Biochimica et Biophysica Acta 939 (1988) 561–568
Elsevier

BBA 73972

Mapping of the field distribution around dielectrophoretically aligned cells by means of small particles as field probes

W. Mehrle [a], R. Hampp [a], U. Zimmermann [b] and H.P. Schwan [b]

[a] *Institut für Biologie der Universität Tübingen, Tübingen and* [b] *Lehrstuhl für Biotechnologie der Universität Würzburg, Würzburg (F.R.G.)*

(Received 27 October 1987)

Key words: Field strength; Aligned protoplast; Protoplast; Electrophoresis; Dielectrophoresis; Field probe

For the prediction of the conditions of electrofusion knowledge about the strength and divergence of the local field around dielectrophoretically aligned cells is required. Bacteria and chloroplasts (showing positive dielectrophoresis) as well as polystyrene and glass beads (exhibiting negative dielectrophoresis) are used as small probes for the field line distribution generated by aligned plant protoplasts. The biological particles gather at the poles of terminal protoplasts and close to the contact zones of adhered protoplasts in the chain, whereas the artificial beads form concentric rings around the individual aligned protoplasts. The position of these concentric rings and of bacterial assembly depends on the volume of the aligned protoplasts and on their location in the chain. These phenomena occur at much lower field strengths than those required for the alignment of these particles in the absence of protoplasts. This suggests that the field strength and the inhomogeneity close to the contact zone of aligned protoplasts may be considerably higher than in the external space. This may also explain why symmetric breakdown leading to fusion can occur in both hemispheres of aligned cells despite the fact that in freely suspended cells an asymmetric breakdown is observed under the experimental conditions.

Introduction

In contrast to chemically or virally induced fusion electrofusion is expected to accurately predict the fusion conditions for any cell fusion system because of the vectorial character of the field parameters [1–3]. However, in practice we are faced with the problem that the equations used for the calculation of cell alignment and of the fusion (breakdown) pulse represent only rough estimations for dielectrophoresis and fusion [1–7]. These equations have been derived for single (or a few), freely suspended cells, but not for the multi-body problems involved in electrofusion. There is experimental evidence that the actual strength and divergence of the field in and around the contact zone of aligned cells during application of the alternating field and the breakdown pulse must be considerably different from the field distribution in the cell-free space between the cell chains [1–3].

Theoretically, it is extremely difficult to calculate the actual field strength and divergence in and around the contact zone of adhered cells. Additional complications arise when differently sized cells are aligned due to the volume dependence of the dielectrophoretic force [4–7] and because the membrane voltage produced by a given field strength is proportional to the cell radius [1–3].

In order to arrive at a better establishment of the critical field conditions for alignment and

Correspondence: W. Mehrle, Institut für Biologie der Universität Tübingen, Auf der Morgenstelle 1, D-7400 Tübingen 1, F.R.G.

fusion of various cells a possible experimental approach would be (at least at the time being) the simultaneous introduction of small particles as indicator probes for the field line distribution in the electrode gap during cell dielectrophoresis. If these particles are small enough and the strength and divergence of the local field around the cells and chains is sufficiently high they will also align in these regions, even though these particles alone may need much higher field strengths for chain formation owing to the volume dependence of the dielectrophoretic force [6]. In particular, a precise picture of the field line distribution and gradients around cell chains may be obtained in this way if two types of particles exhibiting either negative or positive dielectrophoresis are used as field probes. Depending on the ratio of the relative dielectric constants of the particles and the medium these field probes will either move in regions where the dielectrophoretic force has its maximum (positive dielectrophoresis) or its minimum value (negative dielectrophoresis) [6].

In this communication we report on the field line distortion induced by dielectrophoretically aligned plant protoplasts, employing pretreated bacteria and chloroplasts (showing positive dielectrophoresis) as well as glass and polystyrene beads (showing negative dielectrophoresis) for illustration of the field lines. Because of the larger volume of the protoplasts the probes are small enough to avoid significant distortion of the field lines when they are introduced.

