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Photokilling of bacteria by curcumin in different aqueous preparations. Studies on curcumin and curcuminoids XXXVII

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Curcumin has potential as a photosensitiser (PS) in photodynamic therapy (PDT) for localised superficial infections. However, it is a challenge to make an optimal curcumin formulation in which curcumin has acceptable solubility and stability at physiological pH and combined with high selective phototoxic activity towards bacteria. In the present study, the phototoxic effects of curcumin against gram-negative and gram-positive bacteria were investigated in selected aqueous preparations. The gram-positive *Enterococcus faecalis* and *Streptococcus intermedius* and the gram-negative *Escherichia coli* were used as bacterial models. The bacteria were exposed to 1–25 μM curcumin solubilised in DMSO, cyclodextrines, liposomes and surfactants known to interfere with membranes. After 30 min incubation the bacteria were irradiated with fluorescent tubes emitting blue light (emission max 430 nm). The irradiance was 17 mW/cm^2 and the radiant exposure (light dose) was 0.5–30 J/cm^2 . The bacterial survival was calculated as a percentage compared to controls. Various post-irradiation incubation times were tested. Curcumin's native fluorescence was exploited in examination of curcumin uptake in or adherence to bacteria by fluorescence microscopy. Changes in post-irradiation incubation time, curcumin concentration, irradiation dose and preparation strongly influenced the phototoxic efficiency of curcumin *in vitro*. Aqueous preparations of DMSO, polyethyleneglycol and the pluronic block copolymer poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) were the most efficient vehicles for curcumin to exert photokilling of gram-positive and gram-negative bacteria.

1. Introduction

In times of increasing worldwide occurrence of antibiotic-resistant bacteria, antibacterial photodynamic therapy (aPDT) is receiving considerable interest for its potential to efficiently destroy bacterial cells. The method combines the use of visible light or ultraviolet radiation with a light-activated chemical (photosensitising agent: PS) to damage or kill bacteria. The killing is due to phototoxic reactions, in which the PS absorbs photons and induces a series of reactions involving the formation of radicals and reactive oxygen species (ROS). The generated ROS rapidly react non-specifically with multi-targets in their surroundings such as the cell wall, cell membrane, peptides and nucleic acids. The molecular target depends on the localisation of the excited PS. Cellular/bacterial interactions can cause localisation dependent oxidative damage leading to cell damage and/or death. Thus, it appears essential to control the cellular distribution of the PS by properties of the drug formulation (Hamblin and Hasan 2002). Photodynamic therapy is known to kill viruses, bacteria and fungi *in vitro* and has been proposed as treatment against a variety of localised infections. Treatment of localised infections with aPDT requires selectivity of PS for microbes over host cells, delivery of PS into the infected area in close association to the bacteria cells and the ability to efficiently irradiate the lesion with UV or visible light at suitable wavelengths. Gram-negative

bacteria are considered generally less susceptible to aPDT compared to gram-positive bacteria due to a complex outer barrier structure. A positive charge on the PS molecule allows it to bind to, and in some cases penetrate the microbial permeability barrier of gram-negative bacteria (Malik et al. 1992). Hence, most PS utilised in aPDT are complex cationic, polycyclic compounds absorbing radiation from 400 to 800 nm. They are often poorly water soluble, and their tendency to aggregate in solution counteracts the phototoxic potential due to low singlet oxygen quantum yields of PS aggregates (Konan et al. 2002). These challenges call for the use of advanced pharmaceutical formulations for administration of PS in aPDT.

Incorporation of PS in an optimised pharmaceutical formulation will have several advantages: It may lead to an increase in the amount of PS reaching the infection site by solubilisation of the PS or by protection against hydrolytic and enzymatic degradation of the PS *in vivo*. In addition, an optimised formulation of PS may enhance the production of reactive compounds formed in aPDT by influencing the microenvironment of PS *in vivo* and also by reducing the formation of PS aggregates that have reduced photodynamic reactivity. The formulation may also contribute to selective accumulation of PS in the bacterial cells over host cells and thereby, increase the selectivity of the treatment. Further, the formulation can affect the barrier structure of the outer wall of gram-negative cells, thereby enhancing the

permeability of hydrophobic PS. By using a delivery vehicle with a positive charge, tight interactions between the bacterial membrane and the PS may be formed. The feasibility of targeting PS to specific bacteria in order to minimise side effects especially by the use of specific antibodies has been addressed in several studies (Embleton et al. 2004; Bhatti et al. 2000). However, only few attempts have been made to enhance the efficacy of aPDT by modifying the PS formulation (George and Kishen 2008). A satisfactory delivery system must be able to incorporate the PS without loss or alteration of its activity, be biodegradable, and possess a high degree of biocompatibility. Furthermore, the molecules of the delivery system should not absorb photons from the emitted therapeutic source and thereby shield the PS from receiving the proper energy (Konan et al. 2002).

