WATER-SOLUBLE METAL NAPHTHALOCYANINES — NEAR-IR PHOTOSENSITIZERS: CELLULAR UPTAKE, TOXICITY AND PHOTOSENSITIZING PROPERTIES IN NHIK 3025 HUMAN CANCER CELLS

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Abbreviations

$AlPCS_2$	disulphonated aluminium phthalocyanine
DMSO	dimethylsulphoxide
DPCC	dipalmitoyl-diphosphatidyl-choline
HPD	haematoporphyrin derivative
3% MEM	minimum essential medium containing 3% newborn calf serum
10% MEM	minimum essential medium containing 10% newborn calf serum
MNC	metal naphthalocyanine
$MNCS_x$	sulphonated metal naphthalocyanine, where x is the average sulphonation degree per molecule
MPC	metal phthalocyanine
PBS	Dulbecco's phosphate-buffered saline
PDT	photodynamic therapy
PII	photofrin II

Summary

Metal naphthalocyanine complexes (MNCSs) absorb light in the near-IR spectral region (760 nm) where tissue penetration is optimal and they have been proposed as agents for photodynamic therapy (PDT). Sulphonated derivatives of tris-(2,3-naphthalocyanato) bis-chloroaluminium(III) and zinc(II) with various degrees of sulphonation were prepared. Cellular uptake, aggregation in cellular environments, cytotoxicity and photosensitizing properties were studied. Three of the four dyes studied were taken up by cells to a satisfactory degree and were not cytotoxic at the concentration

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used (10 μg ml^{-1}). The least sulphonated sample of zinc naphthalocyanine produced some phototoxic effects (LD_{50} = 1.12 J cm^{-2}). All the other samples of sulphonated naphthalocyanine were found to be aggregated inside the NHIK 3025 cells, preventing any significant PDT effect.

1. Introduction

The sensitizers commonly used in experimental photodynamic therapy (PDT), haematoporphyrin derivative (HPD) and photofrin II (PII) [1], suffer from disadvantages related to their optical absorption properties: their Q band is not particularly strong and is not located at sufficiently long wavelengths. Optimal wavelengths for light penetration through tissue are in the region 700 - 800 nm [2]. This has led to investigations on metal phthalocyanines (MPCs) which have a strong absorption band around 670 nm [3, 4].

Metal naphthalocyanines (MNCs) absorb light even further into the near-IR region than do MPCs (λ = 760 nm). The penetration depth into tissue will be about twice as large at 760 nm than at 630 nm, the wavelength currently used for porphyrins [2]. Indeed, bis(tris-*n*-hexyl-siloxy)-tris-(2,3-naphthalocyanato) silicon (SiNC) incorporated into liposomes (because of its insolubility in aqueous solution) has recently been investigated as a potential PDT agent [5]. Sulphonated derivatives of tris-(2,3-naphthalocyanato) chloroaluminium(III) and zinc(II) (AlNCS and ZnNCS) have the advantage of being soluble in water to some extent. Aspects of the photochemistry of these compounds have already been investigated [6, 7]. Their singlet oxygen quantum yields ϕ_Δ were found to be 0.3 for both compounds in deuterated ethanol:water (9:1) [7]. This compares with ϕ_Δ = 0.35 for SiNC in benzene [5] and ϕ_Δ = 0.4 - 0.6 for porphyrins in D_2O [8]. The degree of sulphonation affects the lipophilicity of a drug and hence its cellular uptake and tendency to aggregate in cells [9, 10]. Parameters such as lipophilicity and aggregation tendency may also be of importance for the tumour localizing ability of a drug [10]. For these reasons NCSs with various degrees of sulphonation were prepared and tested. In this paper we report some of the spectroscopic properties of these MNCSs, their uptake in cells, their lipophilicity as given by the Triton X-114–H_2O partition coefficient, their aggregation in cellular environments, their cytotoxicity and their photosensitizing properties. Since naphthalocyanines tend to be degraded during light exposure we also (in one case) recorded the absorption spectra of cell-bound and protein-bound dye before and after light exposure.

