_m are measured with an electrometer (Keithley 602). The electrical membrane capacity C is measured with a Wheatston bridge. For reasons discussed in Results we developed a 'pulsed voltage clamp' device to measure the stationary current dependence on voltage $I(U)$. This device generates single voltage pulses, the width of which is variable between 1 and 1000 ms. The resulting current pulses are amplified by a current amplifier (Keithley picoamperemeter 417) and recorded on the screen of a storage oscilloscope (Tektronix D 13). This kind of measurement, first developed by Brennecke [5], covers the wide current range of five decades but is impeded by the relatively large membrane capacity. In most cases the current necessary to load or unload this capacitor to a stationary value, overloads the amplifier or at least drastically slows down its settling time. In order to avoid this, the voltage clamp device additionally generates a 'cover' pulse, i.e. a double pulse which coincides with the rise and fall of the voltage pulse. The 'cover' pulse feeds a relais that short-circuits the feed-back of the amplifier, thus loading or unloading the membrane capacity via relais and not via amplifier.

Water fluxes are measured in the double cell. With both compartments being sealed, the fluxes are compensated by the volume regulation and are indicated by the water level in a capillary tube connected to one compartment.

Results

The gel layer, formed according to the procedure described in *Procedure*, scarcely changes the appearance of a 'black' bilayer when observed through a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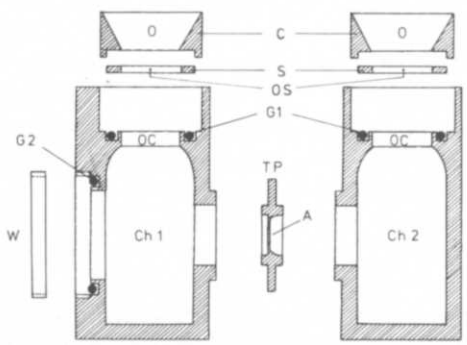
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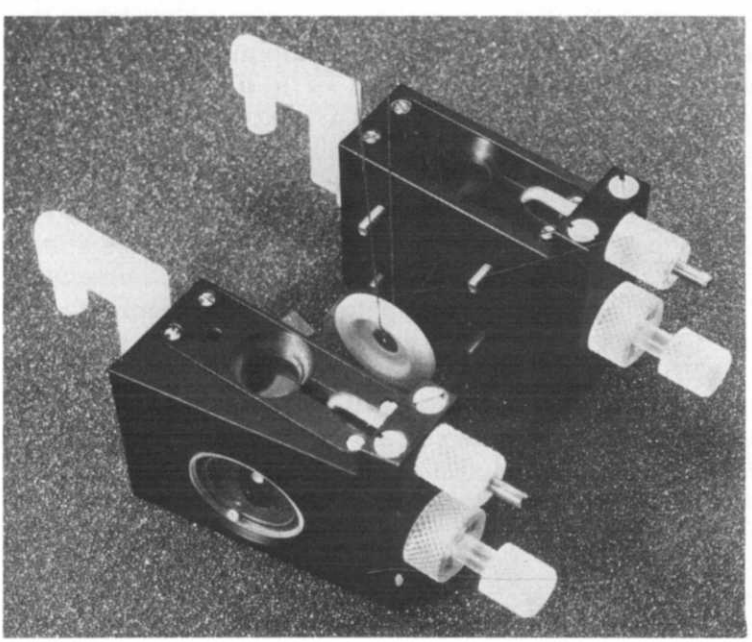

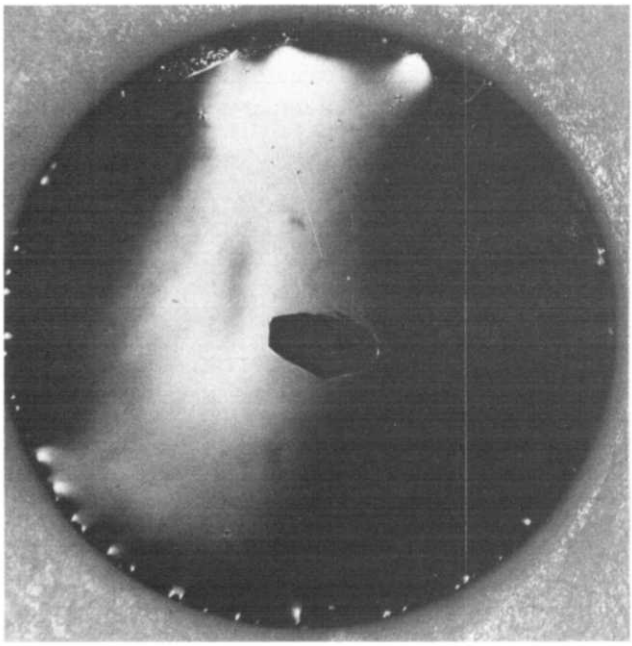

Fig. 2. Photograph of a bilayer-gel membrane which has been perforated by a needle. Diameter of the aperture, 6 mm.