The results demonstrate that this experimental procedure is a suitable approach to get insight into the local field distribution around the cells in a chain, which may pave the ways for further improvement of electrofusion conditions and electrode arrangements.

Materials and Methods

Cell-wall-free mesophyll cells (protoplasts) from primary leaves of *Avena sativa* L. were prepared as reported elsewhere [8], except that instead of cutting leaf segments, the lower epidermis was peeled away. Evacuolated protoplasts were prepared according to Griesbach and Sink [9] with the modifications described in Ref. 10. Purified protoplasts were resuspended in a 0.5 M isopycnic 1:1 solution of sorbitol/sucrose (conductivity about 10 μS/cm). An aliquot of the diluted protoplast suspension (10 μl, $5 \cdot 10^6$ protoplasts/ml) was pipetted between two NiCr wires which served as electrodes if not otherwise stated. The wires (100 or 200 μm diameter) were mounted on a microscope slide 100–800 μm apart. This arrangement causes a slightly non-uniform field.

Electrical fields were applied with a 'Zimmermann Fusion' generator (GCA Corp., Chicago, IL). Voltages were monitored with a Hameg oscilloscope (type HM 203-5). In order to estimate the threshold voltages, that result in chain formation, samples were exposed to an alternating electric field of different strength (10–600 V/cm, rms) at a frequency of 1 MHz. The experimental procedures used here are described in detail by Takashima and Schwan [5].

The distribution of field lines around protoplasts (20 to 50 μm diameter) was illustrated by the use of considerably smaller particles of distinctly different dielectric properties, such as glass beads (3–10 μm diameter), polystyrene microspheres (2.5–5.4 μm diameter, Polysciences Inc.), both in aqueous suspension, chloroplasts (5 μm diameter, set free by mechanical rupture from the leaf protoplasts), or bacteria (1 μm diameter). The latter, *Pseudomonas aeruginosa* (ATCC 10145, German Collection of Microorganisms, Göttingen), were cultured according to Ref. 11. To prevent the bacteria from swimming, suspensions were inactivated by a short microwave irradiation or by keeping them at $-20\,^{\circ}$C for 24 h, in 20 mM CaCl.

Phase contrast photomicrographs were taken with a Leitz microflash (100 W/s).

Results

The threshold field strength of the alternating field required for the alignment of differently sized *Avena* protoplasts containing either a vacuole (average diameter 38 μm) or no vacuole (average diameter 22 μm) is determined to be in the range of 18 V/cm and 25 V/cm, respectively, at a frequency of 1 MHz. Because of their smaller size chloroplasts and bacteria require much higher threshold field strengths of the order of 45 V/cm and 200 V/cm, respectively, to achieve chain formation by positive dielectrophoresis along the field

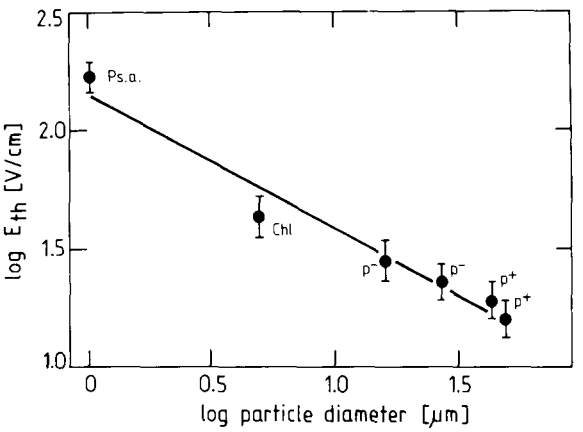

Fig. 1. Double-logarithmic plot of the threshold field strength, E_{th}, for chain formation vs. particle diameter measured at a frequency of 1 MHz. Measurements on four different particle suspensions are shown: P^+, vacuole-containing oat protoplasts (diameter 30–45 μm); P^-, evacuolated oat protoplasts (diameter 10–25 μm); Chl., oat chloroplasts (diameter 5 μm); PS. a., bacteria, *Pseudomonas aeruginosa* (diameter 1 μm). Vertical bars indicate standard deviations. Values are means of ten independent experiments. Fluctuation of data indicates the experimental difficulty of obtaining precise threshold values, even when the experimental system is very well defined.