2. Materials and methods

AlNCS and ZnNCS (see Fig. 1) were prepared by methods reported elsewhere [11]. Degrees of sulphonation were varied according to duration

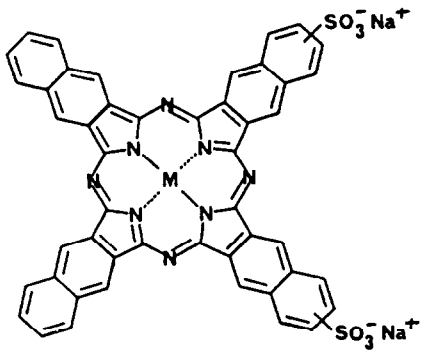


Fig. 1. One possible isomer of MNCS (M ≡ Al or Zn).

of sulphonation [12]. The average number of sulphonates per molecule was determined by microanalysis. Samples of $AlNCS_{1.2}$, $AlNCS_{3.3}$, $ZnNCS_{1.3}$ and $ZnNCS_{3.1}$ were prepared, the subscript denoting the average degree of sulphonation of the molecule. $AlNCS_{1.3}$, due to its low degree of sulphonation, was only partially soluble in phosphate-buffered saline (PBS), so a stock solution was prepared in dimethylsulphoxide (DMSO). $ZnNCS_{1.2}$ was soluble to millimolar concentrations in PBS as were the other compounds. Small aliquots of sterile stock solution were added directly to the culture medium. DMSO concentration was kept at less than 2% by volume of tissue culture medium. This concentration of DMSO had no influence on cell survival or proliferation. Throughout this study human cells of the line NHIK 3025 were used [13]; they were cultivated in minimum essential medium (MEM) with 10% newborn calf serum (Gibco, U.K.; 10% MEM) as described earlier [14].

Cells for uptake studies were incubated at 37 °C in Falcon tissue culture dishes (25 cm^2) for 5 - 6 h in 10% MEM; the medium was then changed to 3% MEM containing the dyes being studied (2 $\mu g\ ml^{-1}$ for $AlNCS_{1.3}$ and 10 $\mu g\ ml^{-1}$ for the others). After incubation for a further 18 h, the dishes were washed five times with cold PBS and brought into suspension in 4 ml PBS by means of a cell scraper (Costar). These samples contained about 10^5 cells ml^{-1}, as determined by Burker chamber counting and by light scattering measurements. The absorption spectra of these cell suspensions were measured with an appropriate cell suspension in the reference cuvette and the concentration of dye within the cell suspension was determined by adding a known amount of the dye to the cell suspension and recording the change in optical density. To calculate intracellular drug concentrations we used the concentration of the drug within cell suspensions of known cell densities and assumed a cellular volume of 2.3×10^{-9} ml [15] (*c*(intra) in Table 1). The concentration outside the cell was assumed to be the concentration of the drug in 3% MEM (*c*(extra) in Table 1). The ratio of these two values is indicative of the drug's tendency to be taken up by the cells and can be compared with values of this ratio for other photosensitizers [10]. To determine whether the dyes were firmly bound within the

TABLE 1

Triton X-114–H_2O partition coefficients K and cellular uptake of the NCSs by NHIK 3025 cells (18 h incubation at 37 °C in 3% MEM; dye concentrations, 10 μg ml^{-1})

Dye	*K*	*c*(intra)/ *c*(extra) *(18 h in 3% MEM)*	*Dye retained after 3 h incubation in 3% dye-free MEM*[a] (%)
$AlNCS_{3.1}$	9.6	14.3	50
$ZnNCS_{1.2}$	19.8	13.2	51
$ZnNCS_{3.1}$	5.4	7.8	63

[a]The cells were first incubated with the dyes for 18 h in 3% MEM.

cell, parallel cell samples were cultivated as above, but after the incubation period, the medium with dye was replaced with fresh 3% MEM and the samples were incubated for a further 3 h. The samples were then analysed as described above and the degree of drug retained by the cell was recorded as a percentage (Table 1).

