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Model of cell electrofusion

Membrane electroporation, pore coalescence and percolation

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High electric field impulses (1–20 kV/cm, 1–20 μs) may trigger fusion between adhering cells or lipid vesicles (electrofusion). In this paper a qualitative model of electrofusion is proposed consistent with both electron and light microscopic data. Electrofusion is considered as a multistep process comprising tight membrane-contact formation, membrane electroporation as well as an alternating series of subsequent fast collective and slow diffusive fusion stages. The following sequence of steps is suggested: (i) The electric field pulse enforces (via polarization) a tight contact between the membranes of the cells or vesicles to be fused. During tight-contact formation between the opposing membrane surfaces the membrane-adherent water layers are partially squeezed out from the intermembraneous space. (ii) Pores are formed in the double membrane contact area (electroporation) involving lateral diffusion and rotation of the lipid molecules in both adhering membrane parts. (iii) With increasing pore density, pore-pore interactions lead to short-range coalescence of double membrane pores resulting in ramified cracks; especially small tongues and loops are formed. (iv) At supercritical pore density long-range coalescence of the pores occurs (percolation) producing one large double membrane loop (or tongue) and subsequently one large hole in the contact area. (v) After switching off the electric field, the smaller pores, tongues and loops reseal and water flows back into the intermembraneous space of the double membrane in the contact area. (vi) As a consequence of the increasing membrane-membrane separation due to water backflow, cooperative rounding of the edges of remaining larger tongues and holes occurs. This results in the formation of an intercellular cytoplasm bridge (channel) concomitant with the disappearance of the contact line between the fusing cells. (vii) The membrane parts surrounded by continuous loop-like cracks may separate from the system and may finally form vesicles. Our electrofusion model comprises a strong linkage between the membrane pore formation by high electric fields (electroporation) and the process of electrofusion. Additionally, both pore-pore interactions as well as protein-protein interactions in the contact area of the fusing cells are explicitly introduced. The model provides a qualitative molecular description of basic experimental observations such as the production of membrane fragments, of smaller inside-out vesicles and the formation of larger intercellular cytoplasm bridges.

1. Introduction

"The observation of transient permeability changes induced by electric impulses in cell membranes [1] is fundamental and opens new perspectives for our understanding of membrane processes."

(Manfred Eigen, 1972 at a Membrane Workshop at Göttingen)

Biological cell membranes, large unilamellar lipid vesicles or planar lipid bilayer membranes are known to become transiently, but dramatically

Dedicated to Professor Manfred Eigen on the occasion of his 60th birthday.

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more permeable by short electric impulses (1–20 kV/cm, 1–20 μs), provided a threshold value of the external electric field strength is exceeded [1–6]. High electric fields appear to induce pores in the membranes. Theoretically, in all thermodynamic and stochastic models of electric pore formation (*electroporation*), it has implicitly been assumed [7–10] that the pores grow and anneal independently of one another.

High electric field pulses may also trigger fusion of cells or lipid vesicles (*electrofusion*) [11–21]. Classically, before pulsing, the cells or vesicles are brought into contact either mechanically by micromanipulation [11,12] or by sedimentation [13,14], or electrically by dielectrophoresis [15,16], or chemically by agents promoting aggregation [17,18] or by specific linkage [19] of the cells. Furthermore, cells in monolayer contacts have been electrofused directly in culture dishes [20]. However, cell fusion may also be obtained when the cell-cell contacts are established after the pulse [21]. The duration of the entire fusion process is less than 1 s in the case of lipid vesicles, while it may last up to 1 h in the case of biological cells [16].

In cell biology and biotechnology the method of electrofusion, i.e., the triggering of the fusion process by a high electric field pulse, has become a powerful tool for cell hybridization and genetic engineering. For instance, viable hybridoma cells producing monoclonal antibody were obtained by the electrofusion technique [19].

Besides the many practical applications, efforts have been made to elucidate the mechanism of electrofusion at the molecular level [22–24]. On the basis of light microscopic data and of physical-chemical reasoning, it was argued that the mechanism of electrofusion should be strongly connected to electroporation [9]. Recently, rapid quench freeze-fracture electron microscopy for the first time provided ultrastructural information about a physically plausible sequence of events during the electrofusion process [25]. These new data necessitate revision of previous fusion models.

In this paper we propose a new model consistent with both electron and light microscopic data. In particular, we focus on mutual pore-pore interactions both in the very electroporation process and in electrofusion. The mechanism of electrofusion is explicitly treated for the case when the cells are brought into contact *before* the application of the electric field pulse. We also discuss the mechanism of electrofusion when cell-cell contacts are established *after* the field pulse.

In our model electrofusion is considered as a multistep process comprising tight membrane-contact formation, membrane electroporation as well as an alternating series of subsequent fast collective and slow diffusive fusion stages. The following sequence of steps is suggested:

(i) The electric field pulse enforces (via polarization) a tight contact between the membranes of the cells or vesicles to be fused. During tight-contact formation between the opposing membrane surfaces the membrane-adherent water layers are partially squeezed out from the intermembraneous space.

(ii) Pores are formed in the double membrane contact area (electroporation) involving the lateral diffusion and rotation of the lipid molecules in both adhering membrane parts.

(iii) With increasing pore density, pore-pore interactions lead to short-range coalescence of double membrane pores resulting in ramified cracks; especially small tongues and loops are formed.

(iv) At supercritical pore density long-range coalescence of the pores occurs (percolation) producing one large double membrane loop (or tongue) and subsequently one large hole in the contact area.

(v) After switching off the electric field, the smaller pores, tongues and loops reseal and water flows back into the intermembraneous space of the double membrane in the contact area.

(vi) As a consequence of the increasing membrane-membrane separation due to water backflow, cooperative rounding of the edges of remaining larger tongues and holes occurs. This results in the formation of an intercellular cytoplasm bridge (channel) concomitant with the disappearance of the contact line between the fusing cells.

(vii) The membrane parts surrounded by continuous loop-like cracks may separate from the system and may finally form vesicles.

As a starting point the main features of the new electrofusion model are outlined on a *qualitative* level with reference to the experimental data. The main result of this programmatic study is that our periodic block model for membrane electropores [9] can also be consistently applied to the electric field-induced membrane fusion. Additionally, both pore-pore interactions as well as protein-protein interactions in the contact area of the fusing cells are explicitly introduced. The present electrofusion model provides a molecular description of basic experimental observations such as the production of membrane fragments, of smaller inside-out vesicles and the formation of larger intercellular cytoplasm bridges.