In the present study, curcumin, bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, was selected as the PS. Curcumin is a yellow-orange pigment which can be synthesised chemically or isolated from the rhizomes of *Curcuma longa* L. Curcumin has been reported to possess a number of biological effects in the absence of light (Aggrawal et al. 2007). Many of these effects can be ascribed to antioxidant and radical scavenging properties, which have been extensively studied (Kunchandy and Rao 1989,1990; Tønnesen and Greenhill 1992). Although the interest in curcumin as a potential drug or drug model is steadily increasing, the use of this compound is limited by its low water solubility and poor stability in solution. Curcumin is practically insoluble in water at acidic or neutral pH, but it is soluble in alkali. At a pH above neutral the compound undergoes rapid hydrolytic degradation (Tønnesen and Karlens 1985). Therefore, one challenge is to make a physiologically compatible topical preparation with acceptable stability and solubility of curcumin. Complex formations of curcumin with cyclodextrins (CD) and micelles have previously been studied (Tønnesen et al. 2002; Tønnesen 2002). The presence of CD increased the solubility of curcumin at pH 5 by a factor of $\geq 10^4$, and a 50–500 fold increase in hydrolytic stability has been obtained above pH 7. Micelle-containing media increased the solubility by approximately 10^5 under the same conditions, while the hydrolytic stability increased 1800-fold (Tønnesen 2005). Curcumin has also been investigated in solutions containing alginate and other viscosity enhancing macromolecules. The solubility of curcumin in an aqueous solution at pH 5 increased by a factor $\geq 10^4$ in the presence of 0.5% (w/v) alginate and gelatin compared to plain buffer, but the macromolecules were not found to stabilise against hydrolytic degradation (Tønnesen 2006).

Curcumin has a rather broad absorption peak in the range 300–500 nm (maximum ~ 430 –435 nm, $\epsilon \sim 30000$ –50000 $M^{-1} cm^{-1}$, depending on the preparation (Bruzell et al. 2005). Due to the low penetration depth of blue light into tissues (Konan et al. 2002), curcumin is suited as a PS for treatment of localised superficial infections in e.g. the mouth and skin. The spectral and photochemical properties of curcumin and hence, the ability to form phototoxic compounds, vary with the surroundings (Khopde et al. 2000; Chignell et al. 1993). As a result, there is a potential for multiple or alternate pathways for the exertion of photodynamic effects. The observed variations can be ascribed to the ability of curcumin to form inter- and intra-molecular bindings. These bindings will then stabilise or destabilise the curcumin molecule towards photochemical degradation. Curcumin is photolabile and decomposes upon irradiation at 425 nm (Tønnesen et al. 1986). The photostability of curcumin was shown to decrease in the presence of micelles, cyclodextrins and alginate (Tønnesen et al. 2002; Tønnesen 2002, 2005). Tønnesen et al. (1987) and Dahl et al. (1989) have previously demonstrated that curcumin dissolved in dimethyl sulfoxide (DMSO) preparations (5–10%) had a bactericidal effect on

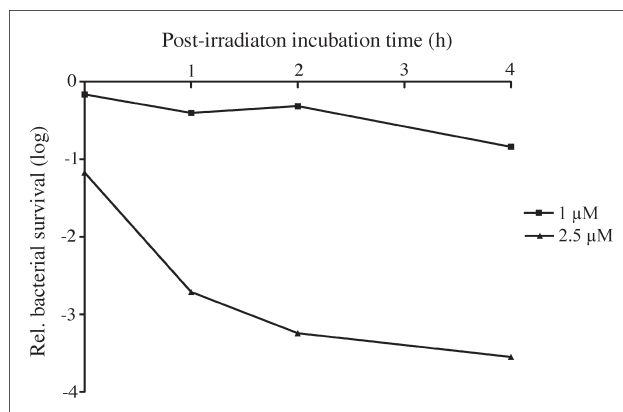


Fig. 1: Effect of the post-irradiation incubation time on the photokilling of *E. faecalis*. 1 μM and 2.5 μM curcumin in 5% DMSO was irradiated with constant radiant exposure of blue light ($0.5 J/cm^2$). Data are log of means, N/N_0 ($n = 12$)

selected gram-positive bacteria in combination with appropriate radiant exposure (the product of irradiance and irradiation time). The effect towards the gram-negative bacteria was less pronounced. The aim of the present study was to examine the influence of different vehicles on the phototoxicity of curcumin against various bacteria species. A screening of several aqueous preparations of curcumin containing both neutral and charged excipients was performed. In the present work, curcumin was synthesised after the method of Pabon (1964), in order to ensure no interference from demethoxy- and bisdemethoxycurcumin on the bacterial phototoxic efficacy. The aerobic species *Enterococcus faecalis* (*E. faecalis*) and the micro-aerophilic species *Streptococcus intermedius* (*S. intermedius*) were used as models for gram-positive bacteria, while *Escherichia coli* (*E. coli*) was selected as a gram-negative bacteria model.

2. Investigations and results

2.1. Phototoxicity of curcumin

Figure 1 presents the influence of various post-irradiation incubation times on survival of *E. faecalis* after treatment with curcumin dissolved in 5% DMSO in PBS at pH 6.1 in combination with a radiant exposure of $0.5 J/cm^2$. As a trend, for a given concentration of curcumin, longer post-irradiation incubation of the samples resulted in lower bacterial survival. The difference in bacterial survival seen in Fig. 1 are all statistically significant except between 1 and 2 h of post-irradiation incubation for the 1 μM curcumin concentration and for the 2.5 μM curcumin concentration seen after 2 and 4 h of post-incubation. A post-irradiation incubation time of 1 h was chosen in the further bacterial phototoxicity experiments.

