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PAPER

Evaluation of photodynamic treatment using aluminum phthalocyanine tetrasulfonate chloride as a photosensitizer: new approach

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Photodynamic therapy (PDT) has been the subject of several clinical studies. Evidence to date suggests that direct cell death may involve apoptosis. T_{24} cells (bladder cancer cells, ATCC-Nr. HTB-4) were subjected to PDT with aluminum phthalocyanine tetrasulfonate chloride (AlS_4Pc-Cl) and red laser light at 670 nm. Morphological changes after PDT were visualized under confocal microscopy. Raman microspectroscopy is considered as one of the newly established methods used for the detection of cytochrome c as an apoptotic marker. Results showed that PDT treated T_{24} cells seem to undergo apoptosis after irradiation with 3 J cm^{-2}. Cytochrome c could not be detected from cells incubated with AlS_4Pc-Cl using Raman spectroscopy whereas AlS_4Pc-Cl seems to interfere with the Raman spectrum of cytochrome c.

Introduction

Photodynamic therapy (PDT) is a treatment modality that has been used in the successful treatment of a number of diseases and disorders, including certain cancers. PDT uses a combination of a selectively localized light-sensitive drug (known as a photosensitizer) and light of an appropriate wavelength used in conjunction with molecular oxygen to elicit cell death.

Phthalocyanines (PC) have received particular attention within the last two decades due to their high molar absorption coefficient (ε *ca.* 10^5 M^{-1} cm^{-1}) in the red part of the spectrum (640–710 nm), which allows increased tissue penetration of the activating light. Low toxicity of phthalocyanines makes them promising for PDT application. Both lipophilic and water-soluble phthalocyanines have been considered as candidates for PDT.[1]

Aluminum phthalocyanine tetrasulfonate chloride (AlS_4Pc-Cl) has been shown to be a particularly promising agent for PDT,[2] where sulphonation significantly increases PC solubility in polar solvents including water, circumventing the need for alternative delivery vehicles. To a great extent, the efficacy of PDT for bladder cancer depends on the ability of the photosensitizer to preferentially accumulate in the diseased tissue and efficiently generate singlet oxygen or other highly reactive species such as radicals, which induce target cell death.[1]

It was demonstrated that confocal laser scanning microscopy can enable rapid image acquisition with sufficient sensitivity to study photoinduced intracellular dynamic action, and the technique can be applied to both cells and tissue sections.[3]

Mitochondria participate in apoptotic signaling by activating caspases *via* release of cytochrome c. Thus, detection of cytochrome c serves as a convenient marker for studying mitochondrial involvement in apoptosis.[4] Different spectroscopic approaches have so far been applied and developed to reach single molecule detection and particular attention has been focused on vibrational spectroscopies[5–7] because they are of fundamental importance for the understanding of internal configurations, structure and dynamics of proteins. In particular, Raman spectroscopy provides significantly detailed information on the structure and dynamics of molecules existing and playing roles in living cells. Raman spectra usually contain many sharp bands at different wavenumbers, whose positions are directly correlated with the vibrations of the molecules in the sample. Raman bands of cytochrome c are strongly resonance enhanced and clearly observed with 532 nm excitation, which can be used to image cytochrome c without any labeling.[8] Therefore, Raman spectroscopy is considered as one of the newly established methods used for the detection of cytochrome c, which shows a characteristic vibrational band at 750 cm^{-1} and other bands in the mid-frequency spectral region (1131, 1312, 1585 cm^{-1}).[6–9] In the following study, dark toxicity and phototoxicity of aluminum phthalocyanine tetrasulfonate chloride were assessed. Confocal laser scanning microscopy was used to study the intracellular phthalocyanine localization and the morphological changes occurring upon photosensitization. Moreover, confocal Raman microspectroscopy, which combines the advantages of confocal microscopy and Raman spectroscopy, was used for the detection of cytochrome c in T_{24} cells.

Materials & methods

Chemicals

Aluminum phthalocyanine tetrasulfonate chloride (AlS_4Pc-Cl) was obtained from Frontier Scientific (Lancashire, UK) and used

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as a photosensitizer. The stock solution of AlS_4Pc-Cl was prepared in double-distilled water (dd H_2O) and filtered with Minisart Filters (0.45 μm). A working solution in the corresponding media was prepared. Cytochrome c from horse heart was purchased from Aldrich (105201) and used without further purification at a concentration of 250 μM in PBS buffer as a reference for the spectroscopic studies. Double-distilled water was used in all experiments.

Cell cultures and treatments

T_{24} cells (bladder cancer cells, ATCC-Nr. HTB-4) were obtained from LGC Standards GmbH, Wesel, Germany. Cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum and 1% glutamic acid in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were cultured in 75 cm^2 cell culture flasks. For experimental purposes, cells were cultured in 24-well plates (1 ml of cell suspension per well). The optimum cell concentration as determined by the growth profile of the cell line was 50 cells mm^{-2}. Cells were allowed to attach for 24 hours before treatment.

Cell monolayers were washed with PBS and AlS_4Pc-Cl prepared from the working solution in growth media was applied in different concentrations and cells were incubated for 24 hours.

For absorption spectra, cells were cultured in 25 cm^2 cell culture flasks for 3 days. AlS_4Pc-Cl was added at 100 μM concentration and cells were incubated for 24 hours. After washing twice with PBS, cells were harvested after a short treatment with 0.25% trypsin/0.1% EDTA and resuspended in PBS. UV-Vis spectra were recorded using untreated cells as reference.

