School of Pharmacy, Department of Pharmaceutics, University of Oslo; Nordic Institute of Dental Materials (NIOM as), Haslum, Norway

Photokilling of bacteria by curcumin in selected polyethylene glycol 400 (PEG 400) preparations
Studies on curcumin and curcuminoids, XLI

T. Haukvik, E. Bruzell, S. Kristensen, H. H. Tønnesen

Received February 12, 2010, accepted March 24, 2010

Tone Haukvik, Department of Pharmaceutics, School of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, 0316 Oslo, Norway
tone.haukvik@farmasi.uio.no

Pharmazie 65: 600–606 (2010) *doi: 10.1691/ph.2010.0048*

Curcumin, bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a yellow-orange pigment which can be synthesised chemically or isolated from the plant *Curcuma longa* L. Curcumin has a rather broad absorption peak in the range 300–500 nm (maximum ~ 430 nm) and has potential as a photosensitiser for treatment of localised superficial infections in *e.g.*, the mouth or skin. Previously, we have demonstrated phototoxic effects of curcumin in selected aqueous preparations against both gram-positive *Enterococcus faecalis* and *Streptococcus intermedius* and gram-negative *Escherichia coli* bacteria *in vitro*. One of the most efficient preparations was curcumin in polyethylene glycol (PEG 400) dissolved in phosphate buffered saline (PBS), pH 6.1. In this study the solubilising effect of PEG 400 on curcumin molecules and the *in vitro* phototoxic effects of these preparations were further evaluated. The effect of varying the curcumin concentration (2.50 μM – 25.00 μM), the radiant exposure (0.5–30 J/cm^2) and the physical state of curcumin against the survival of *E. coli* was investigated. PEG 400 showed an increasing physically stabilising effect towards crystallisation of curcumin in aqueous preparation with increasing concentrations (2.5%–10.0% v/v). Despite a higher solubility of curcumin with increasing PEG 400 concentrations, the surfactant reduced the phototoxicity of curcumin against *E. coli*. The highest phototoxic effect was obtained when curcumin was present in the least physically stable preparation, a stock solution in ethanol added to PBS with or without the lowest test concentration of PEG 400 (2.5% v/v). The obtained phototoxic effect can be increased by increasing the irradiation dose or by choosing an optimal curcumin concentration. *E. faecalis* was efficiently killed by the lowest concentration of curcumin in combination with the lowest radiant exposure when curcumin was dissolved in certain PEG solutions (<0.02% survival), but showed no reduction when exposed to preirradiated curcumin.

1. Introduction

Curcumin is a natural polyphenolic compound isolated from the rhizomes of the plant *Curcuma longa* L. It has a long tradition as a spice and food coloring agent in Indian cooking and is well known from Asian culture as a native medicine against a number of conditions. The natural commercially available curcumin consists of a mixture of three curcuminoids: curcumin, demethoxy- and bisdemethoxy-curcumin. The demethoxy- and bisdemethoxy-compounds can amount to nearly 40% of the sample (Tønnesen et al. 1985). Curcumin has a bright yellow colour and is approved by the FDA as a colouring agent in food, drugs and cosmetics, and has thereby undergone extensive toxicity testing. It has been found to be nontoxic to humans up to a dose of 10 g/day when unexposed to light (Aggrawal et al. 2007). A fast increasing amount of publications suggests that curcumin in the ground state is a powerful anti-inflammatory, anticarcinogenic and antioxidant compound with a therapeutic potential and almost no side effects (Aggrawal et al. 2007). Further, curcumin has been suggested as a photosensitiser (PS) in photodynamic therapy (PDT) (Tønnesen et al. 1987; Dahl et al. 1989). PDT involves the administration of a photosensitising agent (light-activated chemical), followed by irradiation with visible light (380–780 nm) to produce a series of cascade events eventually leading to the generation of toxic reactive oxygen species. PDT may be employed in clinical oncology, for the treatment of other non-malignant conditions, for virus inactivation and for bactericidal purposes (Hamblin and Hasan 2004). The efficacy of PDT is influenced by the properties of the PS: it should be taken up preferentially and/or retained by the diseased tissue in therapeutic concentrations, absorb light in a suitable wavelength range, show little or no dark toxicity and be an efficient generator of phototoxic species. An optimal pharmaceutical formulation can be used to obtain selective delivery of a PS in appropriate concentrations in the diseased area. The formulation may then act as a targeting device. Liposomes, oil dispersions, polymeric particles and hydrophilic polymer-PS conjugates are considered as passive targeting systems as they exploit the natural distribution pattern (passive diffusion and phagocytosis processes) while lipoprotein-mediated or monoclonal antibodies have been used to achieve active targeting systems (Konan et al. 2002). For all systems the formulation must be able to incorporate the

PS without loss or alteration of its activity, be biodegradable and show little or no immunogenicity. Another reason for using vehicles is to increase the solubility and stability of the PS and provide an environment where the PS can be administered in a monomeric form. In fact, due to their hydrophobic chemical structure, most PS tend to aggregate in aqueous media as a result of the propensity of the hydrophobic skeleton to avoid contact with water molecules. This state can hinder the efficacy of the drug *in vivo* by decreasing its bioavailability and limiting its capacity to absorb light and thereby decreasing the generation of phototoxic species (Konan et al. 2002).

