Biochimica et Biophysica Acta, 1066 (1991) 83–89

ADONIS 000527369100262U

BBAMEM 75264

Electroinsertion of xeno proteins in red blood cell membranes yields a long lived protein carrier in circulation

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(Received 12 November 1990)

Key words: Electroinsertion; Erythrocyte; Biotinylation; Flow cytometry; Life-span

Electroinsertion, a novel method of implanting xeno-proteins in red blood cell plasma membranes is applied to the insertion of human glycophorin in mouse red blood cells. The procedures yield erythrocytes with approx. 10^4 glycophorin molecules per cell, displaying the patching phenomenon when reacted with antiglycophorin monoclonal antibodies. Insertion of FITC-labeled glycophorin and subsequent quenching of FITC fluorescence with anti FITC antibody indicated that 70% of the inserted molecules were 'correctly' oriented, displaying the epitopes shown by glycophorin in human red blood cells. Moreover, insertion of FITC glycophorin in the red blood cell membranes yielded, under the fluorescence microscope continuous fluorescence which became patchy after reaction with anti-glycophorin monoclonal antibodies. When injected in mice, biotinylated RBC-Glyc$^+$ were shown to have the same life-span as normal mouse red blood cells, i.e. $t_{1/2} \approx 12$ days. Cytofluorometric assay of human glycophorin inserted in the red blood cell showed the same life-span for the inserted protein as for the red blood cell, with the protein fully 'functional', i.e. capable of binding antibodies. Human glycophorin inserted in mouse erythrocyte did not elicit any immune response in mice, whereas the same concentrations of free glycophorin injected i.v. were highly immunogenic.

Introduction

Due to its relatively long life-span in circulation, the red blood cell may be an ideal carrier of xeno antigens, receptors etc., provided that these molecules can be implanted in the RBC plasma membrane in such a way as to leave the red blood cell's life-span unchanged, while preserving the specific properties of the inserted molecules.

Fusogenic viruses have been successfully used to implant xeno-proteins in plasma membranes of a variety of cells [1,2]. Fusion of liposomes bearing covalently attached lysozyme was observed at acid pH with red blood cell ghosts [3] and with mouse liver nuclei [4]. Brief exposures of RBC with glycophorin or CD4 at acid pH (4.7) led to association of these proteins with the red blood cells [4,5]. However, low pH treatment of the RBC under conditions of maximum protein association results in a significant reduction of the life-span of these RBC-P$^+$, in mouse.

Electroporation was shown to induce transient pores in the plasma membrane of different cell types [6]. The effects of electrical fields on red blood cell membrane, especially the leaks induced by these fields have been recently reviewed [7]. Moreover, it was demonstrated that electroporation did not alter the RBC life-spans [8]. We thought, that by subjecting red blood cells to pulsed electric fields of intensities slightly below those required for electroporation, transient 'perturbation' of the lipid bilayer may be induced, 'perturbations' which would allow the hydrophobic sequence of a protein to partition in the lipid bilayer. Electroinsertion of such a protein was first obtained with glycophorin [9] and then with the CD4 receptor of the T_4 lymphocytes [10] in red blood cell membranes.

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Abbreviations: RBC, red blood cells; NHS-biotin, *N*-hydroxysulfosuccimimide; PE, phycoerythrin; Gam, goat anti mouse antibodies; CD4, antigen on helper-inducer T_4 lymphocytes, monocytes; RBC-P$^+$, red blood cells with xeno-protein inserted in their membrane; RBC-CD4$^+$, red blood cells with CD4 inserted in their membrane; RBC-Glyc$^+$, red blood cells with xeno-glycophorin inserted in their membrane.

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We report the obtention of mouse red blood cells with human glycophorin electroinserted in their plasma membrane with the normal life span in circulation. The presence of the electroinserted protein was assayed by flow cytometry using antiglycophorin monoclonal antibodies and fluorescently stained secondary antibodies.