low-power microscope. After a cross-linking reaction of 1 h some bilayer-gel membranes show slight blue colouring which can only be seen if observed very carefully; this may indicate a gel layer thickness of approx. 1000 Å. This colour is intensified and turns from blue to red if the gel formation is increased for more than 1 h or if the concentrations of the cross-linking reagents are increased. However, there is an amazing change in the mechanical stability: it is possible to punch a hole in a bilayer-gel membrane without changing its remaining, uninjured parts (see Fig. 2). This procedure would destroy unmodified bilayers. Additionally, the average life time of a bilayer, which amounts to 3—4 h for oxidized cholesterol and for a diameter of 6 mm, is increased to several days by the gel formation, i.e. an undamaged bilayer-gel membrane will keep its appearance at least for one week, which was the maximum period of

Fig. 1. Measuring double cell. Each of the compartments can be opened or closed during an experiment without breaking the membrane. (a) Cross-section through one compartment parallel to the front side. (b) Cross-section through both compartments perpendicular to the front side. (c) Photograph of both compartments. C1 and C2, front and back compartments with opening OC; S, sliding sheet with opening OS to open or close the compartment; H, hook that is inserted into hole HM to shift sliding sheet S; K, knob to adjust shifting hook H; C, cover of compartment with opening O, with bearing SV for sliding sheet and bearing SL for hook; TP, replacable Teflon sheet with aperture A; VR, volume regulation with sealing screw SC; W, window; E, electrode pipe filled with agar; G, O-ring gasket for sealing the slides sheet (G1), the window (G2), the electrode (G3), and the volume regulation (G4).

observation. The gel layer not only increases the mechanical stability of the bilayer, as described above, but also changes some of its properties. We therefore studied the electrical capacity C, the electrical resistance R, the membrane potential U_m, the water flux W, and the current dependence on voltage $I(U)$.

Capacity, resistance, membrane potential and water flux

We measured the capacity C at a frequency of 1 kHz, the resistance R for a voltage $U \leqslant 25$ mV, the membrane potential U_m for a concentration ratio $C'_{KCl}/C''_{KCl} = 1 : 10$, and the water flux W for an osmotic pressure caused by a concentration difference $C'_{KCl} - C''_{KCl} = 10$ mM.

During its life time, the unmodified bilayer of oxidized cholesterol shows a monotonous increase of C and R. This may be due to structural changes during an aging process that finally renders the bilayer unstable. Besides its initial increase, U_m of an unmodified bilayer between compartments of different KCl concentrations remains constant. Its sign indicates a selective permeability for cations.

These time courses of C, R and U_m are changed during the cross-linking reaction: the capacity and the resistance stop drifting and the membrane potential changes its sign. After the cross-linking reaction these qualities remain almost constant.

The constancy of the capacity may indicate that some aging processes of the bilayer are stopped or at least slowed down by the cross-linking reaction. During and after this reaction, C keeps almost the same value the bilayer had before the reaction was started. This may imply that the bilayer thickness remains constant, assuming the bilayer to behave as a plate condensor and the resistance of the gel layer itself to be negligible.

In general, the dependence of R of bilayer-gel membranes on time depends upon the salt concentration and the pH of the solution the gel is formed in, and on the sign of the applied voltage. The resistance of some bilayer-gel membranes does not remain constant. For these membranes, the procedure of the gel formation and the following exchange of the solutions, which stops the crosslinking reaction, decrease R by 2 or 3 orders of ten. This 'fall-out rate' was not used for measurements described here. The rate depends, among other things, on the duration of the cross-linking reaction and seems to reach a minimal value of 30—40% for a reaction time of 1 h. Therefore, all following recordings of bilayer-gel membranes were measured after a cross-linking reaction of 1 h.

