Investigation of supersaturated and solubilising preparations of poorly water-soluble photosensitisers intended for antimicrobial photodynamic therapy:

Natural deep eutectic solvents and solid dispersions

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Table of Contents

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Oslo, March 2017

Kristine

Abstract

Antimicrobial resistance is a global concern that threatens our ability to treat common infectious diseases. Measures to reduce the spread of antimicrobial resistance are valuable, but new antimicrobial treatments are also required to slow the current rise in antimicrobial resistance. One potential treatment modality is antimicrobial photodynamic therapy (aPDT), which combines a photosensitiser (PS), visible light and oxygen to produce cytotoxic species such as reactive oxygen species. The aim of the present thesis was to investigate two formulation strategies – natural deep eutectic solvents (NADES) and solid dispersions – of three poorly water-soluble PSs, and evaluate their potential in antimicrobial treatment based on *in vitro* phototoxicity studies in bacteria. Curcumin and two porphyrins were selected as model PSs. The PS-NADES (i.e. PS dissolved in NADES) and the solid dispersions of curcumin (containing a cyclodextrin and one/two polymer(s)) were diluted/dissolved in aqueous solvents to produce supersaturated solutions of the PSs. The physical stability of these aqueous preparations was highly variable, ranging from peak PS concentrations in a supersaturated state which lasted for <1 h to >7 d. A PS intended for aPDT should remain solubilised in a monomeric form for a certain amount of time to allow interactions with the target bacteria, followed by the time required for production of toxic photoproducts by the photoactivated PS. The rapid precipitation of curcumin in diluted NADES (>82% reduction in curcumin concentration within 1 h) was considered inconvenient in bacterial phototoxicity studies, yet this preparation appeared equally (or more) phototoxic as the curcumin solid dispersions *in vitro*. The porphyrins were equally or more phototoxic to the bacteria investigated when dissolved in NADES (diluted 1:200-1:400) than supersaturated solutions of the porphyrins in buffer. The increase in phototoxicity of the porphyrins was ascribed protonation (i.e. their obtaining a positive charge) in the acid-containing NADES, their supersaturated state in the diluted NADES, and/or an additional weakening effect of the NADES on the bacteria. An antibacterial effect of certain NADES without PSs was observed. NADES may be valuable solvents for various PSs intended for aPDT, as they may contribute an antibacterial effect as well as increase the (photo-) toxic potential of the dissolved PS. NADES as formulation strategy was considered intriguing for further studies in the field of aPDT, although the formulation must be tailored to each PS. The findings in this thesis offer a decent platform for the continued investigation of NADES as solvents for PSs (or drugs) intended for antimicrobial treatment.

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

Paper I: Wikene KO, Hegge AB, Bruzell E, Tønnesen HH, 2015.
Formulation and characterization of lyophilized curcumin solid dispersions for antimicrobial photodynamic therapy (aPDT): studies on curcumin and curcuminoids LII.
Drug Development and Industrial Pharmacy 41 (6): 969-977.

Paper II: Wikene KO, Bruzell E, Tønnesen HH, 2015.
Improved antibacterial phototoxicity of a neutral porphyrin in natural deep eutectic solvents.
Journal of Photochemistry and Photobiology B: Biology 148: 188-196

Paper III: Wikene KO, Bruzell E, Tønnesen HH, 2015.
Characterization and antimicrobial phototoxicity of curcumin dissolved in natural deep eutectic solvents.
European Journal of Pharmaceutical Sciences 80: 26-32.

Paper IV: Wikene KO, Rukke HV, Bruzell E, Tønnesen HH, 2016.
Physicochemical characterisation and antimicrobial phototoxicity of an anionic porphyrin in natural deep eutectic solvents.
European Journal of Pharmaceutics and Biopharmaceutics 105: 75-84.

Paper V: Wikene KO, Rukke HV, Bruzell E, Tønnesen HH, 2017.
Investigation of the antimicrobial effect of natural deep eutectic solvents (NADES) as solvents in antimicrobial photodynamic therapy.
Submitted to *Journal of Photochemistry and Photobiology B: Biology*.

Abbreviations

aPDT: antimicrobial photodynamic therapy

C. albicans: Candida albicans

CD: cyclodextrin

C_{eq}: saturation solubility

CFU: colony forming unit(s)

CHA: HA and HPMC combined

DES: deep eutectic solvent(s)

DMSO: dimethyl sulfoxide

DSC: differential scanning calorimetry

E. coli: Escherichia coli

E. faecalis: Enterococcus faecalis

HA: hyaluronic acid

^{1}H-NMR: proton nuclear magnetic resonance

HPβCD: hydroxypropyl-beta-cyclodextrin

HPLC: high-performance liquid chromatography

HPMC: hydroxypropyl methylcellulose

ISC: intersystem crossing

K. pneumoniae: Klebsiella pneumoniae

logD: log distribution coefficient

logP: log partition coefficient

MβCD: methyl-beta-cyclodextrin

M_w: molecular weight

NADES: natural deep eutectic solvent(s)

OD: optical density

P. aeruginosa: Pseudomonas aeruginosa

PBS: phosphate buffered saline

PEG 400: polyethylene glycol 400

PDT: photodynamic therapy

PMNP: polymyxin nonapeptide

PS: photosensitiser

ROS: reactive oxygen species

S. aureus: Staphylococcus aureus

S. epidermidis: Staphylococcus epidermidis

$t_{1/2}$: half-life

T_g: glass transition temperature

T_m: melting point

TCPP: 5,10,15,20-tetrakis(4-carboxyphenyl)porphine

THPP: 5,10,15,20-tetrakis(4-hydroxyphenyl)porphine

TMP: 5,10,15,20-tetrakis(4-methoxyphenyl)porphine

UV-Vis: ultraviolet-visible

1. Aim of the project

The overall aim of the project was to investigate new formulation strategies of curcumin and two poorly water-soluble porphyrins to develop preparations with high bacterial phototoxicity and potential use in antimicrobial photodynamic therapy (aPDT). The main challenges that limit the bioavailability and efficacy of a photosensitiser (PS) in aPDT are low PS solubility in aqueous media, their physicochemical stability and tendency to aggregate. It was therefore of interest to investigate formulations of poorly water-soluble PSs which potentially could result in higher aqueous solubility, if only temporarily (i.e. supersaturation), and increase the *in vitro* phototoxicity of the PSs towards Gram-positive and Gram-negative bacteria. The phototoxicity of the PSs in the different formulations was to be investigated in order to identify the most promising formulation strategy. An increase in phototoxicity could indicate a higher effective PS concentration in solution, i.e. higher aqueous solubility, less tendency to aggregate and precipitate, or close interactions with the target bacteria.

The specific aims of the work were to:

- Develop curcumin solid dispersions through lyophilisation with improved physico-chemical properties and phototoxic effect towards Gram-positive and Gram-negative bacteria compared to previous preparations of curcumin (Paper I).
- Investigate natural deep eutectic solvents (NADES) as solvents for curcumin and evaluate the phototoxicity of curcumin-NADES towards a Gram-negative bacterium (Paper III).
- Investigate NADES as solvents for two poorly water-soluble porphyrins to obtain high *in vitro* phototoxicity towards Gram-positive and Gram-negative bacteria with low concentrations of the porphyrins (Paper II and IV).
- Study the effect of dilution of two organic acid-containing NADES on the toxic effect towards different planktonic microorganisms in the absence and presence of PS and light (Paper V).

2. Introduction

The discovery of antibiotics has had a major impact on human health, and thus population growth and lifetime expectancy. However, within few years of each introduction of a new antibiotic drug to the market, bacteria that had grown resistant to the treatment emerged.[1] The increasing burden of antimicrobial resistance is a major concern and threat to society. As humanity is expected to depend upon antimicrobial treatments for the foreseeable future, new treatments are greatly needed.[1] Preferably, such new treatments should operate by new mechanisms of action, be effective towards several different microorganisms, and show reduced rate and risk of microbial resistance development compared to traditional antimicrobial drugs.