To investigate the rate at which the dyes were initially taken up by the cells, a PBS uptake study was undertaken. Cells (10^5 cells ml^{-1}) were incubated for 18 h in 10% MEM; the MEM was removed, the cells were washed with cold PBS five times and a PBS solution (4 ml) of the dye was added (10 μg ml^{-1} for all but $AlNCS_{1.3}$ which did not dissolve sufficiently in PBS to be studied). The samples were then incubated for 10, 20, 30, 40 and 60 min, after which the cells were again washed with cold PBS and brought into suspension. The amount of dye bound to the cells was determined as described above. Since NHIK cells survive for only 2 - 4 h in PBS we limited the experiment to 1 h.

Cytotoxicity studies were carried out as follows. Cells (400) were inoculated into Falcon tissue culture flasks (25 cm^2). Various concentrations of the sensitizer were introduced in 3% MEM and the cells were incubated with the dyes for 18 h. The 3% MEM with the dyes was removed and the cells were incubated for a further 5 days in 10% MEM. The MEM was then removed, the cells were washed with saline solution, fixed with ethanol, stained with methylene blue and the number of cell colonies was counted. Each colony represented one surviving cell of the previous 400.

Phototoxicity tests were carried out in two ways: (a) 10^5 cells ml^{-1} were inoculated into 25 cm^2 culture flasks with 10% MEM; these were incubated for 5 - 6 h, the MEM was replaced with 3% MEM containing the dye (2 μg ml^{-1} for $AlNCS_{1.2}$ and 10 μg ml^{-1} for the other dyes) and the cells were incubated for 18 h with the dyes; (b) 400 cells were inoculated into 25 cm^2 Falcon tissue culture flasks with 10% MEM; these were incubated for 5 - 6 h, the MEM was replaced with 3% MEM containing the

dye (2 μg ml^{-1} for AlNCS$_{1,2}$ and 10 μg ml^{-1} for the other dyes) and the cells were further incubated for 18 h. The two sets of flasks were then irradiated for periods of 5 min to 1 h using a 900-W Osram high pressure xenon lamp fitted with a Bausch and Lomb grating monochromator with narrow slits (2 mm; bandwidth, 5 nm). A UDT 11a light detector (United Detector Technology, St Monica, CA) was used to measure the fluence rate at the positions of the cells and light flux at the wavelengths used. The cells sensitized with the AlNCS dyes were exposed to 770 nm light and the cells sensitized with the ZnNCS dyes were exposed to 755 nm light. At both wavelengths the fluence rate was 15 mW cm^{-2}. After irradiation, the tissue culture medium containing the dye was replaced with fresh MEM containing 10% serum. The flasks containing 10^5 cells were incubated for 36 h and the flasks containing 400 cells were incubated for 1 week. After incubation, the degree of cell survival was determined by staining with methylene blue, as above, and either counting the remaining cell colonies or using the methods described earlier [16].

The xenon lamp source with monochromator was also used for the photobleaching experiments. During light exposure the sample tube containing suspended cells with ZnNCS$_{1,2}$ in PBS was surrounded by ice–water to prevent any temperature rise. The cell suspension was irradiated at 755 nm at an exposure rate comparable with that used in the survival experiments. Its absorption spectrum was taken after various exposure times up to 20 min. The cell suspension was then incubated in the dark for 30 min at 37 °C and its absorption spectrum was recorded again. In a separate experiment a solution of the dye in MEM containing 3% serum was irradiated and analysed in a similar way.