The measured membrane potential U_m *, generated by a concentration ratio $C'_{KCl}/C''_{KCl} = 1 : 10$, changes its sign during the cross-linking reaction. Inserting the final potential values of a bilayer and of a bilayer-gel membrane into the Goldman equation [8]:

$$U_m = \frac{-RT}{F} \ln \frac{P_{K^+} c''_{K^+} + P_{Cl^-} c'_{Cl^-}}{P_{K^+} c'_{K^+} + P_{Cl^-} c''_{Cl^-}}$$

* The one-side addition of poly-L-lysine, which starts the cross-linking reaction, generates an additional potential U_{PLL} even if the salt concentrations in both compartments are equal [7,8]. We did not correct U_m by U_{PLL} because we assumed U_{PLL} to be short-circuited by U_m.

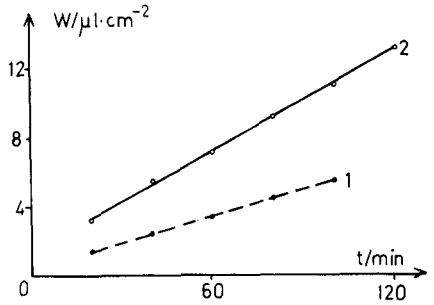

Fig. 3. Time dependences of the water flux W through unmodified bilayers (curve 1) and through bilayer-gel membranes (curve 2). The flux is caused by an osmotic pressure established by a concentration difference $\Delta c_{KCl} = 10$ mM.

we find a permeability ratio $P_{K+}/P_{Cl-} = 1.7$ for the unmodified bilayer and a ratio $P_{K+}/P_{Cl-} = 0.4$ for the bilayer-gel membrane. This indicates that the gel formation turns the membrane permeability from a cationic to an anionic selectivity. Almost simultaneously, the resistance decreases (for $C'_{KCl} = C''_{KCl} = 10$ mM). Both phenomena may be explained by the assumption of a penetration mechanism selective for anions that is built into the membrane during the gel formation.

The water fluxes through a bilayer-gel membrane and through an unmodified bilayer are compared in Fig. 3. The fluxes were caused by an osmotic pressure established by a concentration difference of $c' - c'' = 10$ mM KCl. For this pressure the efflux across unmodified bilayers amounts to $0.9 \cdot 10^{-3}\ \mu l \cdot s^{-1} \cdot cm^{-2}$ (curve 1). The efflux across bilayer-gel membranes is twice as high (curve 2).

The measurements of R of bilayer-gel membranes are afflicted with a huge scattering rate, i.e. R values measured at different membranes can differ more than 300% from their mean value. In contrast to that, recordings of C and U_m at different bilayer-gel membranes differ only by 10% from their mean value. This scattering behaviour is the same as that of unmodified bilayers.

Stationary current-voltage dependences of bilayer-gel membranes

Pure bilayers of oxidized cholesterol and of a diameter of 6 mm burst if a voltage $U > 250$ mV is applied. Bilayer-gel membranes do not burst. These membranes can be exposed to a voltage up to 1000 mV without bursting or changing their appearance. If, however, a voltage of $U > 200$ mV is applied for too long, i.e. for 1 s or more, their electrical conductance increases in a partially irreversible way. Thus, a slow procedure like manually adjusting the clamped voltage and reading the resulting current from the scale of an amperemeter, to measure the current-voltage dependences ($I(U)$ curves) of bilayer-gel membranes yields unreproducible curves showing a marked hysteresis. For measuring the $I(U)$ curves, we applied short voltage pulses generated by the voltage clamp device described in Materials and Methods. This device allows us to measure reproducible $I(U)$ curves of bilayer-gel membranes without any hysteresis up to 1000 mV. According to the settling time of each amplifier

