

# Effect of pH on uptake and photodynamic action of chlorin p6 on human colon and breast adenocarcinoma cell lines

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Received 9th April 2003, Accepted 4th November 2003

First published as an Advance Article on the web 2nd December 2003

The effect of reducing the extracellular pH from 7.4 to 6.0 on the uptake and photosensitivity of chlorin p6, a potential photosensitising drug, has been investigated using two mammalian cell lines, human colon (Colo-205) and breast (MCF-7) adenocarcinoma cells. In Colo-205 cells, the uptake and phototoxicity of chlorin p6 was observed to increase as the pH of the incubation medium decreased. For light doses of up to  $\sim 6 \text{ kJ m}^{-2}$ , although there was no evidence of mitochondrial damage, a significant reduction in Neutral Red uptake was observed, signifying damage to lysosomes. At higher light doses, significant mitochondrial damage was observed, accompanied by saturation of the lysosomal damage. This suggests light-induced relocalization of the photosensitizer from lysosomes to mitochondria. Furthermore, it was found that for a given light dose, lysosomes exhibit greater photosensitivity at lower pH. Since chlorin p6 is known to aggregate at pH 6.0, this observation suggests that the dye accumulation in these cells mainly takes place through endocytosis. In contrast, no significant variation in uptake, photosensitivity, and sites of photodamage was observed for MCF-7 cells at different extracellular pH. Additionally, the lower photosensitivity of lysosomes as compared to mitochondria in these cells suggests chlorin p6 is taken up through diffusion rather than endocytosis.

Photodynamic therapy (PDT) is an innovative cancer treatment modality based on selective accumulation of a photosensitising drug by malignant tissue.<sup>1</sup> Several factors, such as pH, density of low density lipoproteins (LDL), surface charge, and cellular specificity, are believed to influence the selective uptake and retention of the photosensitizer by tumor tissue.<sup>2-5</sup> The effect of pH on the uptake of photosensitising drugs has been investigated in several studies.<sup>6-9</sup> Photodynamic inactivation of cells by hematoporphyrin has been shown to be more efficient at acidic pH than at physiological pH, due to enhanced uptake of the drug.<sup>6</sup> Similarly, under *in vivo* conditions, injection of glucose has been reported to lead to increases in porphyrin uptake and phototoxicity towards tumors, due to a lowering of the tumor pH.<sup>10</sup> The main reason for the increase in cellular uptake of porphyrins and chlorin e6 at low pH is believed to be an increase in the lipophilicity of the drug due to protonation.<sup>8,9</sup> However, increased phototoxicity towards tumors at lower pH has also been observed with drugs for which cellular accumulation is independent of pH. The enhancement of the phototoxicity in this case is believed to be due to inefficient repair of photodamage in acidic conditions.<sup>11</sup> The relative role of these effects in photodynamic inactivation is expected to be dependent on the particular type of tumor cell being targeted. It is therefore of interest to study the pH-dependent photodynamic effects of a photosensitizer on cell lines originated from different tumors.

In this paper, we report results of our investigations on the effect of pH on the uptake and photodynamic action of chlorin p6 on human colon (Colo-205) and breast (MCF-7) adenocarcinoma cells.

## Materials and methods

### Photosensitizer, chemicals, and cell line

Chlorin p6 was prepared following the procedure described in Datta *et al.*<sup>12</sup> Suitable aliquots of a stock solution were added to the culture medium to obtain the concentration used for cell incubation studies.

Eagle's minimum essential medium (EMEM), Rosewell Park Institute medium (RPMI), phosphate buffered saline (PBS),

trypsin, nystatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and fetal bovine serum (FBS) were obtained from Himedia, Mumbai, India. Cetyltrimethylammonium bromide (CTAB) was from SD Fine Chemicals, Mumbai, India. Neutral Red was sourced from Loba Chemie, Mumbai, India. Sodium pyruvate, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and streptomycin were obtained from Sigma, St. Louis, MO, USA.