Materials and Methods

Chemicals

Human glycophorin A type MM ($M_r = 31\,000$) (Sigma, St. Louis, MO) was used throughout. Gel electrophoresis indicated that glycophorin was pure, free of contamination (results not shown). The following anti human glycophorin type A, monoclonal antibodies were used: 10F7 (mouse isotype IgG1) was kindly provided by R.H. Jensen (Biomedical Science Division, University of California) and D2-10 (mouse Isotype IgG1) was from AMAC, Maine. Anti FITC antibodies were from Molecular Probes (Eugene, OR). NHS biotin was purchased from Serva, (New York, NY). Phycoerythrin conjugated, affinipure goat anti-mouse antibodies (Gam-PE) was from Jackson Immuno Research Laboratories (West Grove, PA).

FITC-labeling of glycophorin

FITC was covalently bound to human glycophorin according to Goldman [11]. Briefly, glycophorin, at a concentration of 10 mg/ml in 20 mM Tris-HCl (pH 8.9), was incubated with fluorescein isothiocyanate (Sigma, MO) at a ratio of 100 μg/mg protein. After four hours on ice, free fluorescein was removed by chromatography on Sephadex G-25 (Pharmacia). The FITC-glycophorin fraction was collected, desalted by four washings with distilled water and concentrated (Amicon micropartition system). The immune reactivity of FITC-glycophorin was verified by immunoblotting using 10F7 anti human glycophorin monoclonal antibodies for detection [12].

Electroinsertion

The pulse generator used was a 606 Cober device. The teflon chamber used in electroinsertion experiments is cylindrical, 1.2 cm diameter, with each end formed by 1.2 cm × 2.5 cm stainless steel electrodes, the electrode gap being variable (0.2–1 cm). Potential and current were monitored by a Nicolet 2090 digital oscilloscope. During the experiment, the electroinsertion chamber was held at 37°C using a circulating water thermostat.

Mouse erythrocytes were separated from heparinized fresh whole blood obtained from BALB/C strain mouse by retro orbital sinus puncture. The blood was washed three times with the electroinsertion medium, and an erythrocyte stock suspension was prepared.

Purified glycophorin was dissolved into the electroinsertion medium which consisted of 0.14 M NaCl and 10 mM Na_2HPO_4/NaH_2PO_4 adjusted to pH 8.8 by NaOH. The washing buffer contained 0.145 M NaCl and 5 mM phosphate at pH 7.4 (PBS 7.4). The glycophorin solution was added to the erythrocyte suspension. The final glycophorin concentration was 20 mg/ml and the hematocrit was 70%. After 20 min incubation on ice, the temperature was raised to 37°C and four square electrical pulses of 2.1 kV/cm and 1 ms duration were applied at 15-min intervals. After one hour incubation at 37°C, the cells were then washed three times in PBS 7.4 and kept at 4°C.

A control sample was used as reference, where RBC were subjected to all steps of the described procedure but no glycophorin was added.

Flow cytometry

Flow cytometry was performed on a Coulter EPICS profile instrument and argon laser (488 nm excitation). Two color fluorescence emissions were measured by filtering the passed photons through dichroic mirrors and narrow band filters (500 to 525 nm for green, 575 ± 10 nm for red). Alignment of the instrument was performed using 5 μm immunocheck beads (Coulter). A standard curve of fluorescence intensity vs. the number of fluorescent molecules was obtained at the same photomultiplier-voltage used to measure the fluorescence of labelled RBC. The histograms collected for analysis were 90° side scatter vs. forward scatter and fluorescence vs. number of cells.

Life span measurements of RBC-Glyc$^+$

Biotinylation of erythrocytes was performed according to the method described by Suzuki and Dale [13]. Briefly, mouse RBC-Glyc$^+$ were suspended to a 30% hematocrit in PBS. The RBC suspension (0.5 ml) were incubated at 25°C for 30 min with 250 μg of NHS-biotin. The cells were then washed twice with PBS. Following incubation with saturating avidin-PE (Molecular Probes Inc., Eugene, OR), the average level of biotinylation determined by flow cytometry was 18 000 biotin molecules per RBC.