2. Membrane contact formation

2.1. Cell-cell contact

It appears obvious that the fusion of membranes requires contact between their surfaces. Usually, in electrofusion experiments cells or lipid vesicles are brought into contact before application of the high electric field pulse. Contact may be achieved either by micromanipulation [11,12] or sedimentation [13,14], by means of agglutinating agents [17,18] or by specific chemical linking [19], or electrically by dielectrophoresis [15,16,22, 23,25].

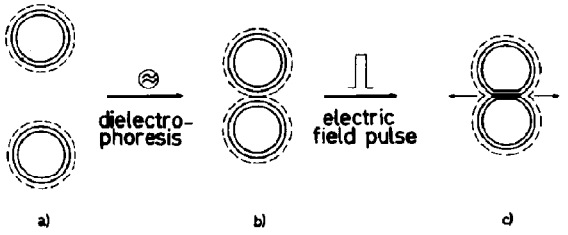

Fig. 1. Membrane contact formation between two cells or vesicles to be fused. Solid lines represent the outer and inner membrane surfaces; dashed lines delineate the borders of the interfacial water layers around the cells; electric fields are applied in the vertical direction. (a) Position at zero external electric fields. (b) Cell-cell contact formation by dielectrophoresis: Effect of an inhomogeneous low-amplitude alternating electric field (e.g., 1.5 MHz, 0.5 kV/cm in the case of erythrocytes [25]). (c) Tight membrane-contact formation by an electric field pulse: Within the contact area (flat part; side view) patches of very tight contact are created by the action of a single high electric field pulse (e.g., 5 kV/cm, 15 μs duration in the case of erythrocytes [25]). The arrows indicate water assumed to be partially squeezed out from the intermembraneous into the extracellular space.

It has been shown that dielectrophoresis [26], i.e., the application of an inhomogeneous alternating electric field of fairly small amplitude, decreases the intermembraneous distance between erythrocytes to about 25 nm. After additional pronase treatment of the cells the distance may be reduced to about 15 nm [25]. Apparently, the repulsive hydration forces between the interfacial water layers of the cell membranes are not overcome solely by dielectrophoresis (fig. 1a and b).

2.2. Tight contact of membrane surfaces

After switching on the high electric field pulse, dielectric and ionic-electric polarization further increases the attractive interaction in the contact area between the cells. The polarization processes in the membrane/bulk interphase (due to Maxwell-Wagner polarization) may play the most important role in this interaction where the attraction energy is proportional to the square of the applied field strength [27]. The field-induced attraction apparently overcompensates both the electrostatic repulsion between the apposed membrane surfaces usually bearing net negative charges and the repulsive hydration forces of the interfacial water layers [28], thereby pushing a part of the water into the bulk extracellular space (arrows in fig. 1c). Thus, by the action of the electric field pulse, an actually *tight membrane-membrane contact* without remaining aqueous boundaries [25] is established at least in patches of the '*double membrane contact area*'. In between these patches water obviously becomes entrapped but appears to remain in connection with the extracellular medium.

3. Membrane electroporation

We assume that a supercritical electric field pulse is able to induce pores not only in single membranes of cells or vesicles, but also in the double membrane contact area of two adhering

cells. According to the theory [7,9], the common driving force of electroporation is enhanced polarization of polar solvent molecules in the region of the larger electric field spreading from the pore wall into the solution of the pore interior. At the beginning of the electroporation process pores can be considered as independent point defects with the probability of lateral pore-pore interactions being negligibly small.

3.1. Single membranes

In the case of spherical cells or vesicles suspended in a conducting medium, the transmembrane potential difference $\Delta\varphi$, induced by an externally applied electric field E_{ex} via ion accumulation at the membrane surfaces, is strongest at the *'pole caps'* in the field direction and decreases with increasing angle θ to the field direction. If the radius a of the cell is large compared to the membrane thickness d ($a \gg d$), and if the electrical conductivity of the membrane is small compared to that of the extracellular and intracellular media, a simple expression for $\Delta\varphi$ is derived [29–31]. The stationary value of the potential drop $\Delta\varphi$ in the direction of E_{ex} is given by:

$$\Delta\varphi = -1.5E_{ex}a|\cos\theta| \quad (1)$$

Eq. 1 applies to spherical, insulating membrane structures; consequently it is a valid approximation only before electroporation. Since living cells have an intrinsic transmembrane potential difference $\Delta\varphi_{in}$, the total transmembrane voltage V_m on both hemispheres of the cells is *asymmetric* with respect to the field direction. At the pole caps ($\theta = 0°, 180°$; $|\cos\theta| = 1$) one obtains:

$$V_m = -1.5E_{ex}a \pm \Delta\varphi_{in} \quad (2)$$

It should be remarked that typically $\Delta\varphi_{in} = -70$ mV, taking the extracellular medium as the reference with the electric potential being zero.

Pore opening (electroporation) takes place if the net transmembrane voltage V_m exceeds a critical value V_{cr} ($|V_{cr}| \approx 0.5$–1 V) [1,3,5–7,32]. Because of the electric asymmetry there is a certain range of the external electric field strength where electroporation only occurs at one of the pole caps. If the asymmetry is only caused by the intrinsic membrane potential, electroporation should start at the hemisphere facing the anode. There is experimental evidence for the pole asym-