The effect of the curcumin concentration on the phototoxicity towards gram-positive bacteria was investigated using 1 μM and 2.5 μM curcumin in 5% DMSO preparation in combination with a radiant exposure of $0.5 J/cm^2$ (Fig. 1; data not shown for *S. intermedius*). For a constant radiant exposure ($0.5 J/cm^2$), 2.5 μM curcumin resulted in statistically significantly less bacterial survival than 1 μM curcumin, irrespective of post-irradiation incubation time (Fig. 1). A concentration of 2.5 μM curcumin was chosen for the further experiments involving gram-positive bacteria. In order to study the phototoxic effect of curcumin against the gram-negative *E. coli*, the concentrations 2.5 and 25 μM were chosen (Fig. 2). At a given radiant exposure treatment with 25 μM curcumin resulted in a statistically significantly lower bacterial survival of *E. coli* than did 2.5 μM curcumin for the

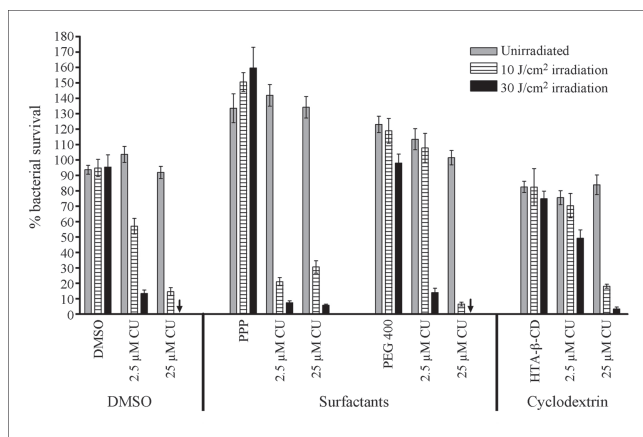


Fig. 2: Phototoxic effects of curcumin in selected aqueous preparations against the gram-negative bacteria *E. coli*. Values represent mean \pm standard error of the mean ($n = 12$). Arrows (\downarrow) indicate less than 0.2% bacterial survival corresponding to ≥ 3 log reductions. (DMSO: 5%; PPP: 0.3% Pluronic L35; PEG 400: 5% Polyethyleneglycol 400; HTA- β -CD: 5% hydroxytrimethylammoniopropyl- β -cyclodextrin)

phototoxic curcumin preparations, with exception of the preparation containing poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol)(Pluronic L35; PPP).

Curcumin preparations that showed phototoxic potential were tested in combination with various radiant exposures on *E. coli*. The results for 10 and 30 J/cm² are presented in Fig. 2. At a given curcumin concentration, a higher radiant exposure resulted in statistically significantly lower *E. coli* survival for the preparations tested, except for 2.5 μ M curcumin solubilised in hydroxytrimethylammoniopropyl- β -cyclodextrin (HTA- β -CD).

Figure 3 presents the phototoxic effects of 2.5 μ M curcumin in selected aqueous preparations in combination with 0.5 J/cm² irradiation, tested against *E. faecalis* and *S. intermedius*. Curcumin in a 5% DMSO preparation resulted in a reduction in CFU/ml at 99.8% for both *E. faecalis* and *S. intermedius* compared to unirradiated controls and samples without curcumin. The efficiency of curcumin to cause a phototoxic reaction in *E. faecalis* was 95–100% in preparations with the following neutral surfactants, the efficacy was slightly different between the three preparations in the following order of ascending bacterial survival: PPP (0.01%) > Triton X-100 (TX-100) (0.14%) > polyethyleneglycol 400 (PEG 400) (4.79%). For *S. intermedius* the effect of these three preparations was identical, resulting in a 100% reduction in CFU/ml compared to controls corresponding to six log reductions. The surfactant TX-100 was not well tolerated by *S. intermedius*, and caused a reduction in CFU/ml even in the absence of curcumin and light. Curcumin (2.5 μ M) solubilised by the neutral surfactants polyoxyethylene 23 lauryl ether and Polysorbat 80 or the anionic surfactant sodium taurodeoxycholate (STC) combined with 0.5 J/cm² irradiation, did not cause any reduction in the bacterial survival of *E. faecalis* (data not shown). The cationic surfactants tetradecyltrimethylammonium bromide (TTAB) and cetylpyridinium bromide (CPB) were found to exert toxic effects to *E. faecalis* in the absence of curcumin and were not suitable as vehicles at concentrations used in the present study (data not shown). Exposure of *E. faecalis* to curcumin in liposomes did not cause any reduction in the bacterial viability (Fig. 3). However, 2.5 μ M curcumin in liposomes in combination with 0.5 J/cm² irradiation resulted in a significant reduction of vial *S. intermedius* bacteria. Curcumin solubilised by 5% hydroxypropyl- β -cyclodextrin (HP- β -CD) or 5% γ -cyclodextrin (γ -CD) combined with light had a stimulating rather than reducing effect on the gram-positive bacterial growth (data not shown).

Table: Phototoxic effects of curcumin against *E. coli*. Combinations of preparation, curcumin concentration and radiant exposure tested that resulted in less than 50% bacterial killing

Type of preparation	Curcumin concentration (μ M)	Radiant exposure (J/cm ²)
DMSO	2.5	0.5 and 10
	25	0.5
Surfactants:		
	TX-100	2.5
PPP	2.5	0.5, 10 and 30
	25	0.5
PEG 400	2.5	0.5 and 10
	25	0.5
STC	2.5	0.5
	25	0.5 and 10
Liposomes	2.5	0.5, 10 and 30
	5	0.5, 10 and 30
Cyclodextrins:		
	γ -CD	2.5
HTA- β -CD	2.5	0.5
	25	0.5 and 10
SBE7- β -CD*	2.5	0.5
	25	0.5 and 10

* Anionic cyclodextrin: Sulfobutylether-hepta- β -cyclodextrin (SBE7- β -CD)