lines between the two cylindrical electrodes. According to the theory developed by Schwan and Sher [4] the square of the threshold field strength is inversely proportional to the volume of the particle (cell). Therefore, a double logarithmic plot of the field strength against the diameter should yield a straight line with a negative slope. As shown in Fig. 1 this theoretically expected relationship is approximately fulfilled for the vacuole-containing and evacuolated plant protoplasts and the biological particles used as field probes.

In the following set of experiments alignment patterns of pretreated bacterial cells and chloroplasts are studied in the presence of vacuole-containing (or evacuolated) plant protoplasts. In agreement with the results obtained above field strengths of 20 V/cm applied to a mixture of protoplasts and bacteria only lead to the alignment of the plant protoplasts. Above a field strength of about 70 V/cm bacteria are, however, attracted to the poles of the protoplasts facing either the opposite electrode or another protoplast aligned some distance apart (Fig. 2A). Above a field strength of 70 V/cm (which leads to a slight elongation of the plant protoplasts) alignment of the pretreated bacteria increases in the close neighbourhood of the contact zones of two adhered cells in a chain. The photograph of Figs. 2B and 2C taken at a field strength of 300 V/cm (for pronounced bacterial accumulation) clearly indicates the preferential orientation of the bacteria around the contact zones of aligned cells. Only when differently sized cells are arranged in a chain can an enveloping of the small protoplast by aligned bacteria be observed (Fig. 2C). Within the contact zone between two protoplasts alignment

Fig. 2. Photomicrographs of the alignment of bacteria and oat mesophyll protoplasts in an alternating electric field generated between two cylindrical electrodes (200 μm apart). (A) At a minimum field strength of 70 V/cm bacteria are attracted to the poles of the aligned protoplasts some distance apart (phase contrast microscopy). (B) Alignment of bacteria close to the contact zone of aligned protoplasts in a continuous chain (phase contrast microscopy, field strength 300 V/cm). Note that the protoplasts are deformed due to the high field strength. (C) Alignment of bacteria in the presence of a protoplast chain of different sized cells. The small protoplast is enveloped by aligned bacteria (phase contrast microscopy, field strength 300 V/cm)). (D) Illustration of the density of the field lines by dark field illumination. Constriction of the field lines at the pole of a single protoplast resulting in a bacteria-free space is obvious. Some distance from the protoplast only chains of bacteria emanating from one electrode to the other are visible.

Fig. 3. (A) Photomicrograph of the arrangement of polystyrene beads (diameter 4 μm) in an alternating field of 100 V/cm strength. The particles are repelled from the electrodes. This effect is the result of negative dielectrophoresis. Only at a high density some of the beads form chains within the gap of the electrodes. (B) Dark field photomicrograph of polystyrene beads and oat protoplasts under conditions as in (A). Most of the light scattering beads move out of the electrode gap. The remaining beads move to the protoplast chains and form (white) concentric rings. These concentric rings are located in the equatorial plane of the protoplasts provided that adjacent cells have the same size. (C) The asymmetric arrangement of concentric polystyrene rings which is observed around terminal protoplasts. The rings are placed in a level perpendicular to the protoplast chains which obviously reflects the region of lowest field intensity and inhomogeneity. (D) Simultaneous alignment of bacteria and polystyrene particles in the presence of chains of evacuolated protoplasts. The rings envelope the protoplasts with the exception of those regions in which bacteria align.