Absorption spectra were measured using a Perkin–Elmer λ15 spectrophotometer equipped with an integrating sphere and quartz cuvettes with a path length of 0.5 cm. Fluorescence spectra were measured using a Perkin–Elmer LS5 spectrofluorometer and a quartz cuvette with a path length of 1.5 mm. In no case was the absorption of the sample in this cuvette greater than 0.1, ensuring negligible inner filter effects. The cell densities in the suspensions were determined by the scattering of 600 nm light; the system was calibrated with cell suspensions of known density as determined by counting in a Burker chamber.

To estimate the lipophilicity of the dyes, an aqueous solution of the non-ionic detergent Triton X-114 was used. This is homogeneous at 0 °C but separates into organic and aqueous phases above 23 °C. The partition coefficients in this Triton X-114–H_2O system have been used previously to determine the relative lipophilicity of dyes [9, 17]. The dye (50 nM) was added to a mixture of PBS (1 ml) and Triton X-114 (0.33 ml) at pH 7.0. Solutions of the appropriate dyes were prepared and left for 2 h in the dark at 0 °C. After checking for homogeneity the solution mixtures were incubated at 37 °C in the dark for 12 h to allow complete phase separation, and the concentration in the aqueous phase was determined spectrophotometrically. The partition coefficients at pH 7.0 were then calculated.

3. Results and discussion

3.1. Spectroscopy

Absorption and fluorescence spectra of $AlNCS_{3.1}$, $ZnNCS_{1.2}$ and $ZnNCS_{3.3}$ in water, aqueous ethanol and MEM containing 3% serum and NHIK 3025 cells were recorded (Fig. 2(a) - (c)). $AlNCS_{1.3}$ was not included in this study because of its low water solubility and high cytotoxic effect (see below). In 90% aqueous ethanol the dyes exist almost completely as monomers as indicated by sharp peaks in the absorption spectra at 755 and 770 nm for ZnNCS and AlNCS respectively (Fig. 2(a)). $ZnNCS_{1.2}$ is slightly more aggregated than the two other dyes as shown by the shoulders in the spectrum around 700 nm. In aqueous solution the dyes are aggregated as shown by the broad absorption band at 700 nm and by the almost complete absence of monomeric bands. Aggregation in aqueous solution has been previously reported to restrict the photosensitizing properties of HPD [18]; it causes a strong reduction in the triplet state quantum yield as the absorbed light energy is dissipated by the formation of ion pairs which quickly recombine. In 3% MEM solution the concentration of the monomer fraction of MNCS increases, judging from the peaks at 755 and 770 nm for ZnNCS and AlNCS (Fig. 2(b)). The fluorescence intensity is also indicative of the degree of monomerization of the naphthalocyanines in the solvent, in that the aggregates of the equivalent phthalocyanines in solution are practically non-fluorescent [19]. From the spectra shown in Fig. 2(b) we conclude that the degree of monomerization in MEM containing 3% serum decreases in the order $AlNCS_{3.1} > ZnNCS_{3.3} > ZnNCS_{1.2}$. Sulphonated aluminium phthalocyanines are well known to be less aggregated in aqueous environments than the equivalent zinc complex [20]. Therefore it is reasonable to assume that the same would be true for sulphonated aluminium and zinc naphthalocyanines. The main reason for this is that AlPCS and AlNCS contain an axial ligand which tends to prevent the ring structures from approaching each other too closely. The reason for the comparatively larger amounts of monomer in 3% MEM than in aqueous solution is probably due to the binding of MNCS to serum proteins producing a greater degree of monomerization.

The spectra of the dyes in NHIK 3025 cells (Fig. 2(c)) indicate that the degree of aggregation of the dyes in the cells decreases in the order $ZnNCS_{3.3} > AlNCS_{3.1} \gg ZnNCS_{1.2}$. The large degree of aggregation and the correspondingly small concentration of monomer would be expected to limit the photosensitizing properties of the dyes in the cells. This is indeed found to be the case.

