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Short communication

Electroporation of cell membranes supporting penetration of photodynamic active macromolecular chromophore dextrans

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Abstract

The aim is to demonstrate that macromolecular chromophore dextrans (Cibacron-dextran) acting as photosensitizers can be transported easily into cancer cells by electroporation of their membranes (short electric pulses on cell suspension between electrodes). There are two possibilities, either:

(A) irradiation starts with the electropulse—showed with easily penetrating thiopyronin—yielding nearly 100% dead cells;

(B) irradiation starts after a resealing time of membrane pores during which macromolecular photosensitizers can penetrate into cells. In this way, fractions of Cibacron-dextran with molecular weights (Mw) 3300, 10,900 and 500,000 are now able to kill.

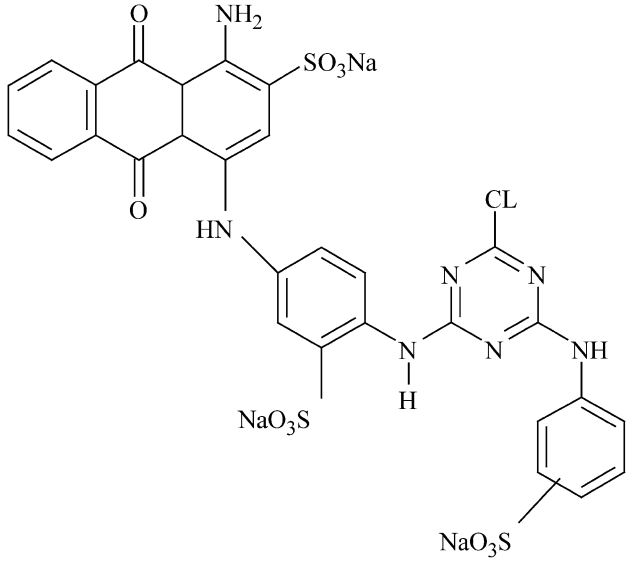
This combination of bioelectrochemistry and photobiology will be suitable also for other biopolymers, connected with photodynamic active chromophores (e.g. chromopeptides) to transport them through cell walls and membranes into cells and tissues. The human cancer cells U-935 and K-562 (pulsed by 1.15 kV/cm field strength) additionally or synergistically reach high rates of necrotic cells (colored by trypan blue) by this combination.

Keywords: Electroporation; Cell membrane; Chromophore dextrans

1. Introduction

Therapeutically used photosensitizers of the first generation are methylene blue, thiopyronin, protoporphyrin IX, "photofrin", etc., with molecular weights (Mw) <1000 penetrate easily through cell membranes. Their mechanism have been studied also by flash photolysis [1–3] with the result that the positive radical of photosensitizers oxidizes among other targets the guanine in DNA. A somewhat larger molecule, the chromopeptide Actinomycin-D or -C (Mw ≈ 1300), was tested recently [4] showing—besides its known cancerotoxic activity—an enhanced photodynamic effect after electroporation of cell membranes.

In combination of electroporation pulses some chromophore dextrans were tested. At these polymer drugs, the dye Cibacron blue (Mw 840) is bound at the α-glucose chain, acting as enzyme inhibitor of cyclic nucleotide phosphodiesterase and lactic dehydrogenase. Up to now, only electrochemical studies on such Cibacron-dextrans have been



Cibacron Blue F3G-A

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presented, namely the reduction currents and diffusion coefficients determined by polarography [5].

This deep blue chromophore is an anthraquinone derivate with an heterocyclic ring bounded by an oxygen bridge—at the position of Cl—to the dextran chain (see formula).

The photochemical effect of anthraquinones (AQ) [6] resulted—according to the overall redox mechanism—via the excited molecule AQ*:

$$AQ \xrightarrow{h\nu} AQ^* \quad (1)$$

$$AQ^* + RH_2 \rightarrow AQH_2 + R^{\cdot} \quad (2)$$

$$AQH_2 + O_2 \rightarrow AQ + H_2O_2 \quad (3)$$

The hydrogen donor RH_2 can be also H_2O. The peroxide H_2O_2, its radicals and $R^{\cdot}$ destroy cell components and inhibit glycolysis of tumor cells; however, in presence of catalase, its lethal effect was diminished [6].