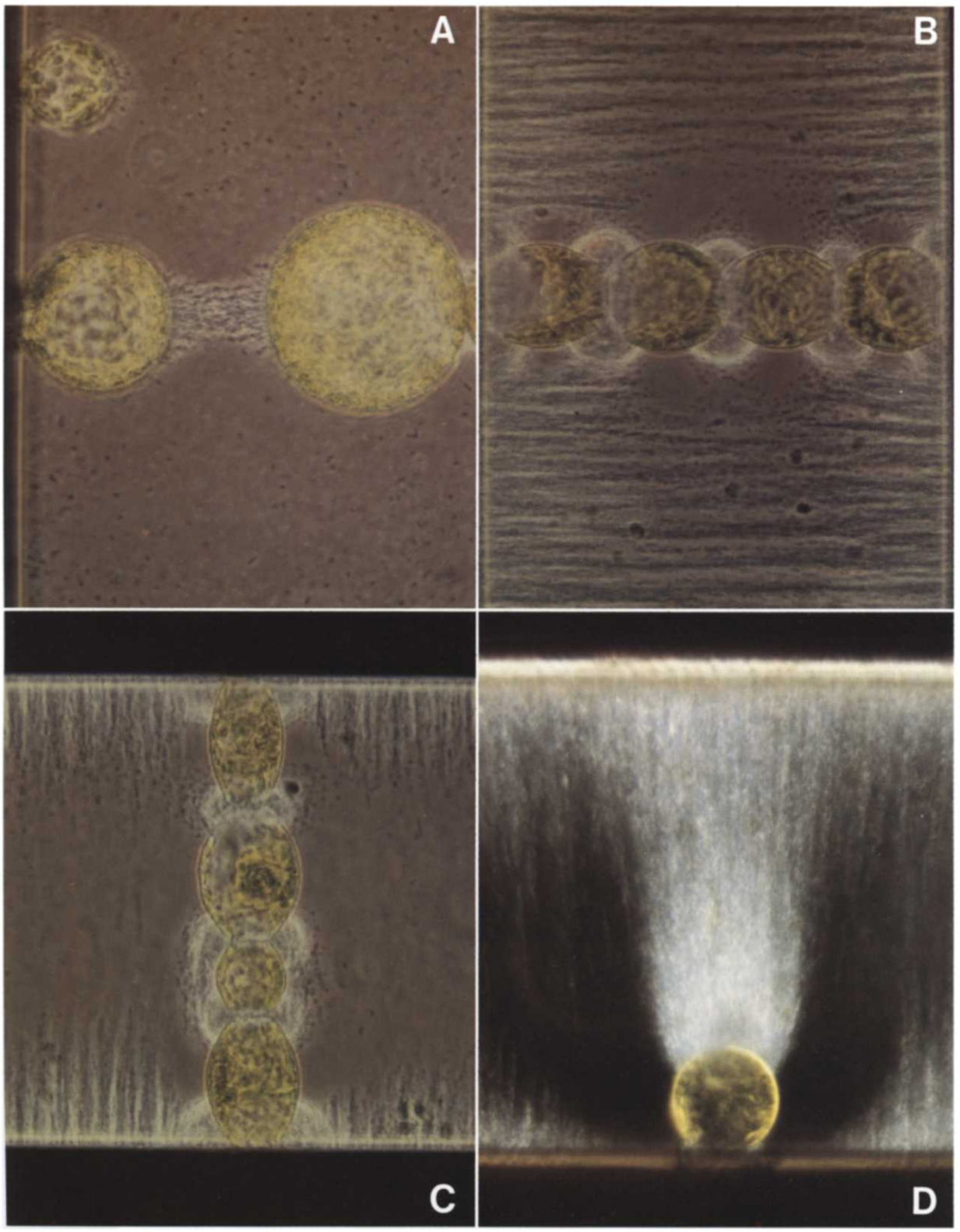

Fig. 2. For legend see p. 563.