The biotinylated erythrocytes were then suspended in a 0.15 M NaCl solution at 50% hematocrit and injected into the mouse through the tail vein. After the injection, samples were collected at different time points and labelled with avidin-PE. The percentage of the fluorescent RBC was determined by flow cytometry.

At the same time point, aliquots of the collected blood were assayed for glycophorin, also by flow cytometry. The RBCs were reacted with 10F7 and D2-10 antihuman glycophorin monoclonal antibodies and then with a Gam-PE secondary antibody and assayed by flow cytometry.

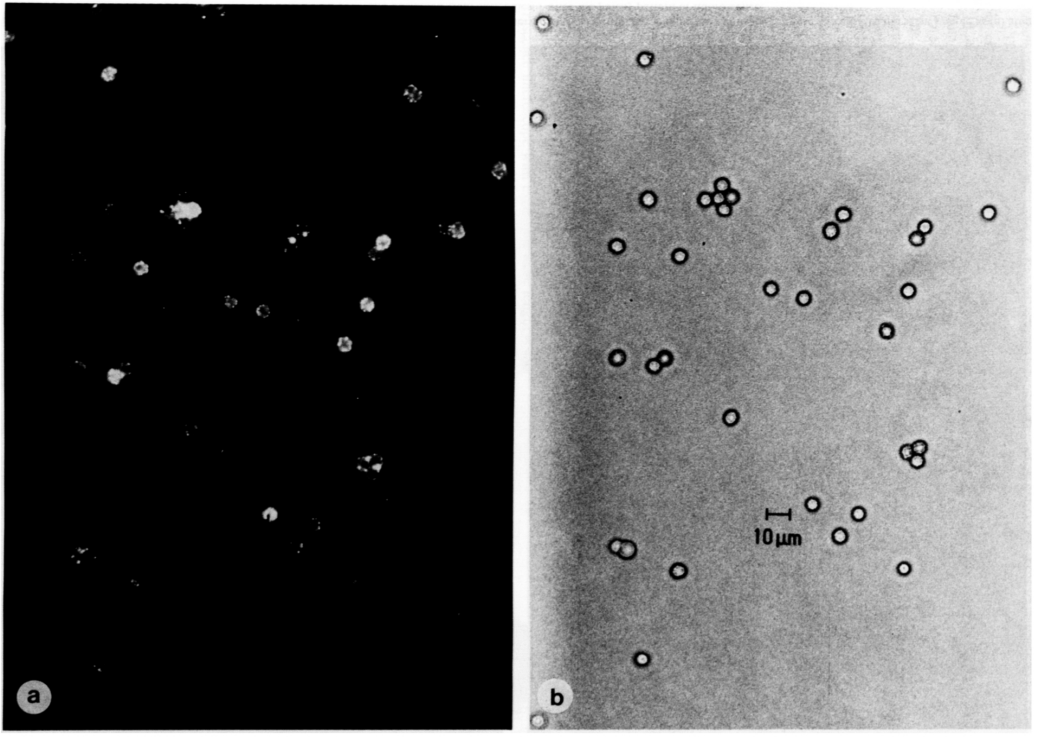

Fig. 1. (a) Immunofluorescence imaging of mouse RBC with human glycophorin inserted in their membrane upon reaction with 10F7 antihuman glycophorin antibodies and Gam-PE secondary antibodies. Notice the patching of glycophorin. Excitation wavelength 488 nm. (b) The same RBC-Glyc$^+$, direct light.