Dextran itself adsorbs at electrodes [5] and cell membranes depending on its Mw in such a way that the adsorbability decreases with increasing Mw.

The photodynamic death (necrosis) of cancer cells U-937 and K-562 according to 1) mechanisms (2) mechanisms (3) [3,4] was counted as percentage of stained cells by the trypan blue method. The aim of this combination between bioelectrochemistry and photochemistry is the utilization of this macromolecular drug, Cibacron-dextran, with a novel chromophore, by the increase of its penetration for its photodynamic action through electroporated cell membranes.

By the way, the model for electroporation–resealing cycle [7] presents data of Serva Blue-G (Mw = 854) comparable with Cibacron blue.

2. Materials and methods

2.1. Substances

Thiopyronine (Mw 198, Fluka), trypan blue (Mw 960.8, Sigma, USA, 0.4% buffer sol.), Cibacron blue F3G-A, Na salt (Mw 840, Fluka), absorption maximum 630 nm. Fractions of Cibacron-dextrans (blue dextran) from Pharmacia (Uppsala) have the following mean molecular weights (Mw) and diffusion coefficients (D), determined by polarography [5]:

Mw	$D \times 10^6$/cm^2 s^{-1}
840 (dye)	5
3300	4.5
10,900	3.4
500,000	0.21

Their dye content is somewhat different and hence such stock solutions were used with the same extinction. Therefore final concentrations contain 3×10^{-5} M Cibacron blue. Trypan blue in the final concentration of 0.2% was used for determination of the cells' viability.

2.2. Cancer cells

Two strains of cancer cells were tested with concentrations of about 5×10^5 cells/ml. The human histolytic lymphoma U-937 cells with mononuclear phagocyte characteristics were from the American Cell Culture Collection, and cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco Life Science, USA), 100 mg/ml streptomycin and 100 U/ml penicillin (Sigma) at 37 °C in a 5% CO_2 incubator of 90% humidity.

The human chronic myeloid, leukemia K-562 cells were from Fujisachi Cell Center (Japan). The culture medium was 90% RPMI (Gibco) with 10% CO_2 at 37 °C, cultivated in the FG Molecul. Cytology (IMB, Jena).

For the measurements, 100 μl cells (5×10^5 cells/ml) in nutrition medium were mixed with 100 μl buffer pH = 5.29 (0.1 M$NaH_2PO_4 \cdot 2H_2O$, 0.1 MKCl, κ = 22.4 mS/cm) for cancer membrane destabilization by hyperacidification. The final suspension has a conductivity of 13.5 mS/cm and a pH = 6.13.

After treatment by electropulse and light, the concentration of dead cells was determined by the trypan blue method [1,9]: to 10 μl cells always 10 μl trypan blue was added at times indicated in the figures.

2.3. Equipment

2.3.1. Exponential pulse—BTX manipulator 600

The electroporation was performed by the BTX Electro Cell Manipulator-600 (Genetronics, San Diego) producing exponentially decaying pulses and a cuvette with embedded aluminum electrodes of 0.2 cm distance [4]. The experiments were carried out in a low output voltage mode at ambient temperatures; 200 μl cell suspension (2.2) plus 60 μl of different dextran fractions were subjected to one pulse (field strength E = 1.15 kV/cm and pulse width of 2–3 ms). After the electropulse, the electroporated cells became colored by trypan blue and also during the resealing time (20 min), where pores can be closed with the exception of already dead cells.

2.3.2. Light irradiation

For light treatment, the BTX cuvettes for experiment and control were positioned in front of a halogen lamp (24 V and 150 W, Osram 64465) at 37 cm distance in the focus area of a lens. The white light beam produced about 55 mW/cm^2 irradiance measured by a thermopile from LASER 2000 (SL-Microtest, Jena). Furthermore, for heat absorption a glass chamber containing 7 cm water layer was positioned between the lamp and the lens.

Possibility (A) was demonstrated in the case of thiopyronin as a model (Fig. 1). The control shows about 20% dead cells after 14 min irradiation, whereas the pulse electroporated 80% of cells and the immediately irradiated cells for 20 min became necrotic to nearly 100%. This technique is the most effective because the photodynamic action prevents the resealing process, otherwise the concentration of "blue cells" should be diminished as shown in Figs. 2 and 3.