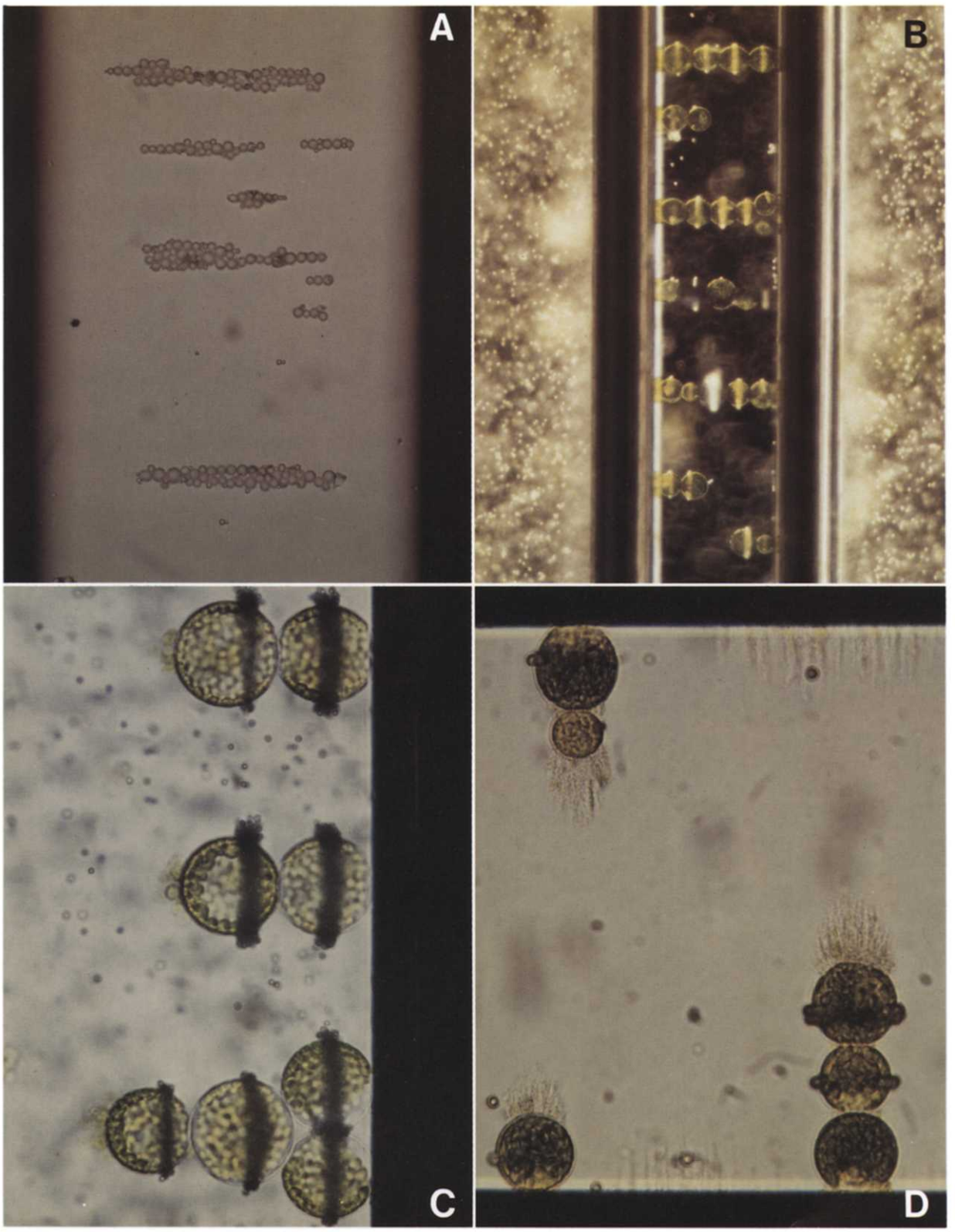

Fig. 3. For legend see p. 563.

of bacteria does apparently not occur, probably because of the close distance between the two apposed membranes which has been found to be of the order of 20 nm [12]. It is obvious that the larger bacteria are excluded from this space. Alignment of bacterial cells in the external space between protoplast chains is not achieved at field strengths below 200 V/cm with the exception that at the cylindrical electrode surfaces small bacteria chains are observed above field strengths of 160 V/cm.