One such treatment is photodynamic therapy (PDT), which combines a dye (i.e. PS), oxygen and visible light to produce reactive photoproducts. Such photoproducts may be free radicals or reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) or hydrogen peroxide (H_2O_2), which are highly reactive.[2, 3] Indeed, $^1O_2$ is reported to have a diffusion distance of ≤ 0.05 μm in cellular medium and generally reacts with the first molecule it encounters.[4] Which reactive photoproducts that are produced depend on the PS, the light source, the formulation and the presence of oxygen.[5] These reactive photoproducts may, if they are close to the target cell or bacterium, damage several biomolecules, e.g. DNA, lipids, amino acids and thereby proteins, which implies a multi-target mode of action.[6-8] The PS should therefore be localised close to or be taken up by the target cells or bacteria before it is photoactivated with light of appropriate wavelengths to achieve the most effective eradicative action.[9] The choice of radiation wavelength depends on the absorption characteristics of the PS used. For topical treatments, it may be advantageous if the light does not penetrate deeper into the tissue than the infected area, e.g. blue light (400-500 nm). A PS which has its absorption maximum in the blue wavelength range may therefore be favourable to reduce photoactivation of PS molecules or other chromophores further down in the tissue (>0.4 mm) where the target microorganisms are not located.[10] This could reduce side-effects and pain during aPDT.[11, 12]

2.1. Photoreactivity of a drug substance

All photoreactions begin with a substance (e.g. a PS) absorbing photons of certain wavelengths and energy, followed by excitation of the substance from its ground state (S_0, the lowest energy level) to excited singlet states (Figure 1). In the singlet states, the valence electrons of a compound (e.g. PS) are antiparallel. The addition of energy raises one valence electron to an outer shell.[6] These excited states are unstable, and the energy may soon be lost as fluorescence from the first singlet state (S_1), by loss of energy in the absence of photon emission (internal conversion or vibrational relaxation), be dissipated as heat or by energy-transfer to nearby molecules (quenching).[6] Alternatively, intersystem crossing (ISC) from S_1 to the first excited triplet state (T_1) may occur (Figure 1). This requires spin conversion of a valence electron of the molecule into parallel spin.[6] From T_1 the energy may be lost by photon emission (phosphorescence), another ISC event followed by delayed $S_1 \rightarrow S_0$ events, or by energy transfer to a substrate.[6] Due to the relatively long lifetime of the PS triplet state (^{3}PS) and its biradical nature, the ^{3}PS can mediate photosensitised reactions, often leading to oxidised products.[2, 6] Photosensitisation can occur in one or two types of reactions, termed Type I and Type II reactions (Figure 1).[2]

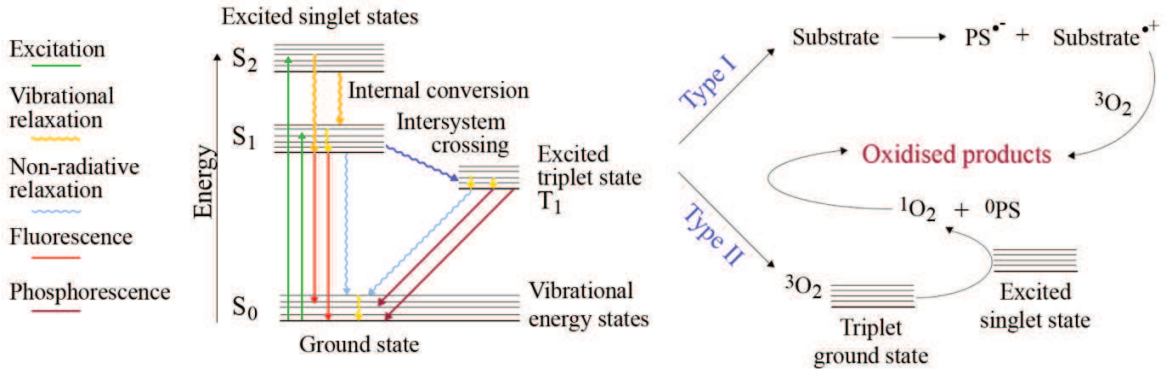

Figure 1 – Jablonski diagram showing photophysical processes that can occur following absorption of photons by a PS. S_0 is the ground state, S_1 and S_2 are excited singlet states and T_1 is the first excited triplet state. From T_1 a drug molecule (i.e. PS) may undergo Type I and/or Type II reactions, both usually resulting in oxidised products.

In the Type I reaction, ^{3}PS reacts directly with a substrate (such as lipids or proteins) in a one-electron transfer reaction, often resulting in the substrate radical cation and the PS radical anion (Figure 1).[13] Both these radicals may then react with other substrates. In the presence of oxygen, the radicals may produce oxidised products, or the PS radical anion may transfer an electron to oxygen, producing the highly reactive superoxide radical anion ($O_2^{\bullet -}$).[13] The radicals produced in a Type I reaction may also react with oxygen to produce a peroxy radical and start a chain reaction.[6]

The ^{3}PS is spin-matched with oxygen in its ground state, which is a triplet ($^3O_2$).[6] The Type II reaction involves energy transfer from the ^{3}PS to $^3O_2$, resulting in the formation of excited singlet oxygen, $^1O_2$, and regeneration of the PS in its ground state (Figure 1). Because $^1O_2$ is now spin-matched with other ground state molecules, it may readily oxidise a variety of substrates susceptible to oxidation, such as DNA, amino acids and lipids.[6, 7]

In a biological system, free radicals and $^1O_2$ can react with the cell wall, enzymes, proteins and DNA depending on their location within the system.[6] Due to its high reactivity, $^1O_2$ has a lifetime of about 3-4.5 µs in water and an estimated diffusion distance of 0.02-0.15 µm.[4, 13] Therefore, the localisation of the PS in a biological system is important for its eventual phototoxic effects. When irradiated, a PS which accumulates on the surface of cells or bacteria tends to produce reactive photoproducts which react with adjacent membrane components or secreted proteins, rather than molecules in the cytoplasm.[13] Some other ROS are more long-lived than $^1O_2$ (e.g. H_2O_2), but will also react with the first molecules they encounter. The initial products of a photoreaction are often peroxides.[2] These are broken down by secondary thermal reactions to induce free radical chain reactions.[2]

2.2. Antimicrobial photodynamic therapy

aPDT utilises PSs to produce ROS and other toxic photoproducts to eradicate infectious microorganisms. Some advantages, disadvantages and current challenges of localised aPDT are listed in Table 1. Due to the photoactivation step, aPDT can only be used to treat infections that can be efficiently reached with light. Further, the PS should only be administered to the defined infection site to reduce harm to other non-target cells. Due to the

localised application of the PS and light to the infected tissue area, aPDT may produce less side effects and microbial resistance development compared to conventional drugs assumed that only target microorganisms are affected.[14] Targeted treatment of localised infections is advantageous because orally or systemically administered antibiotics may have limited efficiency in such cases, either due to limited perfusion of the infected tissue (e.g. burns), or because the bacteria are present as a biofilm.[14]

Table 1 – Advantages, current challenges and disadvantages of aPDT.[8, 9, 14-17]

Advantages	Effective regardless of antibiotic resistance
	Versatile
	Instant results
	No specific target site
	Possibly reduced induction of resistance due to a multi-target mode of action
	Broad therapeutic window (bacteria, yeast, parasites, virus, virulence factors, biofilm)
	Limited side effects
Challenges	Penetration depth
	Selective interactions with target microorganism *in vivo*
	Obtaining the broad therapeutic window (e.g. Gram-positive vs. Gram-negative bacteria) but limiting side effects
	In vitro and *in vivo* correlation
	Formulation to optimise the phototoxic potential of the PS
	Storage- and user-friendly product
Disadvantages	Two-component treatment (preparation and light source)
	Only suitable for localised infections