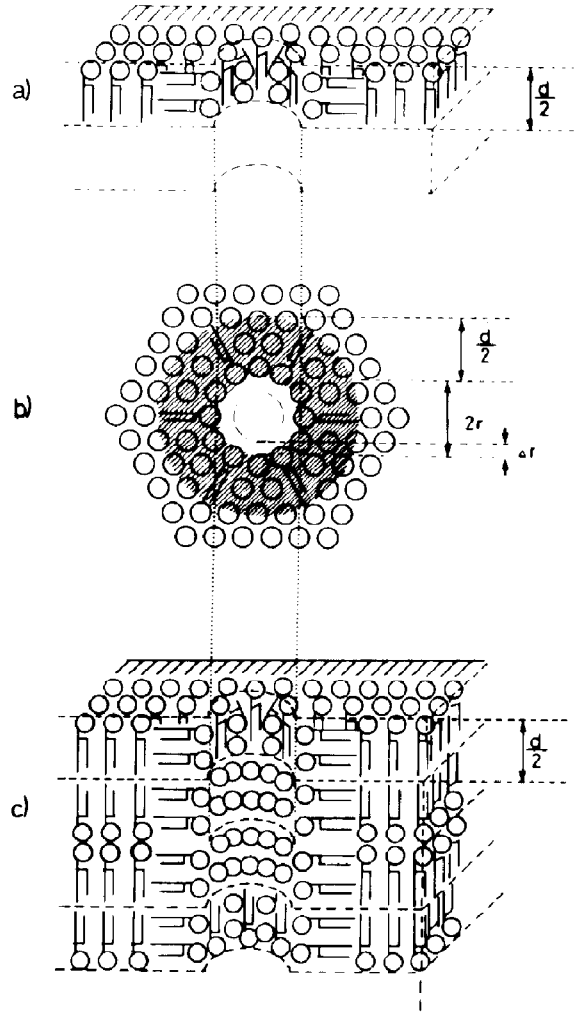

Fig. 2. Periodic block structure of electric field-induced membrane pores (electropores). (a) Cross-section of a single membrane pore. The circles represent the polar head groups of the membrane lipids; only one layer of the lipid bilayer is drawn in detail [9]. (b) Top view of the pore mouth. The hatched area represents the planar part of the pore wall; d, thickness of the bilayer; r, radius of the pore; Δr, thickness of the water layer adjacent to the pore wall edge and experiencing the electric field of the bilayer. (c) Cross-section of a double membrane pore modelled in terms of the periodic block structure.

metry [33–35]. For geometrical reasons the number and density of pores decrease with increasing distance from the poles and vanish at an angle where the net transmembrane voltage decreases below the critical value.

The molecular structure of the electric field-induced membrane pores (*electropores*) is not known. Due to the lack of direct experimental evidence various pore models have been proposed on the basis of *geometric* and *energetic* considerations [7–9]. For the sake of geometrical simplicity pores have been assumed to be cylindric, although this is not the best choice for *entropic* reasons [36]. According to Monte-Carlo simulations of pore shapes the relationship between pore circumference (C) and pore cross-section (S) is fractal, $S \propto C^{1.8}$ [37].

A specific proposal for a pore structure termed a '*periodic block structure*' has been advanced (fig. 2) [9]. The pore wall (shaded area in fig. 2b) is assumed to be a periodic arrangement of lipids in the *normal* bilayer position and of *rotated* lipid blocks. A *block* is defined by two nearest-neighbor lipids within one layer of the bilayer. Energetically there are two advantages of this pore structure: first, it does not require any deformation of the hydrocarbon chains in the pore wall, and second, the periodic structure ensures that the apolar parts of lipid molecules in the membrane/water interface are everywhere surrounded by the polar head groups of the neighbors. This head group environment reduces the extent of direct exposure of hydrophobic groups to water.

The kinetics of pore opening has been calculated in terms of a stochastic model for *stable* and *metastable* planar bilayers [38]. Experimentally, only the delay time between the application of the high electric field and the moment when the membrane conductivity reaches a certain enlarged value was measured for planar membranes [39].

3.2. Double membranes (contact area)

Due to the formation of a tight membrane-membrane contact, pore opening is certainly not independent in both constituent bilayers of the double membrane contact area. Electropores in the constituent single membranes of the contact area induce the formation of '*double membrane pores*', since at the place of a single membrane pore in one membrane the opposing membrane transiently experiences a larger transmembrane electric field strength than an intact double membrane or a separated single membrane. Double membrane pores may primarily form in the patches of tight contact in the contact area. The dynamics of the double membrane pore opening is assumed to be comparable with that of the metastable single planar membrane [38]. This presumption is based on experimental data showing that two contacting lipid vesicles are less stable than the spontaneously fused system [40].

A physically plausible structure of a double membrane pore may be straightforwardly derived from the periodic block model for pores in a single membrane (fig.2a and b) and is presented in fig. 2c. The energy of this double membrane pore is lower than the sum of those of two single membrane pores because the hydrocarbon chains of the two inner membrane layers along the inner pore wall (fig. 2c) are not exposed to the interfacial water. This double membrane pore structure is favorable especially in the case of cylindrical molecules such as lecithin, while in the presence of conical molecules such as lysolecithin the commonly used inverted pore model [7] and its extension for double membranes seems to be more adequate.

4. Pore-pore interaction, pore coalescence and percolation

4.1. Interaction of two pores

Both experimental data and model calculations show [6,7,34,38,39] that not only the size but also the number of pores increase with increasing field strength and pulse duration. This results in an increasing pore density favoring lateral pore-pore interactions (for single membrane pores, e.g., in the pole caps of spherical cells; for double membrane pores in the contact areas). The lateral interaction of two pores starts with contact of the corresponding pore wall edges and is followed by coalescence of the interacting pores. In fig. 3 the

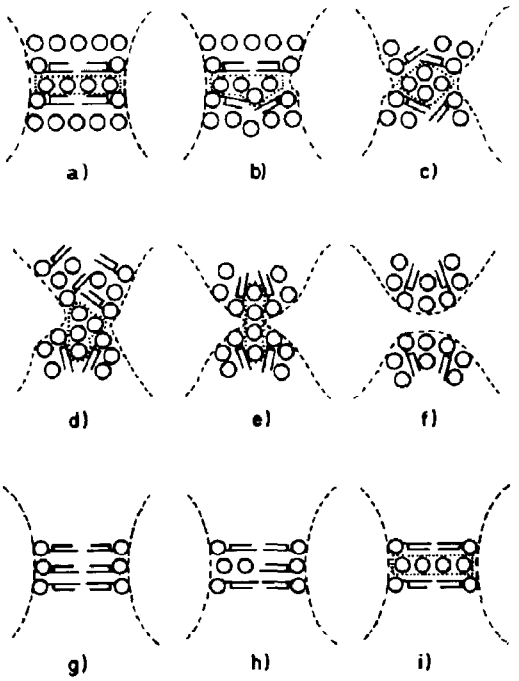

Fig. 3. Stages of pore-pore interaction and pore coalescence within the framework of the periodic block model. (a–f) Sequence of elementary steps of pore coalescence shown for single membrane pores or for sections of the outer layers of two interacting double membrane pores (cf. fig. 2). The dashed lines represent the inner pore edges. The lateral rearrangements of the four lipid molecules considered (dotted boxes) define a fast and energetically favorable way of pore coalescence. (g–i) In the inner layers of interacting double membrane pores block rotations by 90° at the contact point of the pore walls result in a structure which is analogous to structure (a). Subsequently the rearrangements can occur as in (a–f).

sequence of elementary steps of pore coalescence is described within the framework of the periodic block model. Lateral rearrangements of pore wall lipids in single membrane pores are proposed as shown in fig. 3a–f. The same sequence of steps is assumed to occur in the outer layers of interacting double membrane pores as well (cf. fig. 2c). In the inner layers of double membrane pores, however, additional block rotations by 90° [9] at the contact points of the pore walls (fig. 3g–i) have to precede the sequence shown in fig. 3a–f. The lateral rearrangements of the four lipid molecules indicated in fig. 3 by dotted boxes define a fast and energetically favorable way of the coalescence process.

