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Localization Study of Co-Phthalocyanines in Cells by Raman Micro(spectro)scopy

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An investigation of intracellular localization of Co-phthalocyanines is reported. The Raman images of K562 cells stained with phthalocyanine were acquired. To understand the peculiarities of the Raman images, measurements were performed at different *z*-axis positions. The intracellular concentration of phthalocyanine was estimated. A colocalization study was carried out using the fluorescence probes FITC–dextran and acridine orange by means of Raman and fluorescence microscopy. Partial colocalization with both probes was revealed. Copyright © 1999 John Wiley & Sons, Ltd.

INTRODUCTION

Photodynamic therapy of tumors has been applied for more than 10 years.[1] The method is based on the selective accumulation of photosensitizers in tumor tissue. After light irradiation they produce cytotoxic compounds that kill the tumor cells. Phthalocyanines (Pcs) and porphyrins are widely applied as photosensitizers in this method.

Although the method has been known for a long time, the details of the interaction between photosensitizers and cellular organelles are still under intensive study, with a search for new photosensitizers with higher efficiency.

Some of the Pcs are fluorescent, which makes the study of their interaction with living cells by means of conventional fluorescence microscopy fairly easy. However, the fluorescence of some phthalocyanines is strongly quenched and the study of their interaction with cells by means of fluorescence micro(spectro)scopy is difficult. Raman spectroscopy can be a powerful tool in these investigations.

The interaction of AlPcs of different degrees of sulfonation with living cells has been studied previously.[2–4] Colocalization studies by means of confocal fluorescence microscopy showed that four-times sulfonated AlPc is party colocalized with the fluorescence probe acridine orange (AO) and is fully colocalized with fluorescein isothiocyanate (FITC)-dextran. Based on these data, one can suggest the localization of this AlPc in lysosomes and endosomes (the structure of the endocytic pathway). AlPc can be found in lipophilic membranes, whereas AlPcS is detected in lysosome-like structures and in membranes. These studies show that the pathway and distribution inside the cell are determined by the hydrophilic properties of Pcs.

This work was devoted to the study of the localization of CoPcs in living tumor cells and their interaction with cellular structures.

EXPERIMENTAL

We investigated three CoPcs with different peripheral groups (eight pyridinemethyl groups, eight carboxy groups and two sulfonated groups). These Pcs were synthesized at the Institute of Organic Intermediates and Dyes (Moscow, Russia). A stock standard solution (concentration 10^{-2} M) of Pc in phosphate-buffered saline (PBS) was kept at 4 °C and was diluted directly before the experiments to a concentration of 10^{-4} M.

Suspensions growing K562 cells (human leukemic erythroblasts) were used. This cell line was derived from a patient with chronic myeloid leukemia in blastic crisis.[5] After washing, the cells were resuspended in 10^{-4} M Pc solution in cellular medium (RPMI-1640 + 3% fetal calf serum + 2 mM L-glutamine + antibiotics), shaken once and incubated at 37 °C for 12–15 h. They were then washed twice with Pc-free cellular medium. The resulting concentration was 10^8 cells ml^{-1}. This solution was dropped on to a poly-L-lysine-coated cover-glass. After 15 min the cells were attached to the cover-glass as a monolayer. The cover-glass with the cells was put into a container with cellular medium.

In the colocalization studies we used FITC–dextran and AO as fluorescent probes.[6] FITC–dextran accumulates in lysosomes and endosomes[7] and AO accumulates in cellular structures with low pH. The cells (10^6 cells ml^{-1}) were incubated in a mixture of Pc and FITC–dextran solutions (10^{-4} M Pc + 5 mg ml^{-1} FITC–dextran). The cells stained with Pc were incubated in AO solution (2 μg ml^{-1}) for 10 min.

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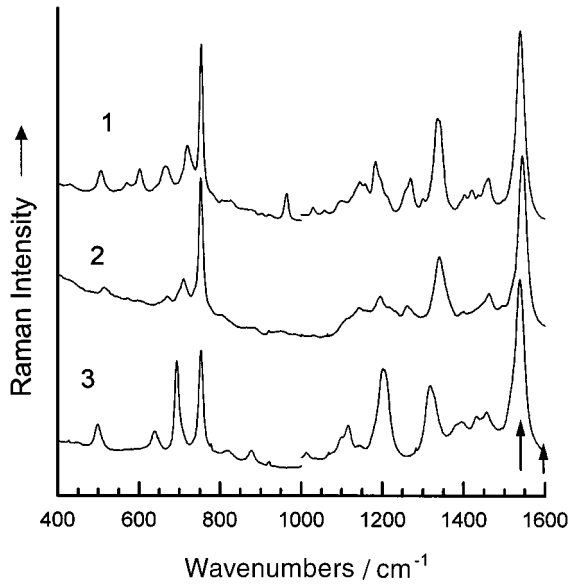

Figure 1. Resonance Raman spectra of CoPcs in PBS (10^{-4} M): 1, disulfonated; 2, octapyridinemethyl; 3, octacarboxy. The arrows show the Raman shifts in which Raman images and Raman background images were measured.