Photodynamic treatment

Dark toxicity of AlS_4Pc-Cl was studied with concentrations ranging from 100 up to 500 μM, while phototoxicity was studied with concentrations 25, 50, 75, 100 μM photosensitizer. Light of wavelength 670 nm (peak absorption for AlS_4Pc-Cl) and 16.5 mW cm^{-2} of a diode laser (Applied Optronics Corp., South Plainfield, NJ, USA) was used for irradiation. Cells were irradiated with energy densities 1, 2 & 3 J cm^{-2}. Cell proliferation was determined 24 hours after PDT and estimated by colorimetric MTT and neutral red assays. Cells that were not incubated with AlS_4Pc-Cl served as control for dark toxicity while cells incubated with 100 μM AlS_4Pc-Cl and not irradiated were used as control for phototoxicity. The data represent mean ± standard deviation (SD) of three separate experiments.

MTT assay

The MTT assay is based on the protocol described for the first time by Mosman (1983).[10] Twenty-four hours after PDT, cells were incubated for 45 minutes with MTT (0.5% w/v) (Sigma-Aldrich, USA) dissolved in DMEM medium. After dropping off the supernatant, the plates were inverted to dry on absorbent paper. Addition of 0.5 ml cold isopropanol followed by gentle shaking for 10 minutes gave complete dissolution. Aliquots (200 μl) of the resulting solutions were transferred to 96-well plates and absorbance was recorded at 560 nm using the microplate spectrophotometer system Lucy 1 (Anthos Labtech Instruments, Krefeld, Germany). Results were analyzed and are presented as percentage of the control values.

Neutral red assay

The neutral red assay is based on the initial protocol described by Borenfreund and Puerner (1984) and determines the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells.[11] Twenty-four hours after PDT, cells were incubated for 2 hours with neutral red dye (0.3% w/v, Biochrom L6313) dissolved in DMEM medium (1 : 200). Cells were then washed with 0.9% NaCl and 1 ml per well of elution medium (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid) was added, followed by gentle shaking for 10 minutes. Aliquots (200 μl) of the resulting solutions were transferred to 96-well plates and absorbance at 540 nm was recorded using the microplate spectrophotometer system Lucy 1 (Anthos Labtech Instruments, Krefeld, Germany). Results were analyzed and are presented as percentages of control values.

Confocal laser scanning microscopy

Cells were visualized under a 40× magnification oil immersion objective using confocal laser scanning microscopy on a LSM 510 (Zeiss, Oberkochen, Germany) in fluorescence mode. Photodynamic treated cells incubated with 100 μM AlS_4Pc-Cl for 24 hours were visualized by excitation with He–Ne laser (632.8 nm). Moreover, T_{24} cells incubated with AlS_4Pc-Cl were washed by PBS and 1 μM DAPI (4′,6-diamidino-2-phenylindole) and 500 nM rhodamine 123 were added in order to label nuclei and mitochondria. Cells were incubated for 30 minutes at 37 °C and visualized by He–Ne laser (632.8 nm). Pictures were edited by LMS image Browser software (Zeiss, Germany) and Adobe Photoshop.

Raman microspectroscopy

Experimental set up. Raman spectra and images of cells from both treated and untreated T_{24} cells have been recorded using an Alpha300 R confocal Raman microscope (WITec alpha300 R, Ulm, Germany). This system was described in detail before.[12] In short, it consists of a confocal microscope which is combined with a Raman spectrometer. The laser source is coupled into the microscope *via* a single mode fiber having a diameter of 50 mm. Sample scanning is achieved by a piezoelectric scan stage. Reflected laser and Rayleigh-scattered light are suppressed by an edge filter while Raman scattering is focused into a multimode optical fiber, which serves as an entrance slit for the spectrometer. The fiber core acts as a pinhole for confocal microscopy. In typical experiments, a frequency doubled Nd:YAG laser (532 nm) was used for excitation with an output of 10 mW at the sample. Culture medium was removed; cells were washed with PBS buffer and observed with a Nikon 60× NA 1.0 water immersion objective.

Data acquisition. Data acquisition was driven by the WITec Project software, version 2.00. At each pixel a complete Raman

spectrum was recorded, covering the region from −131 to 3750 cm^{-1}. Signals were detected by a 1024 × 127 pixel Peltier-cooled CCD detector and a spectrograph with a 600 g mm^{-1} grating allowing a resolution of 4 cm^{-1}. The integration time per pixel varied from 60 to 100 ms, *i.e.* the acquisition time per image was around 15 min. Raman images were generated during data acquisition by integrating over the CH-stretching band (2800–3030 cm^{-1}) to visualize the whole cell.

Data analysis. Data matrices were evaluated by cluster analysis. Cluster analysis is a statistical method which is used to sort data in groups in such a way that the similarity is maximal for data belonging to the same group and minimal for data belonging to different groups. Data sets were submitted to a hierarchical cluster analysis using a multi clustering procedure. Only the spectral region between 500 and 3100 cm^{-1} was taken in consideration.