The preparations in which curcumin showed phototoxicity against gram-positive bacteria were further tested against the gram-negative bacteria *E. coli*. The data for the combinations of preparation, curcumin concentration and radiant exposure that caused a significant reduction of the bacterial viability compared to controls are presented in Fig. 2. Curcumin (25 μ M) in 5% DMSO exposed to an irradiation dose of 30 J/cm² resulted in a very low (0.07%) survival fraction of *E. coli* compared to controls. There was no significant difference between the effects of 25 μ M curcumin in combination with 10 J/cm² irradiation and 2.5 μ M curcumin combined with 30 J/cm² irradiation in a 5% DMSO preparation. Curcumin in the presence of the neutral surfactant PPP and irradiation resulted in a marked decrease in surviving CFU/ml for *E. coli*. Curcumin solubilised by PEG 400 was the most efficient preparation of the samples tested regarding photokilling of gram-negative bacteria. At least a six log reduction in viable bacteria cells was obtained when 25 μ M curcumin was combined with 30 J/cm² light. The cationic HTA- β -CD also showed a potential as a carrier for curcumin in a PDT tested against *E. coli*. Samples containing 2.5 μ M curcumin in 5% HTA- β -CD caused a reduction of 50% of the *E. coli* population compared to controls in PBS, pH 6.1 when exposed to radiant exposure of 30 J/cm². Higher reductions in bacterial survival were obtained using 25 μ M curcumin in HTA- β -CD combined with the same radiant exposure. Bacteria exposed to HTA- β -CD without curcumin showed an average of 20% reduction in bacterial survival compared to controls in PBS, pH 6.1 alone.

Various combinations of preparation, curcumin concentration and irradiation time tested by use of *E. coli* that resulted in $\leq 50\%$ bacterial killing are presented in Table, but will not be further discussed.

2.2. Fluorescence microscopy

Curcumin possesses native fluorescence and the uptake of curcumin in bacteria was examined by fluorescence microscopy. Uptake or adsorption of curcumin to the bacterial surface of

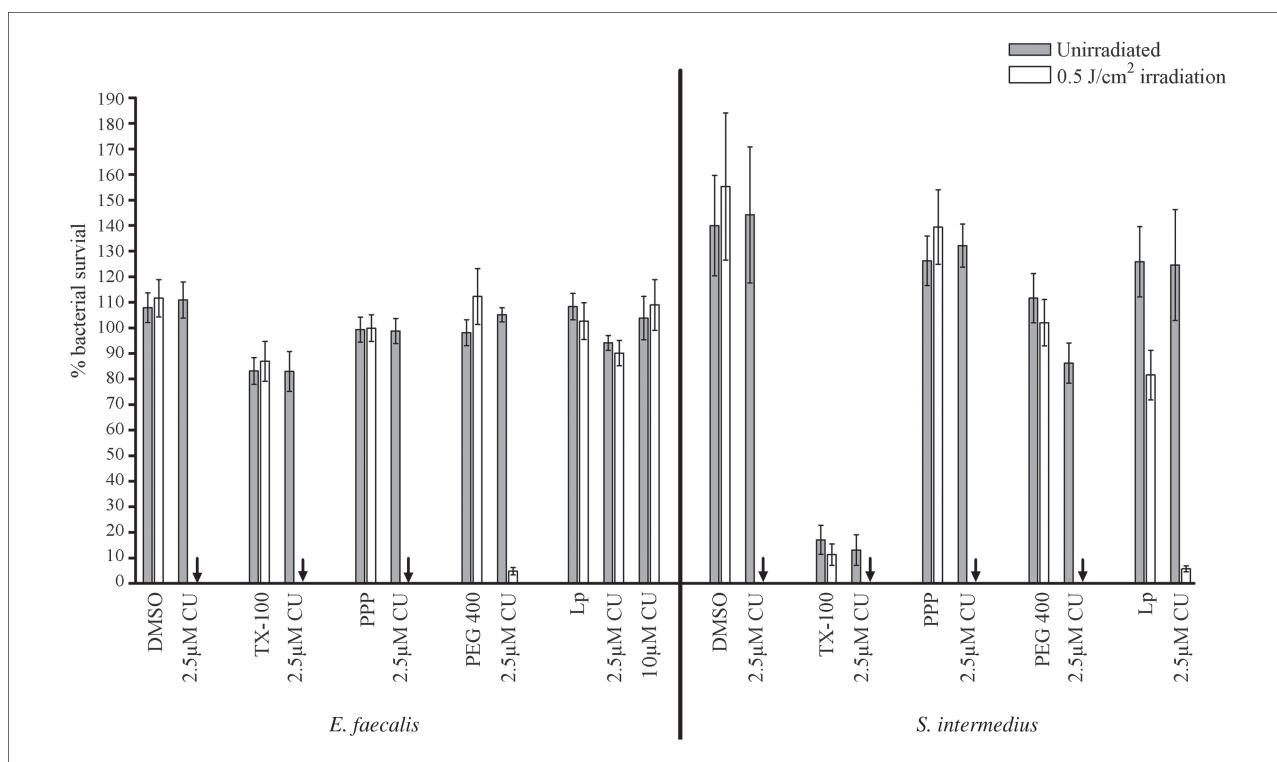


Fig. 3: Phototoxic effects of curcumin (2.5 μM) in selected aqueous preparations against the gram-positive bacteria *E. faecalis* and *S. intermedius*. Values represent mean ± standard error of the mean (n = 12). Arrows (↓) indicate less than 0.2% bacterial survival corresponding to ≥ 3 log reductions. (5% DMSO; TX-100: 0.014% Triton X-100; PPP: 0.3% Pluronic L35; PEG 400: 5% polyethyleneglycol 400; Lp: liposomes)

E. faecalis was confirmed for the preparations containing DMSO, TX-100 and PEG 400 (images not shown). A slightly weaker fluorescence from the bacteria was observed after exposure to curcumin in the PPP preparation. Curcumin in the γ -CD preparation also induced strong fluorescence at the bacterial surfaces, while curcumin in 5% HP- β -CD did not. Adsorption of curcumin to the surface of the gram-negative *E. coli* was observed in the presence of DMSO and PPP. Only a weak fluorescence signal was observed after exposure to curcumin in the PEG 400 preparation, while a stronger signal was obtained by increasing the concentration of curcumin to 25 μM in PEG 400 (images not shown).