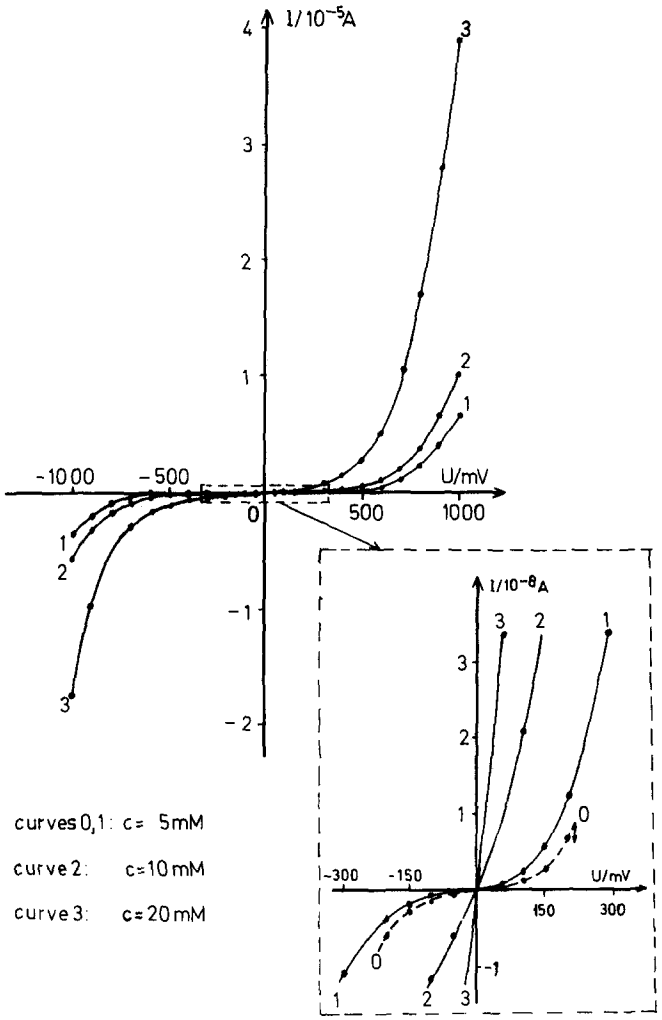

Fig. 4. $I(U)$ curves of bilayer-gel membranes between solutions of equal but varying KCL concentrations (curves 1, 2, 3) measured up to a voltage of 1000 mV. The lower part of the right represents the magnified section around the origin. Curve 0 demonstrates, for comparison, the $I(U)$ curve of unmodified bilayers, which cannot be exposed to a voltage $U > 250$ mV.

range, the length of the voltage pulses was varied from 500 ms for $I = 10^{-7}$ A to 5 ms for $I = 10^{-4}$ A.

Fig. 4 shows $I(U)$ curves of bilayer-gel membranes measured up to a voltage of 1000 mV for different KCl solutions (curves 1, 2, 3) and, for comparison, one $I(U)$ curve of an unmodified bilayer (curve 0). $I(U)$ curves of unmodified bilayers between solutions of equal salt concentrations are symmetrical with respect to the origin ($I(U) = -I(U)$). The $I(U)$ curves of bilayer-gel membranes are not symmetrical: a voltage $U > 0$, i.e. a voltage that moves anions from the *cis*-side and cations from the *trans*-side through the membrane, yields a current that exceeds the reversed current induced by the reversed voltage ($I(U) > -I(U)$), and the inflection point ($d^2I(U)/d^2U = 0$) is shifted from the

origin to negative voltages. This asymmetry may be due to the anion selective 'channels' mentioned above which are built into the bilayer during gel formation, and which partially act like a current rectifier. The current rectifying behaviour results from the structural asymmetry of the bilayer-gel membrane, and mainly from the cationic groups the gel layer is charged with.

The conductance $\lambda(U) = I(U) \cdot U^{-1}$ of the unmodified bilayers increases more than linearly with the voltage. The overlinearity $f(U)$ can be defined by:

$$f(U) = \lambda(U) \cdot \lambda_0^{-1} \text{ with } \lambda_0 = dI(0) \cdot d^{-1}U \tag{1}$$