We have previously demonstrated that curcumin in combination with blue light efficiently can kill mammalian cells (Bruzell et al. 2005) as well as gram-positive and gram-negative bacteria *in vitro* (Haukvik et al. 2009). The effect was shown to be dependent on curcumin concentration, radiant exposure, post-irradiation incubation time and pharmaceutical preparation. Curcumin is highly lipophilic and almost insoluble in water at acidic or neutral pH. At pH values above neutral the solubility increases but the compound then undergoes rapid hydrolytic degradation. It has previously been shown that by decreasing the pH of the solvent from 7.5 to 6.0, the hydrolytic degradation rate was lowered by a factor of 10^3 (Tønnesen and Karlsen 1985). Furthermore the hydrolytic stability and solubility of curcumin can be strongly improved by incorporating curcumin in cyclodextrines (Tønnesen et al. 2002), liposomes (Tønnesen et al. 1993), micelles (Tønnesen 2002) or macromolecules such as alginate (Tønnesen 2006). Further, the photochemical and photophysical properties of curcumin vary with the H-bonding capacity and polarity of the molecular environment (Chignell et al. 1993; Nardo et al. 2009). In a previous study of curcumin phototoxicity against bacteria we tested different aqueous preparations of curcumin containing cyclodextrines, liposomes or surfactants that have been shown to increase the solubility and hydrolytic stability of curcumin in solution. One of the most efficient aqueous solutions of curcumin to induce phototoxicity against gram-negative bacteria, was a preparation of curcumin in 5.0% polyethylene glycol 400 (PEG 400) in PBS pH 6.1 (Haukvik et al. 2009). Polyethylene glycols are stable, water-soluble and hydrophilic addition polymers of ethylene oxide and water. Aqueous PEGs can be used either as suspending agents, to adjust the viscosity and consistency of other suspending vehicles or to enhance the aqueous solubility of poorly soluble compounds (Rowe et al. 2003). They are regarded as nontoxic and non-irritant materials and PEG 400 has been used in concentrations up to approximately 30% v/v in parental dosage forms (Rowe et al. 2003). However, the solubilising effect of PEG 400 on curcumin molecules is not much investigated and was one of the aims in the present study. A further aim of the study was to establish the optimal combination of curcumin concentration, PEG 400 concentration and irradiant exposure to be used in photokilling of gram-positive *E. faecalis* and gram-negative *E. coli* bacteria. In order to optimize the stability of curcumin and eliminate the effect of hydrolytic degradation products on the bacterial cells, the preparations of curcumin used in the present study were made in PBS, pH 6.1. However, for the final dilution of the bacterial suspensions a saline buffer of pH 7.0 was applied.

2. Investigations and results

2.1. Solubility and stability of curcumin in PEG 400 solutions

2.1.1. Quantification of the solubility

The amount of curcumin dissolved in a saturated solution containing 2.5% and 10.0% PEG 400 in PBS without ethanol was determined by HPLC. Quantification of curcumin in 2.5% PEG 400 was not possible because the saturation concentration was below the detection limit of the analytical system. The concentration of curcumin in the preparation containing 10.0% PEG 400 was determined to 8.4×10^{-8} M (data not shown).

For the further experiments, unless otherwise stated, curcumin was added to the preparations as a stock solution prepared in ethanol (2 mM).

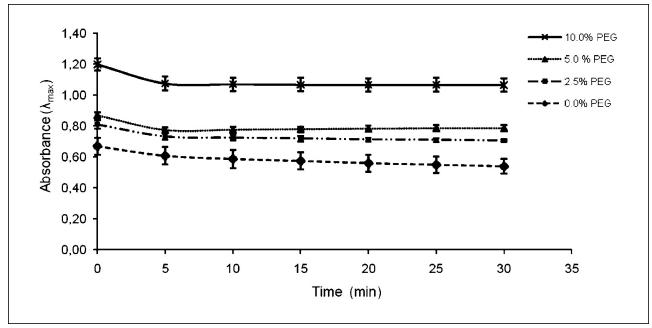
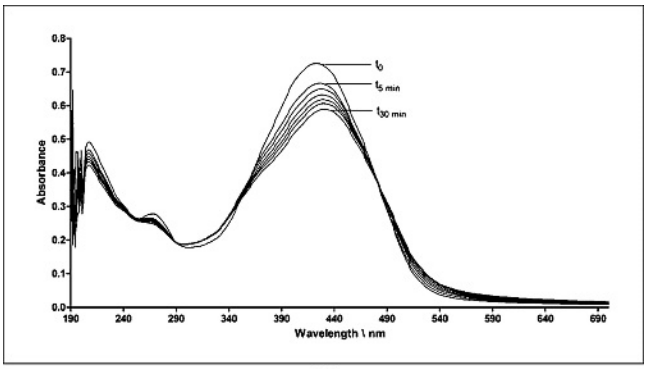


Fig. 1: Absorbance (λ_{max}) of 25.0 μM curcumin in 0.0% (diamonds) 2.5% (dots), 5.0% (triangels), 10.0%(solid black line) PEG 400 v/v in PBS measured as a function of time. Error bars represent standard deviations

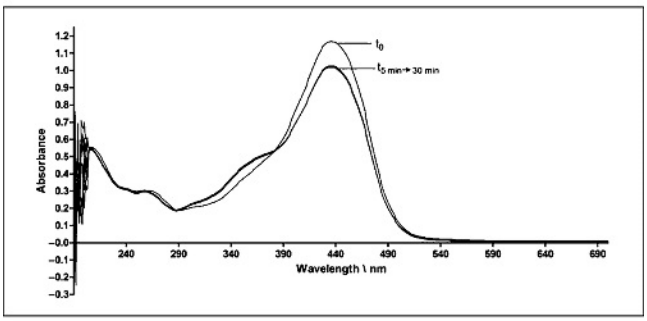
2.1.2. UV-VIS Absorption measurements and HPLC analysis