2.3. Singlet oxygen measurements

The production of singlet oxygen (¹O₂) induced by 25 μM curcumin in PEG 400 and PPP was measured, as curcumin demonstrated a strong phototoxic effect against both gram-positive and gram-negative bacteria in the presence of these surfactants. The singlet oxygen quantum yields obtained were very low (<0.003). The addition of 25 μM FeSO₄ to the samples in order to evaluate the phototoxic potential of a tentative Fe – curcumin complex, had no influence on the production of singlet oxygen (data not shown).

3. Discussion

The mechanisms by which curcumin causes 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 effects have been ascribed to the excited state of curcumin (Tønnesen et al. 1987). The present study demonstrates that curcumin in combination with blue light had a phototoxic effect on both gram-negative and gram-positive bacteria *in vitro*. This effect was dependent on curcumin concentration, radiant exposure (light dose),

post-irradiation incubation time, bacteria species and properties of the curcumin preparation (Fig. 2 and Fig. 3). A six-fold log reduction in viable bacterial cells was obtained at a relatively low curcumin concentration (2.5–25 μM) and radiant exposure (0.5–30 J/cm²). The phototoxic effect at similar concentrations of curcumin and a constant radiant exposure was shown to change from 100 to 0% survival by variation in the excipients of the curcumin preparation. Curcumin in the examined preparations had a rather broad absorption peak at 300–500 nm (maximum at 420–440 nm, depending on the preparation). There was complete spectral overlap between the curcumin absorption spectrum in the preparations and the emission spectrum of the light polymerization unit. Further, the curcumin concentrations used in this study offered no toxicity in the absence of light to any of the bacteria employed. This finding is consistent with an earlier report on the (dark) toxicity of curcumin (Lutomski et al. 1974). In this study, concentrations 100 times higher than those employed in the present work were required to kill *Staphylococcus aureus* and *Sarcina lutea*, while no reduction was observed in the survival of *Salmonella typhimurium* or *E. coli* even at 300 times higher concentrations.

The observed phototoxicity of curcumin in a DMSO preparation (2.1.) is consistent with previous findings, demonstrating that curcumin in 5–10% of DMSO causes photokilling of both gram-positive and gram-negative bacteria (Tønnesen et al. 1987 and Dahl et al. 1989). Therefore, the phototoxicity of curcumin solubilised by 5% DMSO was used as a basis when comparing the effect of varying preparation, curcumin concentration and radiant exposure throughout the study. Although DMSO has exceptional solvent properties and is known to increase permeability of drugs, it may cause tissue damage and even systemic effects (Rowe et al. 2003). Therefore, DMSO is not optimal as a pharmaceutical excipient, which calls for development of alternative drug formulations. The results obtained when varying the post-irradiation incubation times from 0 to 4 h (Fig. 1), indicate that the toxic species formed during irradiation had long

life-times or induced cascade reactions with the formation of other toxic species (*e.g.* free radicals) that continued to exert their effects post irradiation. This observation may have an impact on the efficiency in an *in vivo* situation. Alternatively, the phototoxic effect may have been caused by the superoxide radical and other radicals produced in dark reactions by photodegradation products of curcumin as suggested by Dahl et al. (1994).

PEG 400 is well tolerated and widely used in pharmaceutical formulations including parenteral, topical, ophthalmologic, oral and rectal preparations. In the present study, curcumin in a 5% PEG 400 preparation was an efficient PS against gram-positive and gram-negative bacteria (Fig. 2 and Fig. 3). In comparison the PS methylene blue solubilized in PEG has previously been shown to cause significant bacterial reduction in combination with light (George and Kishen 2008). As presented in Fig. 2, even without curcumin 5%, PEG 400 combined with radiant exposure of 30 J/cm² showed a phototoxic effect against *E. coli* compared to the bacterial survivals seen for lower exposures. This effect will be further examined and discussed in an upcoming publication. Curcumin was also an efficient PS when solubilised by the neutral surfactant PPP (Fig. 2 and Fig. 3) which is a lipophilic block copolymer with short PO-block (Pluronic L35). The CMC of Pluronic L35 is reported to be 5.3×10^{-3} M at 37 °C, pH 7.4 (Batrakova et al. 1999). At block copolymer concentrations below the CMC, which are used in this study, the unimers form molecular solutions rather than micelles in water. Hydrophobic interactions may then occur between unimers of pluronics and curcumin. The unimers of Pluronic L35 may further adhere to the bacterial outer surface and increase the permeability. Both lipophilic and hydrophilic pluronics are known to adhere to cell membranes and cause fluidisation and/or membrane solidification (Kabanov et al. 2002). The singlet oxygen quantum yield in curcumin preparations with PEG 400 or PPP was measured as these preparations showed the highest degree of phototoxicity against gram-negative bacteria in the present study. Previously, curcumin is found to photosensitise ¹O₂ formation in aprotic environments, but ¹O₂ has not been detected after excitation of curcumin in either water or D₂O (Chignell et al. 1994). As both PEG 400 and PPP have protic properties our results showing a very limited formation of ¹O₂ (2.3), are consistent with previous findings. However, these results may not fully represent what will happen during the phototoxic experiments. If curcumin or some photoreactive intermediates are partitioned between the aqueous and lipophilic phases in the bacterial wall, *e.g.* the cytoplasmic membrane, protic and aprotic mechanisms could occur simultaneously and singlet oxygen may be formed within the bacteria.