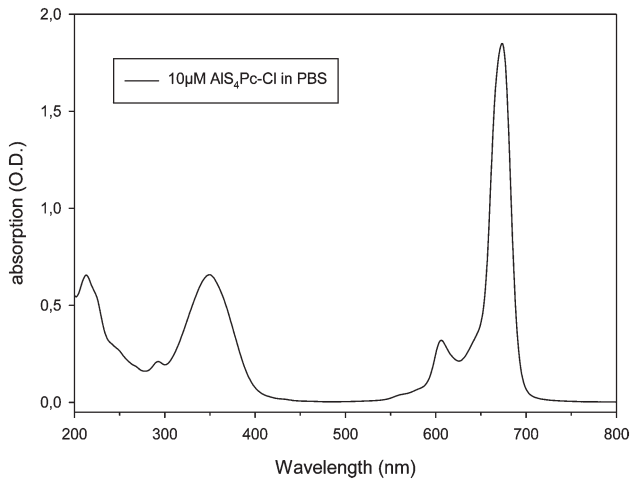

Fig. 1 UV-Vis absorption spectrum of 10 µM AlS_4Pc-Cl in PBS buffer. AlS_4Pc-Cl: aluminum phthalocyanine tetrasulfonate chloride.

Results & discussions

Photodynamic therapy using aluminum phthalocyanine tetrasulfonate is an effective strategy to attain a tumor-selective behavior

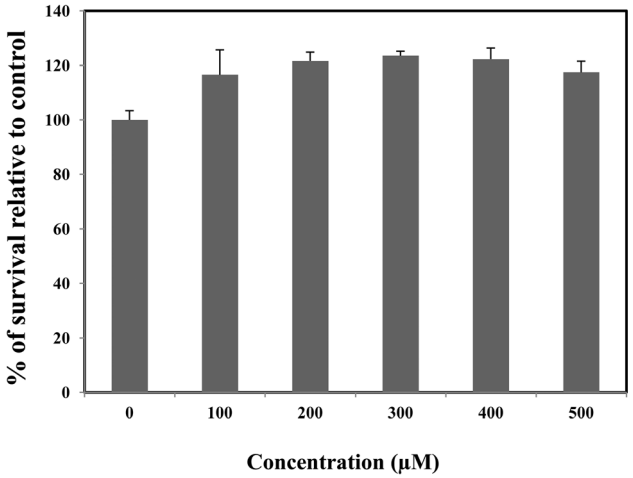

Fig. 2 Dark toxicity of AlS_4Pc-Cl on T_{24} cells. T_{24} cells incubated for 24 hours with different concentrations of AlS_4Pc-Cl. Cells proliferation were determined 24 hours after incubation and estimated by MTT assay. The data represent mean ± standard deviation (SD) of three separate experiments. AlS_4Pc-Cl: aluminum phthalocyanine tetrasulfonate chloride; T_{24}: bladder cancer cells.; MTT: a colorimetric assay used to assess the cell viability by measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color.

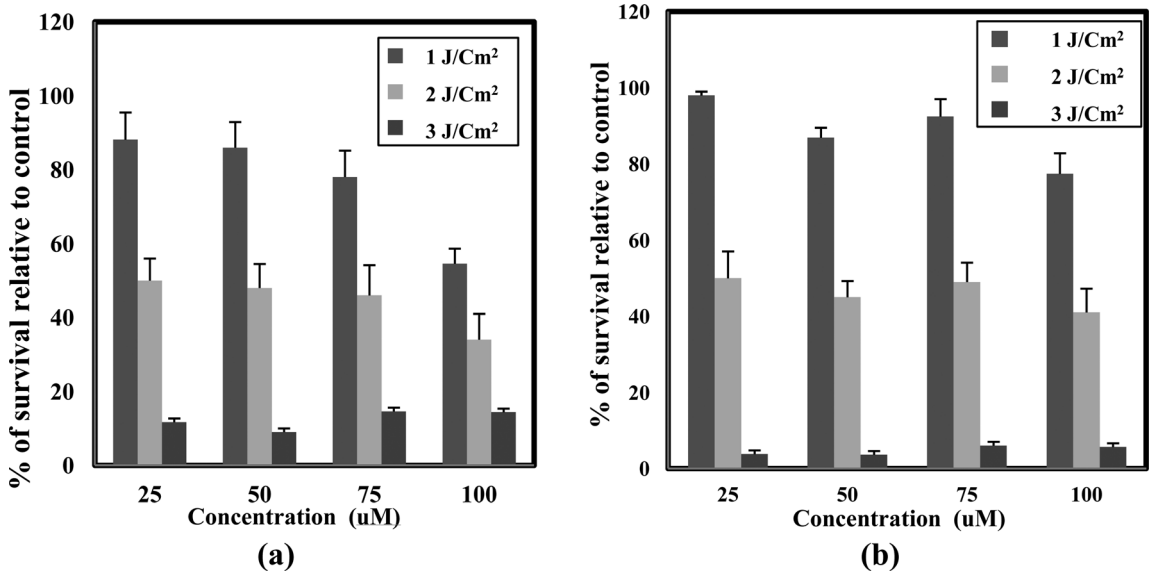

Fig. 3 Phototoxicity of AlS_4Pc-Cl on T_{24} cells. T_{24} cells incubated for 24 hours with different concentrations of AlS_4Pc-Cl and irradiated with different energy densities of 670 nm diode laser. Cell proliferation was determined 24 hours after PDT and estimated by (a) MTT assay and (b) NR assay. The data represent mean ± standard deviation (SD) of three separate experiments. AlS_4Pc-Cl: aluminum phthalocyanine tetrasulfonate chloride; T_{24}: bladder cancer cells; SD: standard deviation; PDT: photodynamic treatment; MTT: a colorimetric assay used to assess the cell viability by measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color; NR: Neutral Red.