Human colon (Colo-205) and breast (MCF-7) adenocarcinoma cells were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Colo-205 cells were maintained in RPMI supplemented with 10% FBS and antibiotics. MCF-7 cells were grown in EMEM supplemented with 10% FBS, 1mM sodium pyruvate, and antibiotics. Media used for growth of both Colo-205 and MCF-7 cells were buffered with  $2.2 \text{ g L}^{-1}$  sodium bicarbonate. The cells were grown in monolayers at 37 °C in a 5% CO<sub>2</sub> humidified incubator. The doubling times for the Colo-205 and MCF-7 cells were 25 and 28 h, respectively.

### Effect of pH on chlorin p6 uptake

For pH dependency studies, Colo-205 ( $1.5 \times 10^5$ ) and MCF-7 ( $2 \times 10^5$ ) cells were grown in plastic dishes in appropriate media for 48 h at 37 °C. Subsequently, the growth medium containing sodium bicarbonate was replaced with medium buffered with HEPES (20 mM), as bicarbonate reacts with CO<sub>2</sub> and prevents adjustment of specific pH value. The pH of the medium was varied from 7.4 to 6.0 by adding 80  $\mu\text{l}$  of 1 N hydrochloric acid to 5 ml of medium. Addition of this amount of hydrochloric acid resulted in a change in the osmolarity of the medium from 280 to 312 mosmol. In order to detect any possible adverse effect of the change in pH, the viability of cells was determined after 1, 3, and 6 h of incubation by Trypan Blue assay. The loss of viability in the case of both Colo-205 and MCF-7 cells incubated at low pH (pH 6.0) for up to 3 h was 4% as compared to cells incubated for the same period at pH 7.4. After 6 h, the viability of Colo-205 and MCF-7 cells reduced by 12 and 10%, respectively. To study the pH-dependent uptake of chlorin p6, cells were incubated in darkness for 3 h with 10  $\mu\text{M}$  chlorin p6 in media of varying pH. Chlorin p6 at this concentration did

not cause any dark toxicity. Subsequent to incubation with chlorin p6, the culture medium containing the sensitizer was removed and the monolayer was washed three times with cold PBS. Cells were harvested using 0.25% trypsin and resuspended in PBS. Total uptake of photosensitizer by cells was measured by solubilising the cells using CTAB, as described by Joshi *et al.*<sup>13</sup> In brief, cell suspension ( $0.1 \times 10^6$ ) was dissolved in 1.5 ml of CTAB. The fluorescence intensity of the chlorin p6 in the solubilized cells was measured with a Spex Fluorolog spectrofluorimeter using 400 nm excitation wavelength. In order to study the binding of chlorin p6 in Colo-205 and MCF-7 cells, the fluorescence intensity of cell-bound chlorin p6 was measured without solubilization in some cases.

### Phototoxicity assay

Cells were inoculated at the concentration of  $2 \times 10^4$  cells per well in a 96-well microplate. After overnight incubation, cells were incubated for 3 h in darkness with chlorin p6 in a medium containing 10% FBS. The pH of the incubation medium was adjusted in the range 6.0–7.4. Subsequently, the medium containing chlorin p6 was removed, the monolayer was washed with medium without serum, and fresh growth medium of the required pH was added. Cells were then exposed to white light from two fluorescent tubes covered with a perspex sheet. The fluorescent intensity at the position of the cells was  $9.6 \text{ W m}^{-2}$ , as measured by a Scientech 372 power meter. Following irradiation of the cells, the media in all the wells were replaced with fresh growth medium at pH 7.4. After 24 h, MTT assays were performed to determine the cell survival following the method described by Mosman.<sup>14</sup> Briefly, 100  $\mu\text{l}$  of medium containing 10  $\mu\text{l}$  MTT (5 mg  $\text{ml}^{-1}$ ) was added to each well and the cells incubated with the mixture for 4 h. The culture medium was removed and the formazan crystals formed were dissolved using isopropanol containing 0.4 N hydrochloric acid. The optical density was measured at 570 and 690 nm using a Biotec Instruments Power Wave 340 microplate reader. Each experiment included two controls, one in which cells were treated with dye but not exposed to light, and another where cells were exposed to light but not treated with dye. In some experiments, the MTT assay was performed immediately following irradiation to assess mitochondrial damage.<sup>15</sup>

