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Red blood cell ghosts and intact red blood cells as complementary models in photodynamic cell research

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Abstract

Recent research on erythrocytes as model cells for photodynamic therapy showed differing behaviour of certain photosensitisers in erythrocytes compared to other cells. Differences of dye accumulation in the cell membrane were proposed to be the reason for the distinct photodynamic effects. Using pheophorbide *a* as an example, the combination of erythrocyte ghosts as models to follow the dye accumulation in the cell membrane and intact erythrocytes as model cells to show the photodynamic damage is provided. Evidence for the correctness of the combination of erythrocyte ghosts and intact erythrocytes as a functioning model system in photodynamic cell research is provided using the confocal laser scanning microscopy on intact, pheophorbide *a* loaded erythrocytes.

Keywords: Human erythrocytes; Erythrocyte ghosts; Photodynamic therapy; Pheophorbide *a*; Confocal microscopy

1. Introduction

Recently, distinct differences of photodynamic cell damage in human erythrocytes after incubation with a variety of photosensitisers (phthalocyanines, a porphyrin and pheophorbide *a*) were reported [1]. These results were surprising since some of the applied photosensitisers showed a similar efficiency in cutaneous cell lines [1,2], as well as in in vivo experiments [2]. As a possible explanation, differences in the accumulation of the dye in the cell membrane were proposed. Due to widely overlapping spectra of haemoglobin and the photosensitiser and the predominance of haemoglobin, a spectral determination of the photosensitiser in the erythrocyte membrane is very difficult in most cases. In the past, erythrocyte ghosts were used to show singlet oxygen generation from inside a membrane [3]. The aim of the present report is to provide the complementary model system of erythrocyte ghosts and intact erythrocytes for photodynamic cell research.

As in other studies concerning the principal testing for photodynamic applications, like photosensitisers in Langmuir–Blodgett films [4] or microcrystalline cellulose as a carrier for potosensitisers [5], the prominent dye pheophorbide *a* was chosen to be the sample photosensitiser.

2. Experimental

Pheophorbide *a* was extracted from young leaves of *Urtica urens* according to Willstätter and Stoll [6].

Freshly drawn blood from healthy human donors was used throughout all experiments. The blood was washed three times by centrifugation ($1500 \times g$, 8 min) at room temperature in phosphate-buffered solution (150 mM NaCl, 5.8 mM NaH_2PO_4–Na_2HPO_4), pH 7.4. Plasma and buffy coat were removed by aspiration.

For the erythrocyte ghost experiments, the cells were prepared according to the modified Dodge method [7]. For absorption measurements, a Shimadzu UV-160A absorption spectrometer was used. The fluorescence was measured using a set-up combining a xenon lamp with a polychromator (Oriel 77250) and a spectrograph (L.O.T. Oriel Instaspec IV). The singlet-oxygen detection was realised by a set-up for time-dissolved luminescence measurements. The excitation source was an optical parametric oscillator (OP-901.355, BMI) pumped by the third harmonic of a Nd:YAG laser and the detector was a liquid nitrogen cooled germanium photodiode (EO-817P, Northcoast Optical Systems). For experimental details concerning the erythrocyte ghost experiments, see Ref. [8].

For the confocal laser scanning microscopy, a confocal head (PCM 2000) was attached to an inverted microscope

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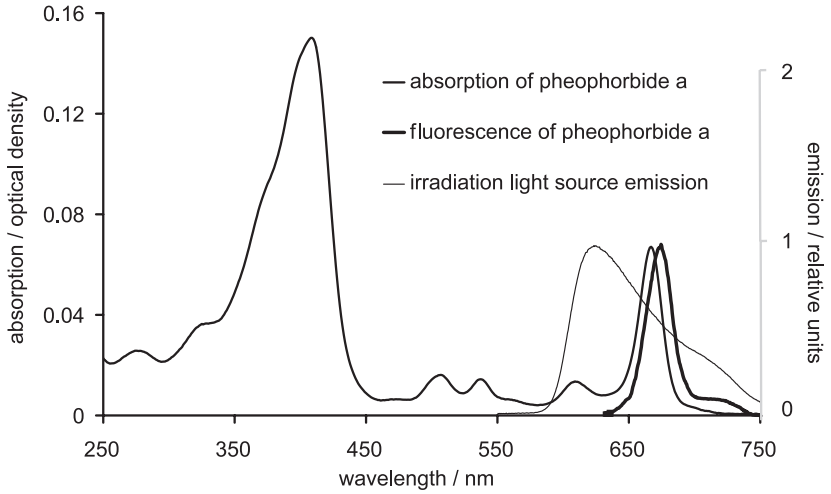

Fig. 1. Absorption and fluorescence spectra of pheophorbid *a* in ethanol. The concentration of pheophorbid *a* was 1.5×10^{-6} M. Each spectrum is the mean of three spectra from three independent solution preparations. Additionally, the measured emission spectrum of the irradiation source is given.