The UV-VIS absorption spectrum of 25.0 μM curcumin in PBS with concentrations of PEG 400 in the range 0.0 – 10.0% v/v was recorded every 5th minute for 30 min in order to investigate the solubility and stability of curcumin in these preparations prior to incubation with bacteria. The investigations were performed at room temperature in the absence of bacteria. There was a statistical significantly increase in absorbance of curcumin samples with increasing PEG 400 concentration (Fig. 1). A plot of the curcumin absorbance at 430 nm after 30 minutes storage versus the PEG 400 concentration showed a linear correlation ($r^2 > 0.99$) (data not shown). Further, the absorption maximum was slightly red-shifted with higher PEG 400 concentration, from 428 nm in the absence of PEG 400 to 436 nm in the presence of 10.0% PEG 400 in the preparation (data not shown). In the absence of PEG 400 the curcumin absorption at λ_{max} decreased linearly ($r^2 > 0.99$) as a function of time (Fig. 2a). This decrease in curcumin absorbance was accompanied by an increase in absorbance at 500 nm to 700 nm (Fig. 2a). Curcumin in a monomeric form does not absorb above 560 nm. The absorption may therefore be due to the formation of curcumin aggregates in solutions without the cosolvent. A drop in absorbance at λ_{max} after 5 min was also observed in a curcumin solution containing 10.0% PEG 400 (Fig. 2b). The spectrum does however, not undergo any further changes during the time of the experiment in opposite to samples without PEG 400. A shoulder at approximately 355–360 nm was observed in curcumin samples containing PEG 400 (Fig. 2b).

Further, preparations of 25.0 μM curcumin containing 0.0, 2.5% and 10.0% v/v PEG 400 in PBS were examined spectrophotometrically and by use of HPLC over a period of 4 h. The samples were withdrawn at specified time intervals and centrifuged prior to analysis. A steady decrease in curcumin concentration was observed for all three preparations throughout the 4 h (data not shown). This observation indicates that all samples were supersaturated and that curcumin aggregates were present prior to centrifugation. The addition of increasing amounts of PEG 400 seemed to reduce the formation of curcumin aggregates in the preparations in a concentration dependent manner. Preparations without PEG 400 showed a low initial curcumin concentration



(a)



(b)

Fig. 2: a) Absorption spectra of 25.0 μM curcumin in PBS without PEG 400 measured every 5th minute for 30 min.
b) Absorption spectra of 25.0 μM curcumin in 10.0% PEG 400 v/v in PBS measured every 5th min for 30 min

in the centrifuged sample (5.1 μM) and a slightly linear decrease during the next 4 h ($r^2 = 0.98$). The preparation containing 2.5% PEG 400 had an initial curcumin concentration of 13.0 μM. After a decrease to 8.1 μM within the first hour the concentration seemed to stabilize and showed only a slight decrease during the next hours (data not shown). The higher initial curcumin concentrations (21.5 μM) measured in samples containing 10.0% PEG 400 indicated that nearly all the curcumin was kept in solution; i.e., supersaturation was not initially obtained. The curcumin concentration then decreased slightly during the 4 h with a negative slope of 1×10^{-6} M/hour (data not shown). The standard curve was prepared in ethanol instead of PBS, because of the low solubility of curcumin in PBS. The reason that the initial curcumin concentration measured in 10.0% PEG 400 did not equal the theoretical concentration (25.0 μM) may be due to the formation of aggregates or curcumin's lower molar absorbtivity in PBS compared to ethanol. HPLC analyses (detection at 254 nm) were performed to exclude hydrolytic degradation as the cause of the observed decrease in curcumin concentration. No degradation products could be identified at low PEG 400 concentrations (0.0–2.5%) (data not shown). In the samples containing 10.0% PEG 400 a small new peak (retention time, $t_r = 7.4$ min) was observed in the HPLC chromatograms after 1 h storage, but it disappeared after 3–4 h. The supernatant obtained after centrifugation was diluted with organic solvent and analyzed spectrophotometrically for all samples. The results demonstrated an increase in absorbance (data not shown). The increase was less pronounced in samples containing 10.0% PEG 400. This observation further emphasizes that supersaturation is likely to occur in aqueous media.

2.2. Antibacterial phototoxicity of curcumin

2.2.1. Effect of radiant exposure

Curcumin samples (25.00 μM in PBS) containing various concentrations of PEG 400 were incubated with suspensions of

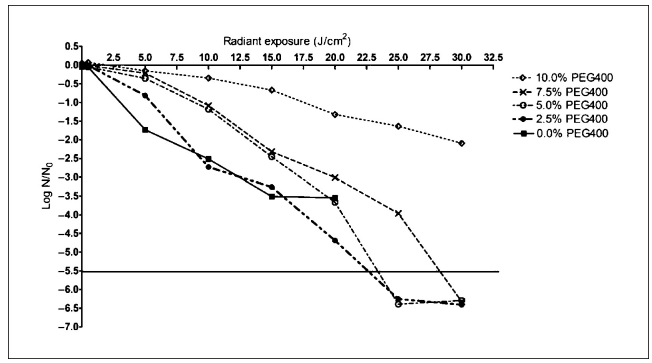


Fig. 3: Phototoxic effects of 25.00 μM curcumin in 0.0% to 10.0% v/v PEG in PBS combined with blue light of various radiant exposures against *E. coli* (n = 12). N: number of CFU/ml at the exposed plates; N_0: number of CFU/ml at the plates in the absence of PEG 400, curcumin and light. The horizontal line intersecting −5.5 log N/N_0 indicates the lower detection limit of the phototoxicity experiments (no surviving bacteria). N is set to 1 below this line

E. coli bacteria. The samples were exposed to various radiant exposures from 0 to 30 J/cm²; corresponding to 0, 5, 10, 15, 20, 25 and 30 min exposure in a light chamber. Plots of the log bacterial survival against light exposure at each PEG 400 concentration are presented in Fig. 3. The plots demonstrated a nearly linear correlation which indicates first order kinetics, with a regression coefficient varying from 0.74 to 0.92. The bacterial survival decreased by increasing irradiation time (Fig. 3).