and amounts to

$$f(250\ \text{mV}) = 4 \text{ for } C_{KCl} = 5\ \text{mM} \tag{2}$$

It is remarkable that for bilayer-gel membranes this overlinearity continues up

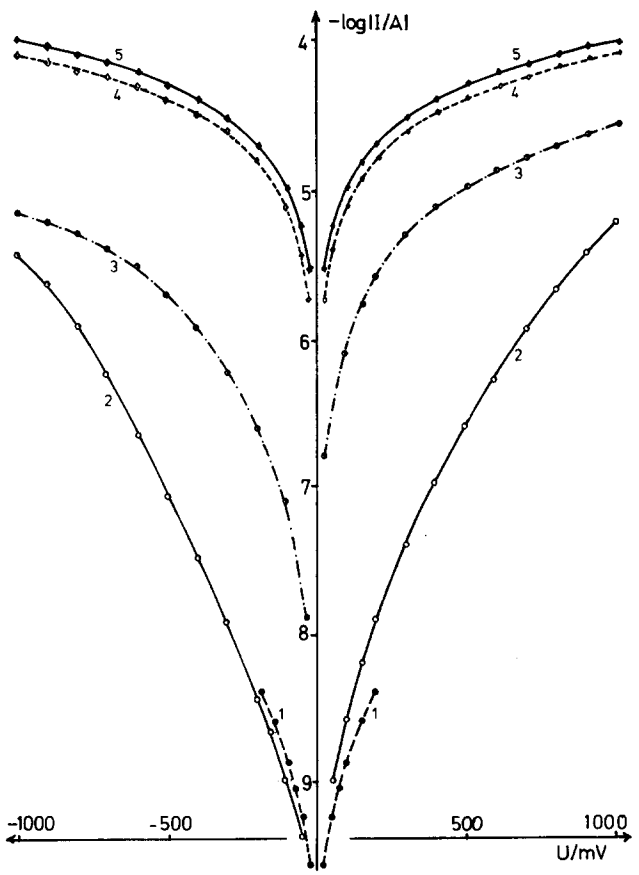

Fig. 5. Different $I(U)$-curves for $c'_{KCl} = c''_{KCl} = 5$ mM in logarithmic current scale. Curve 1, unmodified bilayers; curve 2, bilayer-gel membranes; curve 3, bilayer-gel membranes with solutions of 10^{-4} mM amphotericin B; curve 4, bilayer-gel membranes treated with Triton X-100. This curve may be considered to be the $I(U)$ curve of the gel layer itself as Triton X-100 dissolves the bilayer probably without affecting the gel layer; curve 5, compartments with solutions and agar bridges, but without a membrane (electrode curve).

to 1000 mV, and that $f(U)$ reaches extremely high values because of the extension of the voltage range. For instance:

$f(1000\ \text{mV}) = 550$ for $C_{\text{KCl}} = 5$ mM

i.e. the conductance of a bilayer-gel membrane at 1000 mV is 550 times higher than that at 1 mV.

The bilayer is the main ion barrier of the bilayer-gel membrane, even though the crosslinking reaction decreases this barrier to some extent. This is demonstrated by the $I(U)$-curves of Fig. 5.

The difference between the bilayer curve and the curve of the bilayer-gel membrane as well as the difference between the electrode curve and the curve of the gel layer itself is very small compared to the enormous difference between the bilayer curve and the electrode curve. This implies that the bilayer is the main ion barrier of the bilayer-gel membrane, and the resistance of the gel layer is negligible.

Curve 3 of Fig. 5 represents the $I(U)$ curve of a bilayer-gel membrane between equal solutions of KCl which both contain 10^{-4} mM amphotericin B. This curve is an example of lowering the resistance of an asymmetrical bilayer-gel membrane by introducing an additional penetration mechanism.

All $I(U)$ curves described here are recorded at bilayer-gel membranes between aqueous solutions with a pH of 10 and an ionic strength of $c < 0.1$ M. A change of the pH of more than ±1 or an increase of the ionic strength to $c > 0.1$ M destroys the electrical resistance of bilayer-gel membranes. This behaviour may be caused by the lysine chains which remain positively charged after the gel formation. The positive charges of the amino groups interact with the ions of the solutions. A change of their concentration may alter the conformation and the volume of the gel layer. This in turn leads to a change of the bilayer that drastically decreases its resistance.

Conclusions

The procedure, which we developed to form bilayer-gel membranes, induces the reagents poly-L-lysine and glutardialdehyde to cross-link only at one interface between a black bilayer and the adjacent water phase. Thereby a gel layer is 'attached' to one bilayer side. During the gel formation, some bilayer properties remain unchanged. Other properties, however, are changed and therefore the bilayer-gel membrane may be partially considered a new membrane system. Compared to unmodified bilayers, this membrane system has an ion barrier of the same magnitude, the same capacity, and similar overlinearities of the $I(U)$ curves. Yet, bilayer-gel membranes are asymmetrical and differ in the ion selectivity and in the increased mechanical and electrical stability from an unmodified bilayer.

But the main ion barrier of the bilayer-gel membrane is represented by the bilayer and can only be penetrated by two kinds of penetration mechanisms. The first kind is poorly cation selective and originates from the bilayer. The second kind is anion selective, is formed during the cross-linking reaction, and partially acts as a current rectifier. Both penetration mechanisms are characterized by an overlinear $I(U)$ dependence.

The gel layer bears fixed charges whose density influences its conformation. A conformational change of the gel layer influences the bilayer and is able to make it electrically unstable. The fixed charges could be eliminated by using cross-linking reagents which yield a neutral gel layer, but these reagents must meet some basic requirements which restrict the wide range [9,10] of available multifunctional reagents, e.g., one reagent must have more than two functional groups, one reagent has to be amphiphilic, both reagents must be water-soluble, and they must cross-link under conditions which neither destroy nor damage the bilayer.

Acknowledgments

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