The PS and the photoproducts produced upon irradiation of the PS display passive targeting, resulting in a non-specific attack on e.g. the cell wall, ribosomes, enzymes or DNA.[6, 7] The multi-target mode of action may delay or reduce the development of bacterial resistance to aPDT. However, bacteria have genetic responses and defence mechanisms to oxidative stress and protein damage, which may be relevant in potential aPDT resistance development.[7] Attempts to induce bacterial resistance by repeated PDT or ROS exposure of surviving bacteria have not shown reduced sensitivity to the treatment, nor to treatment with

antibiotics.[18, 19] However, induction of tolerance to H_2O_2 during exponential growth of *Escherichia coli* has been observed.[20] aPDT is effective regardless of the antibiotic resistance pattern of a bacterial strain, and antibiotic resistant bacteria have shown to be equally sensitive to aPDT as naïve strains.[14, 21, 22] However, different bacteria are not equally sensitive to a specific photodynamic treatment, and bacteria growing in biofilm are generally more resistant to any treatment than planktonic bacteria.[8, 23, 24]

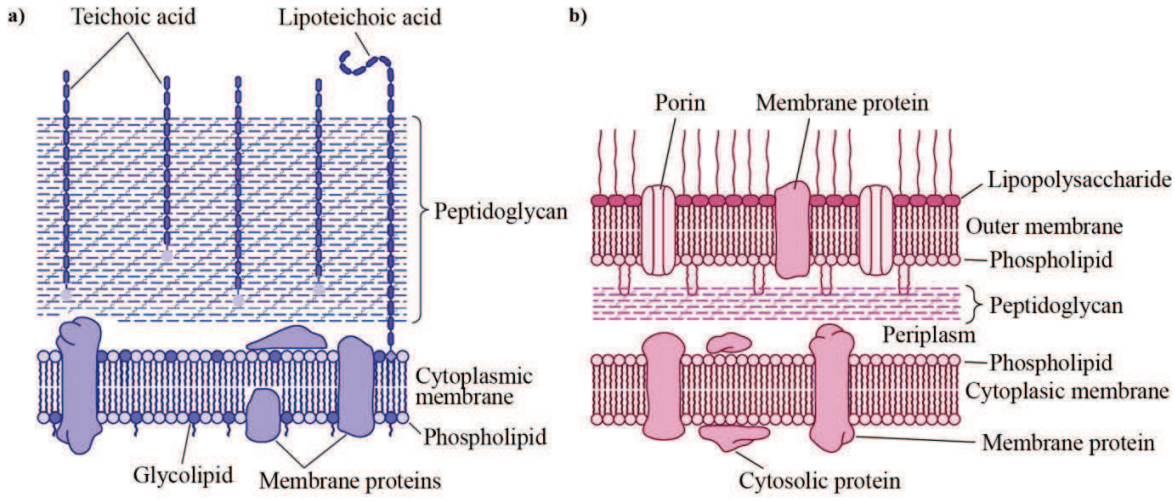

Figure 2 – Structure of a) the Gram-positive cell wall and b) the Gram-negative cell wall. Modified from ref. [25].

Gram-negative bacteria are generally regarded as less sensitive to aPDT than Gram-positive bacteria.[9, 15, 26-31] This is explained by their different cell wall structures: The cell wall of Gram-positive bacteria consists of a phospholipid bilayer (cytoplasmic) membrane and a thick, fairly porous peptidoglycan layer which frequently contains teichoic acids and surface proteins (Figure 2a). The structure of the Gram-negative cell envelope consists of a cytoplasmic membrane, less peptidoglycan than the cell wall of Gram-positive bacteria, and an asymmetrical, highly organised outer membrane composed of proteins, lipoproteins, phospholipids and lipopolysaccharide (Figure 2b).[15, 28] A higher PS concentration and light dose are often needed to obtain similar phototoxic effect in Gram-negative bacteria as in Gram-positive bacteria, or pre-treatment of the Gram-negative bacteria with a membrane-

disrupting agent.[27, 29, 32] Cationic PSs are reported to photoinactivate both Gram-positive and Gram-negative bacteria, while neutral and anionic PSs are less or non-phototoxic by themselves.[14, 15, 26, 30, 33-36] Neutral or anionic PSs may require the use of a positively charged vehicle or change in the microenvironmental pH to obtain acceptable phototoxicity towards Gram-negative bacteria.

Bacterial infections such as infected wounds and periodontal infections are not the only infections suited for aPDT. This treatment modality may also be useful to deal with *inter alia* fungal infections, localised tuberculosis, cutaneous leishmaniasis and otitis media.[9] The broad therapeutic window is one of several advantages of aPDT (Table 1).

2.3. Formulation of products intended for topical aPDT

A drug delivery system for use on localised infections will come in contact with damaged or sensitive tissue. The formulation should therefore have a high degree of biocompatibility, be non-immunogenic and preferably biodegradable. Development of a formulation intended for aPDT depends on the physicochemical characteristics of the PS, and must be tailored to optimise selective interactions between the PS and the target microorganisms. Further, it should not absorb or scatter light of wavelengths intended for photoactivation of the PS. Because delivery of visible light is a localised process, aPDT is most likely to be carried out by local delivery of the PS into the infected area, followed by irradiation of the same area.[14] Selectivity towards bacteria over mammalian cells have been suggested to be obtainable with a combination of short PS incubation time prior to irradiation (5-10 min), low concentrations of PS (< 5 μM), low fluence rate (< 50 mW/cm^2) and low radiant exposure (< 5 J/cm^2).[9, 28]

Most second-generation PSs (e.g. porphyrins, phthalocyanines and chlorins) as well as curcumin (*2.4.1. Curcumin*) are hydrophobic in nature which means that systemic or topical administration to aqueous sites is greatly hampered. In the absence of an appropriate formulation, the PS will aggregate in contact with aqueous media, resulting in poor aPDT efficacy due to a reduced ability to absorb light, limited bioavailability and decreased lifetime

of the excited PS.[36, 37] The stability of a PS in aqueous media may also be an issue in the absence of an appropriate formulation.

Certain biological surfaces, such as the oral mucosa or topical wounds, are covered with an aqueous layer.[38] The formulation should be compatible and miscible with exudate or other bodily fluids present at the site of infection. The PS delivery system should be able to promote selective uptake of the PS and not cause discomfort to the patient. Further, the formulation should keep the PS in a monomeric state which possesses higher bioavailability and is more photoactive than large aggregates.[36, 37] As photoactivation of the PS is the key to aPDT, the *in vivo* photostability of the PS in a certain drug delivery system should be considered. Some PSs have low hydrolytic stability and may rapidly degrade in aqueous media of a certain pH.[24, 39] Solid or non-aqueous preparations of the PS to be dissolved or diluted before administration and irradiation may be suitable in such cases. The drug delivery system should provide appropriate shelf-life and *in vivo* stability of the PS for the entire duration of the treatment.

2.4. Model photoreactive drug substances

2.4.1. Curcumin

Curcumin, a natural yellow pigment in turmeric, is derived from the rhizomes of *Curcuma longa* L. It has been commonly used as a food colourant, in traditional medicine and in cosmetics.[40] This phenolic compound is widely investigated as anti-inflammatory, antioxidant, anticarcinogenic, antiinfectious and as a wound healing agent.[41] Additionally, curcumin has shown potential as a PS intended for aPDT.[23, 24, 42-50] Commercially available curcumin is a mixture of curcumin (≥77%), demethoxycurcumin and bisdemethoxy-curcumin.[40] Curcumin used in the present work was therefore synthesised according to the method of Pabon[51] in order to avoid interference from the other two curcuminoids present in the commercial product.