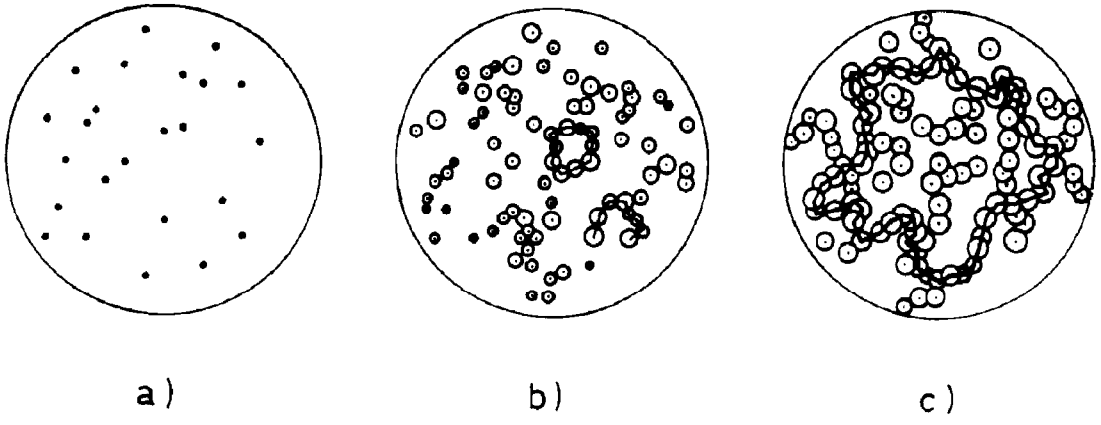

Fig. 4. Evolution of short- and long-range interactions between membrane pores (pore coalescence and percolation). The overall picture is valid for single membranes (e.g., in the pole caps of cells or vesicles) as well as for the double membrane contact area of two adhering cells (top views of membrane surfaces; fractional pore density σ). (a) Noninteracting pores ($\sigma \ll \sigma_{cr}$): Snapshot taken at the beginning of the electric field pulse application; dots indicate independent membrane pores. (b) Subcritical pore density ($\sigma < \sigma_{cr}$): Increasing pore number and pore size under field pulse action; circles represent the outer wall edges of membrane pores. Within small clusters of interacting membrane pores cracks are formed by short-range pore coalescence leading to small tongues and small loops (one small tongue and one small loop are indicated schematically). (c) Supercritical pore density ($\sigma \geq \sigma_{cr}$): Long-range pore coalescence occurs (percolation); the thick solid line indicates the outermost closed chain of successively interacting pores (percolation line) and is called the large loop (the remaining cracks are not drawn).

Table 1

Membrane defect structures and fragments resulting from pore coalescence (zero or low membrane protein density)

(a) Single planar or vesicular membranes; (b) double membrane in the contact area of adhering cells or vesicles.

Fractional pore density (σ)	Class of primary defects	Crack surface topology	Membrane fragments	
			Intermediates	Vesicles
$\sigma \ll \sigma_{cr}$ (low)	point defects	independent pores	no	no
$\sigma < \sigma_{cr}$ (subcritical; short-range pore coalescence)	line defects (short cracks)	small tongues	no	no
		small loops	(a) small disk micelles (b) small double bells	(a) small (b) small; inside-out
$\sigma \geq \sigma_{cr}$ (supercritical; pore percolation)	line defects (short and long cracks)	one large loop (enclosing smaller loops)	(a) small disk micelles (b) small double bells	(a) small (b) small; inside-out

4.2. Short-range pore coalescence (small cracks)

With increasing pore density (σ) the initially independent membrane pores (fig. 4a) may form finite clusters of interacting pores (fig. 4b). As a result of multiple pore-pore coalescence within these clusters short *'cracks'* (line defects) are opening. Topologically, one may differentiate between open cracks of tongue-like structure and closed cracks forming loop-like structures. We denote these structures resulting from short-range pore coalescence *'small tongues'* and *'small loops'*, respectively (in single membranes as well as in the double membrane contact area of adhering cells; see fig. 4b, cf. table 1).

4.3. Long-range pore coalescence (percolation)

At a critical surface density of the pores (σ_{cr}) long-range pore coalescence proceeds and the longest chain of successively interacting pores (*'percolation line'*) becomes comparable with the circumference of the electroporated area (fig. 4c). Theory predicts that long-range pore coalescence already occurs well before the whole area is closely covered by pores.

4.3.1. Percolation theory

The phenomenon of long-range coalescence has been extensively studied in mathematics and physics as a part of the *percolation theory* [41,42]. According to this theory a *'path'* is said to exist between two pores A and B (called *'sites'* in the general mathematical theory) if a sequence of sites (pores) can be found, beginning with A and ending with B, such that successive sites interact with each other. There may be many paths between a given pair of sites. If there is at least one path the sites are said to be *connected*. The sites may be partitioned into *'clusters'* such that pairs of sites in the same cluster are connected; there is no path between sites in different clusters. The cluster size increases with increasing number of sites. This size variation, as a function of the system parameters, is the essential variable of percolation theory.

Most systems to which percolation theory is applicable contain so many sites that boundary effects may be ignored. Hence, the actual finite system (e.g., double membrane pores in the contact area) can be replaced by a model system with an infinite number of sites on an unbounded surface. In such a system the *cluster size* or *'extent'* may become *infinite* at some *critical site density* σ_{cr}. If $\sigma > \sigma_{cr}$, the system is said to be in a *'percolating state'*. The transition from a non-percolating ($\sigma < \sigma_{cr}$) to percolating state is a kind of *phase transition*. Using the nomenclature of percolation theory, membrane pore percolation is a kind of *site percolation* since the place of the pores (sites) is random but the contacts between the pores (called *'bonds'* in the general theory) are determined by the relative position and size of the

pores. Since the pores can be situated everywhere on the continuous surface of the contact area long-range pore coalescence is a *'continuum percolation'*.

Percolation theory provides an estimate of the critical pore density where long-range coalescence occurs. Assuming that the size of each individual pore is represented by the ensemble average of the pore size, the problem of pore percolation is analogous to the well-studied problem of *'disk percolation'* on a two-dimensional surface [43,44]. As a result, disk percolation is initiated if the surface fraction of disks is 0.44 for non-overlapping disks, and 0.67 for overlapping disks [41,44]. Non-overlapping disks may be taken as a model for pores with a hard core interaction potential, while overlapping disks refer to coalescent pores with zero interaction. The actual pore-pore interaction potentials should be between these extremes; pore percolation may perhaps start at $\sigma_c \approx 0.5$.