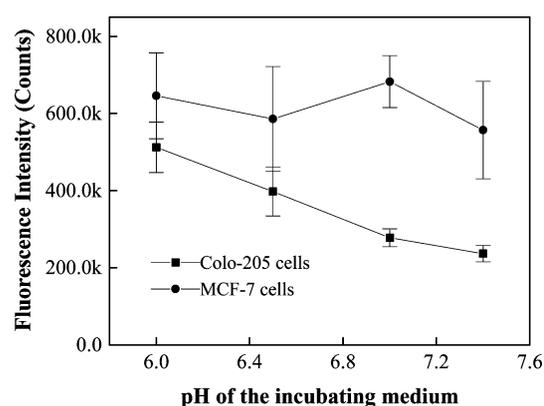
### Neutral Red uptake

To assess the photoinduced damage to lysosomes, uptake of Neutral Red (a lysosomal specific probe) by cells was studied.<sup>16,17</sup> Cells were plated in a 96-well microplate treated with photosensitizer and irradiated as described above. Following irradiation, the growth medium was replaced with medium containing Neutral Red (50  $\mu\text{g ml}^{-1}$ ) and the cells incubated for 3 h in darkness. Subsequently, cells were washed with PBS and the Neutral Red taken up by the cells was extracted using acidified ethanol. Uptake of Neutral Red was monitored using the microplate reader at 540 nm.

## Results and discussion

### Effect of pH on cellular uptake of chlorin p6

Fig. 1 shows the total uptake of chlorin p6 by Colo-205 and MCF-7 cells following incubation with the drug for 3 h in growth medium at different pH. The reason for choosing an incubation period of 3 h was that the uptake of chlorin p6 in Colo-205 and MCF-7 cells showed a linear increase up to 3 h in a medium at pH 7.4. Beyond this time, the rate of uptake decreased (data not shown). As can be seen from Fig. 1, the uptake of chlorin p6 by Colo-205 cells increases with decreasing pH of the incubation medium. Here, it is pertinent to note that chlorin p6 exists in more than one anionic form at physiological pH and, on decreasing the pH, the protonated form



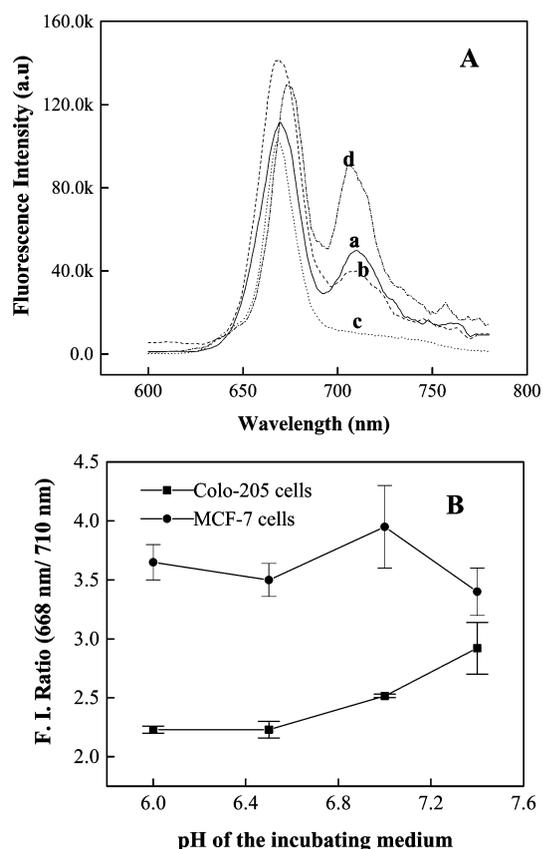
**Fig. 1** Cellular uptake of chlorin p6 as a function of pH. Cells were incubated with 10  $\mu\text{M}$  chlorin p6 in growth medium at pH 6.0, 6.5, and 7.4 for 3 h. Fluorescence from cell-bound chlorin p6 was measured by solubilising the cells with CTAB. Each data point represents the average  $\pm$  SE of three experiments.