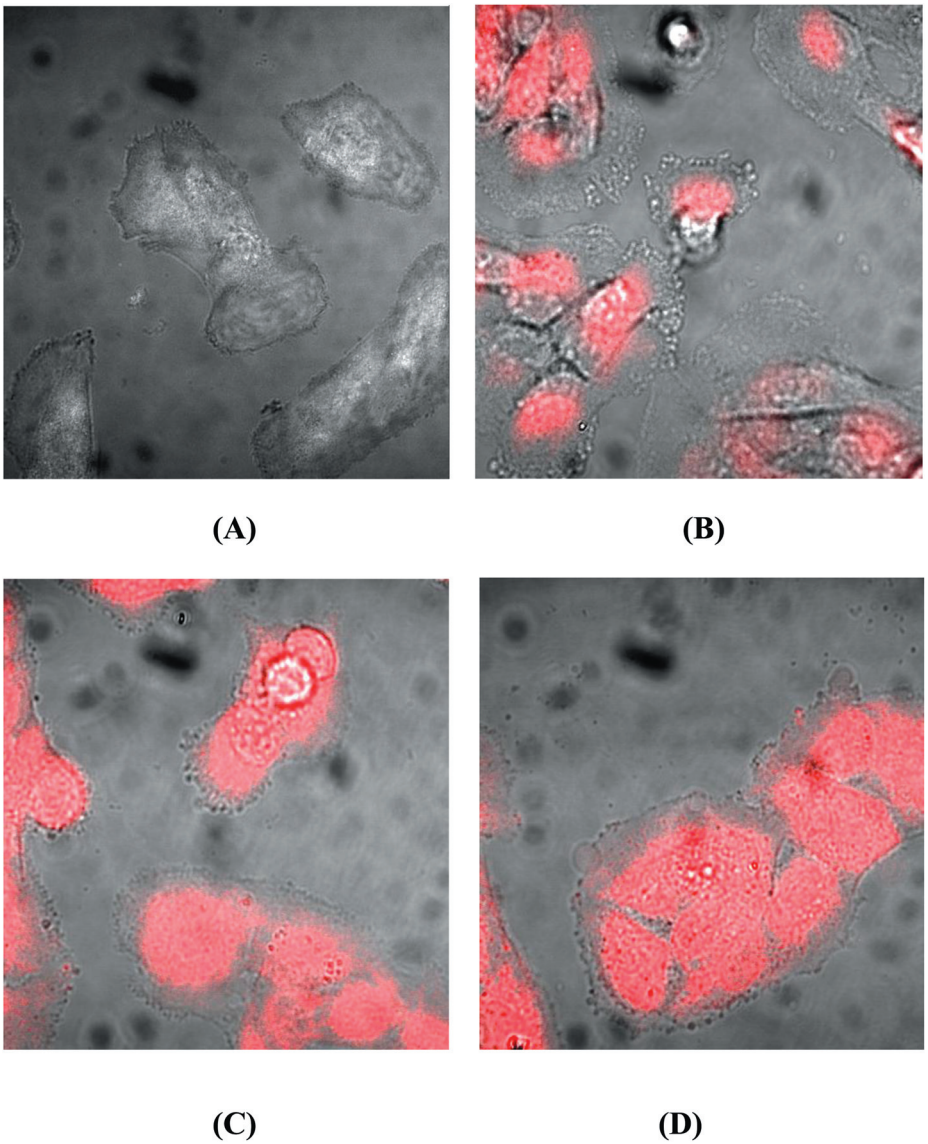

Fig. 4 Confocal microscopic imaging of T_{24} cells scanned with 633 nm. All T_{24} cells were incubated for 24 hours with 100 μM AlS_4Pc-Cl. A: T_{24} cells were kept in dark and scanned immediately; B: T_{24} cells were scanned with 1 J cm^{-2}; C: T_{24} cells were scanned with 3 J cm^{-2}; D: T_{24} cells were scanned with 5 J cm^{-2}.

and this could be due to its hydrophilic character which permits stable encapsulation in the aqueous internal compartment.[13]

The UV-Vis spectrum of AlS_4Pc-Cl (10 μM) showed two bands in the range of 600–691 nm (Fig. 1), therefore a diode laser of 670 nm was used for irradiation. This agrees with the findings of Moan *et al.*, who mentioned that aluminum phthalocyanine tetrasulfonate exhibits high molar absorption at 672 nm, a wavelength that is not absorbed or dispersed by endogenous tissue components.[14]

Cytotoxicity of AlS_4Pc-Cl was studied within a range of concentrations and results showed that T_{24} cells incubated for 24 hours with AlS_4Pc-Cl ranging from 100 μM up to 500 μM had no significant effect on cell viability in non-irradiated cultures (Fig. 2). Therefore, sulfonated aluminum phthalocyanine has no dark toxicity.[15]