4.3.2. Percolation in double membranes

At sufficiently high electric field strength (and pulse duration) the critical pore density is exceeded ($\sigma \geq \sigma_{cr}$) and percolation of the double membrane pores proceeds. As a result, within the contact area of adhering cells a long closed chain of coalescent pores (percolation line) may form (fig. 4c). Such a loop enclosing an entire network of smaller cracks (fig. 5a) is called a *'large double membrane loop'*. If the percolation line is not completely closed for some reason (see section

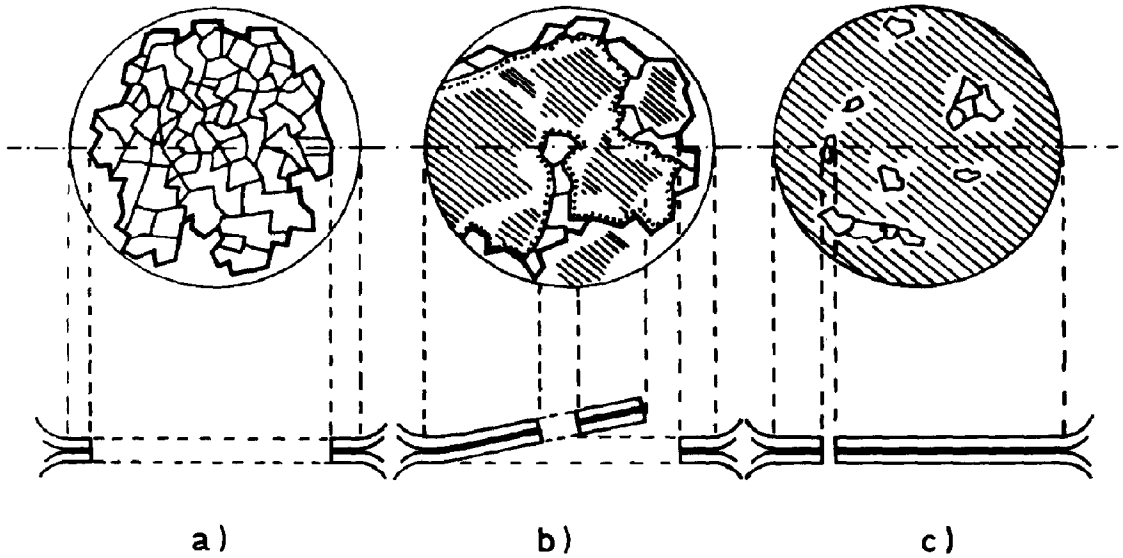

Fig. 5. Effect of membrane proteins on pore coalescence in the double membrane contact area of two adhering cells (fractional protein density β). (Upper part) Top views of the contact area; (lower part) cross-sections of the contact area taken along the dashed-dotted line (in the top views); thick solid lines, outermost percolation lines; thin solid lines, small double membrane loops; hatched areas, protein-rich regions. (a) Zero or low protein concentrations ($\beta \ll \beta_{cr}$): There is no protein-protein interaction. At supercritical pore density fragmentation of the whole area within the large double membrane loop (thick solid line) occurs. After removal of the enclosed membrane fragments (which become small inside-out vesicles) one large hole remains (see cross-section). (b) Subcritical protein concentration ($\beta < \beta_{cr}$): Finite clusters (hatched areas) of interacting proteins limit fragmentation of the area enclosed by the outer percolation line. Additionally, there arises an inner percolation line (· · · · · ·) defining the edge of the large double membrane tongue remaining after removal of the fragmented regions between the outer and inner percolation lines. The large double membrane tongue may bend from the plane of the contact area (see cross-section). (c) Supercritical protein concentration ($\beta \geq \beta_{cr}$): Long-range pore coalescence cannot occur due to extended and ramified protein percolation lines (within the hatched areas). Therefore, except for some smaller loops, the contact area remains intact during and after the field pulse application (see cross-section).

4.3.4.2), the contact area exhibits a *'large double membrane tongue'*. The double membrane area surrounded by the large loop may fragment and disappear resulting in one 'large hole' within the double membrane region (fig. 5a, lower row). Subsequently, an *intercellular cytoplasm bridge* (channel) may form (see section 6.3). The experimentally observed vehement mixing of the intracellular contents of fusing cells [45] is consistent with at least one large hole at the contact area. The *'uniqueness theorem'* of percolation theory [46] would predict the occurrence of just one large loop and consequently of just one large hole.

4.3.3. Percolation in single membranes

In single membranes initial short-range pore coalescence also grows into long-range coalescence at supercritical pore density ($\sigma \geq \sigma_{cr}$). In the case of vesicular membranes the pore density decreases with increasing distance from the poles (cf. section 3.1). Thus, besides small tongues and loops due to short-range coalescence at some distance from the poles, a large loop (or tongue, see section 4.3.4.1) may form by percolation within the pole regions. The openings at the sites of bending tongues (experimentally observed in the membrane of previously pulsed single erythrocytes [25]) may be the reason why macromolecules such as hemoglobin [4,47] or DNA [48,49] are able to permeate cell membranes after electroporation. These experimental observations suggest that pore coalescence and percolation presumably are similar in double and single membranes.

4.3.4. Membrane proteins and pore coalescence

4.3.4.1. Zero or low protein concentrations. In the case of electrofusion of large lipid vesicles (zero protein concentration) pore coalescence may develop everywhere in the contact area. At supercritical pore density a very ramified network of cracks is finally surrounded by one large loop practically following the edge of the contact area (fig. 5a). Double membrane fragments enclosed by small loops can leave the plane of the contact area and may appear as small inside-out vesicles in the intracellular space of the fusing cells (cf. table 1; see section 6.2). This membrane fragmentation may considerably reduce the size of the surface area in the double membrane part (fig. 5a). Indeed, during the electrofusion of artificial lipid vesicles there is a 20% reduction in membrane surface area [50]; in this case the formation of inside-out vesicles is, however, not visible by light microscopy. Electrofusion of membranes with low protein content, i.e., without noticeable protein-protein interactions, probably involves the same sequence of events as at zero protein concentration.