It is difficult to explain why curcumin did not exert phototoxic antibacterial effects in preparations containing the other neutral surfactants tested *i.e.* Polysorbat 80 and polyoxyethylene 23 lauryl ether (tested against *E. faecalis*) and triton X-100 (ineffective against gram-negative bacteria). There are structural similarities between them and PEG 400 and PPP, all containing a polyoxyethylene group of variable chain length, with a hydroxyl group attached at the end (Rowe et al. 2003). However, in addition both polyoxyethylene 23 lauryl ether and Polysorbat 80 have a hydrophobic moiety which may cause a different interaction with curcumin in these preparations and hence, reduce the phototoxic potential. The cationic surfactants TTAB and CPB were chosen as vehicles for curcumin because previous studies have shown their ability to solubilise and stabilise curcumin (Tønnesen 2002). However, due to their toxicity towards the bacterial cells tested they were not suitable as vehicles for curcumin under the present experimental conditions. The anionic bile acid STC is known for its ability to permeabilise membranes by increasing the membrane fluidity (Albalak et al. 1996).

Despite this fact, curcumin in a STC preparation did not induce phototoxic effects against bacteria in the present study.

The phototoxic effect of curcumin incorporated in liposomes was limited when tested against gram-negative and gram-positive bacteria in the present study (2.4). Liposomes are known to protect drug substances against hydrolytic and enzymatic degradation. They are used to promote monomerisation of photosensitisers and are used as targeting devices in PDT (Derycke and de Witte 2004). Curcumin is lipid soluble and will be incorporated in the membrane of liposomes (Iwunze 2003). Bruzell et al. (2005) have previously demonstrated a cytotoxic effect of curcumin against rat submandibular acinar cells (SM 10-12 cells) when curcumin was incorporated in liposomes of corresponding composition and size as where used in the present study combined with a low radiant exposure (1.6–6 J/cm²). The lower efficiency seen against bacteria compared to SM 10–12 cells in aPDT using curcumin as a PS is probably due to the differences in the outer layer of the prokaryotic bacterial cells and the eukaryotic mammalian cells. The liposomes may fuse and thereby release the PS in the eukaryote lipid bilayer cell membrane of the SM 10–12 cells. On the contrary, most bacterial cells have a low affinity for lipophilic compounds, in particular the gram-negative bacteria (Hancock and Bell 1988). Curcumin will then have a higher affinity for the core of the liposomes than the bacterial outer surface and will not be released from the liposomes. If the curcumin molecules are still incorporated in the liposome membrane during irradiation the probability for photoperoxidation of the liposomes seems to be greater than damage of the bacterial cells (Tønnesen et al. 1993).

Several neutral cyclodextrins have previously been described as excellent vehicles to solubilise and stabilise curcumin in aqueous solutions and are also demonstrated to cause cytotoxic effects against SM 10–12 cells (Tønnesen et al. 2002; Bruzell et al. 2005). However, in the present study not any phototoxic effect of 2.5–25 μM curcumin neither in neutral 5% CD sample (HP-β-CD and γ-CD) nor in the anionic SBE7-β-CD sample against gram-positive or gram-negative bacteria was observed (2.4). However, use of the cationic HTA-β-CD as a vehicle for curcumin at the highest concentration (25 μM) did result in an efficient killing of the gram-negative bacteria when exposed to the highest radiant exposure (30 J/cm²) (Fig. 2). The different effects obtained may be due to the high affinity of curcumin for the central cavities of the CDs, which was previously demonstrated to follow the order: SBE7-β-CD > HP-β-CD > HTA-β-CD (Tønnesen 2002). Thus, the affinity of curcumin for the lipophilic inner cavity of SBE7-β-CD and the neutral CDs is likely higher than for the outer bacterial surfaces. Therefore, the curcumin molecules will probably reside in the cavity rather than being attached to the outer wall of the bacteria, which is required for phototoxic efficiency. The ability of the different CDs to interact with the outer bacterial membrane may also be of importance. A positive charge on the outer surface of the CD will bring the curcumin-CD complex in close association with the negatively charged outer surface of the gram-negative bacteria. Phototoxic species may then be formed close to the bacterial wall. Another possibility is disorganisation of the bacterial outer membrane layer by intercalation of the cationic CD into the lipopolysaccharide layer, similar to other polycationic substances that are known to increase the permeability of gram-negative bacteria (Malik et al. 1992). Curcumin molecules may then permeate the lipopolysaccharide layer and reach the cell membrane where it can exert its effect when exposed to light.

An important aim of the present work was to develop an aqueous preparation of curcumin in which curcumin has an acceptable solubility and stability and at the same time is an efficient PS against gram-positive as well as gram-negative bacteria. No curcumin phototoxicity was observed in preparations known to

solubilise and stabilise the compound, such as liposomes and CD. Surfactants which form flexible and dynamic networks in water seemed to be the best vehicles for curcumin as a PS. The solubility and stability of curcumin in these surfactant preparations will be further examined.