(Eclipse TE2000-U, Nikon). The mode of confocal measurement of erythrocytes is given in Ref. [9]. In the presented experiments, the light source was an argon laser (model 161C, Spectra Physics Lasers). The transmission microscopy picture was recorded using a digital camera (DN100, Nikon)

In the irradiation experiments, the absorption was measured using the Perkin Elmer Spectrophotometer Lambda 2. For irradiation a slide projector (Kodak Ektopro 3010) containing a 300-W xenon bulb equipped with a red filter (B + W, Schneider Kreuznach) was used. The final emission spectrum of the light source is plotted in Fig. 1 and the light fluence was measured by an optical power meter (S371 with sensor head model 247; United Detector Technology).

It has previously been shown that the photodynamic treatment of erythrocytes causes the release of potassium ions, on an identical, but time-shifted behaviour, when compared with haemolysis [10]. The latter has been taken to define the cell death of erythrocytes. In order to determine the percentage of haemolysis the optical density at a wavelength of 414 nm (maximum of the absorption spectrum of haemoglobin) of the supernatant of photodynamic treated erythrocytes was related to samples where 100% haemolysis was induced by placing the erythrocytes in distilled water. For experimental details concerning the irradiation experiments, see Ref. [2].

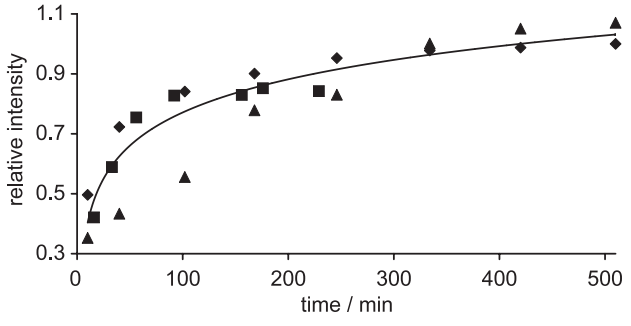

Fig. 2. Accumulation of pheophorbide *a* during its incubation in erythrocyte ghosts. The concentrations were 0.5 mg/ml for the ghosts and a total of 8 μM for pheophorbide *a*. The absorption (♦) was followed at 675 nm, the fluorescence (■) at 680 nm and the singlet oxygen luminescence (▲) at 1270 nm. The values were normalized in order to follow the same logarithmic regression line.

3. Results and discussion

In order to provide a comparison to the erythrocyte ghost measurements, the absorption spectrum as well as the fluorescence spectrum of pheophorbide *a* in the organic solvent ethanol was measured and is given in Fig. 1. The accessory extinction coefficient of pheophorbide *a* at a wavelength of 667 nm was measured to be approximately 44,500 ± 1000 M cm^{-1}.

Fig. 2 documents the accumulation of pheophorbide *a* in the membrane of erythrocyte ghosts. The incubation was followed by the independent determination of the absorption maximum at 675 nm,[1] the fluorescence at 680 nm and the singlet oxygen luminescence over time. The values were normalised in a way that the logarithmic regression lines of the single data rows came closest. The average regression line of all three rows is then plotted in Fig. 2.

Although the over all behaviour of the absorption, fluorescence and singlet oxygen luminescence is very similar, small differences, especially at the beginning of the incubation occur. One reason is likely to be the presence of dye aggregates at the initial state of the membrane accumulation. These dye aggregates contribute, for example, to measured absorption values, but not to the singlet oxygen luminescence values. Due to the photophysical properties of pheophorbide *a* in aqueous solution [11], where pheophorbid *a*

[1] The maximum of the absorption spectrum (and hence also the flourescence spectrum) of pheophorbide a in a lipid environment is about 8 nm bathochromatically shifted compared to the one in the organic solvent ethanol (e.g., Fig. 1).

occurs in a non-monomeric state, the present measurements of the singlet oxygen must originate from the monomeric photosensitiser [12] and hence from the cell membrane.

However, comparing the measured intensities with the intensities of pheophorbide *a* in well defined solutions (e.g., Fig. 1), it is safe to estimate that, after an incubation time of 120 min, at least 80% of the totally provided pheophorbide *a* is membrane bound (approximately 6.5 μM in the presented experiments). Therefore, an incubation time of 2 h is a minimum requirement, especially because it is a minimum time to enable a dye distribution up to a monomeric occurrence of the dye in the membrane. On the other side, it is enough time for most applications to allow a sufficient amount of the dye to accumulate.

In order to check the data obtained from the erythrocyte ghost experiments concerning the accumulation of the dye exclusively in the cell membrane, confocal images of pheophorbide *a* incubated erythrocytes were taken. Fig. 3 shows representative images from laser scanning confocal microscopy. Although the pictures show a clear enrichment of the dye in the membrane and no fluorescence signal from inside the cells, it is impossible to provide a quantitative analysis. However, the measurement of the confocal sections supports the legitimacy to transfer the data obtained from erythrocyte ghost experiments to intact erythrocytes.