Possibility (B) for Cibacron-dextrans let cells reseal their electroporated membranes [7] during the uptake of photosensitizer, which was tested already with thiopyronin [9] some time ago.

The following procedure let cells reseal their membranes during the uptake of photosensitizer.

(a) *for experiment*: until 20 min after the pulse, the cell suspension was kept in the dark for resealing the membrane pores, verified by decreased number of blue stained cells, which were determined by the trypan blue test at each point in the figures. After 20 min waiting, most electropores are closed and the dye is inside.
(b) *for control*: suspension kept only 20 min in the dark (as it is shown in Figs. 2 and 3) and then the cuvettes became irradiated. All experiments were repeated at least three times with S.D. = ± 4% for each determination. The responses of U-937 and K-562 cells are in the same order of magnitude.

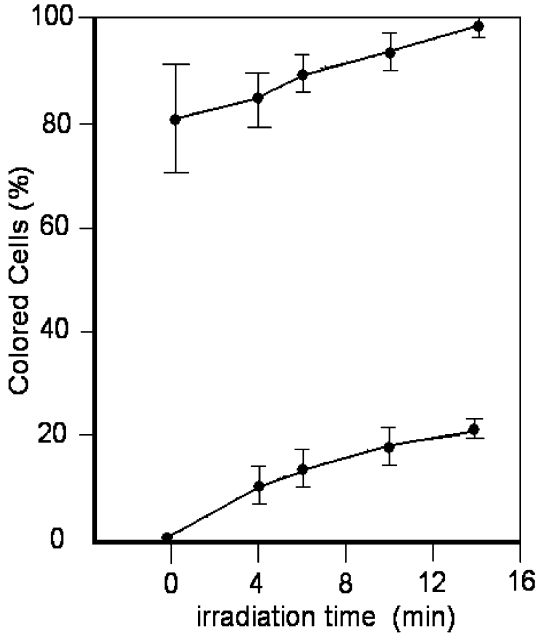

Fig. 1. Possibility (A): Combination of electroporation and photodynamic effect on U-937 cells with the classical sensitizer thiopyronine (1×10^{-5} M) in 1:1 nutritional medium and 0.6 M mannitol, 22 °C. Curve below control: colored (dead) cells by irradiation without field. Curve above experiment: 0.5 min after the pulse (2 kV/cm, 800 pF, time constant 11 ms) the irradiation starts. Because there is no time available for resealing membranes, most cells were killed immediately. Increasing intensities of electropulse and light, thus all cells can be destroyed.

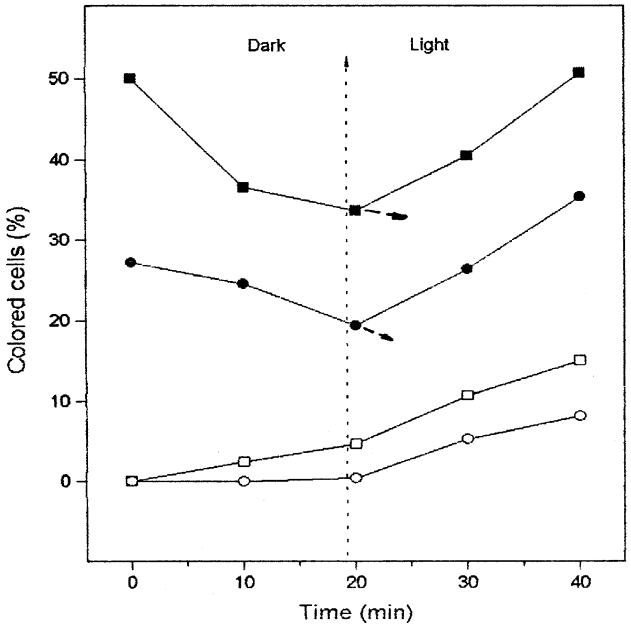

Fig. 2. Synergism of electropulse and photodynamic effect on U-937 cells by two fractions of blue dextrans Mw = 3300 (■) Mw = 500,000 (●). After the pulse 20 min waiting for resealing (process of electroporated membrane not finished completely →) and uptake of dextran fraction the irradiation started by white light. Control for Mw = 3300 (□) and Mw = 500 000 (○) without pulse. Percentage of stained cells by trypan blue during ≤ 20 min means they are electroporated; however, ≥ 20 min later cells are killed by synergism of both agents.