The differences in response between gram-negative and gram-positive bacteria towards curcumin are most likely due to the structural differences in the outer bacterial surfaces. The gram-positive bacteria outer cover consists of a relatively permeable peptidoglycan layer which offers little protection against aPDT. In contrast, the outer lipopolysaccharide (LPS) layer of gram-negative bacteria is electrostatically linked to divalent cations and produces a very efficient barrier against lipophilic compounds. Therefore, gram-negative bacteria are generally considered less susceptible to aPDT unless the structure of the outer membrane is altered by pretreatment with chelators like EDTA or by using polycations as PS. The ability of chelators (e.g. EDTA) to destabilise the LPS coating by removing the Mg^{2+} and Ca^{2+} ions that act as bridges between neighboring LPS molecules, is well accepted (Hamblin and Hasan 2004). This destabilisation renders the outer barrier more permeable to lipophilic molecules such as PS and is known to increase the efficiency of PDT. Curcumin, being a diketone, can act as a chelating agent and interact with cations as Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Fe^{3+} and Al^{3+} (Began et al. 1999). The iron-chelating ability of curcumin is confirmed by the observed formation of mixed hydroxo species in which curcumin acts 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, thus destabilising the lipopolysaccharide layer of gram-negative bacteria and facilitate the uptake of unbound curcumin into the bacterial membrane. This chelating ability of curcumin may explain the efficient phototoxic effect against gram-negative bacteria, despite the fact that curcumin is a neutral compound. The fluorescence microscopy investigations performed in this study (2.2) confirmed uptake or adsorption of curcumin to the bacteria, both to the gram-positive and the gram-negative, for the most efficient preparations tested: DMSO, PEG 400 and PPP. However, with fluorescence microscopy it was not possible to separate the uptake and the adsorption of curcumin to the outer bacterial surface. Further studies are required in order to determine where the curcumin molecules are located when they exert the aPDT effect both in gram-negative and gram-positive bacteria, and the differences between them.

The photochemical properties of curcumin are dependent on the environment, in particular by the H-binding capacity of the media (Chignell et al. 1994). The hydrogen atoms of the phenolic hydroxyl (OH) groups in the curcumin structure are intramolecularly H-bonded to the adjacent methoxy groups, allowing the oxygen atoms of the phenolic OH-groups to participate in hydrogen bond formation with the solvent or the bacterial outer membrane as a hydrogen acceptor (Tønnesen et al. 1995). Destabilisation of the excited state will occur when the non-bonding electrons on the oxygen atom of the OH-group become engaged in intermolecular hydrogen bonding instead of being donated to the ring. In general, this would lead to an increase in destabilisation of the excited state by an increase in hydrogen-bonding donor capacity of the medium (Nardo et al. 2008). Curcumin has previously been demonstrated to form a complex with the anionic polysaccharide alginate induced by electrostatic interactions and/or H-bond formation (Tønnesen 2006). It is possible that a similar complex is formed between curcumin and the lipopolysaccharide layer of gram-negative bacteria, keeping the curcumin molecules closely attached to the wall, destabilising the excited state and thereby enhancing the phototoxic efficacy of the compound.

An upcoming study will include investigations of the mechanisms responsible for the observed phototoxicity, optimization of the experimental conditions, and evaluation of solubility, hydrolytic stability and photochemical and photophysical properties of curcumin in selected aqueous preparations.

4. Experimental

4.1. Materials

The samples were protected from light throughout the experiments. Pure curcumin was synthesised following the procedure given by Pabon (1964). The purity 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) (BioWittaker, Lonza, Verviers, Belgium) was adjusted to pH 6.1 by adding HCl and sterile filtered before use. All chemicals used were of analytical grade.

4.2. Curcumin preparations

A stock solution of curcumin (2 mM) was prepared in ethanol. The solution was stored in the refrigerator (+4 °C) and used for the aqueous preparations.

4.2.1. DMSO preparations

Solutions of curcumin in PBS, pH 6.1 containing 5% dimethylsulfoxide (DMSO; Sigma Aldrich, Steinheim, Germany) were prepared in concentrations 1.0 μ M, 2.5 μ M and 25 μ M.

4.2.2. Surfactant preparations

Solutions of curcumin in concentrations 2.5 μ M and 25 μ M were prepared in PBS, pH 6.1 in combination with various surfactants. The non-ionic surfactants used were Triton X-100 (TX-100; Sigma Chemicals Co, St. Louis, MO, USA; 0.014%), polyoxyethylene 23 lauryl ether (Brij 35; Sigma - Aldrich; 0.015%), Polysorbat 80 (Norsk Medisinaldepot, Oslo, Norway; 0.005% and 0.010%), polyethyleneglycol 400 (PEG 400; Sigma - Aldrich; 5%) and poly (ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (PPP; Pluronic[®]L35; Sigma - Aldrich; 0.3%). The ionic surfactants used were the cationic tetradecyltrimethylammonium bromide (TTAB; Sigma Chemicals Co; 0.202%) and cetylpyridinium bromide (CPB; Sigma Chemicals Co; 0.058%) and the anionic sodium taurodeoxycholate (STC; Sigma Chemicals Co; 0.313% and 0.189%).

4.2.3. Cyclodextrin preparations

5% solutions of hydroxypropyl- β -cyclodextrin (HP- β -CD; Wacker Chemie AG, München, Germany), γ -cyclodextrin (γ -CD; Cavamax[®] W8; Wacker Chemie AG), anionic sulfobutylether-hepta- β -cyclodextrin (SBE7- β -CD; CAPTISOL, CyDex Pharmaceuticals Inc., Lenexa, KS, USA) and cationic hydroxytrimethylammoniopropyl- β -cyclodextrin (HTA- β -CD; Wacker Chemie AG) were prepared in PBS pH 6.1. Aliquots of the curcumin stock solution were added to give a final curcumin concentration of 2.5 μ M and 25 μ M (only 2.5 μ M for HP- β -CD).