As we have seen the dyes are mainly present in an aggregated form in aqueous solution. The lipophilicity of these aggregates is supposedly related to their Triton X-114–H_2O partition coefficient K (Table 1) which decreases in the order $ZnNCS_{1.2} > AlNCS_{3.1} > ZnNCS_{3.3}$. The partition coefficients found here are much smaller than expected for monomeric and lipophilic compounds [10], an observation that is certainly related to the strong

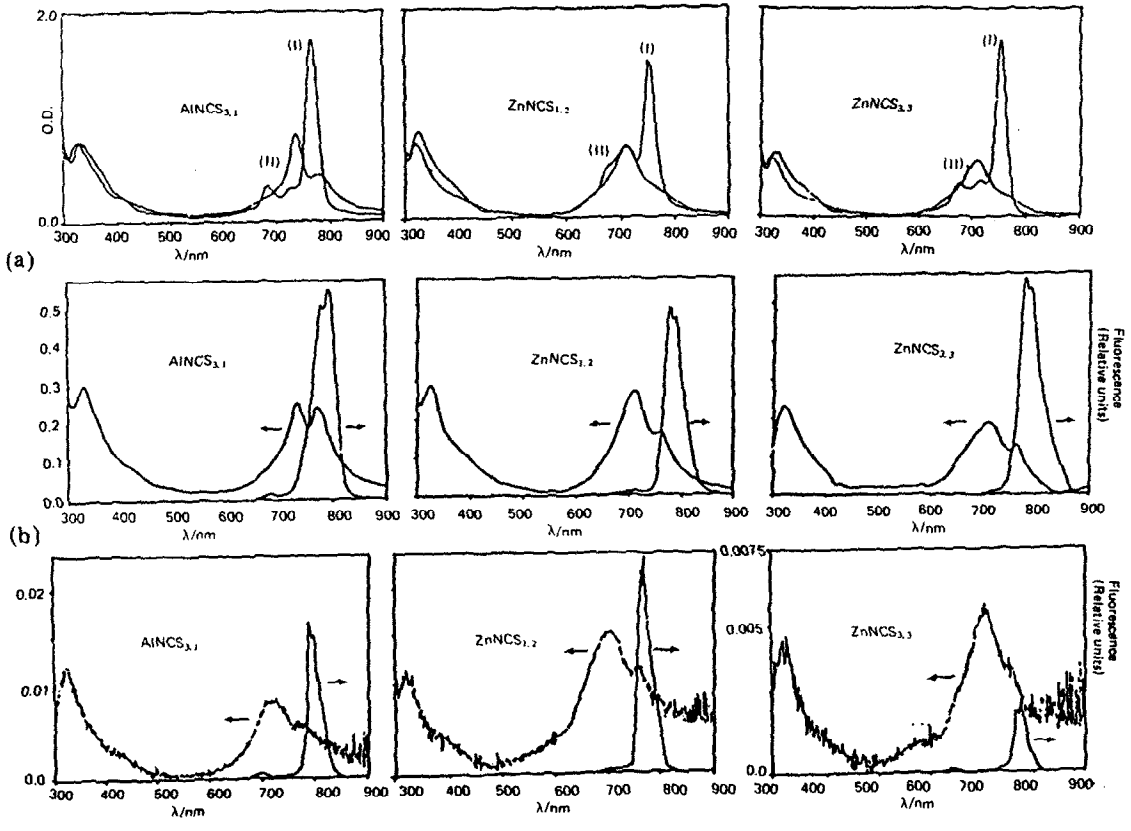


Fig. 2. UV–visible absorption spectra and fluorescence emission spectra of the MNCS dyes in various media: (a) absorption spectra in 90% aqueous ethanol (I) and water (II) ([Dye] = 10 μg ml^{-1}); (b) absorption spectra and fluorescence emission spectra (λ_{ex} = 340 nm) in 3% MEM solution ([Dye] = 10 μg ml^{-1}); (c) absorption spectra and fluorescence emission spectra (λ_{ex} = 340 nm) in NHIK 3025 cells.

tendency of MNCS to aggregate. Not surprisingly, since lipophilic dyes supposedly localize in lipid-rich regions of cells (membranes), the dye with the highest K value ($ZnNCS_{1.2}$) tends to be least aggregated in the cells (Fig. 2(c)) in contrast with that found for the same dyes in aqueous solution (Fig. 2(a)).