Curcumin is composed of two phenyl moieties symmetrically linked through a β-diketone moiety (Figure 3). The molecule exhibits tautomerism between keto- and enol-structures, which may exist in *cis* or *trans* conformation. In solution, curcumin mainly adopts

the *cis* enol form which is characterised by the formation of a strong intramolecular hydrogen (H)-bond (Figure 3, right). In protic solvents, the keto-enol equilibrium might be shifted towards the keto form (Figure 3, left) due to rupture of the intramolecular H-bond in favour of intermolecular H-bonds, or the *trans* enol form favoured over the *cis* form for the same reason.[52]

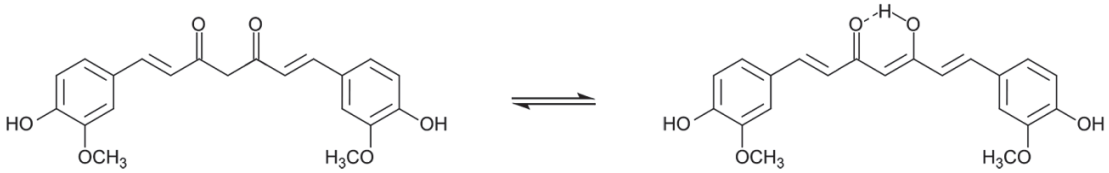

Figure 3 – Chemical structure of curcumin in keto-enol tautomeric equilibrium.

Curcumin is a small lipophilic molecule (M_w 368.38 g/mol) which is practically insoluble in water at acidic and neutral pH values ($\leq 3 \times 10^{-8}$ M at pH 1-7).[39, 53] It is soluble in alkali but is rapidly degraded.[39] At physiological pH (7.4) 10-25% of curcumin is in its monoanionic form (dissociation of the enolic hydrogen) and the remaining is in the neutral state.[52, 54] Curcumin's absorption maximum is usually in the blue wavelength range, depending on the solvent. In most of the polar solvents, the absorption maximum is at ~420 nm and the molar absorption coefficient is ~20 000-50 000 $M^{-1}cm^{-1}$.[52] Curcumin is photolabile and degrade upon exposure to UV-Vis radiation mainly through breaking of the β-diketone link to form smaller phenolic compounds such as vanillin and ferulic acid.[52, 55]

Upon irradiation with visible light, curcumin may produce reactive photoproducts concurrently with its photodegradation, hence its use as a PS. The exact mechanism of the production of photoproducts is partly unknown and is solvent dependent. The phototoxicity of curcumin has been reported to be oxygen dependent, i.e. whether it undergoes a Type I or a Type II reaction, oxygen is required to induce lethal damage to bacteria or cells.[44, 45] Dahl *et al.* (1989) attributed the antibacterial phototoxicity of curcumin to long-lived photoproducts such as H_2O_2 as no $^1O_2$ was detected in deuterated water, dimethyl sulfoxide (DMSO) or acetonitrile.[45] The presence of a $^1O_2$ scavenger has been reported not to affect the

phototoxicity of curcumin towards *Candida albicans*.[23] Production of $^1O_2$ does not seem to be a major part of the mechanism of phototoxicity induced by curcumin in aqueous media.[23, 45, 46, 56]

Low (micromolar) concentrations of curcumin are sufficient to photoinactivate several Gram-positive bacteria, Gram-negative bacteria, and fungi *in vitro*.[23, 24, 42, 45-49] Bacteria have been found to be more susceptible to the phototoxic effect of curcumin than fungi (*C. albicans*), and Gram-positive bacteria more susceptible than Gram-negative bacteria.[23, 42, 45-48] These results are consistent with the general observations of sensitivity of bacteria and fungi to *in vitro* photoinactivation.[9, 15, 26-31] Microbial cells were reported to be more susceptible to photoinactivation by curcumin than mammalian cells, although significant phototoxicity towards mammalian cells has been reported *in vitro*.[23, 43] A clinical study on mouth disinfection by aPDT with curcumin reported no dark toxicity or major adverse effects after millimolar concentrations of curcumin and 20 J/cm^2 blue light, but an average of 68% reduction in the amount of salivary pathogens.[57] The broad spectrum of antimicrobial action of photoactivated curcumin (*in vitro*) and low toxicity towards mammalian cells make it an attractive PS for topical aPDT combined with blue light, except for deep (>0.4 mm) infections.

The challenges related to formulation of curcumin for topical aPDT include making physiologically compatible preparations with acceptable physical and chemical stability and solubility of curcumin. The excipients should increase the hydrolytic stability of curcumin and counteract aggregation of curcumin upon contact with aqueous media. The drug delivery system should also provide acceptable photostability of curcumin, and preferentially encourage interactions with the target microorganisms. A number of approaches for formulation of curcumin have been explored including cyclodextrins, surfactants, liposomes, and alginate.[24, 42, 43, 45-49, 58] In the present thesis (Papers I and III), solid dispersions containing a cyclodextrin (see *2.5. Cyclodextrins as solubility enhancers*) and polymer(s) for *ex tempore* preparation of supersaturated solutions (Paper I), and novel eutectic solvents (Paper III; see *2.6. Eutectic solvents*) were investigated. Both formulation strategies resulted in supersaturated solutions of curcumin after dissolution (of curcumin solid dispersions) or dilution (of curcumin-eutectics) in aqueous media (see *2.7. Supersaturating drug delivery systems intended for topical aPDT*).

2.4.2. Porphyrins

Porphyrins are a group of macrocyclic compounds consisting of the same core structure, the π-conjugated porphine ring. Reduction of double bonds in the porphine ring (22 π electrons) produces the related chlorins (20 π electrons) and bacteriochlorins (18 π electrons), with increasing absorbance in the red wavelength range compared to the porphyrins.[59] These groups of related dyes gained increased attention following the reports on the phototoxicity of Photofrin (haematoporphyrin derivative, HpD) for experimental treatment of certain cancers in the 1970s and 80s.[3, 60] HpD had the disadvantage of being a complex mixture of porphyrin dimers and oligomers, and it has not been possible to isolate a single highly active component.[3, 61] This led to the investigation of the second generation PSs. Among these, porphyrins have gained much attention, and chlorins even more due to their higher absorbance in the red wavelength range compared to porphyrins, which is advantageous in PDT of tumours. The absorption maximum (Soret band) of porphyrins and chlorins is in the blue wavelength range (400-445 nm), and the first Q-band is in the red wavelength range which shifts to lower energy of increasing intensity with the reduction of the porphine ring.[59] Porphyrins have been found to be more phototoxic to various bacteria in the presence of blue light compared to green or red light of similar intensity, which is probably related to the higher absorbance at the Soret band compared to the Q-bands.[62, 63] Porphyrins were chosen as model PSs in the investigation of solubilising and supersaturated preparations intended for aPDT in the present thesis.

Meso-tetraphenylporphyrins are synthetically accessible and can be easily substituted to modify the hydrophilicity.[3] The neutral porphyrin 5,10,15,20-tetrakis(4-hydroxyphenyl)-porphine (THPP; Papers II and V) and the anionic porphyrin 5,10,15,20-tetrakis(4-carboxyphenyl)porphine (TCPP; Paper IV) were chosen as model porphyrins in the present work (Figure 4). Additionally, the neutral porphyrin 5,10,15,20-tetrakis(4-methoxyphenyl)-porphine (TMP; Figure 4) was briefly investigated (only reported in the thesis). Cationic PSs are believed to produce superior phototoxicity towards Gram-negative bacteria due to charge-charge interactions with the negatively charged bacterial surface.[14, 15, 26, 27, 35, 36] Therefore, porphyrins with a high potential for phototoxic improvement were investigated to explore the impact of the formulation on the phototoxicity of the model PSs.

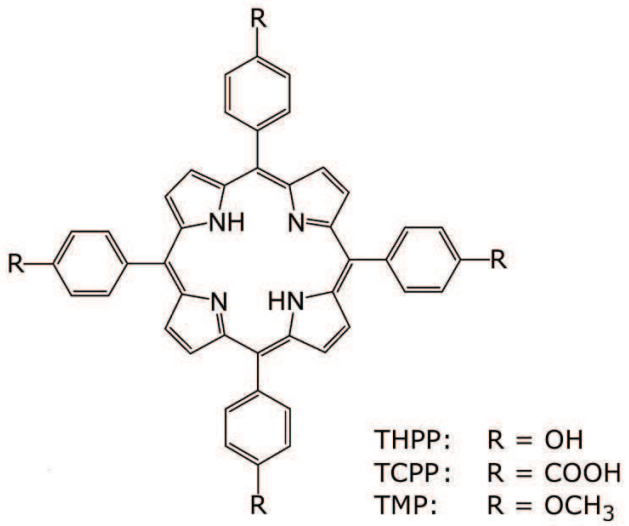

Figure 4 – Molecular structure of the investigated porphyrins, THPP, TCPP and TMP.