Analysis of the data of the phototoxicity of AlS_4Pc-Cl on T_{24} cells using MTT assay showed that an increase in the concentration of AlS_4Pc-Cl was associated with a slight decrease in the number of living cells after irradiation with 1 or 2 J cm^{-2} (Fig. 3a). This agree with the results of other authors,[2] who found that there was a relative inefficiency of AlS_4Pc compared with the less sulphonated aluminum phthalocyanine even at high concentrations for the PDT of murine colorectal carcinoma cells, at light doses of 2.16 J cm^{-2}. Since the degree of sulphonation is correlated inversely to the lipid–water partition coefficient, an increase in the number of sulphonation groups would lead to a decrease in PC lipophilicity. Therefore, the uptake of the higher sulfonated PC is lower than the less sulphonated aluminum phthalocyanine. By contrast, at light dose (3 J cm^{-2}), increasing the concentration of AlS_4Pc-Cl had no significant effect on the number of living cells, whereas at all AlS_4Pc-Cl concentrations (25, 50, 75 & 100 μM) the surviving of T_{24} cells was reduced by greater than 90% (Fig. 3a). Evaluation of cytotoxicity by NR assay (Fig. 3b) gave results almost comparable with those

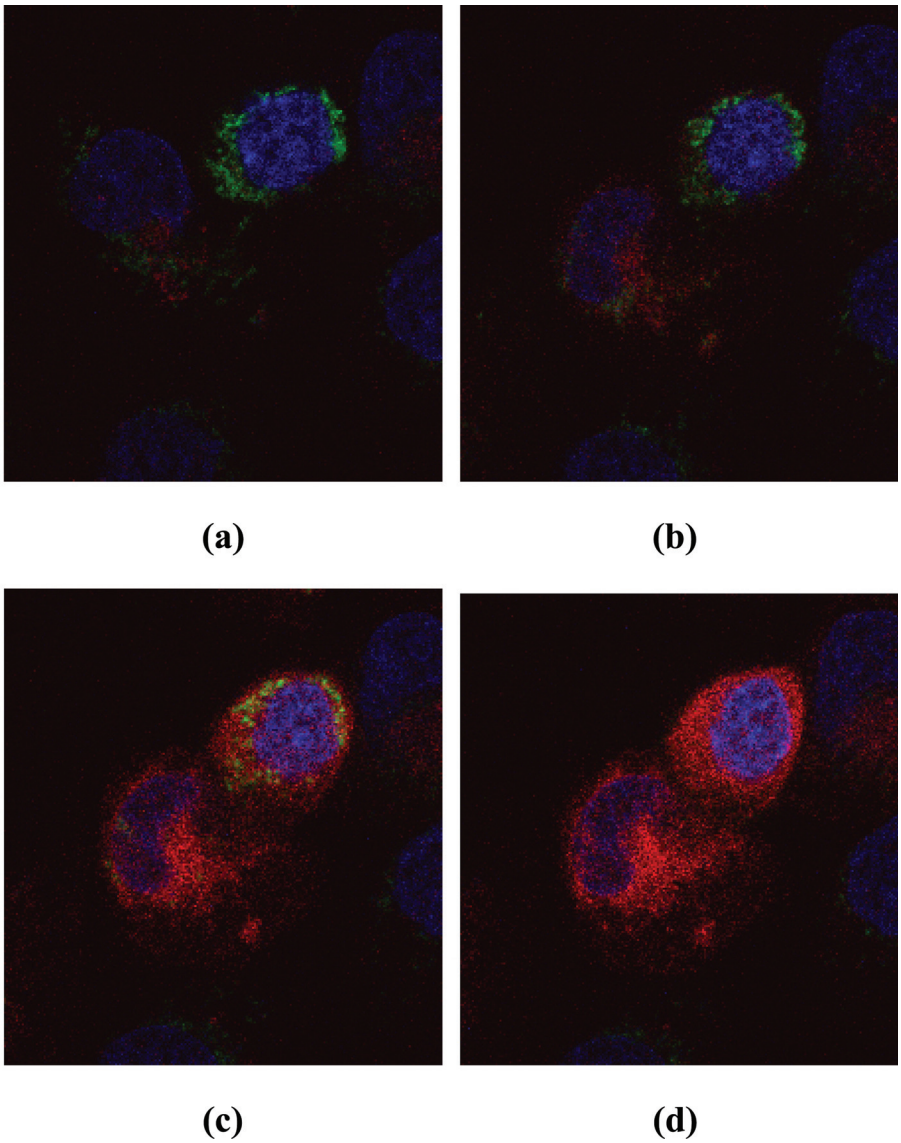

Fig. 5 Confocal microscopic imaging of labeled T_{24} cells scanned with 633 nm. All T_{24} cells were incubated for 24 hours with 100 μM AlS_4Pc-Cl. Nuclei were labeled with 1 μM DAPI while mitochondria were labeled with 500 nM rhodamine 123. a: T_{24} cells were kept in dark and scanned immediately; b: T_{24} cells were scanned with 1 J cm^{-2}; c: T_{24} cells were scanned with 3 J cm^{-2}; d: T_{24} cells were scanned with 5 J cm^{-2}. PDT: photodynamic treatment; T_{24}: bladder cancer cells; AlS_4Pc-Cl: aluminum phthalocyanine tetrasulfonate chloride; DAPI: 4,6-diamidino-2-phenylindole.