3.2. Cell uptake

In PBS the rate of uptake of the dye by the cell is very much enhanced, so that over a comparatively short time, the kinetics of dye uptake can be investigated (Fig. 3). Similar kinetics have been observed for the cell uptake of porphyrins under comparable conditions [21, 22]: an initial rapid rate of uptake is observed, supposedly related to binding to the plasma membrane, followed by a slower rate which may be related to uptake and binding to intracellular structures.

For both short-term uptake of the dyes in PBS and incubation for 18 h with the dyes in MEM containing 3% serum, the order of uptake is $AlNCS_{3.1} \approx ZnNCS_{1.2} > ZnNCS_{3.3}$ (Fig. 3, Table 1). $AlNCS_{1.3}$ was not tested for PBS cell uptake because of its low solubility. In 3% MEM it is more soluble, probably because of binding to serum proteins, but it is not taken up by cells to any measurable extent. The absolute cell uptake, as judged by the ratio c(intra):c(extra) (Table 1), is comparable with that found for photofrin II under similar conditions, namely 12.5 [10].

In the case of more monomeric compounds the cellular uptake is generally found to increase with increasing Triton X-114–H_2O partition coefficient [10]. Of the present dyes, $AlNCS_{3.1}$ is taken up more efficiently by the cells than might have been expected from its Triton X-114–H_2O partition coefficient. $AlNCS_{3.1}$ may be taken up more efficiently than $ZnNCS_{3.3}$ because aluminium has an axial ligand (Cl) which may increase cellular uptake. Furthermore, $AlNCS_{3.1}$ seems to be slightly less aggregated in

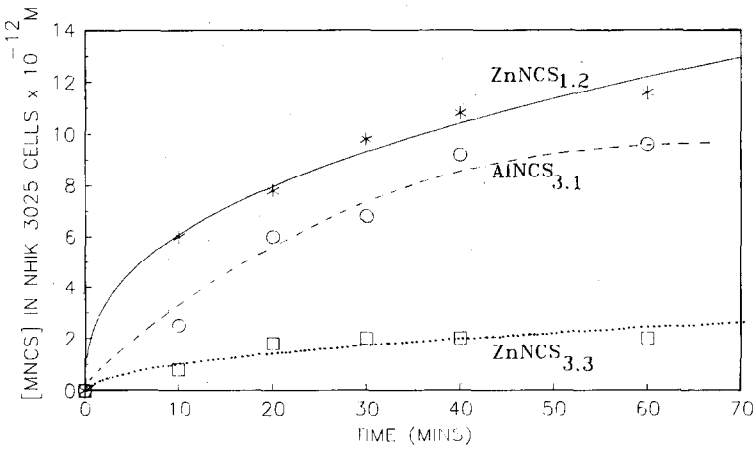


Fig. 3. Cellular uptake of NCSs by NHIK 3025 cells from PBS ([Dye] = 10 μg ml^{-1}).

the 3% MEM medium (Fig. 2(b)) and this may also facilitate the cell uptake. It does not seem that the extra valency results in any chemical binding to cellular components, since $AlNCS_{3.1}$ is not better retained by the cells than the other dyes (see below).

After washing for 3 h in fresh MEM containing 3% serum, 50% or more of the drug is retained which indicates that this proportion of the drug in the cell is firmly bound, perhaps inside cell organelles. This is a slightly lower reduction than found for photofrin II (approximately 85%), but is comparable with that found for some of the haematoporphyrin ethers [23].