Para-THPP was chosen over *ortho*- and *meta*-THPP due to the reported skin and cerebral sensitivity induced in mice by the latter two.[60] Bacterial phototoxicity studies with THPP have shown higher phototoxicity towards Gram-positive than Gram-negative bacteria, although the Gram-negative phototoxicity increased in the presence of a membrane-disorganising agent (polymyxin nonapeptide (PMNP)) to 3 log reductions in viable *E. coli* with 29 μM THPP (3 h irradiation with two unfiltered tungsten lamps with a combined irradiance of 140 W/m^2).[36] Nitzan *et al.* (1995) also found that the photoinactivation was positively correlated with porphyrin monomerisation.[36] Anionic porphyrins structurally related to TCPP (i.e. tetra-(4-sulfonatophenyl)porphine and TCPP-Pd(II)) have been reported to induce limited phototoxicity towards Gram-positive bacteria and not be phototoxic towards Gram-negative bacteria and mycobacteria.[35, 36, 64] This is generally believed to be due to electrostatic repulsion between the negatively charged PS and the negatively charged bacterial surface. Therefore, anionic porphyrins are not often investigated as PSs in aPDT.

With a calculated logD at pH 7.4 of 4.8, the solubility of THPP in aqueous media is poor.[65] TCPP adapts different ionic states depending on the pH of the solvent. Its calculated logD value at pH 7.4 is -2.8 which should indicate high aqueous solubility.[66] It was found, however, that TCPP was practically insoluble in water and phosphate buffer pH 7.4 (Paper IV).

The latter was probably due to interactions with ions in the buffer and aggregation, which is a common challenge with porphyrins.[67] Due to hydrophobic interactions, H-bonds or ionic interactions, porphyrins may stack to form small or large aggregates with different photo-physical properties.

THPP and TCPP dissolved in methanol have their Soret bands at 419 nm and 415 nm, respectively, in addition to four Q-bands at longer wavelengths (512-649 nm). Their molar absorptivity at the Soret band varies depending on the solvent, but is usually in the range 300 000-600 000 $M^{-1}cm^{-1}$. Porphyrins are generally believed to follow a Type II reaction pathway upon irradiation, after ISC to a long-lived triplet state, usually with a fairly high $^1O_2$ quantum yield depending on the solvent and microenvironment.[3] However, Type I mechanisms may also occur alongside the formation of $^1O_2$.[3]

The challenges related to formulation of these porphyrins when intended for aPDT include the making of physiologically compatible preparations with acceptable physical-, chemical- and photostability of the porphyrins. Further, as many porphyrins exhibit limited solubility and aggregation, the formulation should keep the porphyrins solubilised as monomers, even after application, to ensure high phototoxicity. The monomeric state of a porphyrin is the most phototoxic state due to interactions with the target cells, possibly enhanced cellular uptake and efficient generation of ROS.[36, 37] Aggregation of the PS might therefore lead to reduced photosensitisation and reduced uptake by target cells. Usually, poorly water-soluble *meso*-tetraphenylporphyrins are prepared in DMSO or aqueous alkali for investigation of aPDT effect.[36, 60] In the present work, novel eutectic solvents were explored as solvents for THPP and TCPP intended for aPDT (see *2.6. Eutectic solvents*). Such solvents were selected to investigate the possibility of enhancing the phototoxicity of the porphyrins by maintaining the monomeric state, due to intermolecular interactions with the solvent (possibly at supersaturated concentrations), and thereby produce solutions that could be suitable for topical application.

2.5. Cyclodextrins as solubility enhancers

Cyclodextrins (CDs) are cone-shaped, cyclic oligosaccharides with a hydrophobic interior and a hydrophilic exterior commonly used in pharmaceutical preparations. Three CDs are readily available, namely the αCD, βCD and γCD comprising six, seven and eight glucopyranose units, respectively.[68] Their cavity size increases with the number of units. The water-solubility of unsubstituted CDs is, however, limited, especially that of βCD, but may be increased by substitution of the hydroxyl groups and subsequent breaking of intramolecular H-bonds.[38]

In aqueous solutions, the hydrophobic CD cavity is occupied by water molecules, though these interactions are energetically disfavoured due to the polarity of water and the slightly apolar CD cavity. The water molecules may therefore be replaced by other guest molecules with more favourable interactions with the CD cavity, forming CD inclusion complexes.[68] The entire guest molecule or just parts of it may be included in the CD cavity depending on the size of the molecule versus the size of the CD cavity. The aqueous solubility of a hydrophobic drug may be enhanced in the form of an inclusion complex. However, in solution, equilibrium is established between the dissociated and associated CD and guest (D) molecules (Eq. 1):

$$CD_{free} + D_{free} \rightleftharpoons CD{:}D_{complex} \qquad \text{Eq. 1}$$

By increasing the solubility of a hydrophobic drug through complexation with a CD, the bioavailability of the drug may increase if diffusion through an aqueous layer is the rate-limiting step.[38] As previously stated, many infection sites are covered with exudate or other aqueous fluids which may serve as an effective barrier for diffusion of hydrophobic drugs, such as most PSs. Only PS molecules adjacent to the microorganisms can be adsorbed or absorbed to elucidate the most effective phototoxic events (cf. *2.1. Photoreactivity of a drug substance*). Hydrophilic CDs can act as permeation enhancers for hydrophobic drugs through aqueous layers, thereby increasing the drug availability at the surface of biological membranes. For optimal phototoxicity, a PS should dissociate from the CD complex upon contact with its

target, i.e. the CD:D complex must not be too stable, otherwise it will not release the PS in favour of the target cells.[46, 48] Free drug molecules may penetrate cellular membranes more efficiently than the CD complex due to lipophilicity and cellular membrane molecular weight cut-off values.[38] Also, for optimal phototoxicity, it is desirable if the photoproducts attack cellular targets in their close proximity and not simply the CD vehicle with which the PS may be associated. For pharmaceutical formulations, the amount of CD should be optimised since both insufficient amounts and excess amounts of CD may result in suboptimal drug bioavailability.[69] In addition to an increase in guest molecule solubility and bioavailability, CDs may also affect the physical and chemical stability of drugs.[53, 69]

Several CDs have been investigated as solubilisers of curcumin, such as hydroxypropyl-αCD (HPαCD), HPβCD, HPγCD, methyl-βCD (MβCD), the anionic sulfobutylether-βCD (SBEβCD) and the cationic hydroxytrimethylammoniumpropyl-βCD (HTAβCD).[24, 43, 46-49, 53] Cationic PSs or PS vehicles show superior phototoxicity in aPDT due to close interactions between the PS and the negatively charged bacterial membrane.[14, 15, 26, 27, 30, 33, 35, 36, 46] HTAβCD could be beneficial as a solubiliser of a neutral PS to improve its phototoxic potential. Indeed, the phototoxic effect of curcumin solubilised by HTAβCD has been reported to be higher against *E. coli* than when it was solubilised in HPβCD (at 25 μM curcumin, 5% CD and 30 J/cm^2 blue light), although inferior to preparations in polyethylene glycol 400 (PEG 400).[46] However, the HTAβCD complexation constant with curcumin was inferior compared to the other CDs investigated by Tønnesen *et al.* (2002).[53] Due to the poor aqueous solubility of curcumin, a certain affinity for the CD cavity is expected to be necessary for solubilisation of this PS. HPβCD has previously been investigated as solubiliser of curcumin in solid dispersions (i.e. a supersaturated drug delivery system) intended for aPDT, resulting in complete eradication (>6 log reductions) of *E. coli* after exposure to 25 μM curcumin combined with 14 J/cm^2 blue light.[49] In the present work, to evaluate another neutral βCD for which curcumin had shown moderate affinity,[53] MβCD was investigated as solubiliser of curcumin in solid dispersions (Paper I).