obtained with MTT. Therefore, irradiation with 3 J cm^{-2} is the minimum irradiation dose required for inducing lethal photosensitization effect on T_{24} cells in agreement with the previous study which concluded that induction of apoptosis could be observed after irradiation with fluencies of 3 J cm^{-2} from diode laser.[16]

Since confocal laser scanning microscopy could be used to study the intracellular distributions of a series of phthalocyanines, PDT can be employed for both therapeutic and imaging purposes when the photosensitizer (PS) fluoresces.[17] Therefore, imaging of T_{24} cells incubated with 100 μM Al_4Pc-Cl scanned with 633 nm by using confocal laser scanning microscopy was done. AlS_4Pc-Cl was shown to be spread diffusely over the cytoplasm upon scanning with 633 nm from confocal microscopy (Fig. 4A–D). This could be due to the fact that photochemical reactions induced by irradiation give rise to lipid peroxidation, which may impair membrane integrity and thereby cause PS release from their primary loci and redistribution.[18–20] In addition, it was mentioned that the redistribution of aluminum phthalocyanine tetrasulfonate from lysosomal compartments can be very rapid, taking minutes or even seconds.[21] Moreover, it was observed that PDT-treated T_{24} cells induce membrane blebbing as an apoptotic sign of cell death, in agreement with the other authors who stated that, by using aluminium phthalocyanine tetrasulfonate chloride as a photosensitizer, cells were found to undergo apoptosis after irradiation with an average 3 J cm^{-2}.[16,22]

Confocal laser scanning microscopy images of T_{24} cells incubated for 24 hours with 100 μM AlS_4Pc-Cl and labeled with 1 μM DAPI and 500 nM rhodamine 123 were recorded. Results

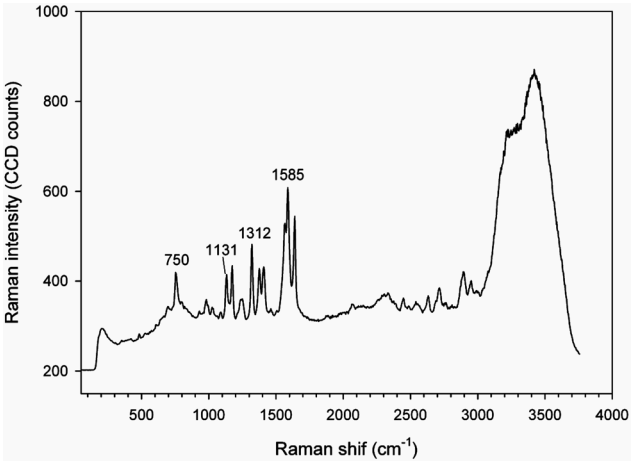

Fig. 6 Raman spectrum of pure cytochrome C 250 μM in PBS buffer. A Raman spectrum of cytochrome c shows characteristic vibrational bands at 750, 1131, 1312 and 1585 cm^{-1}. Cytc: cytochrome c, PBS: phosphate buffered saline.

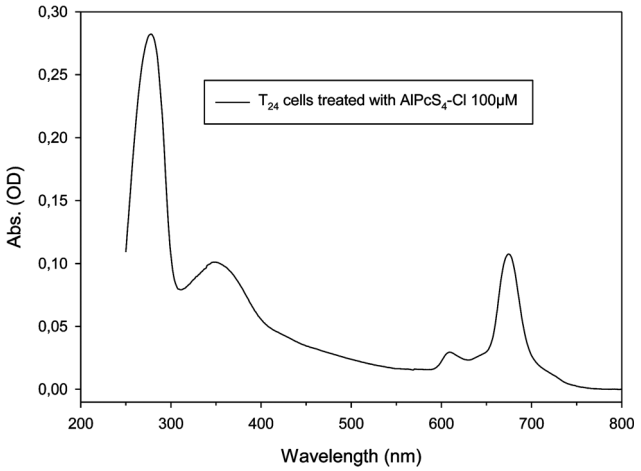

Fig. 7 UV-Vis absorption spectrum of AlS_4Pc-Cl in T_{24} cells. T_{24} cells incubated with 100 μM AlS_4Pc-Cl for 24 hours. AlS_4Pc-Cl: aluminum phthalocyanine tetrasulfonate chloride.

showed that AlS_4Pc-Cl seemed to be initially accumulating in cellular compartments other than mitochondria, eventually lysosomes. The fluorescence of rhodamine 123 (green) as an indicator for intact mitochondria does not decrease dramatically during irradiation, while AlS_4Pc-Cl spread rapidly over the cytoplasm upon scanning with 633 nm (Fig. 5a–c). This finding is in agreement with others who stated that aluminum phthalocyanine tetrasulfonate were found to be accumulated in granular lysosomal structures at the cell periphery.[23,24] Although AlS_4Pc-Cl seemed to be accumulated in lysosomes, an inhibitory effect seemed to be transmitted to the mitochondria (Fig. 5d). It was stated that, however photosensitizers may be accumulated in other cellular compartments (*e.g.* lysosomes, endoplasmic reticulum (ER), Golgi apparatus), local damage induced by photosensitization may be propagated to the mitochondria by various means.[4] On the other hand, it was suggested that mitochondria quite specifically accumulate AlS_4Pc-Cl.[25]