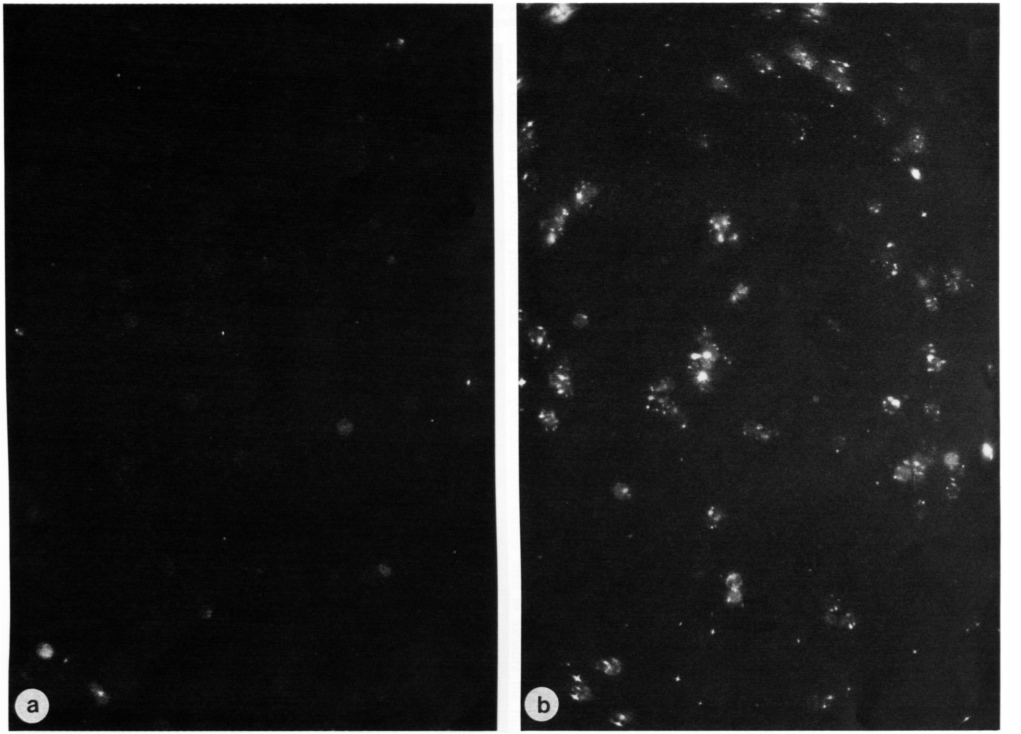

Fig. 2. Immunofluorescence imaging of (a) mouse RBC with FITC labeled human glycophorin inserted in their membrane. Notice the uniformly fluorescent cells; (b) the same sample as (a) after reaction with 10F7 and unstained Gam antibodies. Notice the patchy pattern of fluorescence.

Humoral immune response

The sero conversion of the mice injected with free or electroinserted glycophorin was assayed on days 7, 14 and 21 post injection by flow cytometry. $5 \cdot 10^6$ human RBC suspended in 50 μl of PBS were incubated for 30 min at 24°C with 10 μl of mouse serum. The RBC, after being washed twice by PBS, were incubated with Gam-PE secondary antibodies. After washing, the RBC were analyzed by flow cytometry (red fluorescence).

Results

Electroinsertion of glycophorin

The glycophorin molecules inserted into the RBC membrane were able to react with two different anti-human glycophorin monoclonal antibodies: 10F7 [14] and D2-10. In a preliminary study of these antibodies, and using a flow cytometric assay, labeling with 10F7 and Gam-PE evidenced 4500 epitopes; labeling of another aliquot of the same sample with D2-10 and Gam-PE showed 4100 epitopes. Using 10F7, D2-10 and Gam-PE, 8200 epitopes were detected. These data demonstrate that these two epitopes are not overlapping and that the electroinserted glycophorin identically exposes these two different epitopes.