These results clearly show that the dielectrophoretic forces arising from the distorted field around the contact zones of protoplasts in chains must exceed the critical value for the given electrode arrangement. Since the (effective) dielectrophoretic force is not only proportional to the square of the field strength, but also to the divergence (inhomogeneity) of the field [6] it is difficult to decide whether the occurrence of bacteria alignment at 70 V/cm around the contact zone of adhered protoplasts is due to an increase in strength and/or in inhomogeneity of the local field. Fig. 2D (see also Fig. 2A) demonstrates that the field line density as illustrated by bacteria alignment at field strengths of 300 V/cm is much higher between the pole of a (terminal) protoplast and the opposite electrode as well as at the contact zones of protoplasts than in the external space. On the other hand, it is also evident that the field is much more distorted in the neighbourhood of the poles and at the contact zones between adhered protoplasts than far away from the protoplast chain. The influence of the inhomogeneity of the electric field can also be demonstrated by using plate electrodes for protoplast alignment. Under these conditions a field strength of 30 V/cm does not lead to the formation of protoplasts chains emanating from the flat electrodes. Obviously, in this electrode arrangement the field is too homogeneous (also at the electrode surface) to create the necessary dielectrophoretic force. In this case chains are only formed in the middle of the electrode gap. This alignment occurs because of the inhomogeneity of the local fields introduced by the generated dipoles within the protoplasts which result in a sufficient force for attraction of the cells (for theory, see Ref. 7).

Similar results are obtained by means of chloroplasts. Alignment of these particles in the neighbourhood of the contact zones and at the poles of the protoplast chains are, however, observed at slightly lower field strengths (25 V/cm, instead of 45 V/cm in the absence of protoplasts) because of their larger radius compared to that of bacteria.

In a second set of experiments the biological particles were replaced by polystyrene (or glass) beads. These particles are injected into the electrode gap in the absence of protoplasts. At the very beginning, this results in the formation of chains along the field lines far away from the electrode surfaces, that means in regions of weakest field intensity and divergence within the electrode gap (Fig. 3A). After a couple of minutes these chains start to migrate into the space outside the electrodes (Fig. 3B). Chains emanating from the electrode surface cannot be observed. These particles apparently show negative dielectrophoresis at this frequency of field. Application of an alternating electric field of 80 V/cm to a mixture of polystyrene (or glass) beads and protoplasts results in a similar phenomenon with the notable exception that a part of these particles also start to migrate into those regions of the protoplast chains where no bacteria alignment is observed. The result is the formation of concentric rings of particles around the individual protoplasts within the chains (Figs. 3B–D). It is interesting to mention that these rings of particles are located in the 'equatorial' plane of the protoplasts if the adjacent cells are of comparable size. However, in the case of considerable differences in radius of two adjacent protoplasts or for terminal protoplasts (Fig. 3C) these rings are asymmetrically arranged. The width of these particle rings depends on the amount of particles injected. At higher concentrations these rings envelop the whole protoplast with the exception of the region in which the biological particles align. This can be also demonstrated by experiments, in which both kinds of particles are injected together with the protoplasts into the electrode gap (Fig. 3D).

Glass beads yield the same results as polystyrene particles. Polystyrene particles have, however, the advantage that their density is similar to that of the medium so that they do not sediment so quickly. It is interesting to note that after

application of a breakdown pulse of 700 V/cm intensity and 20 μs duration as required for protoplast fusion the polystyrene rings of these two cells move and merge into each other at the former membrane contact zone during the fusion process.