The solid dispersions may be applied directly on a moist infection or dissolved *ex tempore* if the target site is not adequately moistened by aqueous fluid. The advantages of the former technique include ease of application and retention at the target site. However,

application of a solid preparation to be dissolved at the target site requires the presence of sufficient liquid, and may prevent light from reaching the PS molecules unless the preparation is removed prior to irradiation. The light may be prevented from reaching the PS molecules due to an inner filter effect (preclusion of a light wave propagating through the medium due to high absorbance at the excitation wavelength) which is due to a high PS concentration, or light attenuation, which is the gradual loss of energy through a medium (may be due to absorption or scattering of the incoming light by the formulation). If inner filter effect and/or light attenuation is an issue, the preparation should be removed from the target site before irradiation. The contact time should allow maceration of the formulation and interaction between the PS and the target microorganisms. However, removal of the preparation may reduce the amount of PS in close proximity of the target bacteria due to washing or swabbing, and will be painful for the patient. Advantages of *ex tempore* dissolution include accurate and reproducible dosing of the PS – if performed correctly by healthcare professionals – and limited need for removal prior to irradiation assumed that the PS concentration is fairly low. Disadvantages of *ex tempore* dissolution of solid dispersions include the decrease in viscosity, making them more difficult to retain at the target site, and the preparation procedure required before application. After treatment, the preparation does not have to be removed if the PS and the excipients in the drug delivery system are biocompatible and biodegradable and do not hamper the wound healing process.

2.6. Eutectic solvents

A eutectic mixture is a mixture of two or more components which, at certain ratios, result in a system having a lower melting point than either of the individual components (Figure 5). Any deviation from the specific component ratios may result in precipitation of the individual components. The melting point (T_m) of a component (A) gradually declines upon the addition of increasing amounts of another component (B), and *vice versa*, until the two curves meet (Figure 5). This point is termed the eutectic point and specifies a certain temperature and component ratio. The eutectic is formed based on interactions between the components, resulting in a product with unique properties relative to the individual

components. After formation of the eutectic, the eutectic temperature denotes the temperature at which the eutectic transforms between a solid and a liquid state.[70]

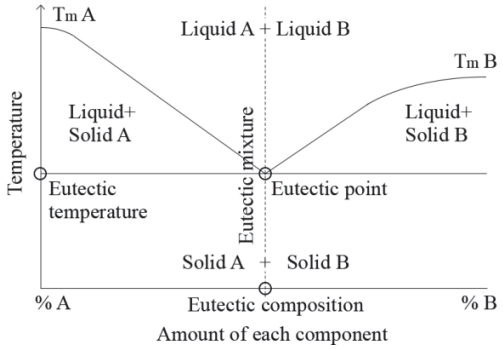

Figure 5 – Phase diagram showing the melting point (T_m) of two components (A and B) as a function of their ratio at constant pressure. During heating, the components go from a solid state, to a state at which both liquid and solid states coexist, to an entirely liquid state above T_m. At the eutectic point, all three phases (solid A, solid B and liquid) exist at the same time. After preparation of a eutectic mixture, it will appear in its liquid state above the eutectic temperature.

Eutectic systems with a very large depression of the melting point (about 200 °C) are called deep eutectic solvents (DES).[71] These may be composed of a combination of metal salts (e.g. $ZnCl_2$), organic salts (e.g. choline chloride, formerly known as vitamin B_4) and/or a H-bond donor (e.g. ethylene glycol).[71] The large decrease in melting point can be explained by strong intermolecular H-bonds which limits crystallisation of the components.[72] DES have found use in *inter alia* organic synthesis, dissolution and extraction processes, replacing the more expensive, less environmentally friendly ionic liquids.[71]

The cytotoxic and antibacterial potential of various DES have recently been investigated.[73-79] The results demonstrate that various eutectic solvents show highly variable toxicity towards different cell lines, bacteria, fish and plants, and may be toxic upon ingestion.[73-78] The eutectics are reported to be more toxic than their individual components, even upon dilution with aqueous solutions.[74, 76, 77, 79] However, the opposite may also occur.[75, 78] Dilution of DES could theoretically rupture the supramolecular structure, but the cytotoxic response indicates that the eutectic network remains upon dilution.[75-79]

2.6.1. Natural deep eutectic solvents

Natural-based DES can be obtained using abundant cellular constituents (e.g. primary metabolites) such as amino acids, sugars, choline derivatives and organic acids (i.e. interactions between an H-bond donor and an H-bond acceptor).[73, 80, 81] These NADES are potential new "green" solvents in many fields.[73] Indeed, when used as solvent for extraction of gluten from foods, their "greenness" was considered excellent.[82] Their advantages as solvents are reported to include their low cost, low vapour pressure, low flammability, biodegradability, potentially high degree of biocompatibility, and extensive solvent potential for various solutes.[70, 73, 76, 81-84]

NADES may be produced by simply melting its components together (heating method), or by dissolving the components in water followed by evaporation (the vacuum evaporation method).[81] The resulting eutectics are colourless, transparent liquids with extensive intermolecular interactions that can lead to the formation of a supramolecular network.[80, 81, 85] A large number of H-bonds has been reported to form between the NADES components after preparation of the eutectic, indicating that the potential for H-bonds plays a major role in the formation of NADES.[81, 85, 86] Water may also participate in the supramolecular H-bond structure.[81, 85, 86] The water remaining after vacuum evaporation is tightly bound in the eutectic.[80] The NADES may be lyophilised to remove more of the residual water, but the resulting eutectics may be extremely viscous or be present in a solid state (unpublished results). Depending on the composition and water content, NADES with different properties (e.g. polarity, viscosity) may be produced.[82, 85, 86]

NADES (with and without included water) are reported to have a glass transition temperature (T_g) usually in the range -60 °C to -90 °C, and to decompose at 100-200 °C, but some have decomposition temperature even above 200 °C.[70, 81, 87] The wide temperature range of the liquid state of NADES suggests their role as naturally occurring cryoprotectants in certain cells.[81] Additionally, NADES have been reported to increase the thermostability of enzymes such as lysozyme.[86] These eutectic solvents may solubilise a wide range of hydrophilic and hydrophobic compounds; both small molecules and macromolecules have been reported to be soluble in certain NADES (see next section).[70, 80-84, 88] Their possible natural origin in certain cells (i.e. potential biocompatibility), "greenness", and ability to

solubilise poorly water-soluble compounds (without encapsulation in a drug delivery vehicle such as liposomes or CDs) indicated that these eutectics could be potential solvents for the hydrophobic PSs investigated in this thesis. It was suggested that the application of NADES as solvents for certain PSs could increase their phototoxic potential by solubilising them in a monomeric state without sterical hindrance of the PSs upon interaction with the target bacteria. Further, the use of potentially biocompatible eutectics could allow the PS-NADES product to remain at the infection site after treatment, without causing harm to mammalian cells (*in vivo*).