4.3.4.2. Subcritical protein concentrations. The presence of membrane proteins reduces the effective lipid area available for pore coalescence. Be-

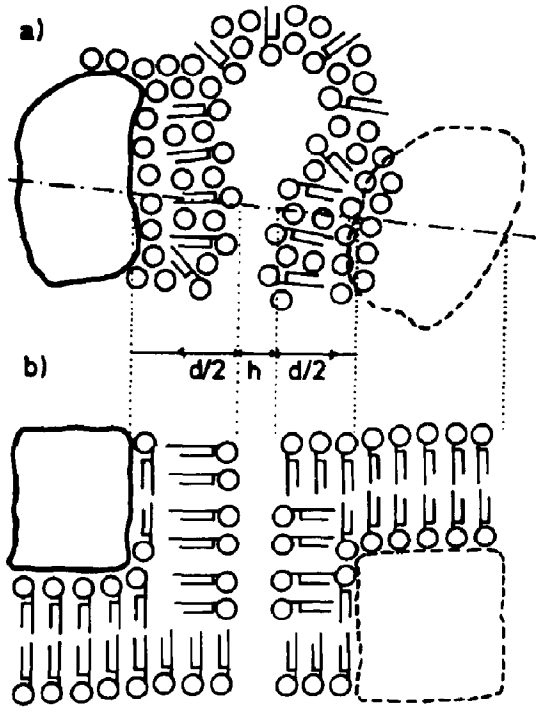

Fig. 6. Line of interacting double membrane pores (cf. fig. 3) between two membrane proteins in the contact area. (a) Top view; (b) cross-section taken along the dashed-dotted line (in the top view). Thick solid line, the edge of a protein in the upper membrane; dashed line, the edge of a protein located in the lower membrane; $d/2$, thickness of the lipid pore wall; h, width of the double membrane line defect (crack) in the contact area. There is no room for the formation of lipid pore walls between two membrane proteins which are closer to each other than the lipid membrane thickness d.

cause of the restricted mobility of the boundary lipid molecules near the membrane proteins [51,52] the probability of pore formation in the vicinity of protein molecules is smaller. In addition, the cracks will be less ramified. Because of the finite thickness of the pore walls the pore percolation line cannot form between two proteins the surfaces of which are closer to each other than twice the lipid pore wall thickness (fig. 6). Therefore, within finite clusters of interacting proteins (shaded in fig. 5b) the formation of long cracks is excluded.

Due to protein clustering the outermost percolation line may not be closed such that the contact area exhibits a *large double membrane tongue*. Cracks leading to fragmentation can only form in the lipid-rich regions between the protein clusters, provided the borderlines of the clusters are sufficiently separated from each other. Thus, in addition to the *outer percolation line* (thick solid line in fig. 5b) an *inner percolation line* arises (dotted in fig. 5b). It defines the edge of the double membrane tongue remaining after removal of the fragments between the outer and inner percolation lines. These small and medium double membrane fragments (including fragments from protein-rich regions) finally form inside-out vesicles (cf. table 2). The large double membrane tongue may bend from the plane of the contact area (fig. 5b, lower row).

4.3.4.3. Supercritical protein concentrations. The long chains of apparently aggregated intramembraneous particles seen in freeze-fracture micrographs of human erythrocyte membranes [53,54] may be viewed as protein percolation lines. Above a threshold value (β_{cr}) of the protein fraction (β) in the overall membrane surface, long chains of interacting, apparently connected proteins appear in the cell membrane. These chains of proteins are considered to result from *protein percolation*, which may be treated analogously to disk percolation [41,42] (cf. section 4.3.1).

Proteins in the membrane are called to be *'connected'* if a continuous line of interacting pores *cannot* form between them. Since the effective protein fractional density in the double membrane contact area is twice the value in either of the constituent single membranes, the threshold value in the protein fraction leading to protein percolation in the double membrane should be about 0.20–0.25. The existence of protein percolation depends on the type of membrane; in the studies on erythrocytes [53,54] it is not caused by electric field pulses.

If membrane proteins have percolated in the contact area, long-range coalescence of the double membrane pores cannot occur across the extended and ramified protein percolation lines. Thus, pore percolation is inhibited by protein percolation (cf. table 2). In this case small double membrane tongues (or loops) can only form in the protein-free parts of the contact area (fig. 5c), as is really evident from freeze-fracture micrographs [25].

Table 2

Membrane proteins and long-range pore coalescence (percolation) at supercritical pore density ($\sigma \geq \sigma_{cr}$)

(a) Single planar or vesicular membranes; (b) double membrane in the contact area of adhering cells.

Fractional protein density (β)	Crack surface topology	Membrane fragments	
		Intermediates	Vesicles
$\beta < \beta_{cr}$ (subcritical)	one large tongue	no	no
	small loops	(a) small disk micelles	(a) small
		(b) small double bells	(b) small; inside-out
	medium loops	(a) medium disk micelles	(a) medium
	(surrounding protein patches)	(b) medium double bells	(b) medium; inside-out
$\beta \geq \beta_{cr}$ (supercritical)	pore percolation inhibited by protein percolation (only short-range pore coalescence phenomena possible; cf. table 1)		

5. Resealing of small defects

5.1. Lipid bilayers and single cell membranes

After termination of the electric field pulse *resealing* of the membrane defects is observed. In planar lipid bilayer membranes the resealing of the field-induced pores is completed within 2–20 μs, and is strongly dependent on temperature and on the type of lipid molecules [39]. In the case of independent pores the mechanism of pore resealing in stable and metastable planar lipid membranes has been described quantitatively [38]. Pore resealing in a *metastable membrane* takes place if all of the pores are below a critical size. If one of the pores exceeds the critical size, the opening process continues and finally causes membrane rupture. In the case of a *stable membrane* pore resealing always occurs if the electric field is switched off. The kinetics of the resealing process has been measured for metastable planar membranes of oxidized cholesterol [39]; the model calculations [38] consistently reproduce the experimental data.

The theoretical results on planar lipid bilayers are not directly applicable to resealing of electroporated spherical membranes. Here, curvature effects and, in the case of single cells, the presence of membrane proteins and coupling to intra- and extracellular structures must be taken into account. Additionally, pore-pore interactions must be included. The membranes of large lipid vesicles or of single cells can be considered as stable objects as long as they do not interact with other membranes. Therefore, in analogy to stable planar membranes [55,56], one can expect pore resealing to be independent of pore size.

In the case of single *cell* membranes the resealing process lasts from several minutes up to 1 h [16,57]. Probably membrane proteins and their boundary lipids restrict the lateral diffusion of lipid molecules. The resealing of interacting pores or of holes most likely requires a longer time than that of independent pores because the resealing time is proportional to the surface area of the defect while the flow of lipid molecules towards the defect is proportional to the circumference. The occasional presence of cytoskeletal elements, in particular in the larger holes, may appreciably reduce the rate of the annealing process.