Examination of mouse RBC-Glyc$^+$ under the fluorescence microscope with excitation wave length = 480 $\pm$25 nm yielded the pictures shown in Fig. 1a. The punctate fluorescence pattern observed after reaction of the RBC-Glyc$^+$ with 10F7, anti-human glycophorin mAbs and with secondary Gam-PE antibodies is due to the patching of the inserted glycophorin upon reaction with the monoclonal antibodies. In order to verify whether the patching phenomenon observed was due to reaction with the mAbs, FITC-labeled glycophorin was electroinserted in RBC membranes. The fluorescence shown by the cells is continuous (Fig. 2a). When reacted with 10F7 and unstained Gam secondary antibody, patching is observed (Fig. 2b). This indicates that the patchy distribution of the electroinserted protein is not due to the insertion procedure but is a genuine phenomenon, occurring after reaction of the inserted protein with monoclonal antibodies.

Quantitation of the electroinserted glycophorin was made by flow cytometry. When reacted with 10F7 anti human glycophorin monoclonal antibodies and with a secondary antibody stained with phycoerythrin, flow cytometry analysis yielded the histogram presented in Fig. 3.

By comparing this histogram (Fig. 3b) with that of mouse red blood cells control (Fig. 3a), it appears that all cells subjected to the electroinsertion procedure expose human glycophorin on their membranes. It could be shown that an average of about 10^4 epitopes of human glycophorin per red blood cell could be

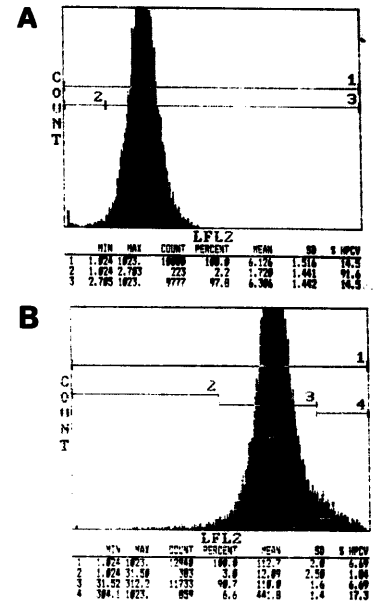

Fig. 3. Flow cytometry histograms (red fluorescence): (a) Control RBC; (b) mouse RBC-Glyc$^+$ stained with 10F7 and Gam-PE antibodies. LFL2 corresponds to the logarithm of the red fluorescence intensity. These RBC-Glyc$^+$ had a mean peak channel corresponding to 10^4 molecules per RBC. Histograms were generated from 10^4 RBC.

detected using the fluorescent beads standards (see Materials and Methods).

Due to the very nature of the electroinsertion process, it is obvious that not all the inserted glycophorin molecules would be oriented in the membrane in proper positions, exposing the epitopes which human glycophorin naturally exposes. In order to check the orientation of the inserted molecules, insertion of FITC labelled glycophorin in mouse RBC membranes was performed. The number of fluorophores inserted was assayed by flow cytometry (Fig. 4, solid curve). The RBC-Glyc$^+$ were then reacted with anti-FITC antibodies and the fluorescence again measured (Fig. 4, dashed curve).

RBC-Glyc-FITC showed in the flow cytometer a fluorescence mean peak shift of 33.33% with respect to

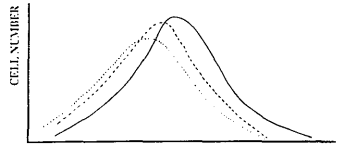

Fig. 4. Flow cytometry histograms (green fluorescence):(·····), Control RBC: ———, mouse RBC with FITC labelled human glycophorin inserted in their membrane; - - - - -, quenching of the fluorescence of the same RBC-Glyc$^+$-FITC after reaction with anti FITC antibodies. Histogram was generated from 10^4 RBC.

the control. After reaction with anti FITC antibodies, this shift was reduced to 10% with respect to the controls. Thus, it appears that approx. 70% of the fluorescence of electroinserted FITC-glycophorin is quenched by the anti-FITC antibodies; this suggests that this is also the proportion of 'correctly' exposed molecules among the electroinserted ones.

Attempts to electroinsert in mouse RBC membrane recombinant, soluble CD4, β_2-microglobulin or human serum albumin consistently failed (results not shown).