predominates.<sup>12</sup> Since protonated drug is more hydrophobic in nature, the rate of its uptake by Colo-205 cells increases. These results are consistent with the enhanced uptake of porphyrins and chlorin e6 observed in cells at low pH.<sup>7,8</sup> In contrast, no pH-dependent change in uptake was observed for MCF-7 cells, although uptake at all pH values investigated was more than for Colo-205 cells.

To understand the differences observed in uptake of the drug by Colo-205 and MCF-7 cells at different pH, binding of chlorin p6 to these cells was studied by fluorescence spectroscopy. Fig. 2(A) shows the fluorescence spectra of chlorin p6 bound to Colo-205 and MCF-7 cells following incubation with the drug for 3 h in growth medium at pH 6.0. Upon binding to cells, chlorin p6 shows emission peaks at 668 and 710 nm. This indicates that chlorin p6 binds to two different micro-environments in the cells. To assess the influence of the environment on the fluorescence of chlorin p6, fluorescence spectra of the drug in polar and non-polar solvents were obtained. In a polar solvent (CTAB), the fluorescence peak appears at 668 nm. However, in toluene (a non-polar solvent), in which chlorin p6 is sparingly soluble, the emission maxima are observed at 671 and 710 nm. These results suggest that the peaks observed at 668 and 710 nm arise due to binding of chlorin p6 to hydrophilic and hydrophobic environments, respectively. The observed decrease in the ratio of the fluorescence intensity at 668 and 710 nm in Colo-205 cells at lower pH (<7.4) is consistent with increased accumulation of protonated chlorin p6 at lower pH Fig. 2(B). In contrast to Colo-205 cells, MCF-7 cells do not show any increase in accumulation of protonated chlorin p6 at lower pH. However, the fluorescence intensity at 710 nm for MCF-7 cells is lower compared to Colo-205 cells, suggesting reduced accumulation of protonated chlorin p6 in these cells.

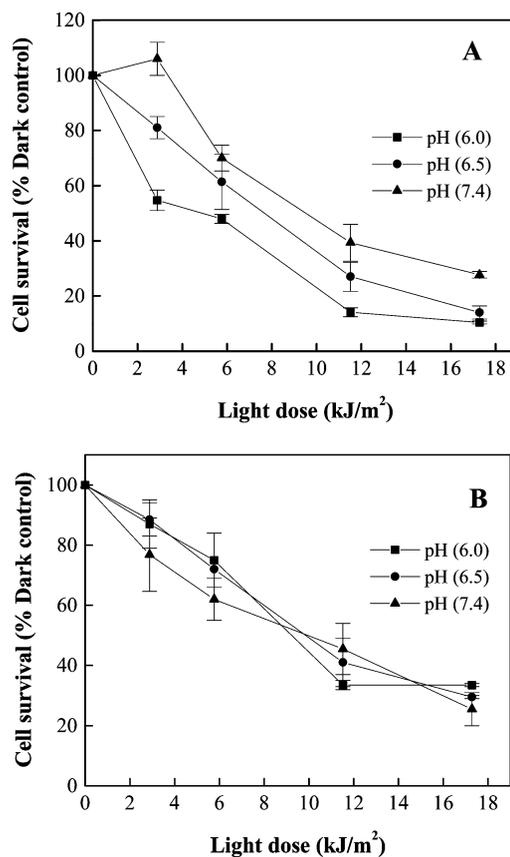
### Effect of pH on photosensitization

Many cellular processes, such as proliferation of cells, transport of metabolites, and the activity of enzymes, are known to depend on pH. It is therefore expected that the effect of therapeutic agents will also depend on both extracellular as well as intracellular pH.<sup>2</sup> Thus, the effect of photoirradiation on the survival of dye-treated cells was investigated at different extracellular pH using MTT assays. Changing the pH of the incubation medium from 7.4 to 6.0 by itself did not have any significant effect ( $p > 0.3$ ) on the survival of Colo-205 and MCF-7 cells incubated with or without chlorin p6 in darkness. The survival of drug-treated Colo-205 and MCF-7 cells on photoirradiation was found to decrease as a function of light dose (Fig. 3). The observed increase in the phototoxicity of chlorin p6 in Colo-205 cells with decreasing extracellular pH correlates