5.2. Double membranes

In contrast to single membranes, resealing of double membrane defects comprises *two simultaneous processes* in the contact area: changes in size of the defects (pores, cracks and holes) and backflow of previously squeezed-out water into the intermembraneous space of the double membrane. In addition, the state of the adhering cells is *metastable* relative to the stable fused state. After termination of the pulse lasting direct cell-cell contact is ensured by the edges of the double membrane defects in the contact area. Along the walls of the *'defect lines'* the cell membranes interact via *van-der-Waals* bonds (fig 2b). Thus, the *double membrane edges* also serve as permeability barriers for water flow between the extracellular (intermembraneous) and intracellular spaces.

In the presence of an external electric field the chemical potential difference between the water in the extracellular and intermembraneous spaces is counterbalanced by an the electric field-mediated attractive interaction between the cells [27]. After the field pulse, however, extracellular water is driven back and tends to separate the cells. This separation is against the fusion tendency of the whole system. When a double membrane pore is closing, the two constituent membranes are separating at that point. If every defect reseals completely, the whole contact area separates and there is no fusion. This is the case when the contact area is very small (point-to-point contact of the cells), the number and size of the defects also being small as a result [16].

6. Cooperative edge rounding and swelling

If the contact area is sufficiently large, the double membrane holes and tongues interfere with the backflow of water. Larger holes and tongues do not reseal to form separated bilayers. Experimental evidence [16] suggests that edge rounding and swelling processes dominate.

The membrane contact angle at the edge of a

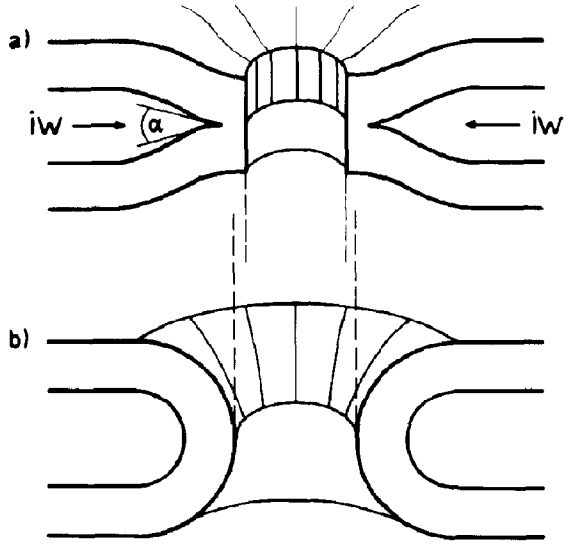

Fig. 7. Formation of an intercellular cytoplasm bridge (cross-sections). (a) A double membrane loop in the contact area. The membrane contact angle (α) at the edge of the defect increases with increasing amount of extracellular water flowing in (arrows iw). (b) Cooperative rounding of the initially sharp edge results in an intercellular channel enabling the formation of a cytoplasm bridge.

double membrane defect (α in fig. 7a) increases with increasing amount of inflowing extracellular water. In the presence of a large double membrane tongue (see section 4.3.4.2) the water flows into the intermembraneous area leading to tongue swelling (fig. 8a).

6.1. Tongue swelling

During the electrofusion of mesophyll protoplasts [22] swelling of the contact area of adhering

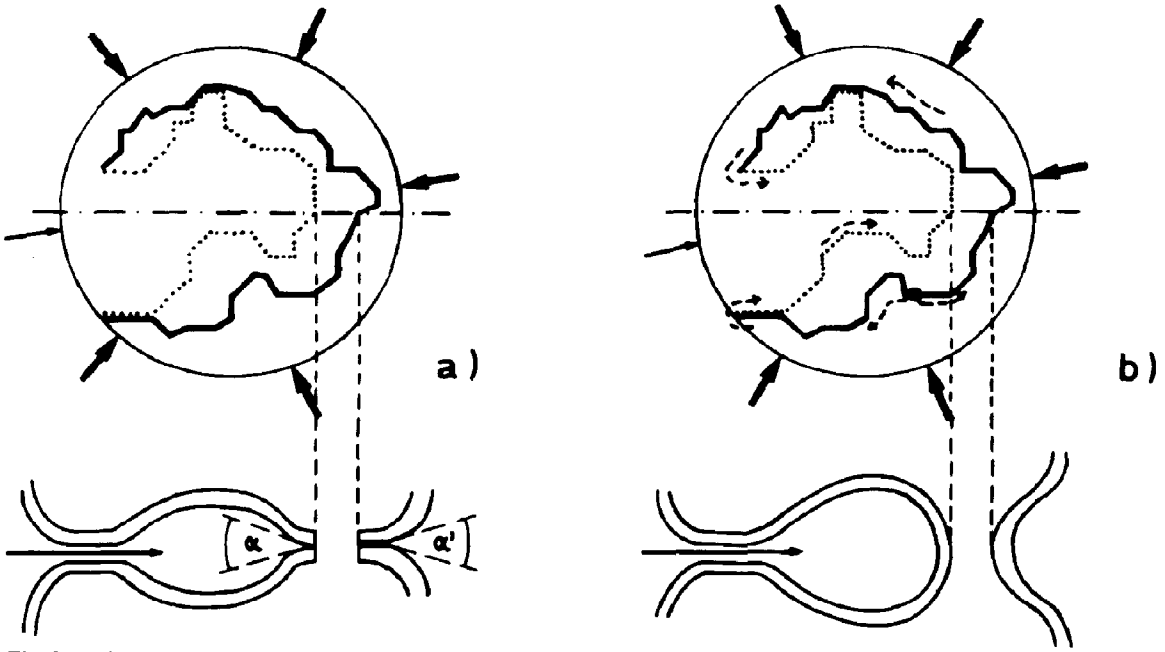

Fig. 8. Fusion steps due to the backflow of extracellular water into the intermembraneous space of the double membrane contact area. Thick solid line, outer percolation line in the contact area; dotted line, edge of the large double membrane tongue (cf. fig. 5b). The thick arrows represent the water flow towards the contact line. The thin arrow marks the water flow into the intermembraneous space of the large double membrane tongue. (Upper part) Top views of the contact area; (lower part) cross-sections of the contact area taken along the dashed-dotted lines (of the top views). (a) Swelling of the large double membrane tongue. A large inside-out vesicle is formed which in cross-section transiently looks like a double bell (lower row). With increasing amount of water in the intermembraneous space the membrane contact angles α and α' increase resulting in a tension on the edge regions. (b) Cooperative rounding of the edges along the percolation lines. (Upper part) The large black dot represents the nucleus of the rounded edge structure which spreads (dashed arrows) over the whole double membrane edge. (Lower part) Cross-section of the rounded edges.

cell membranes has been observed; this swelling was called *'vesicle formation'*. The size of the swelling object was slightly smaller than that of the entire contact area. Intracellular stain did not appear in the swelling object [22], which looked like a *'double bell'* apparently symmetrically located along the plane of the contact area. Sometimes several smaller swelling objects have been observed within one contact area. However, swelling objects have not been found in electrofusion of large artificial lipid vesicles [50].