It was known that cytochrome c, a protein that exists inside the mitochondria, plays major roles in respiration and apoptosis.[4,26] Raman microspectroscopy is considered as one of the newly established methods used for the detection of cytochrome c, since this molecule exhibits a characteristic vibrational band at 750 cm^{-1} and other bands in the mid-frequency spectral region (1131, 1312, 1585 cm^{-1}); therefore this technique can be used to image cytochrome c without any labeling.[6–9] Raman spectra of pure cytochrome c from horse heart dissolved in PBS buffer at 250 μM have been recorded and results showed the expected vibrational bands (Fig. 6), in agreement with those published in literature.[6,27,28]

Living T_{24} cells were analyzed as monolayers with the above described Raman setup. The green excitation light of the frequency doubled Nd:YAG laser (532 nm) does not excite typical cellular fluorescent molecules.[8]

UV-Vis absorption spectrum of T_{24} cells incubated with 100 μM AlS_4Pc-Cl for 24 hours was recorded (Fig. 7). Results showed that AlS_4Pc-Cl localized in T_{24} cells has no absorbance at 532 nm, therefore the laser used for Raman microspectroscopy does not excite AlS_4Pc-Cl.

In order to design our experiments, T_{24} cells were divided into four groups as follows: (i) control group (untreated T_{24} cells), (ii) irradiated control group (T_{24} cells irradiated with 6, 14 & 19 J cm^{-2} of 670 nm diode laser), (iii) PDT group (photodynamic treated T_{24} cells incubated with 25 μM AlS_4Pc-Cl and irradiated with 3 J cm^{-2} of 670 nm diode laser) and (iv) dark control group (T_{24} cells incubated in dark with 25, 50, 75 & 100 μM AlS_4Pc-Cl).

Data sets were analyzed by integrating over the selected vibration bands and intensity images were calculated by the WITec Project software. Raman spectra of all investigated cells were recorded from −131 to 3750 cm^{-1}. Raman images were obtained by integrating over the C–H stretching band (2800–3030 cm^{-1}) during data acquisition. In this simple way, several cellular compartments can be distinguished, in particular nuclei show different intensities compared to the cytoplasm (Fig. 8A). Data matrices were evaluated using cluster analysis where only the spectral region between 500 and 3100 cm^{-1} was taken in consideration.

The results of cluster analysis of Raman microspectroscopy data sets of T_{24} cells showing the distribution of the calculated clusters are presented in Fig. 8B. The distribution of clusters containing proteins at different concentrations is represented in gray color and the distribution of cytochrome c cluster is represented in red. In addition, the average Raman spectra of the most prominent cell cluster are shown in Fig. 8C.

Raman data sets from untreated living T_{24} cells (control group) showed a cluster with typical protein bands (1450, 1655 cm^{-1}) and with a characteristic vibrational band at 750 cm^{-1} besides other bands in the mid-frequency spectral region (1131, 1312, 1585 cm^{-1}) which are comparable to those of pure cytochrome c [Fig. 8(i, C)].

Data sets from T_{24} cells irradiated with 6, 14 & 19 J cm^{-2} of 670 nm diode laser (irradiated control group), in the absence of AlS_4Pc-Cl, have been recorded and analyzed by cluster analysis, and the characteristic vibrational bands of cytochrome c were detected in one cluster in these cells [Fig. 8(ii, C)].

On the other hand, in Raman data sets from PDT-treated T_{24} cells incubated with 25, 50, 75 & 100 μM AlS_4Pc-Cl and