2.2.2. Effect of PEG 400 concentration

The samples with the highest *in vitro* phototoxicity were 25.00 μM curcumin in PBS with or without 2.5% PEG 400 as demonstrated in Fig. 3. Higher concentrations of PEG 400 caused a statistically significantly reduced phototoxic effect of curcumin against *E. coli* independent of irradiation dose.
Samples of 10.0% PEG 400 in PBS in the absence of curcumin had no phototoxic effect against *E. coli* combined with the radiant exposures applied in the present study. However, a preliminary study (n = 4) demonstrated that after exposure to higher PEG 400 concentrations, 15.0% and 20.0%, in combination with 30 J/cm² resulted in an *E. coli* survival of 2.2% and 0.0% respectively, in the absence of curcumin. Even in the absence of light these PEG concentrations caused a reduction in bacterial survival (90.4% and 28.9% survival respectively) (data not shown).

2.2.3. Effect of curcumin concentration

Curcumin samples (2.50 – 25.00 μM) prepared in PBS with or without the addition of 2.5% PEG 400 were incubated with *E. coli* for 30 min prior to irradiation with 10 J/cm² to evaluate the influence of curcumin concentration on the phototoxicity. Phototoxicity increased statistically significantly with increasing curcumin concentrations up to 12.50 μM when PEG 400 was present in the preparation (Fig. 4). At this concentration the effect reached a plateau, and no further increase in effect was observed for the higher concentrations. In preparations without PEG 400 the plateau was reached at 6.25 μM and lasted up to 18.75 μM. At higher concentrations (from 18.75 to 25.00 μM) there was a significant reduction in the phototoxic effect. Further, only for the two lowest concentrations of curcumin (2.50 and 6.25 μM) there was a statistically significant difference in the phototoxic effect between the preparation with or without 2.5% PEG 400. Preparations without PEG possessed the highest phototoxic potential as demonstrated by a higher reduction in CFU/ml.

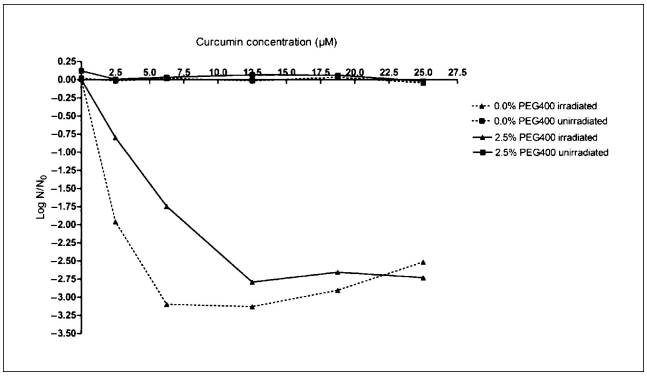


Fig. 4: Phototoxic effect of 0.00 µM to 25.00 µM curcumin in PBS without or with the addition of 2.5% PEG 400 combined with a radiant exposure of 10 J/cm^2 against *E. coli* (n = 12). N: number of CFU/ml at the exposed plates; N_0: number of CFU/ml at the plates in the absence of PEG 400, curcumin and light

The phototoxic effect of curcumin against *E. faecalis* was tested for samples of curcumin in PBS without the addition of PEG 400. Even a tenfold reduction in curcumin concentration in combination with radiant exposure of 0.5 J/cm^2 – 20 J/cm^2 caused no colony forming units (CFU) to be detected after the treatment. In contrast controls without curcumin or unirradiated controls caused no reduction in CFU (data not shown).

2.2.4. Effect of the lag time between sample preparation and incubation with bacteria

Supersaturation of curcumin might be expected in samples without or at low concentrations of PEG 400 as described above (2.1.2). This condition might have influenced the phototoxic effect of the preparations. To investigate the supersaturation effect the solutions were stored for 1 or 3 h at room temperature prior to incubation with *E. coli.* These investigations were performed using 25.00 µM curcumin in PBS with and without 2.5% PEG 400 combined with radiant exposure of 10 J/cm^2. Phototoxicity of stored samples without PEG 400 was not significantly different from freshly prepared samples (data not shown). However, for samples containing 2.5% PEG 400 the lag time had a significantly reducing effect on the phototoxicity of curcumin compared to freshly prepared samples (1.3% and 0.2% bacterial survival, respectively). There was no statistically significant difference between 1 and 3 h lag time. The differences in phototoxicity between samples with and without 2.5% PEG 400 were statistically significant only in the cases when there were a lag time between the preparation of the curcumin solution and the incubation with *E. coli* (data not shown).

2.2.5. Toxicity testing of photochemical degradation products of curcumin

The potential toxicity of photochemical degradation products of curcumin were tested by pre-irradiation of curcumin solution immediately before dark incubation with *E. faecalis* (10^6 CFU/ml). This particular strain was chosen for the experiment because of its sensitivity to phototoxic effects of curcumin (see 2.2.2). However, the colony-forming ability of this strain was not affected in the presence of a pre-irradiated curcumin solution (data not shown).