The direct image Raman microscope used has been described elsewhere.[8] A Spectra-Physics Model 375B tunable dye laser was operated with DCM (4-Dieyanomethylene-2-methyl-6-(p-dimethylaminostyryl)-4H-pyran) and was pumped by an argon ion laser (Coherent Radiation Innova 90). The laser power at the sample was 40–50 mW. A water immersion 63 × microscope objective (Zeiss Plan Neofluar, numerical aperture 1.2) was used for focusing the laser beam and collecting the scattered light. The scanning of the laser beam with two mirrors allowed us to obtain images of area 24 × 24 μm in the object plane. The scanning rates for the mirrors were 1 and 10 Hz. A dielectric dichroic mirror was used for optical coupling of the microscope with the detection arm of the set-up. It had high transmittance (95%) in the wavelength interval 620–680 nm and above 690 nm its reflection rose steeply to > 98%. A Raman holographic edge filter (Kaiser Optical System) was applied to suppress the laser light. The key element of the design was a narrowband dielectric transmission filter (Omega Optical). The transmission was centered at 735 nm with an FWHM of 0.5 nm (10 cm^{-1}). The Raman image could be recorded at the desired Raman shift by tuning the wavelength of the dye laser in such a way that the wavelength of the Raman scattered signal coincided with the transmission wavelength of the narrow band dielectric filter. The image was detected by a slow-scan liquid nitrogen charge coupled device (CCD) camera (Model AT1, Wright Instruments) with an accumulation time of 10 min. The total signal detection efficiency of the setup was estimated to be *ca.* 15%.

Test measurements on 1 μm polystyrene beads allowed us to estimate the axial resolution of the set-up as not worse than 2.5 μm.

When the set-up was used in the fluorescence mode, an additional mirror was inserted in the path of the laser beam directing the light from the excitation source towards the sample. For excitation in the blue region we used a high-pressure mercury lamp of a Nikon Optiphot fluorescence microscope with a 450–490 nm excitation filter and a dichroic mirror (DM 510, Nikon). A 520 nm barrier filter (Nikon) was used to detect fluorescence. The accumulation time for fluorescence images was 10 s.

The white light bright-field images of cells were acquired during 1 s, using the in-base illumination of the microscope.

RESULTS

Raman images of cells were recorded at 1538–1549 cm^{-1} detuning (the most intense bands in the CoPc Raman spectra, Fig. 1). The background was measured at 1600 cm^{-1} detuning. The excitation at 660 nm corresponded to the Raman image detection at 1549 cm^{-1} and the excitation at 658 nm corresponded to 1600 cm^{-1} detuning. To estimate the Pc concentration in cells, the Raman 'image' of 100 μM Pc solution was measured.

We estimated the intracellular concentration of Pc to be 50–100 μM (variation from cell to cell).

The Raman images of cells stained with different Pcs are very similar. We observed some small, bright 'spots' inside cells and a 'cloud' around them. To understand the origin of this 'cloud' we measured the Raman images at different *z*-axis positions. The results are presented in Fig. 2. The positions of the bright spots are different for each of these images.

In order to specify the type of intracellular structures in which the Pc is localized, we carried out colocalization measurements. Both Raman (of Pc) and fluorescence (of FITC–dextran and AO) images were detected in succession for the same cell. The Raman and fluorescence images are presented in Fig. 3. Both images show a few small spots inside the cell. All of the spots are in the same part of the cell but their positions and relative intensities are not identical.

DISCUSSION

It is known that phthalocyanines of different hydrophobicity penetrate into the cell and accumulate therein in different ways. The most hydrophilic molecules penetrate inside the cell with the help of endocytosis,[9] whereas the hydrophobic molecules diffuse through the membranes.[10] Hence the former tend to accumulate in lysosomes and the latter in the membranes and cytoplasm.