4.2.4. Curcumin liposomes

Curcumin liposomes is prepared as described by Bruzell et al. (2005). The lipid concentration was 4 mg/ml in all samples and the concentration of curcumin was 0.0921 mg/ml. The particle size (~100 nm) was controlled by photon correlation spectroscopy using a Coulter N4Plus Sub-Micron Particle analyzer (Beckman Coulter, Fullerton, CA, USA). The liposome preparations were freeze-dried; freezing temperature -22 °C; drying phase 30–35 h with a temperature steadily increasing from -22 °C to 20 °C. The vials were subsequently sealed under vacuum and stored in a refrigerator (-20 °C). The exact concentration of curcumin in the liposome preparation was measured at 420 nm by reversed phase HPLC; method described by Tønnesen et al. (2002). A stock solution was prepared immediately before use by resuspending and diluting the freeze-dried liposomes in PBS, pH 6.1 to final curcumin concentrations of 2.5 μ M and 10 μ M.

4.3. Incubation and irradiation

4.3.1. Microorganisms

Enterococcus faecalis (*E. faecalis*) (A197A) and *Escherichia coli* (*E. coli*) (ATCC 25922) were maintained by three times weekly subculture in tryptone soya broth (TSB). The bacteria were incubated at 37 °C. *Streptococcus intermedius* (*S. intermedius*) (ATCC 27335) was maintained by twice weekly subculture in brain heart infusion media (BHI) and incubated at 37 °C under

micro aerobic conditions (5% CO₂) obtained by addition of AnaeroGen™ (Oxoid, Basingstoke, Hampshire, UK).

4.3.2. Irradiation and dosimetry

The light source was a light-polymerisation unit equipped with three fluorescent tubes emitting blue light in the wavelength range 400–500 nm (Ralutec 9W171) with an emission maximum at 430 nm (Polylux PT, Dreve Dentamid GmbH, Unna, Germany). The mean irradiance of nine points at the cell dish level inside the irradiation chamber was 17.3 mW/cm² (±5%). Irradiance was kept constant through all experiments. Irradiation duration was 0.5–30 min corresponding to a radiant exposure of 0.5–30 J/cm². The irradiance was monitored with 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) and UVA (268 UVA) part of the spectrum.

4.3.3. Testing of phototoxicity

The bacterial test strains were grown overnight in nutrient broth at 37 °C. Aliquots of the overnight culture were then diluted in PBS pH 6.1 and transferred to test tubes containing curcumin in a given carrier system to a final bacterial concentration of 10⁶ CFU/ml. These mixtures were then incubated for 30 min in the dark at 37 °C to allow penetration of curcumin into the bacterial cells or interaction with the outer bacterial wall prior to irradiation. The suspensions were irradiated for a selected time (see 4.3), and subsequently diluted 40x with PBS pH 7.0 and incubated in the dark for 1–4 h to allow the reaction between toxic products and cellular targets. Aliquots of the suspensions were plated onto TSB and BHI agar, respectively, with a Whitley automatic spiral plater (Don Whitley Scientific LTD, Shirley, West Yorkshire, UK) to determine the survival by colony-forming ability. The colonies were counted with an Acolyte colony counter (Synbiosis Europe Office, Cambridge, UK) after 24 h (*E. faecalis* and *E. coli*) and after 48 h (*S. intermedius*) of incubation at 37 °C. Dark and vehicle controls were included in the study. The bacterial survival was calculated as percentage compared to dark controls in PBS or as log N/N₀ where N = CFU/ml of the exposed and N₀ = CFU/ml in the dark controls in PBS. All bacterial tests were performed three times with four replicates in each experiment.

4.4. Fluorescence microscopy

A volume of 0.5 ml of the bacterial overnight cultures was added to 2 ml of each of the aqueous curcumin carrier systems containing 12.5 μM curcumin (and 25 μM curcumin for the 5% PEG 400 preparation). These mixtures were incubated for 30 min at 37 °C to allow addition of curcumin to the bacterial surface or uptake of curcumin to the bacteria. The solutions were centrifuged at 5000 rpm for 10 min, followed by washing with 1 ml of PBS, pH 6.1 and a second centrifugation at 13000 rpm for 5 min. The bacteria were transferred to 100 μl of sterilised water, and one drop was prepared for microscopic examination using a fluorescence microscope (Olympus BX51, Olympus Europe, Hamburg, Germany) with an excitation filter 420–480 nm. Images were taken by a camera (Olympus DP70; Olympus Europe, Hamburg, Germany) coupled to the microscope.

4.5. Singlet oxygen measurements

Samples of 25 μM curcumin were prepared in 5% PEG 400 and in 0.3% PPP, both in PBS, pH 6.1. The absorption at the excitation wavelength was ≤ 0.9 as determined by a Shimadzu UV-2401 PC UV-VIS scanning spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The generation of singlet oxygen was measured by direct detection of singlet oxygen luminescence at 1270 nm in a steady state mode, after excitation at 433 nm (PEG 400 preparation) and 440 nm (PPP preparation) corresponding to the wavelengths of maximum absorbance. The excitation source was a 75 W xenon lamp. The luminescence from singlet oxygen was detected on a PTI (London, Ontario, Canada; with Model 101 monochromators (f/4 0.2 m Czerny-Turner configuration) modular fluorescence system equipped with an EQ-817 germanium detector system (North Coast Optical Systems and Sensors, Santa Rosa, CA, USA) operated under liquid nitrogen conditions. An average of three luminescence spectra for each sample was taken and the area under the curve (AUC) was calculated by the use of Felix™ for Windows software. The quantum yield of singlet oxygen was calculated using a standard of riboflavin in methanol. The measurements were repeated after addition of 25 μM FeSO₄ to each sample.

4.6. Statistics

Student's *t*-test for independent samples (two – tailed) was used to evaluate the results and estimate differences. A *P*-value less than 0.05 was chosen

as statistically significant. Each value was expressed as the mean or the mean ± standard error of the mean.

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