2.2.6. Effect of curcumin saturation

A saturated curcumin sample was prepared by dispersing an amount of solid curcumin corresponding to a final concentration of 25 µM in PBS. A part of the solution was filtered (0.22 µm) prior to incubation with *E. coli* and irradiation (10 J/cm^2). Neither unfiltered nor filtered samples showed any phototoxic effects against *E. coli* (data not shown).

3. Discussion

The solubility of solid curcumin in solutions containing 2.5% and 10.0% PEG 400 in PBS was very low (2.1.1). However, the UV-VIS absorption measurements demonstrated that the addition of PEG 400 to a certain extent kept curcumin dissolved in PBS when curcumin was pre-dissolved in ethanol and slowly added to the preparations (2.1.2). An increase in absorption at 430 nm with increasing PEG 400 concentration as well as HPLC analysis of samples stored for 4 h indicated that curcumin solubility increased as a function of surfactant concentration (Fig. 1 and 2.1.2). The aromatic rings of the curcumin molecule could be involved in hydrophobic interactions (Began et al. 1999). The polymeric chain of any PEG is alternately hydrophilic by the oxygen and hydrophobic by the ethylene units. PEG 400 displays both H-bond accepting and H-bond donating groups and has among 10 potential interaction sites per monomer (Derkaoui et al. 2007). It exhibits specific interactions with water leading to e.g., a helical structure that appears under certain temperature and concentration conditions (Derkaoui et al. 2007). From the UV-VIS absortion spectra of curcumin in PEG 400 in PBS it was apparent that curcumin interacted with PEG 400 (Fig. 2ab). According to Fig. 1 and Fig. 2b the absorption of curcumin in 10.0% PEG 400 decreased the first 5 min after which it was constant, indicating that the complex was formed within the first 5 min after addition of curcumin stock solution to the PEG 400 preparations. While for the preparations with lower PEG 400 concentration (2.5% and 5.0%) the absorbance varied slightly more with time (Fig. 1 and Fig. 2a). Further, as the concentration of PEG 400 in the preparations increased, a shoulder was formed in the absorption spectrum around 355–360 nm (Fig. 2b). A similar shoulder is previously observed in the absorption spectrum of curcumin in a preparation containing 3% hydroxypropyl -β-cyclodextrin (unpublished data). However, what causes the shoulder in the spectra is still unknown and will be further evaluated in an upcoming study. Previous findings have demonstrated a red shift in the curcumin absorption maximum when the polarity of the medium was increased (Chignell et al. 1994; Tønnesen et al. 1995; Khopde et al. 2000). The slight red shift in the absorption maximum with increasing PEG 400 concentration observed in the present study might be due to interactions between PEG 400 and curcumin. Based on the overall results in the present study (2.1.2) it seems likely that a supersaturated curcumin solution was formed initially when the PEG 400 concentration was below a critical limit.

Despite a higher solubility of curcumin observed with increasing PEG 400 concentrations, the surfactant had a negative influence on the phototoxicity of curcumin against *E. coli* (Fig. 3). In the preparations containing PEG 400 the curcumin molecules may form various states by different interactions e.g.: free curcumin ↔ curcumin-curcumin ↔ curcumin-bacterium ↔ curcumin-PEG 400. In preparations without PEG 400 fewer interactions may occur, e.g.: free curcumin ↔ curcumin-curcumin ↔ curcumin-bacterium or larger aggregates of curcumin. Since an increase in the PEG 400 concentration reduced the phototoxic potential of curcumin (2.2.1 and Fig. 3) and saturated curcumin prepared in PBS did not exert any phototoxicity against the bacteria (2.2.6) the curcumin in interaction with PEG 400 or larger aggregates of curcumin could not be responsible for the observed phototoxic effect. A prerequisite for photosensitization of a microbial cell is the binding of the PS to the outer membrane (Malik et al. 1992; Jori and

Coppellotti 2007). Therefore it was likely that curcumin molecules present in the bacterial membrane or adhered to the bacterial surface, were involved in the phototoxic reactions. According to Dahl et al. (1989) the antibacterial photoeffect may also be dependent on a reservoir of curcumin dimers/aggregates or free curcumin molecules surrounding the bacteria during irradiation. The investigators showed that the phototoxic effect was reduced when the curcumin solution/preparation was removed from the samples prior to irradiation. Therefore the reduced effect observed when PEG 400 was added to the preparations may be due to a decreased amount of curcumin molecules free to interact with the bacterial membranes. The molecules may be either sterically trapped in large PEG 400 aggregates or have higher affinity for the PEG 400 than the bacterial membrane. Large aggregates of PEG 400 in water may be formed in concentrations above the critical aggregation concentration (cac), which is temperature dependent in a way described by Derkaoui et al. (2007). Aggregates of PEG 400 will exist in water unless the concentration is below ~0.05 – 0.10 mol/L (= 1.8% – 3.6% v/v) and the temperature differ from 35 °C (Derkaoui et al. 2007). According to this work, in our study there will mostly be free unimers of PEG 400 at room temperature at the lowest concentration of the surfactant (0.07 mol/L = 2.5% v/v). An increasing amount of PEG aggregates might be expected to be formed at the three higher concentrations of PEG 400 (0.14, 0.21 and 0.28 mol/L = 5.0%, 7.5% and 10.0% v/v respectively). However, for temperatures above 35 °C, e.g., during incubation and irradiation, aggregate formation might be contradicted in a temperature dependent manner. The most likely unimer configuration of PEG 400 in water is a helical structure, with oxygen atoms stuck inside the helix, linked together by H-bonds through water molecules, ethyl groups displayed outside the helix and hydroxyl end groups in free contact with water (Derkaoui et al. 2007; Tasaki 1996). It seems possible that curcumin molecules with their H-bonding capacity form tight interactions with the PEG 400 structure, thereby displacing the water molecules inside the helix or connecting with the free hydroxyl ends.