Since NADES is a newly discovered group of DES (Choi *et al.* in 2011),[80] little is currently known of their toxicity compared to the related DES. The observed toxicity may depend on the composition and concentration of NADES, as well as the type of microorganism and method used.[78, 79, 89, 90] Addition of water to DES or NADES is reported to weaken the H-bond network.[85, 86] However, it is currently debated whether the eutectic solvents would become a simple solution of their individual components or result in a weakly bound network upon dilution to certain concentrations.[77, 79, 85, 86] It has been reported that the supramolecular H-bond network is broken upon addition of more than 50% water, as seen from shifts in the ^{1}H-NMR spectra of the NADES.[85, 86] However, a resulting weakly bound NADES network after dilution with aqueous media was suggested to explain several results on e.g. cytotoxicity, bioavailability and physical properties of compounds dissolved in diluted NADES.[76, 77, 79, 80, 82-84]

2.6.2. Natural deep eutectic solvents as solvents for photoreactive drugs intended for aPDT

Investigation of NADES in general, and as solvents for photoreactive drugs intended for aPDT, is in its infancy (Figure 6). While the related DES have been researched for several years, none of these eutectic systems have been employed as experimental solvents for PSs, possibly due to a high melting point of many DES and an observed toxicity towards bacteria, brine shrimp, fish, and different cell lines.[73-78] The cytotoxic mechanism of DES has been proposed to be due to an increase in membrane porosity, increase in ROS concentration, oxidative stress and ultimately apoptosis.[75-77] The key to reducing this cytotoxicity may reside in the use of primary metabolites as constituents, i.e. NADES, and beware of dose-dependent

effects. Indeed, if some cells produce and utilise NADES in germination or cryopreservation, as suggested by Choi *et al.* (2011),[80] their cytotoxicity should be minimal compared to DES. However, the concentration, duration of exposure and biological system must be taken into account. Studies on certain choline chloride-based NADES have shown that these solvents possess low cytotoxicity (EC_{50} >2000 mg/L) and are readily biodegradable.[76, 83] It has also been reported in *in vitro* and *in vivo* studies of various choline chloride-based DES that the one containing urea (i.e. a (NA)DES) was less toxic than the other DES towards several normal and carcinoma cell lines.[77] Most cancer cell lines were even resistant to this (NA)DES, though it caused immediate death in mice after oral administration.[77] Recent studies have found that NADES may be less cytotoxic than DES to various cell lines *in vitro*, although they were more destructive than DES *in vivo* (which might be attributed to their higher viscosity, i.e. the NADES may have failed to circulate properly in mice).[79] Similar to DES, an increase in cell membrane porosity and an increase in intracellular ROS concentration have been observed after exposure of cells to diluted NADES *in vitro*.[79] The presence of primary (or secondary) metabolites as constituents of NADES may therefore not automatically indicate that the NADES have similar biocompatibility as their individual components. As demonstrated for the DES, the cytotoxicity of NADES may be lower or higher than their individual components, and must be investigated for each individual eutectic mixture to assess the degree of biocompatibility.[74-79, 91]

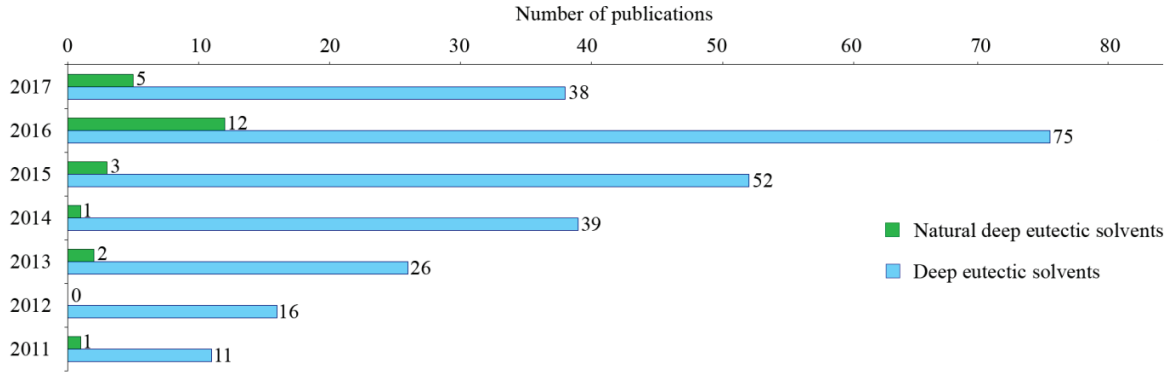

Figure 6 – Number of publications with «natural deep eutectic solvent(s)» or «deep eutectic solvent(s)» in the title, abstract or keywords per year (PubMed, accessed on 09.03.2017)

NADES are interesting as potential solvents for PSs intended for aPDT based on the ease of synthesis/manufacturing, low cost due to the low cost of the raw materials, low toxicity of their individual components in solutions at relevant concentrations, their liquid state, their high polarity and their high solvability. The observation that different NADES with polarity higher than water or similar to that of methanol can solubilise a wide range of water-soluble and -insoluble small and large molecules (e.g. biomolecules such as proteins, enzymes and DNA, and pharmaceuticals such as quercetin, griseofulvin and rutin) is unique.[70, 80-84, 88] The polarity of the NADES does not necessarily correspond to their solubilising properties of various solutes. This indicates that extensive intermolecular interactions between the solute and the NADES are required for optimal solubilising properties.[81] As proposed in Paper III, the difference in solubilising properties of different NADES may be due to a difference in H-bond donating and H-bond accepting properties and the spatial arrangement of interacting groups potentially involved in the solubilisation process. These unique solubilising properties of NADES suggest their role as alternative media to water and lipids in biological systems.[80, 81] Additionally, NADES have been proposed to be formed intracellularly to reduce the cytotoxic effect of their individual constituents.[79]

Little is known about the effects of NADES on intact or damaged skin. Upon combination of components to form NADES, the H-bonds formed affect the components' physical and chemical properties, which could render them more or less toxic than when present as individual components.[74, 76, 79, 91] Topical administration of NADES is regarded as more safe than oral administration, as demonstrated by the hepatic injury observed in mice following oral administration of some DES and NADES.[77, 79] However, oral administration is applicable through the identification of a non-toxic NADES: the NADES proline:glutamic acid (2:1) was used to dissolve rutin (10 mg in 0.5 ml) and the product was administered orally to mice. Rutin-NADES resulted in a 2-fold increase in plasma bioavailability due to improvement in rutin absorption in the presence of NADES compared to a water suspension, and no toxicity was reported by the authors.[84]

It has become evident since NADES were first mentioned in 2011 that the use of an organic acid as the H-bond donor may increase the toxicity of the NADES.[73, 76, 89, 90] High viscosity (>600 mPa·s) of some NADES has also been reported to contribute to an increase in

cytotoxicity (*in vitro*), although the results were not entirely consistent.[89] High viscosity of a NADES indicates a rigid supramolecular structure with a strong H-bond network. Addition of water to the NADES will affect the viscosity and polarity, and the toxicity of NADES may also be tuned by the water content.[85, 89] The affinity of NADES for H-bond donors and acceptors in cell membranes may result in their accumulation on cell surfaces and affect the cells adversely.[89] Similarly, cations of NADES (e.g. choline chloride-based NADES) may interact with negatively charged groups present in cell or bacterial membranes, leading to loss of membrane integrity.[75] In topical aPDT, selective increase in bacterial membrane porosity without affecting the adjacent cells would be advantageous. However, in what manner NADES affect bacterial membranes is unknown at present.

A general challenge with topical aPDT is the required close interaction between the PS and the target microorganism, which is important due to the short diffusion distances and high reactivity of ROS (cf. *2.1. Photoreactivity of a drug substance*). Disruption of the bacterial membrane could result in an increase in bacterial uptake of a PS solubilised in NADES, thus increasing the detrimental photodamage during aPDT. Even if the NADES itself does not induce damage to the bacterial membrane, the increase in PS solubility in NADES, and thereby bioavailability, may increase its phototoxic potential (cf. *2.3. Formulation of products intended for topical aPDT).* PS-NADES preparations may be used undiluted or diluted with possible synergistic or additive antibacterial effect between the NADES and the photoactivated PS. If extensive dilution of the selected NADES is needed to ensure the absence of cytotoxicity, the reduction in viscosity can make it more difficult to retain the preparation at the target site.