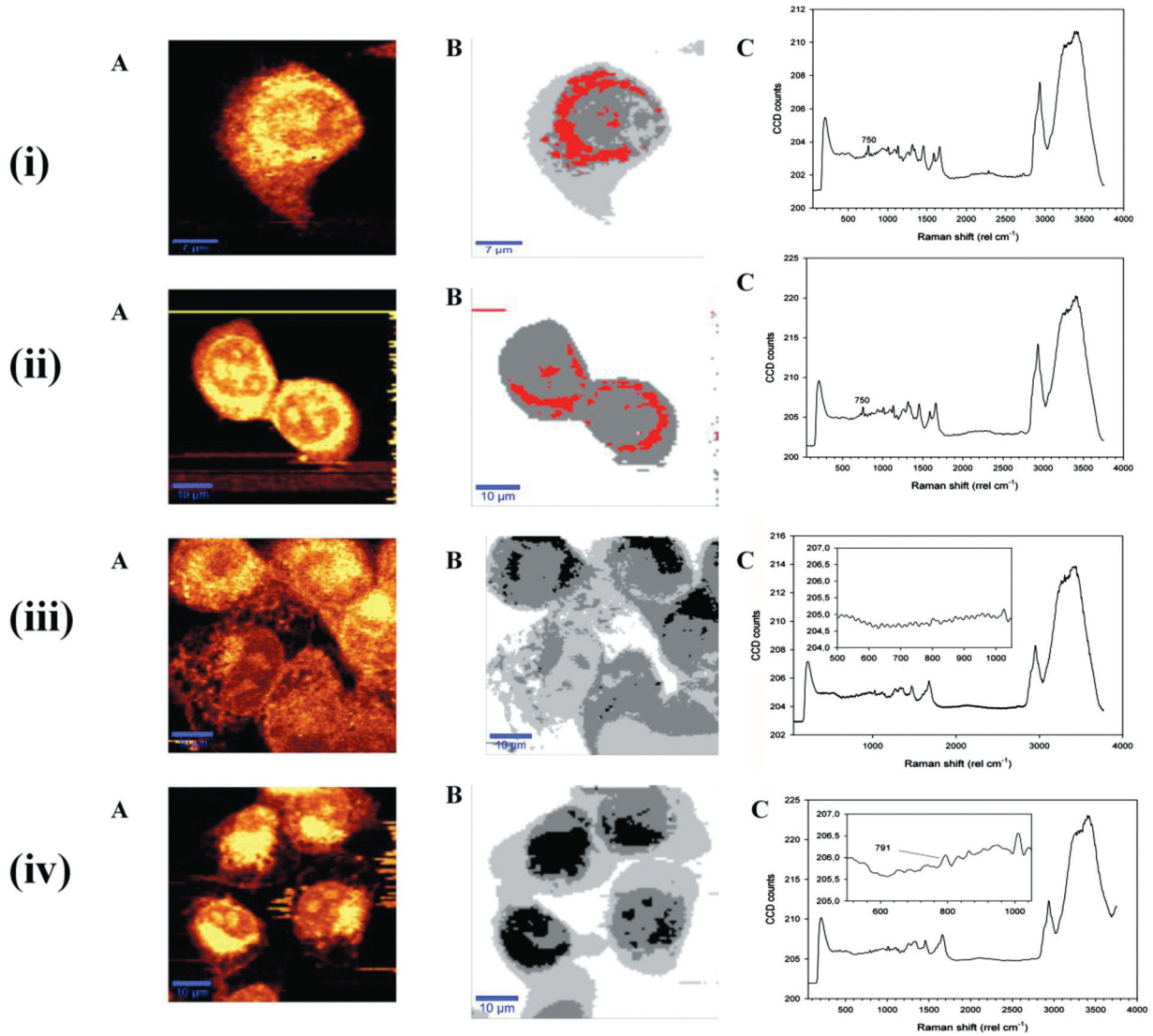

Fig. 8 Raman microspectroscopy of T_{24} cells under investigation with the excitation wavelength of 532 nm. A: False color images of T_{24} cells from confocal Raman microscopy showing distribution and intensity of the C–H stretching band (2800–3030 cm^{-1}). B: Cluster analysis of Raman microspectroscopy data sets of T_{24} cells showing the cluster distribution; distribution of clusters containing proteins at different concentration (gray color); distribution of cytochrome c cluster (red color); a further cluster represents water and is not shown. C: Average spectrum of the most prominent cell cluster. (i) Control group (untreated T_{24} cells); (ii) irradiated control group (T_{24} cells irradiated with 6, 14 & 19 J cm^{-2} of 670 nm diode laser); (iii) PDT group (photodynamic treated T_{24} cells incubated with 25 µM AlS_4Pc-Cl and irradiated with 3 J cm^{-2} of 670 nm diode laser) and (iv) dark control group (T_{24} cells incubated in dark with 25, 50, 75 & 100 µM AlS_4Pc-Cl). T_{24}: bladder cancer cells; AlS_4Pc-Cl: aluminum phthalocyanine tetrasulfonate chloride.

irradiated with energy density 3 J cm^{-2} (PDT group), no cluster could be assigned to cytochrome c [Fig. 8(iii, C)].

Similarly, in Raman data sets from T_{24} cells incubated in the dark with 25, 50, 75 & 100 µM AlS_4Pc-Cl (dark control group) no cluster corresponding to cytochrome c could be detected [Fig. 8(iv, C)].

This could be explained by the finding of Laia *et al.*[29,30] who stated that AlS_4Pc indeed forms complexes with cytochrome c, leading to decay of the fluorescence of sulfonated aluminum phthalocyanines due to an electron-transfer process from the phthalocyanine to the cytochrome c and in addition AlS_4Pc is adsorbed on the cytochrome c at more than one location due to electrostatic interactions. Although AlS_4Pc-Cl was localized predominantly at lysosomes[23,24] and quite specifically at mitochondria,[25] cytochrome c could not be detected in T_{24} cells incubated with AlS_4Pc-Cl and not irradiated. However, further Raman studies should be followed up in the future using Nd: YAG lasers with different parameters in order to ensure that Raman spectroscopy did not have any effect that could influence the localization of the photosensetizers in the cells.

In conclusion, AlS_4Pc-Cl is an effective photosensitizer for T_{24} cells, whereas it has no dark toxicity up to a concentration of 500 µM. The cytotoxicity of photodynamic treatment using AlS_4Pc-Cl followed both the concentration of the sensitizer as well as the irradiation energy in a dose-dependent manner and 3 J cm^{-2} is the minimum irradiation dose required for inducing

lethal photosensitization effect on T_{24} cells. Moreover, PDT-treated T_{24} cells seem to undergo apoptosis after irradiation with that dose. Raman microspectroscopy could be used for the detection of cytochrome c in cells, as long as they were not treated with AlS_4Pc-Cl. When AlS_4Pc-Cl was used as a photosensitizer, cytochrome c could not be detected, since AlS_4Pc-Cl seems to interfere with the Raman spectrum of cytochrome c due to binding between AlS_4Pc-Cl and cytochrome c. In general, our work is a preliminary step toward assessing the efficiency of Raman microspectroscopy in evaluation of photodynamic treatment *via* detection of cytochrome c. Assessment of the efficiency of this technique will require further work on different types of photosensitizers and different cell types.

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