Supersaturation can lead to an increase in membrane penetration of poorly soluble drugs due to an increase in thermodynamic activity of the drug substance outside the membrane (Moser et al. 2001; Gao et al. 2003). If supersaturation plays a major role for the absorption rate of curcumin in the bacteria the lag time between the sample preparation and incubation with bacteria should influence the results, i.e., the extent of supersaturation vs. precipitation prior to incubation. However, the results obtained indicate that the lag time is of minor importance for the phototoxic effects (2.2.4). The only significant difference was seen in the case of samples containing 2.5% PEG 400, where a lag time of $\geq$1 h slightly reduced the phototoxic effect. This result was probably due to the instabilities observed by the absorption spectra during the first 5 min for the different PEG 400 solutions before more stable complexes were formed (Fig. 1). The curcumin molecules will probably be more available to the bacteria during this time interval. The temperature increase that occurs during incubation and irradiation might be sufficient to prevent or reverse the precipitation of curcumin during the experiment. The results from the curcumin dose – phototoxic response experiments (2.2.3) showed that the phototoxic effect reached a threshold at curcumin concentrations of approximately 12.50 μM and 6.25 μM for solutions with 2.5% and without PEG 400, respectively (Fig. 4). These curcumin concentrations correspond to initial curcumin concentrations in those media (without bacteria present) quantified to 13.0 μM and 5.1 μM, respectively (2.1.2), indicating that the phototoxic effect was exerted by curcumin which was solubilised when the bacteria was added. The number of surviving CFU/ml above the respective threshold concentrations was similar for the two preparations. The significant decrease in phototoxic effect observed when the curcumin concentration was increased from 18.75 to 25.00 μM in a preparation without PEG 400 cannot easily be explained.

The phototoxic effect can be increased by increasing the irradiation dose independent of the PEG 400 concentration. The first order kinetics of the phototoxic effect observed (Fig. 3), was previously described by Dahl et al. (1994), for photokilling of rat basophilic leukemia cells with curcumin as a photosensitizer. The mechanisms by which curcumin causes light induced cell death have not yet been established. Since the main photodegradation products of curcumin (e.g., vanillin and ferulic acid) do not show any photobiological activity, the photobiological effect has been ascribed to the excited state of curcumin (Tønnesen et al. 1987). In the present study this theory was confirmed by testing the toxicity of pre-irradiated curcumin sample against *E. faecalis* (2.2.5). No reduction in surviving CFU was observed when the bacteria were added to pre-irradiated samples immediately after light exposure. Even the presence of bacteria in the preparation during irradiation, resulted in 100% killing of *E. feacalis* (2.2.1). A more pronounced sensitivity towards curcumin-induced phototoxicity was demonstrated in gram- positive (*E. faecalis*) compared to gram-negative (e.g., *E. coli*) bacteria (data not shown). This observation is consistent with the general assumption that gram-positive bacteria with their relatively permeable outer surface have little protection against antibacterial PDT. Cationic or neutral PS in combination with polycationic carriers or chelators which may function as permeability enhancers of the outer wall of gram-negative bacteria (Jori et al. 2007), are believed to be a necessity to obtain efficient photo-killing of gram-negative bacteria. Both curcumin and PEG 400 are neutral compounds. However, curcumin being a diketone, can act as a bidentate chelating ligand through the β-diketo moiety in the dissociated enolic form (Borsari et al. 2002). Curcumin in this form may bind to divalent cations (Began et al. 1999), thus destabilising the lipopolysaccharide layer of gram-negative bacteria and facilitate the uptake of free curcumin into the bacterial membrane. Further studies on the mechanisms leading to photokilling by curcumin will be published separately.

Based on the findings of the present investigations curcumin still shows potential as an efficient PS in antibacterial PDT *in vitro*. The phototoxic effect was dependent on radiant exposure, curcumin concentration and curcumin preparation. In aqueous preparations an excipient is necessary to dissolve and stabilize curcumin. PEG 400 in high concentration (10.0% v/v) demonstrated potential as a solubiliser and a physical stabilizer of curcumin in aqueous preparations. However, as the concentration of PEG 400 was increased and the stabilizing effect was improved, the phototoxic efficiency of the curcumin decreased (Fig. 3). We suggest that this observation was due to reduced availability of the curcumin molecules for the bacteria and hence, reduced uptake or adsorption to the outer bacterial wall. A cosolvent that does not form structures which trap the curcumin molecules, but keeps them available for the bacteria would probably represent a more suitable excipient for antibacterial PDT formulations.

4. Experimental

4.1. Materials

Curcumin was synthesized following the procedure given by Pabon (1964). The purity level of the product was controlled by HPLC (Tønnesen et al. 2002), TLC (Tønnesen et al. 1986) and Differential Scanning Calorimetry (DSC) (Tomren et al. 2007). Phosphate buffered saline (PBS; Lonza, Verviers, Belgium) was adjusted to pH 6.1 by adding HCl (Sigma Aldrich,

USA) and filtered (0.22 μm; Millipore S.A.S., Molsheim, France) prior to use. All chemicals used were of analytical grade. The water used where purified and deionized.