The CoPcs under study have high solubility in water and we believe that the molecules are hydrophilic. We may assume that the bright 'spots' in the images correspond to lysosomes. This is proved to some extent by the colocalization studies, where correlation of the Raman and fluorescence images was observed. The same results (Raman images of cells stained with Pc) were obtained by Freeman *et al.*[11]

By comparing the signals levels we could estimate only the 'effective' intracellular concentration of Pc, i.e. the level of concentration that could be observed in the

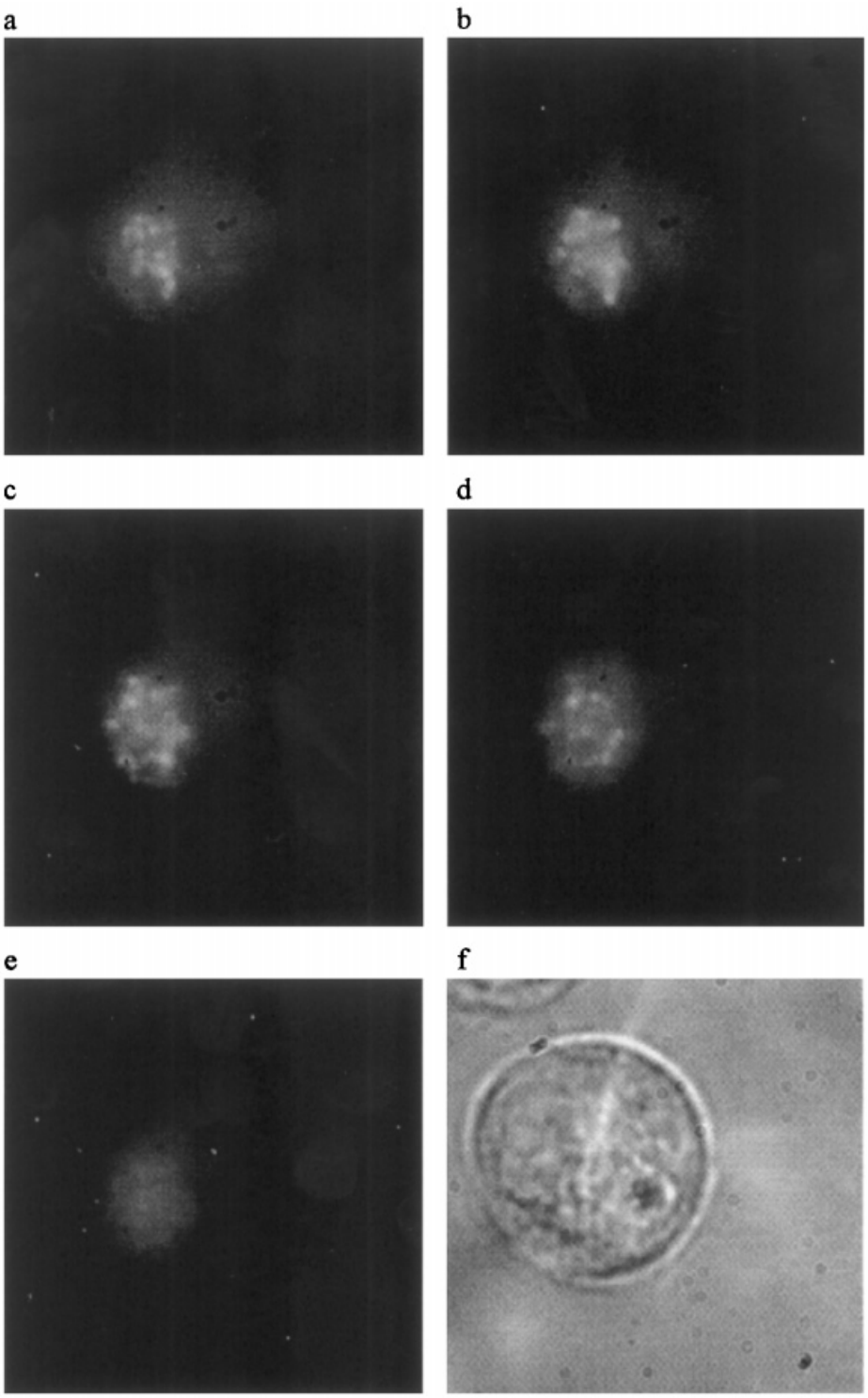

Figure 2. Raman images of a cell stained with octacarboxy-CoPc taken at different position of the focal plane: (a) 0; (b) 3; (c) 4.5; (d) 7.5; (e) 11 μm; (f) white light bright-field image.

case when the Pc was distributed homogeneously inside the cell. However, it can be seen from the data presented that the distribution of Pc inside the cell is far from homogeneous. The signal that we detect in the case of cell measurements presumably comes from several lysosomes in which Pc is really accumulated. The volume of several lysosomes is smaller than the measuring volume. Hence the local concentration of Pc must be higher than the estimated 'effective' concentration.