3.3. Cytotoxicity

The cytotoxicities of the dyes are shown in Fig. 4. $ZnNCS_{1.2}$ is the least toxic over the dose range studied. $AlNCS_{3.1}$ and $ZnNCS_{3.3}$ are a little more toxic, but still not significantly cytotoxic at the dosages used in this study (10 $\mu g\ ml^{-1}$). However, compared with the equivalent phthalocyanines, these compounds are significantly more cytotoxic. In the laboratory where this work was carried out, a dosage of 200 $\mu g\ ml^{-1}$ of $AlPCS_2$ is regularly used with no cytotoxic effect. The increase in the cytotoxicity of naphthalocyanines may be due to the much larger size of the molecule compared with the equivalent phthalocyanines, which may disrupt the cell physiology. $AlNCS_{1.3}$ is very cytotoxic. This is surprising since it is not efficiently bound to the cells. The cytotoxic effect may be due to its high degree of aggregation which may result in slight precipitation of the dye on the cells during the 18 h of incubation. As a result of the solubility and cytotoxicity problems this dye was not studied further.

3.4. Phototoxicity

According to the results of both methods of determination of phototoxicity, only $ZnNCS_{1.2}$ demonstrates significant phototoxicity. An

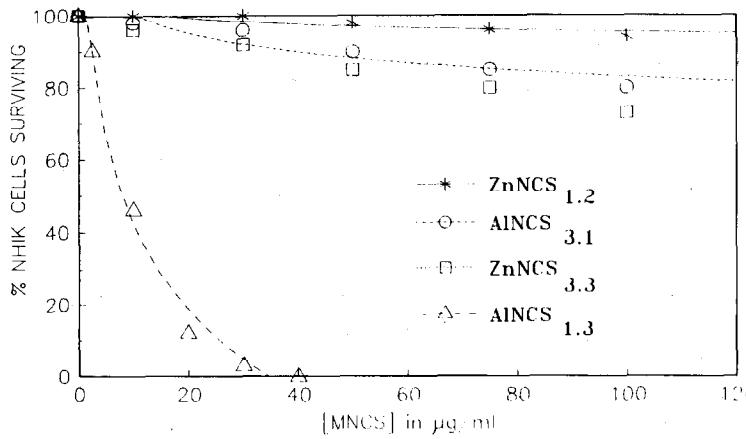
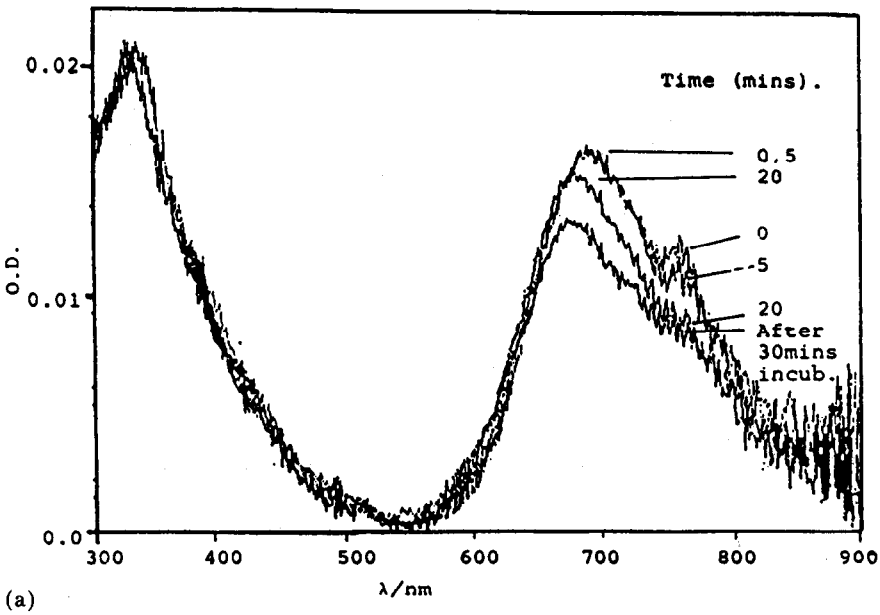
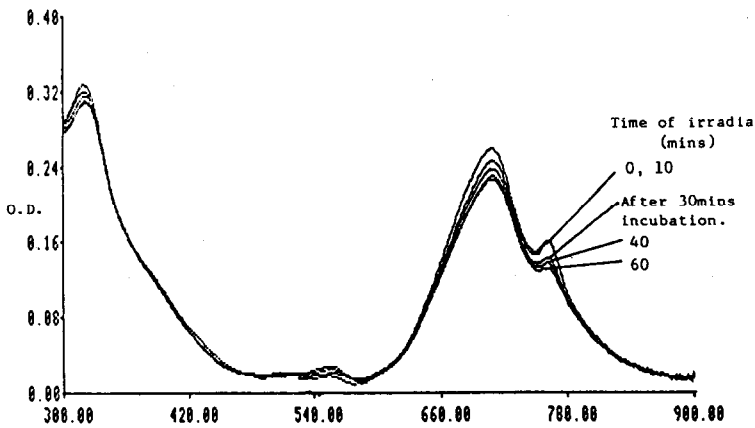