These observations can be readily rationalized in terms of our fusion model: The swelling objects of the protoplast fusion are identified with swelling double membrane tongues (fig. 8), the lack of swelling objects in lipid vesicle fusion being due to complete fragmentation of the area enclosed by a large loop (see section 4.3.4.1 and fig. 5a). If tongue swelling indeed arises from inflow of extracellular water, the swelling objects are expected to exclude the stain introduced into the cells prior to fusion. It should be mentioned that previous electrofusion models fail to interpret both experimental observations: the lack of stain in the swelling objects as well as the exclusive location of the objects just in the plane of the contact area.

6.2. *Vesicle formation*

Freeze-fracture electron micrographs of the electrofusion process of large vesicles derived from the mitochondrial inner membrane exhibit larger and smaller membrane fragments which were identified as inside-out vesicles located within the larger main electrofusion products [23]. According to our model these fusion products originate from disconnected double membrane fragments (cf. fig. 5a and b; tables 1 and 2). These fragments may swell by inflow of intracellular water and transiently look like double bells. Edge rounding finally leads to spherical vesicles.

6.3. *Formation of intercellular cytoplasm bridges and disappearance of contact lines*

The continuous inflow of water into the intermembraneous space increases the membrane contact angles (α and α' in fig. 8a) and enhances the tension on the double membrane edges of the defects (figs. 7 and 8a). This increasing tension triggers the *cooperative rounding* of the double membrane edges (fig. 8b). Edge rounding may finally result in the opening of an *'intercellular channel'* of less (energetically unfavorable) membrane curvature (cf. fig. 7b). The formation of a cytoplasm bridge (channel) apparently is the decisive irreversible step in electrofusion. Such cytoplasma bridges have actually been observed in electrofusion experiments [16,25,45].

The role of the contact angle in the rounding process and in fusion itself was demonstrated in experiments with giant phosphatidylcholine/phosphatidylethanolamine (PC/PE) unilamellar vesicles [58]. In the case of point-to-point contact of these vesicles the fusion could be induced by mechanically pulling the vesicles apart for a moment along their poles. The fusion took place in spite of the small contact area because the mechanical deformation increased the otherwise zero contact angle inducing the rounding of the defect edges.

In the case of a large contact area the backflow of water also enlarges the contact angle at the outer percolation line (α' in fig. 8a). Thus, the rounding process occurs spontaneously without any micromanipulation. The rounding process involves the *disappearance of the contact line* as observed by light microscopy as one of the final stages in electrofusion [16].

When the large double membrane tongue remains intact and swells within the contact plane, the cooperative edge rounding process runs along both the outer and inner percolation lines (see fig. 8b). Thus, the contact lines disappear simultaneously at the cell-cell contact and at the swollen tongue. These consequences of our model are consistent with the experimental observations of mesophyll protoplast fusion where the swelling of the objects is concomitant with the fading of the contact line [22].

7. Membrane contact after pulsing

Electrofusion has so far been discussed in detail for the case where cells at first are brought into

contact and then the electric field pulse is applied. However, cell fusion may also be obtained if at first the high electric field pulse is applied to a suspension of noncontacting cells (not lipid vesicles), and subsequent contact established by dielectrophoresis [21,59] or sedimentation [60]. As yet, no ultrastructural data are available concerning this fusion process. However, its mechanism should be different from that discussed above because, after pulsing, dielectrophoresis or sedimentation alone cannot produce the envisaged tight membrane-membrane contact over a sufficiently large contact area (cf. section 2.2, fig. 1c).

According to experimental data the fusion yield will be much higher if the cell poles (exposed in the field direction) are subsequently brought into contact in an oriented manner [59]. It is known that electroporation may lead to a fairly prolonged permeability increase in single cell membranes [47,49,61]. This means that field-induced pores, cracks and other kinds of defect structures (especially located in the pole caps) may be fairly long-lived. Local cell-cell contacts obviously are then capable of initiating some fusion events. Starting from the field-induced, long-lived single membrane defects we may envisage this fusion process as follows:

(i) The ordered interfacial water structure is broken or loosened along the line of membrane defects resulting in a local reduction of the repulsive hydration forces.

(ii) As a result of thermal fluctuations the membrane can deviate from the average membrane shape; especially along the pore and crack edges local bending out from the membrane plane may occur.

(iii) The ramified defect structures increase the chance of direct contact between the edges belonging to defects at opposing membranes of cells contacting after the field pulse.

(iv) At the direct contact sites of the edges the formation of rounded edge structures is energetically favored. The rounded edge structure involves the reestablishment of both the bilayer structure and the ordered interfacial water structure.

(v) Because of the energetic reasons mentioned above the local edge-edge contact cooperatively spreads over the remaining part of the edges of small tongues and loops. The formation of an intercellular cytoplasm bridge (fig. 7) may also be the decisive step in this fusion process subsequently leading to the final rounded fusion product.

8. Concluding remarks

The general point in every fusion mechanism is the requirement of sufficiently tight contact between the fusing cells or vesicles, either established locally by fluctuations (in the absence of a field pulse) or enforced by electrically induced attraction. The opposing membranes interact through spontaneously formed or induced membrane pores and cracks. The actual fusion process occurs spontaneously proceeding from the metastable state of the contacting cells to the stable fused state.

Fusion will be favored by increasing the contact area as well as the concentration of membrane defects and the extent of ramification of the membrane cracks. Interestingly, tongue-like cracks preferentially are detected in apparently protein-free membrane areas of electrofused erythrocytes [25]. According to our model high concentrations of membrane proteins should prevent electrofusion.

The electrofusion model, treated here on a purely qualitative level, aims at stimulating quantitative experimental and theoretical investigations. Since electrofusion phenomena are rather complicated, partial aspects of electrofusion events may be tackled separately. In any case, the further elucidation of the underlying mechanisms may provide a basis for a more goal-directed application of the electrofusion technique in cell biology and biotechnology.

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