Comparing the images with different z-positions, we may suggest that the signal at each position consists of two parts. The first one comes from the organelles (presumably lysosomes) that are exactly in the focal plane (bright 'spots'). The second one is from the out-of-plane organelles, i.e. from those lying below or above the focal plane. The latter give rise to the 'cloud' in the image.

There may be several reasons for only a partial correlation between Raman and fluorescence images. First, we use lenses with not completely compensated chromatic aberrations. Images at different wavelengths have different positions in the imaging plane. Second, we

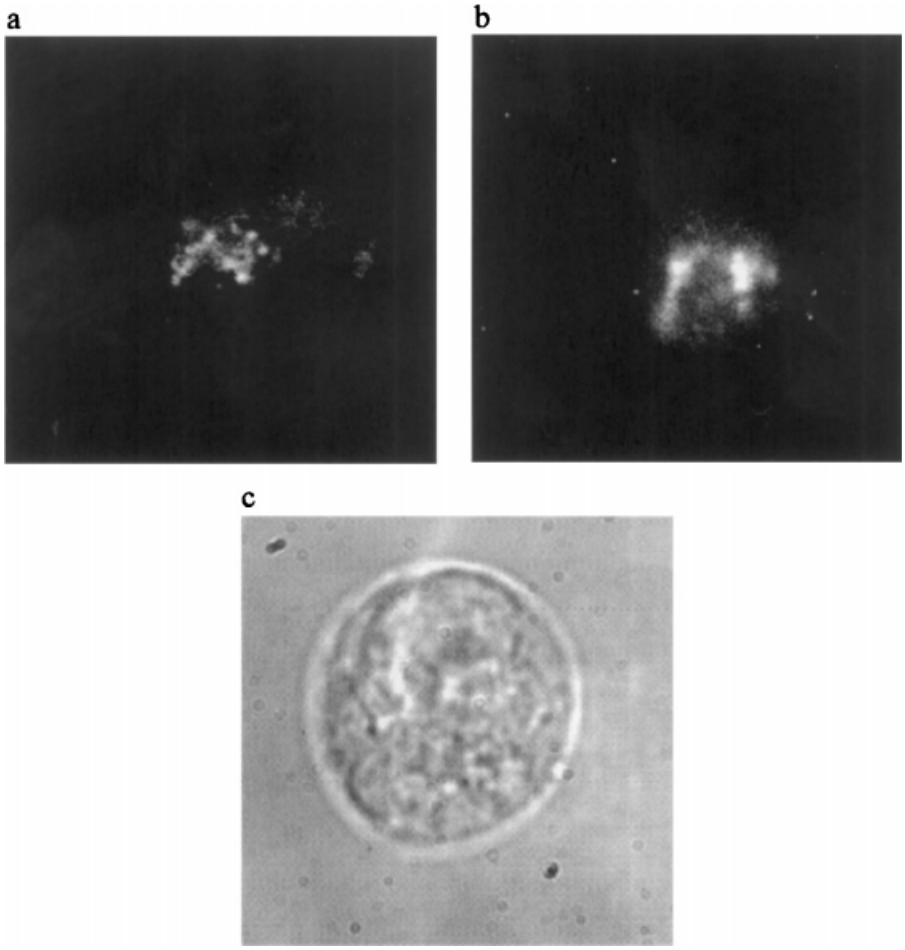

Figure 3. Fluorescence and Raman images of a cell stained with octacarboxy-CoPc and FITC–dextran: (a) fluorescence (of FITC–dextran) image; (b) Raman (of CoPc) image; (c) white light bright-field image.

measured the Raman images with a narrow band filter (about 0.5 nm) and fluorescent images with a broad filter.

CONCLUSION

Raman images of cells incubated in CoPcs were measured. The Raman images of different CoPcs are very similar, which can be explained by the nearly identical hydrophobicities of Pcs. A colocalization study of CoPcs, FITC–dextran and AO was carried out. We suggest that these Pcs are localized in lysosomes. We estimated the intracellular concentration to be 50–100 μM. Raman images at different z-positions were measured. It is likely that Pcs, localized in lysosomes, are distributed in the cell volume, which makes colocalization studies difficult.

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