**Fig. 2** (A) Fluorescence emission spectra of chlorin p6 in Colo-205 cells (a), MCF-7 cells (b), CTAB (c), and toluene (d). Cells were incubated for 3 h in darkness in growth medium (pH 6.0) containing 10  $\mu$ M chlorin p6. Subsequent treatments were carried out as described in Materials and Methods. Fluorescence spectra of cell-bound chlorin p6 were measured using an excitation wavelength of 400 nm. (B) Change in the ratio of fluorescence emission intensity (668 nm/710 nm) of cell-bound chlorin p6 as a function of the pH of the incubating medium. Each data point represents the average  $\pm$  SE of three experiments.

with the increased drug uptake at low pH. The survival curve of Colo-205 cells photosensitized at physiological pH shows a shoulder at lower light doses, suggesting repair of photodamage. This shoulder disappears when the cells are irradiated at lower pH, indicating a reduction of the repair processes. Similar pH-dependent photosensitivity has been observed in human colon carcinoma (WiDr) cells photosensitized with *meso*-tetrakis(hydroxyphenyl)chlorin, even though drug uptake was independent of extracellular pH.<sup>11</sup> Although the photosensitivity of MCF-7 cells exposed to light at different pH is comparable to that of Colo-205 cells irradiated at pH 7.4, the survival curves for MCF-7 cells do not show a shoulder, even at physiological pH. This indicates poor repair of photodamage in these cells. The absence of pH-dependent photosensitivity in MCF-7 cells may not only arise because drug uptake in these cells does not depend on pH, but it is also possible that these cells have more efficient homeostatic mechanisms for regulation of intracellular pH to physiological pH. It has been reported that mammalian cells in culture exposed to acidic pH for short periods (<6 h) maintain intracellular pH to a value  $\sim$ 0.25 higher than extracellular pH through different regulatory mechanisms.<sup>2,18</sup> However, it is possible that the Colo-205 and MCF-7 cells may not have the same capacity to regulate intracellular pH and therefore their intracellular pH may vary slightly when exposed to low pH. This observation is consistent with the fact that breast cancer cells have large intracellular acidic vesicles. These vesicles have very high acidification potentials as compared to typical lysosomal values found in normal cells and are able to remove excess H<sup>+</sup> ions to maintain optimum cytosolic