Fig. 4. Cytotoxicity of NHIK 3025 cells with various concentrations of dye in 3% MEM (incubated for 18 h with the dye then for 5 days in 10% dye-free MEM).



(a)



(b)

Fig. 5. (a) Photodegradation of $ZnNCS_{1.2}$ in NHIK 3025 cells by 755 nm light ([Dye] in cell suspension = 1.2×10^{-12} M). (b) Photodegradation of $ZnNCS_{1.2}$ in 3% MEM by 755 nm light ([Dye] = 10 μg ml^{-1}).

exposure of cells to about 1 J cm^{-2} of 760 nm light is needed to inactivate 50% of the cells. This is an exposure which is more than a factor of 20 larger than that needed to inactivate cells incubated with photofrin II (18 h incubation, 10 μg dye ml^{-1}, MEM containing 3% serum). No phototoxic effect is detected for $AlNCS_{3.1}$ or $ZnNCS_{3.3}$. Thus in a cellular system, the MNCSs tested in this work appear to be rather inefficient photosensitizers. This is probably due to two or more factors. Firstly, the strong aggregation of the

dyes in the cells reduces the yield of singlet oxygen to a very low value [19]. Secondly, monomers of the MNCSs are rapidly photodegraded [7]. The photodegradation of $ZnNCS_{1.2}$ in cells and in MEM containing 3% serum was studied in separate experiments. The results are shown in Fig. 5. Under both conditions the small amount of monomer absorbing at 755 nm is progressively photodegraded over a period of 20 min. Incubation of light-exposed cell samples for 30 min in the dark does not result in any significant restoration of the monomers. Thus even though the aggregates are much less photolabile than the monomers (Fig. 5), the equilibrium between aggregates and monomers is restored too slowly to be of any importance in most practical therapeutic situations. The reason for the slow restoration of the equilibrium may be that aggregates and monomers are localized in separate parts of the cell and so are not in strict dynamic equilibrium. This is not the case for $ZnNCS_{1.2}$ in MEM containing 3% serum (Fig. 5(b)): the monomers absorbing at 755 nm are degraded on irradiation, but more and more aggregates dissociate to form monomers, which are again degraded. Incubation of the solution for 30 min in the dark results in a return to the pre-irradiation equilibrium between monomer and aggregate.

4. Conclusions

Although these dyes have good optical properties with respect to PDT and are taken up by cells to a satisfactory degree, aggregation and photodegradation problems prevent them from producing a significant phototoxic effect. A possible way of increasing their photosensitizing effect may be to introduce them to the cell in a monomeric form using, for instance, DPPC liposomes. If the drugs are monomeric on irradiation they may produce a significant phototoxic effect before degrading. Evidence discussed elsewhere [7] suggests that this may be the case.

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