Due to the previously mentioned results in erythrocyte ghosts, the incubation time for the irradiation measurements was chosen to be 2 h. The pheophorbide *a* concentration during the incubation was 10.7 μM and hence according to the above considerations at the time of the irradiation approximately 8.5 μM. Fig. 4A,B shows the cell survival of the red blood cells against the irradiation time and the fluence rate, respectively. In the presented experiments, the lysis of the cells is taken as equivalent to the cell death.

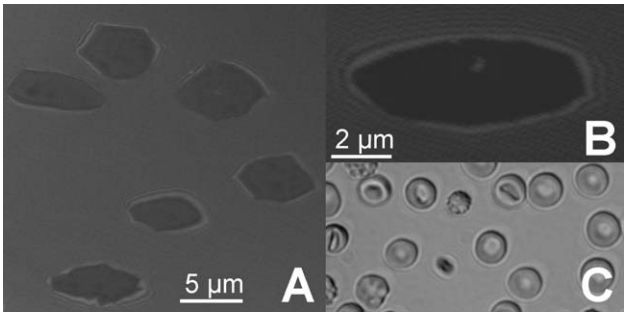

Fig. 3. Confocal fluorescence images of human erythrocytes incubated with pheophorbide *a* for 2 h at 37 °C are shown in parts A and B (100× Plan Apo lens). The light regions represent fluorescence signals and hence the presence of the photosensitiser pheophorbide *a*. The fluorecent dye is clearly accumulated exclusively in the cell membranes. In part A, the cell surrounding medium still contains approximately 100 nM pheophorbide *a*, while in part B the dye is completely removed by washing and centrifugation. The confocal section of part B shows a piece of membrane from the dent in the middle of the discocyte. For comparison, part C shows a transmission microscopy picture (40× Hoffman modulation lens) of the same batch of pheophorbide *a* loaded erythrocytes as used in parts A and B.

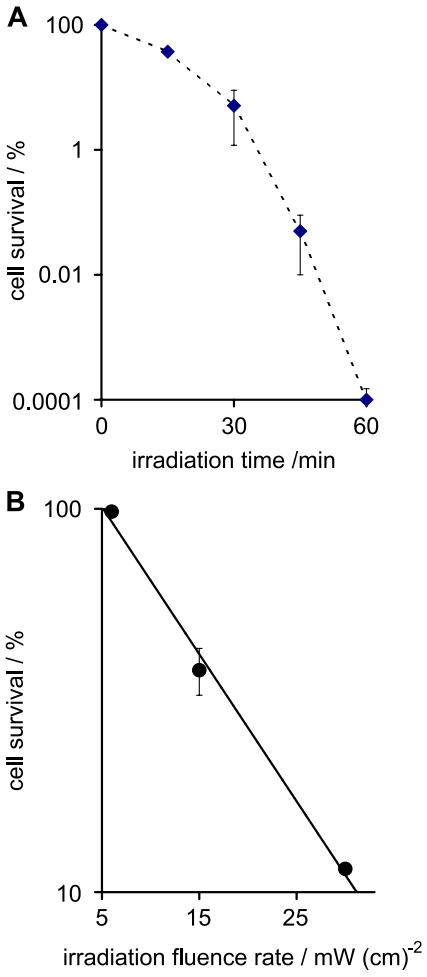

Fig. 4. (A) Cell survival curve after incubation with pheophorbide *a* (final concentration of approximately 8.5 μM) for 2 h and irradiation with red light at a fluence rate of 15 mW/cm^2 at the indicated irradiation time. Each point is the mean of at least three independent experiments and the error bars represent the standard deviation. (B) Cell survival after incubation with pheophorbide *a* (final concentration of approximately 8.5 μM) for 2 h and 15-min irradiation with red light at fluences like indicated. Each point is the mean of four independent experiments and the error bars represent the standard deviation. The regression curve shows an exponential dependence. Points without a drawn error bar have a standard deviation that is within the size of the symbol.

Increasing the irradiation time at a constant fluence of 15 mW/cm^2, from zero to 60 min, the cell survival decreases super-exponentially from 100% to almost zero (Fig. 4A). Varying the irradiation fluence between 6 and 30 mW/cm^2, while keeping the irradiation time constant at 15 min, the cell survival shows an exponential dependence (Fig. 4B).

These results suggest that with high irradiation fluences photodynamic effects on erythrocytes can be achieved with short irradiation intervals, while for irradiations where erythrocytes must not be damaged, for example photodynamic erythrocyte concentrate sterilisation [13], low fluences should be preferred.

In summary, the erythrocyte ghost experiments allow a determination of the dye content in the erythrocyte mem-

brane while the confocal laser scanning microscopy provides the evidence that data from erythrocyte ghosts can be transferred to intact erythrocytes. Finally, the intact erythrocytes provide a convenient model to control the cell survival after photodynamic treatment of the cells.

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