Life-span measurements

After electroinsertion of glycophorin, the RBC-Glyc$^+$ were biotinylated [13] and 200 μl were injected i.v., in the tail vein of mice. Blood was drawn at regular time points, separated in two aliquots which were assayed, one with phycoerythrin-avidin and the other for glycophorin by reaction with 10F7, D2-10, mAbs and PE-stained secondary antibodies, using the flow cytometer.

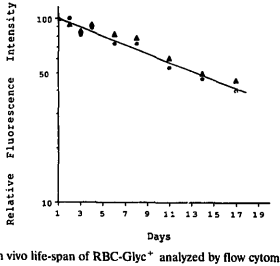

Fig. 5. In vivo life-span of RBC-Glyc$^+$ analyzed by flow cytometry. ●, The percentage of the biotinylated RBC-Glyc$^+$ was determined by labelling biotinylated RBC with avidin-PE. ▲, The glycophorin epitopes of RBC-Glyc$^+$ were detected using 10F7, D2-10 and Gam-PE secondary antibodies. These measurements show a half life-span of 12 days.

Fig. 5 shows the time course of the decrease of PE's fluorescence (RBC-biotin-avidin) and glycophorin epitopes detected by 10F7, D2-10 and Gam-PE. The two decays are practically identical, indicating:

(a) the half-life time of the RBC subjected to electroinsertion is approx. 12 days, the normal value for mouse RBC [8], and

(b) the electroinserted glycophorin is present on the mouse RBC, in a functionally 'active' state (as evidenced by its reaction with the mAbs) for the whole duration of the RBC life.

Immune response

No humoral immune response to RBC-Glyc$^+$ could be detected for three weeks, whereas normal seroconversion against human glycophorin was detected, when equivalent amounts of glycophorin dissolved in phosphate-buffered saline were injected in mice.

Discussion

We have presented evidence that subjecting red blood cells to pulsed electrical fields, in the presence of foreign membrane proteins, can result in the insertion of these proteins in the RBC membrane. Detected by immunofluorescence and by flow cytometry, the inserted human glycophorin was shown to expose two epitopes after insertion which are normally exposed by glycophorin in human red blood cells [14]. The inserted glycophorin molecules display lateral motion in the mouse erythrocyte membrane, sufficiently in order to form patches after reaction with monoclonal antibodies. It could be ruled out that patching may have been induced by the process of insertion itself, since FITC-glycophorin shows a continuous fluorescence when electroinserted in the mouse RBC membrane; upon binding to antiglycophorin mAbs and secondary unstained antibodies, it becomes punctate, indicating patching. This may be viewed as a significant argument against adsorption as mechanism of association of the protein with the RBC plasma membrane. Moreover, adsorbed fluoresceing material would not display lateral mobility [15]. A number of electroinsertion attempts made with proteins without a hydrophobic transmembrane like BSA, β_2-microglobulin and soluble CD4 [10] failed.

The relatively large number of human glycophorin epitopes detectable after electroinsertion indicates that red blood cells may possibly act as a sort of targeted carrier, if antigens or receptors are inserted in their membranes. The experiments aimed at evaluating the proportion of molecules inserted with 'proper' orientation, i.e. exposing the usual human glycophorin epitopes, showed a relatively high proportion of such molecules, namely 70%. Only ..5% of the fluorescein fluorophores in the inserted FITC-glycophorin could

not be quenched by the anti-FITC antibodies. Assay of the number of epitopes yields thus the true number of 'active' inserted molecules.

The measurement of the life-span in circulation of the mouse erythrocytes subjected to electroinsertion and of the human glycophorin inserted in their membrane yielded clear results. Whereas it had been demonstrated that the life-span of RBC in circulation was not affected by electroporation [8], it is somewhat unexpected to find that the electroinserted glycophorin should have the same life-span as the RBC. This result suggests that, as already suggested by the patching experiments, human glycophorin is not adsorbed on the RBC surface. It appears very unlikely that an adsorbed molecule should circulate for 24 days in the mouse blood vessels, with a daily disappearance rate equal to that of the mouse erythrocytes.