4.2. Preparation of samples

A stock solution of curcumin (2 mM) was prepared in ethanol and stored in the refrigerator (+4 °C). Samples of curcumin in final concentrations of 2.50 μM, 6.25 μM, 12.50 μM, 18.75 μM and 25.00 μM were prepared in PBS, pH 6.1 from the stock solution combined with 0.0% – 10.0% (v/v) polyethylene glycol 400 (PEG 400; Aldrich). All curcumin preparations were made immediately before experiments and kept protected from light throughout the experiments.

4.3. Analysis

4.3.1. UV-VIS absorption spectra

The UV-VIS absorption spectra of curcumin were measured by UV-VIS scanning spectrophotometers (Shimadzu UV-2101 PC Shimadzu Corp., Kyoto, Japan and Specord 200, Analytik Jena AG, Jena, Germany). As reference, identical samples without curcumin were used. All absorption measurements were carried out in triplicate.

4.3.2. Quantification by HPLC

Samples of curcumin in preparations containing 0.0, 2.5% and 10.0% PEG 400 v/v in PBS, pH 6.1 were withdrawn every hour for 4 h. The curcumin concentration was measured at 254 nm by the previously described reversed phase HPLC method (Tønnesen et al. 2002) with the following modifications: The mobile phase was composed of 0.5% citric acid (adjusted to pH 3 with KOH) and methanol (38:62). The flow rate was 0.8 ml/minute and the retention time of curcumin was approximately 11 min. The method was linear in the concentration range 1.00 μM – 25.00 μM ($r^2 = 0.999$). The experiments were carried out in triplicate.

4.4. Incubation and irradiation

4.4.1. Microorganisms

Enterococcus faecalis (A197A) and *Escherichia coli* (ATCC 25922) were maintained by three times weekly subculture in tryptone soya broth (TSB; Oxoid Ltd., Bassingstoke, UK). The bacteria were incubated at 37 °C.

4.4.2. Irradiation and dosimetry

The light source was a light-polymerisation unit (Polylux PT, Dreve Dentamid GmbH, Unna, Germany) equipped with three fluorescent tubes emitting blue light in the wavelength range 400–500 nm (Ralutec 9W/171, Radium, Germany) with an emission maximum at 430 nm. The mean irradiance of nine points at the cell dish level inside the irradiation chamber was determined to 17.3 mW/cm^2 (± 5%). The irradiance was kept constant throughout the experiments. Irradiation duration was 0.5 – 30 min corresponding to 0.5–30 J/cm^2 radiant exposure. The irradiance was monitored at regular intervals with a UDT 271 radiometer (United Detector Technology, San Diego, CA, USA) calibrated towards a spectroradiometer. The radiometer was equipped with probes sensitive in the blue (268 BLU) part of the spectrum.

4.4.3. Testing of phototoxicity

The bacterial test strains were grown overnight in nutrient broth at 37 °C. Aliquots of the overnight culture were diluted in PBS pH 6.1 and transferred to test tubes with curcumin solution to give a final bacteria concentration of 10^6 CFU/ml. 1 ml from each test tube where transmitted to steril plastic petri dishes of 35 mm × 10 mm. The samples were incubated for 30 min in the dark at 37 °C to allow penetration of curcumin into the bacterial cells or interaction with the outer cell wall prior to irradiation. After incubation the samples were irradiated for a given time, diluted 40 times with PBS pH 7.0 and incubated in the dark for 1 h to allow for the reaction between possible toxic photoproducts and cellular targets. Aliquots of the bacterial suspensions were plated onto TSB agar with an automatic spiral plater (Whitley, Don Whitley Scientific LTD, Shirley, West Yorkshire, England) to estimate the survival by counting the colony-forming ability. The colonies were counted by the use of a colony counter (Acolyte, Synbiosis Europe Office, Cambridge, UK) after 24 h of incubation at 37 °C. Dark and reference sample controls were included in the study. The bacterial survival was calculated as percentage survival compared to dark controls in PBS or log N/N_0, where N is the number of CFU/ml at the exposed plates and N_0 is the number of CFU/ml at the plates in the absence of PEG 400, PS and light. All bacterial tests were performed three times with four replicates in each experiment.

4.5. Solubility of curcumin in samples containing PEG 400

Solubility was determined by adding an excess of curcumin to a PBS, pH 6.1 solution containing 2.5% or 10.0% PEG 400. The saturated solutions were equilibrated under continuous agitation for 1 week, centrifuged at 6.5 rpm for 5 min and filtered through a Spartan 13/0.45RC filter (Schleider & Schull, Germany). An aliquot of the filtrates was quantified for curcumin content by reversed-phase HPLC (Tønnesen et al. 2002). The studies were carried out in triplicate.

4.6. Statistics

Student's *t*-test for independent samples (two – tailed) and univariate analysis of variance were used to evaluate the results and estimate significant differences. A *P*-value less than 0.05 was chosen as statistically significant. Each value was expressed as the mean.

Acknowledgment: The authors thank Inger Sofie Dragland, NIOM as, Norway for technical assistance and John E. Tibballs, NIOM as, Norway for assistance with the statistical analysis.

References

Aggrawal BB, Sundaram C, Malani N, Ichkawa H (2007) Curcumin: the Indian solid gold. Adv Exp Med Biol 595: 1–75.

Began G, Sudharshan E, Sankar KU, Rao AGA (1999) Interaction of curcumin with phosphatidylcholine: A spectrofluorometric study. J Agric Food Chem 47: 4992–4997.