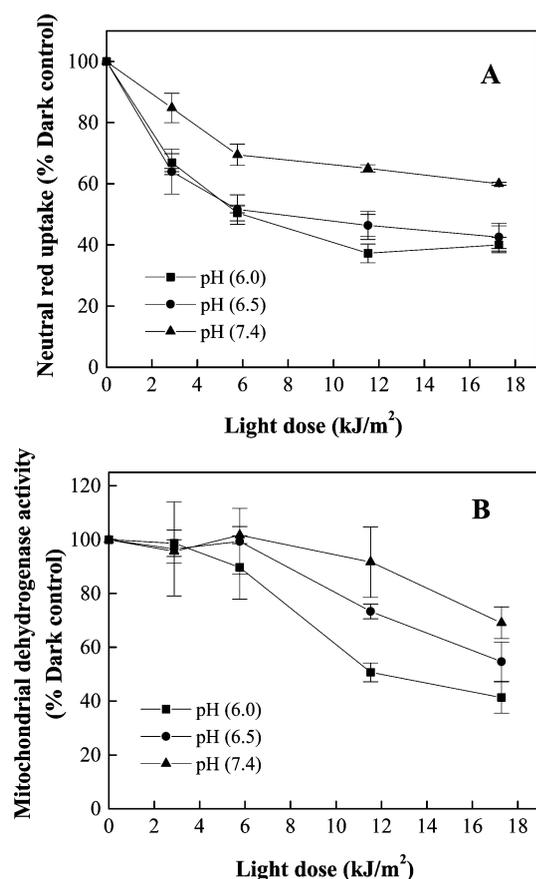


**Fig. 3** Survival of Colo-205 (A) and MCF-7 (B) cells following incubation with chlorin p6 (10  $\mu$ M) in growth medium for 3 h in darkness and irradiation with light at pH 6.0, 6.5, and 7.4. Each data point represents the average  $\pm$  SE of three experiments.

pH levels.<sup>19</sup> This may, in part, be responsible for the observation that the phototoxicity of chlorin p6 in MCF-7 cells did not show significant pH dependence.

#### Sites of photodamage

The sites where photodamage occurs within the cell depend on the subcellular localization of the photosensitizer, which is determined by chemical properties such as hydrophobicity, charge, and amphiphilic character.<sup>20</sup> Since changing the pH alters the degree of protonation of chlorin p6 and thus modulates its hydrophobicity, the effect of varying the extracellular pH on the photodamage sites was also investigated. Photodamage to lysosomes and mitochondria was assessed by Neutral Red uptake and mitochondrial dehydrogenase assays, respectively. The uptake of Neutral Red and dehydrogenase activity in both Colo-205 and MCF-7 cells incubated with and without chlorin p6 in darkness at different pH did not vary significantly ( $p > 0.4$ ). However, on irradiation of cells incubated with chlorin p6, a reduction in uptake of Neutral Red is observed up to a light dose of 5.8 kJ m<sup>-2</sup> [Fig. 4(A)] and the decrease is larger at pH values below physiological pH. This indicates that lysosomes are more susceptible to damage at lower pH. However, at higher light doses, the decrease in uptake of Neutral Red levels off. This could be due to release of the photosensitizer from the lysosomes into the cytoplasm as a result of photodamage. It is also pertinent to note that no significant change in mitochondrial dehydrogenase activity is seen in cells irradiated with light doses of up to 5.8 kJ m<sup>-2</sup>. However, substantial mitochondrial damage is evident at higher light doses throughout the pH range studied [Fig. 4(B)]. This suggests relocation of the photosensitizer from lysosomes to mitochondria. The redistribution of the photosensitizer from lysosomes to other cell organelles at non-toxic light doses has been reported by many investigators.<sup>21,22</sup>

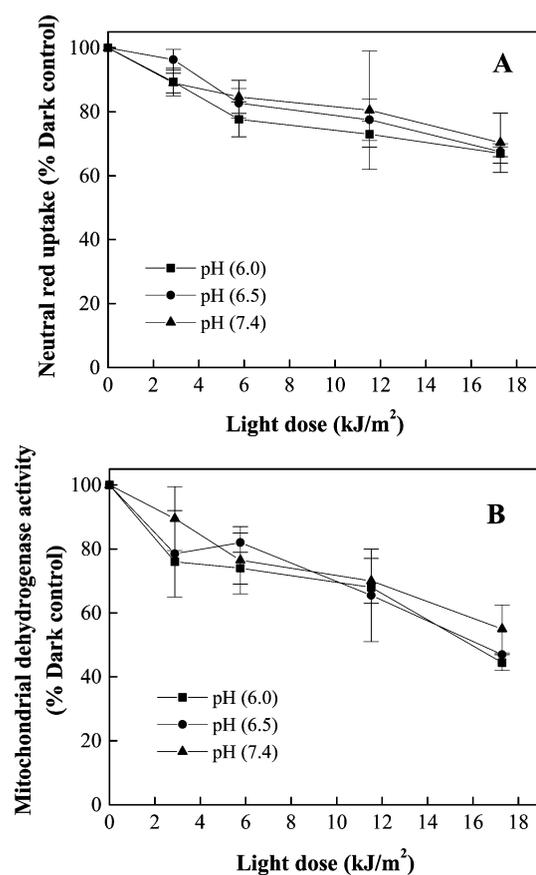


**Fig. 4** Effect of irradiating Colo-205 cells at pH 6.0, 6.5 and 7.4 following incubation with chlorin p6 (10  $\mu$ M) in growth medium for 3 h in darkness as judged by Neutral Red uptake (A) and mitochondrial dehydrogenase assay (B). Each data point represents the average  $\pm$  SE of three experiments.