Discussion

The use of field probes demonstrate that the presence and alignment of cells for electrofusion considerably changes the field line distribution within the electrode gap. Provided that the introduced particles do not significantly change the field pattern we can conclude that both the field strength and the inhomogeneity (divergence) around the cells in an aligned cell chain considerably differs from that in the cell-free space. The high density of bacterial chains in the close neighbourhood of the contact zones and at the poles of single or terminal cells in a chain suggests that the local field strength should be considerably higher than that in the external space. Furthermore, electro-rotation measurements on large plant protoplasts suggest, that at the frequency of 1 MHz used here both membranes (tonoplast and plasmalemma) present a low impedance [13]. Because of the high conductance of the cell to 1 MHz current it is expected that the field strength in the gap between the two apposed membranes of adhered cells must be greatly enhanced compared to that in the space between the cell chains. However, the results also show that the distortion of the field around the cells creates a high inhomogeneity which may contribute considerably to particle alignment.

In any case, if we consider the complementary alignment of biological and artificial particles we can conclude that the term ∇E^2 which controls dielectrophoresis for a given particle system must assume values in the external space close to the chains which are between those of the region at the contact zone of two adhered cells and those of the equatorial plane of a cell in the chain. Otherwise, the formation of equatorial rings of artifical particles cannot be understood.

The increase of both the field strength and the inhomogeneity in and around the contact zone of adhered cells in a chain may help to explain the discrepancy between breakdown of membranes of freely suspended cells and of adhered cells. In non-electrolyte solutions as used here breakdown occurs asymmetrically, i.e. only in one hemisphere of freely-suspended cells, if field pulses of critical or slightly supercritical strengths are applied [14,15]. The asymmetry of the breakdown event arises from the superposition of the generated field on the intrinsic field. In the hemisphere facing the anode the two field vectors are in parallel, in the other one antiparallel. Since comparable field strengths of the breakdown pulse are required for fusion, breakdown would occur only in one of the membranes of the adhered cells. The results obtained here suggest, however, that both the field strength and the inhomogeneity in and around the contact zone are sufficiently high to induce local breakdown in the membranes of both hemispheres. Thus cytoplasmic connections between the two adhered cells can be generated as has been seen by electron microscopy [12]. The inhomogeneous field pattern around the contact zone may be one of the driving forces for the intermingling of the two adhered membranes. From the photographs in Fig. 2 it is immediately clear that the two adhered membranes are contracted due to the induced charges at the apposed membrane areas. This result explains why the alternating field which has to be switched off during application of the breakdown pulse must be switched on again for a couple of seconds just after electrically induced permeabilisation of the membrane area in the contact zone [2,3].

Osmotic driving forces as suggested by Lucy and Ahkong [16,17] may be involved in a subsequent step of the intermingling of the membranes of two adhered cells. The complimentary orientation and arrangement of the biological and artifical particles along the protoplast chains also support the assumption that electropermeabilisation only occurs in a restricted area of the cells in contrast to chemically and virally induced fusion [2,3]. The formation of broad rings of artificial beads around the protoplasts clearly indicates that in these regions the breakdown voltage is not exceeded at the field strengths used for fusion. This has the important consequence that release of intracellular solutes must be very small under mild electrofusion conditions and that a possible release is only restricted to the pole areas of termi-

nal protoplasts in the chain.

Finally, the finding that significantly smaller cells in the chain produce a larger increase in field strength and inhomogeneity in their surrounding than equal-sized cells may be important for the improvement of fusion conditions of differently sized cells.

This problem is particularly evident in electrofusion of small lymphocytes and large myeloma cells for formation of hybridoma cells. Indeed, as shown recently [18], the size distribution and, in particular, the modal volume determines the yield of hybrids. One promising way to overcome this problem is to change the growth conditions of the myeloma cells in order to produce smaller sized cells. Likewise, it is evident why stimulated lymphocytes which have a comparable size to myeloma cells preferentially fuse with myeloma cells so that most of the hybridoma cells obtained by electrofusion produce antibodies (Refs. 19, 20, and Schmidt, Zimmermann, Neil, unpublished data).

Acknowledgments

The authors are indebted to the Bundesministerium für Forschung und Technologie (Grant No. 01QV85203 to R.H.) and the Deutsche Forschungsgemeinsschaft (SFB 176 to U.Z.) for financial support.

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