Borsari M, Ferrari E, Grandi R, Saladini M (2002) Curcuminoids as potential new iron-chelating agents: spectroscopic, polarographic and potentiometric study on their Fe(III) complexing ability. Inorganica Chim Acta 328: 61–68.

Bruzell EM, Morisbak E, Tønnesen HH (2005) Studies on curcumin and curcuminoids. XXIX. Photoinduced cytotoxicity of curcumin in selected aqueous preparations. Photochem Photobiol Sci 4: 523–530.

Chignell CF, Bilski P, Reszka KJ, Motten AG, Sik RH, Dahl TA (1994) Spectral and photochemical properties of curcumin. Photochem Photobiol 59: 295–302.

Dahl TA, McGowan WM, Shand MA, Srinivasan VS (1989) Photokilling of bacteria by the natural dye curcumin. Arch Microbiol 151: 183–185.

Dahl TA, Bilski P, Reszka KJ, Chignell CF (1994) Photocytotoxicity of curcumin. Photochem Photobiol 59: 290–294.

Derkaoui N, Said S, Grohens Y, Olier R, Privat M (2007) PEG400 novel phase description in water. J Colloid Interface Sci 205: 330–338.

Gao P, Rush BD, Pfund WP, Huang T, Bauer JM, Morozowich W, Kuo MS, Hageman MJ (2003) Development of a supersaturable SEDDS (S-SEDDS) formulation of paclitaxel with improved oral bioavailability. J Pharm Sci 92: 2386–2398.

Hamblin MR, Hasan T (2004) Photodynamic therapy: a new antibacterial approach to infectious disease? Photochem Photobiol Sci 3: 436–450.

Haukvik T, Bruzell E, Kristensen S, Tønnesen HH (2009) Photokilling of bacteria by curcumin in different aqueous preparations. Studies on curcumin and curcuminoids XXXVII. Pharmazie 64: 666–673.

Jori G, Coppellotti O (2007) Inactivation of pathogenic microorganisms by photodynamic techniques: Mechanistic aspects and perspective applications. Anti-Infective Agents in Med Chem 6: 119–131.

Khopde SM, Priyadarsini KI, Palit DK, Mukherjee T (2000) Effect of solvent on the excited-state properties of curcumin. Photochem Photobiol 72: 625–631.

Konan YN, Gurny R, Allémann E (2002) State of the art in the delivery of photosensitizers for photodynamic therapy. J Photochem Photobiol B 66: 89–106.

Kunchandy E, Rao MNA (1989) Effect of curcumin on hydroxyl radical generation through Fenton reaction. Int J Pharm 1989: 173–176.

Malik Z, Ladan H, Nitzan Y (1992) Photodynamic inactivation of Gram-negative bacteria: problems and possible solutions. J Photochem Photobiol B 14: 262–266.

Moser K, Kriwet K, Froehlich C, Naik A, Kalia YN, Guy RH (2001) Permeation enhancement of a highly lipophilic drug using supersaturated systems. J Pharm Sci 90: 607–616.

Nardo L, Paderno R, Andreoni A, Másson M, Haukvik T, Tønnesen HH (2008) Role of H-bond formation in the photoreactivity of curcumin. Spectroscopy 22: 187–198.

Pabon HJJ (1964) A synthesis of curcumin and related compounds. Rec Trav Chim Pays-Bas 83: 379–386.

Rowe RC, Sheskey PJ, Weller PJ (ed.) (2003) Handbook of Pharmaceutical Excipients, 4th ed., London, Chicago, p. 454–459.

Tasaki K (1996) Poly(oxyethylene)-water interactions: A molecular dynamics study. J Am Chem Soc 118: 8459–8469.

Tomren MA, Másson M, Loftsson T, Tønnesen HH (2007) Studies on curcumin and curcuminoids XXXI. Symmetric and asymmetric curcuminoids: Stability, activity and complexation with cyclodextrin. Int J Pharm 338: 27–34.

Tønnsen HH, Karlsen J (1985) Studies on curcumin and curcuminoids V. Alkaline degradation of curcumin. Z Lebensm Unters Forsch 180: 132–134.

Tønnesen HH, Karlsen J, van Henegouwen GB (1986) Studies on curcumin and curcuminods VIII. Photochemical stability of curcumin. Z Lebensm Unters Forsch 183: 116–122.

Tønnesen HH, de Vries HD, Karlsen J, Beijersbergen van Henegouwen G (1987) Studies on curcumin and curcuminoids IX: Investigation of the photobiological activity of curcumin using bacterial indicator systems. J Pharm Sci 76: 371–373.

Tønnesen HH, Smistad G, Ågren T, Karlsen J (1993) Studies on curcumin and curcuminoids XXIII: Effects of curcumin on liposomal lipid peroxidation. Int J Pharm 90: 221–228.

Tønnesen HH, Arrieta AF, Lerner D (1995) Studies on curcumin and curcuminoids. Part XXIV: Characterization of the spectroscopic properties of the naturally occurring curcuminoids and selected derivatives. Pharmazie 50: 689–693.

Tønnesen HH (2002) Solubility, chemical and photochemical stability of curcumin in surfactant solutions. Pharmazie 57: 820–823.

Tønnesen HH, Másson M, Loftsson T (2002) Studies on curcumin and curcuminoids. XXVII. Cyclodextrin complexation; solubility, chemical stability and photochemical stability. Int J Pharm 244: 127–135.

Tønnesen HH (2006) Solubility and stability of curcumin in solutions containing alginate and other viscosity modifying macromolecules. Pharmazie 61: 696–700.