Relocalization of the photosensitizer was also observed in cells irradiated at lower fluence (3.2 W m<sup>-2</sup>). The results indicate that there is no difference in the response of cells irradiated at lower fluence with respect to redistribution of the photosensitizer, except that the redistribution occurs at a much lower light dose (3.8 kJ m<sup>-2</sup>). Interestingly, at a given dose, photodamage to lysosomes and mitochondria was found to be higher at lower fluence than higher fluence (data not shown). A possible reason for this difference may be the fact that photobleaching of the photosensitizer will be lower at lower fluence. However, this question needs to be investigated further.

The effect of irradiation on photodamage to lysosomes and mitochondria in MCF-7 cells incubated with chlorin p6 at different extracellular pH is shown in Fig. 5. In contrast to the results obtained for Colo-205 cells, for MCF-7 cells, the change in uptake of Neutral Red was significantly smaller as compared with the decrease in mitochondrial dehydrogenase activity at all the light doses. This suggests that lysosomes are not the primary site of photodamage in MCF-7 cells.

One possible reason for the observed differences in the intracellular sites of photodamage in Colo-205 and MCF-7 cells may be the different mechanisms of uptake. The results presented in Fig. 4(A), suggest that in Colo-205 cells, dye accumulation mainly takes place through endocytosis. This is consistent with the fact that protonation of chlorin p6 at lower pH values leads to aggregation,<sup>12</sup> and it is believed that aggregated photosensitizer is internalized through an endocytic pathway into lysosomes.<sup>20</sup> The lower photosensitivity of the lysosomes and the absence of significant pH dependence observed in MCF-7 cells suggest that endocytosis does not play an important role in this case. The photosensitizer may enter these cells *via* diffusion. The reason for the different uptake mechanisms of the two cells may be the intrinsic differences in



**Fig. 5** Effect of incubating MCF-7 cells with chlorin p6 and irradiating at different extracellular pH on A) Neutral Red uptake and B) Mitochondrial dehydrogenase assay. Cells were incubated with chlorin p6 in growth medium at pH 6.0, 6.5 and 7.4 for 3 h in darkness prior to irradiation. Each data point represents the average  $\pm$  SE of three experiments.

the structural composition of the membranes of the two cells. Indeed, Maman and Braut<sup>23</sup> have shown that the uptake of porphyrin derivatives by vesicles is strongly influenced by several factors, *e.g.* phospholipid content of the membrane, thickness of bilayer, *etc.*

To conclude, we have shown that the pH dependence of the uptake and photodynamic action of chlorin p6 is significantly different in colon carcinoma (Colo-205) and breast carcinoma (MCF-7) cells. In Colo-205 cells, the uptake and phototoxicity of chlorin p6 was observed to increase with decreasing pH of the incubation medium. Significant photodamage to lysosomes was observed at low light doses and the damage levelled off at higher doses. In contrast, while there was no significant mitochondrial damage at low light doses, pronounced damage was observed at higher light doses. This suggests light-induced relocalization of the photosensitizer from lysosomes to mitochondria. Furthermore, the observation that lysosomes are more photosensitive at low pH, where enhanced accumulation of protonated chlorin p6 takes place, suggests that drug accumulation in these cells occurs mainly through endocytosis. In contrast, for MCF-7 cells, no significant pH dependence of the uptake and photosensitivity of chlorin p6 was observed. In addition, the observation that damage to mitochondria was greater than to lysosomes at all the light doses suggests chlorin p6 is taken up in MCF-7 cells through diffusion rather than endocytosis.

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