Counting always of about 200 cells from electroporation as well as from irradiation treatment was performed on 40% of dead cells using the microscope (Olympus, Japan).

3. Results and discussions

All three fractions of Cibacron-dextran produce a negligible effect in the control measurement during 20 min waiting time in the dark. However, after electroporation by similar pulses the penetration of Cibacron blue dextran and the resealing of membrane pores during 20 min (in the dark) takes place and the following irradiation by 66 J/cm^2 produces a distinct photodynamic effect on U-937 cells (rising part in Fig. 2 after 20 min).

A somewhat stronger, synergistic effect occurs with K-562 cells (Fig. 3, compare Mw = 3300). The percentage of cells stained by trypan blue after resealing 20 min in the dark and at the end of irradiation (dead cells by photooxidation) decrease with increasing molecular weight, which means the efficacy of electropulse as well as the penetration ability is still hindered by adsorption [10] and slower diffusion (see Section 2.1). Only the low-molecular dye Cibacron blue itself, which penetrates easily (Fig. 3), yields

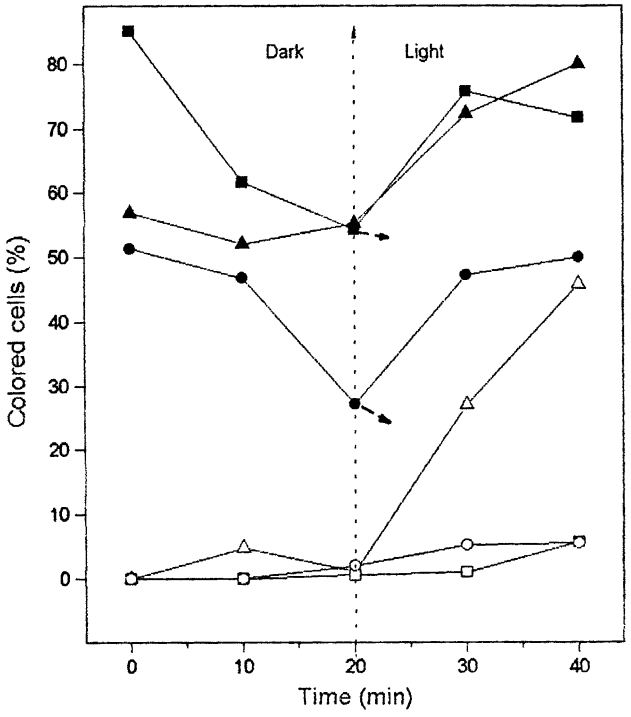

Fig. 3. Synergism of electropulse and photodynamic effect on K-562 cells by two fractions of blue dextrans Mw = 3300 (■) Mw = 10,900(●) and the chromophore Cibacron blue Mw = 840 (▲) itself. After 20 min waiting for resealing (not finished completely →) irradiation started by white light. Below the Control (without electropulse) for Mw = 3300 (□), Mw = 10,900 (○) and Cibacron blue (Δ, 3×10^{-5} M), which penetrates faster through membranes than Cibacron-dextrans (like other anthraquinones [8]) and produces about 45% lethality of K-562 cells.

a stronger photodynamic effect of 45% lethality without preceding electroporation, comparable with other anthraquinone derivatives [6,8].

Nevertheless, the killing effect of Cibacron blue increases further by the electropulse until 80%. The killing of cells by low-molecular sensitizers can be enhanced to 100%, if irradiation starts at the same time as the electropulse without waiting for resealing [7] of membrane pores (possibility A), as shown with thiopyronin in Fig. 1 [9].

In spite of electrostatic repulsion between the negatively charged cell membrane and the Cibacron-dextrans, their penetration will be facilitated always by electroporation, because at lower temperatures the pores remain open for minutes. Therefore also high-molecular Cibacron-dextrans can diffuse into cells or even into tissues in spite of their large dimensions and low diffusion coefficients $< 0.5 \times 10^{-6}$ cm^2/s. The same will be possible for any other biopolymers or artificial macromolecules with excitable chromophores reacting according to different photodynamic mechanisms. In such cases, a synergism of electroporation and photodynamics can occur as in the current tumor therapy according to the invasive needle-electrode technique [11] for bleomycin.

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