During the life span measurements of RBC-Glyc$^+$ and glycophorin epitopes, one of our concern was to monitor an eventual anti human glycophorin immune response. It was obvious that no complement mediated or cellular immune response occurred because the life span of the RBC-Glyc$^+$ was unaffected, even in mice boosted with a second RBC-Glyc$^+$ injection.

One μg of glycophorin dissolved in PBS when i.v. injected in mice elicited humoral response. (detectable antibodies seven days p.i.). The mice injected with RBC-Glyc$^+$ remained sero negative, even if boosted by a second injection of RBC-Glyc$^+$. While glycophorin dissolved in PBS and injected i.v. elicited a significant immune response in mice, the same amounts of glycophorin electroinserted in the red blood cell membrane did not show any antigenic activity. Further studies of the immune response to xeno antigens inserted in RBC could provide information about the immunological tolerance to these RBC-P$^+$.

These observations suggest that the RBC can serve not only as a long lived carrier exposing the inserted molecules but also that these molecules do not elicit the usual immune response which they do when they are injected free. Similar results were observed with inserted CD4. Should this be valid for a variety of proteins electroinserted in RBC membranes, such RBC-P$^+$ might find a use as long-lived, targeted carriers in some specific therapies, more so since insertion of CD4 in the RBC-membrane, at much lower concentrations (1 mg/ml) yielded a biologically active entity [16].

References

1 Volsky, D.J., Cabantchik, Z.I., Beigel, M. and Loyter, A. (1979) Proc. Natl. Acad. Sci. USA 76, 5440–5444.
2 Projanski-Jakobvits, A., Volsky, D.J., Loyter, A. and Sharon, N. (1980) Proc. Natl. Acad. Sci USA 77, 7247–7251.
3 Arvinte, T., Hildenbrand, K., Wahl, P. and Nicolau, C. (1986) Proc. Natl. Acad. Sci. USA 83, 962–966.
4 Arvinte, T., Schulz, B., Cudd, A. and Nicolau, C. (1989) Biochim. Biophys. Acta 981, 51–60.
5 Arvinte, T., Cudd, A., Schulz, B. and Nicolau, C. (1989) Biochim. Biophys. Acta 981, 61–68.
6 Neumann, E. and Rosenheck, K. (1972) J. Membr. Biol. 10, 279–290.
7 Deuticke, B. and Schwister, K. (1989) in Electroporation and Electrofusion in Cell Biology, eds. (Neumann, E., Sowers, A.E. and Jordan, C., eds.), pp. 127–148, Plenum Press, New York and London.
8 Kinosita, Jr., R. and Tsong, T.Y. (1978) Nature 272, 258–260.
9 Mouneimne, Y., Tosi, P.-F., Gazitt, Y. and Nicolau, C. (1989) Biochem. Biophys. Res. Commun. 159, 34–40.
10 Mouneimne, Y., Tosi, P.-F., Barhoumi, R. and Nicolau, C. (1990) Biochim. Biophys. Acta 1027, 53–58.
11 Goldman, M. (1968) Fluorescent Antibody Methods pp. 101–103; 123–124, Academic Press, New York.
12 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
13 Suzuki, K. and Dale, G.L. (1987) Blood 70, 791–795.
14 Bigbee, W.L., Langlais, R.C., Vandelaan, M. and Jensen, R.M. (1984) J. Immunol. 133, 3149–3155.
15 Szoka, F., Jacobson, K. Derzko, Y. and Papahadjopoulos, D. (1980) Biochim. Biophys. Acta 600, 1–18.
16 Zeira, M., Tosi, P.-F., Mouneimne, Y., Lazarte, J., Sneed, L., Volsky, D.J. and Nicolau, C. (1991) Proc. Natl. Acad. Sci. USA 88, 4409–4413.