If the amount of pure NADES for antibacterial treatment or the combined product of NADES and PS applied is biocompatible and biodegradable, the formulation should not have to be removed after treatment. However, that would also depend on whether the formulation affects the photoactivation of the PS (i.e. light attenuation by absorption or scattering) during treatment. Additionally, if the PS concentration is high, the light may be prevented from reaching the PS molecules due to an inner filter effect. Removal of the preparation after a certain period after application could circumvent the issue of the inner filter effect. However, removal of the preparation will also reduce the amount of PS in close proximity to the target

bacteria and potentially reduce the aPDT effect. Additionally, removal of the formulation may be painful for the patient. Inner filter effect should not be an issue if the PS concentration is sufficiently low. However, a too low PS concentration could be insufficient for interactions with the target bacteria. Still, the effective PS concentration may be reduced by the use of supersaturating drug delivery systems which may increase the phototoxic potential of a PS (see next section).

2.7. Supersaturating drug delivery systems intended for topical aPDT

Drugs may be in solution at concentrations above their saturation solubility (C_{eq}), in a supersaturated state (Figure 7). This state is thermodynamically unstable, and the concentration of the drug will return to its saturation concentration through precipitation of excess drug. Supersaturating drug delivery systems intended for topical aPDT may produce a higher phototoxic effect of a poorly water-soluble PS than conventional drug delivery systems.[48, 49] It was therefore of interest to investigate supersaturating drug delivery systems of the PSs curcumin, THPP and TCPP. The formulations investigated in this thesis were, as mentioned, solid dispersions (Paper I) and eutectic solvents (Papers II-V).

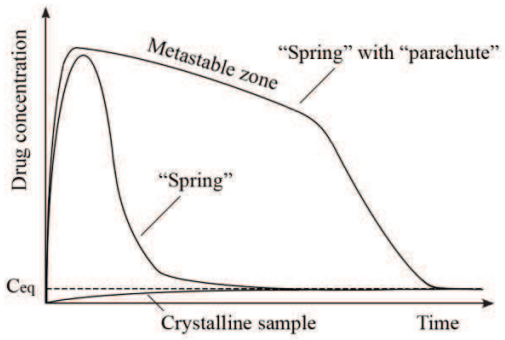

Figure 7 – The drug concentration-time profile following dissolution of a crystalline sample, and a higher energy "spring" form of a drug in the presence and absence of precipitation inhibitors which act as a "parachute". C_{eq} represents the saturation solubility. Diagram adapted from ref. [92].

The supersaturated state can only be generated by a higher energy (e.g. amorphous) form of the drug (a "spring"), as compared to the crystalline state (Figure 7).[92] To temporarily maintain the supersaturated state of a drug, precipitation inhibitors, or "parachutes", may be added which interfere with the precipitation process (Figure 7).[92] The polymer hydroxypropyl

methylcellulose (HPMC) has been employed for this purpose.[49, 92] If the supersaturated state of a PS lasts for a sufficient amount of time, the increased drug concentration at the target site may result in an enhanced flux across e.g. wound exudate or biological membranes, resulting in higher PS concentrations at the target site.[92] Further, if the dissolved PS molecules in a supersaturated solution remain in the monomeric state during irradiation with light, the PS may be readily excited to produce toxic photoproducts.

The absorption of a PS by a bacterium can be assessed in the context of Fick's first law, where the diffusion flux (J) of dissolved PS molecules across a plane of unit area is proportional to the negative diffusion coefficient (D) of the molecule and the concentration gradient across the plane ($\frac{dC}{dx}$) (Eq.2):[93, 94]

$$J = -D\ \frac{dC}{dx} \qquad \text{Eq. 2}$$

The negative sign indicates that the diffusion flux is down the concentration gradient. A supersaturated state of a PS may induce higher phototoxic effect than a simple solution due to an increased PS concentration at the bacterial membrane and possibly an increase in interactions between the PS and the target.[48, 92] Thus, a supersaturated drug delivery system of a PS may produce superior phototoxicity compared to a solubilising drug delivery system, provided the same quantum yield for the formation of reactive species. This phenomenon has been demonstrated for curcumin.[48, 49] Low concentrations of CDs have been reported to produce supersaturated solutions of curcumin, while higher amounts of CDs simply solubilise curcumin and lead to less free curcumin in solution available for interaction with target bacteria.[47, 48] The supersaturated solutions of the PSs investigated in this thesis were therefore expected to induce high bacterial phototoxicity.

Several formulation options have been employed to generate metastable supersaturated solutions of poorly water-soluble drugs.[92] One of these is solid dispersions. Solid dispersions have been reported to be a promising strategy for formulation of curcumin.[49, 95] The high melting point (183 °C) of curcumin reflects strong crystal lattice energy. A major improvement in apparent solubility of curcumin can therefore be expected upon disruption of its crystalline structure.[95] Indeed, the phototoxicity of 10 μM curcumin towards the Gram-

negative bacterium *E. coli* was the highest in the presence of 0.01% (w/v) MβCD compared to higher MβCD concentrations or ethanol, demonstrating the positive influence of supersaturation on curcumin phototoxicity.[48] However, additional excipients are needed to prolong the metastable supersaturated state of curcumin in the presence of small amounts of MβCD. For this purpose, hyaluronic acid (HA) and/or HPMC were included in the preparation of curcumin solid dispersions (Paper I) to prolong the supersaturated state (i.e. the metastable zone displayed in Figure 7) after rapid dissolution.

NADES were investigated as solvents for poorly water-soluble PSs in Papers II-V. Due to rapid aggregation of such PSs upon contact with aqueous media, formulation in a drug delivery system that solubilises the PS in a monomeric form may improve its phototoxic potential. A solution of a PS in NADES may form a supersaturated or a solubilising drug delivery system. However, even though the PS may be solubilised in a monomeric form, the resulting phototoxic effect on bacteria may not be high if the PS does not preferentially interact with the bacteria or if the PS absorbance (i.e. concentration) is too high. It was suggested that the phototoxic potential of the PSs investigated in this thesis could be enhanced by preparing supersaturated PS-NADES samples through dilution with aqueous media. However, for the supersaturation strategy to be useful, the supersaturated solution must be temporarily stabilised to allow for significant absorption of the drug through a membrane.[92] Therefore, conventional supersaturating drug delivery systems contain precipitation inhibitors.[92] Dissolution of a PS in NADES and/or dilution of PS-NADES solutions may produce a supersaturated solution of the PS (Papers II-IV), but no additional precipitation inhibitor except for the (diluted) NADES network (or free, individual NADES components in aqueous solution) is present. Therefore, without additional formulation strategies, a challenge with PS-NADES supersaturated solutions is precipitation of the PS. To produce predictable and reproducible phototoxicity of a PS solubilised in diluted NADES, one is therefore dependent on the remaining NADES network to delay aggregation and precipitation of the PS, or to add small amounts of a stabilising polymer to the NADES solution. The former strategy was investigated in this thesis to avoid any sterical hindrance between the solubilised PS and the target bacteria induced by the excipients, except for the presence of the NADES. Most of the pure NADES (i.e. in the absence of PS) investigated in this thesis were highly bactericidal

when they were used as undiluted solutions (i.e. complete eradication of the bacteria).[91] Hence, undiluted PS-NADES samples were not investigated in the phototoxicity studies.

3. Summary of papers

Paper I

The aim of the first study was to develop and characterise lyophilised curcumin solid dispersions (i.e. foams) for use in *in vitro* aPDT. The foams should have improved drug load and physical stability in the dissolved, supersaturated state and high availability to Gram-positive and Gram-negative bacteria in *in vitro* aPDT compared to previous aPDT preparations of curcumin. In this manner, bacterial phototoxicity could be improved. The solid dispersions were prepared with MβCD, HA and/or HPMC. MβCD was chosen as a solubilisation enhancer of curcumin, and the polymers should function as stabilisers of the supersaturated solution of curcumin which was formed upon dissolution of the foams in aqueous medium. The solid dispersions of curcumin had a drug load in the range of 1.4-5.5% (w/w) and contained < 5% (w/w) water. The foams were further characterised by differential scanning calorimetry (DSC), photo- and thermal stability, dissolution rate in water and the concentration of dissolved curcumin after dissolution of the foams in water. Blue light in the 400-550 nm range was used to photoactivate curcumin in phototoxicity studies on *Enterococcus faecalis* and *E. coli*. The solid dispersions appeared thermally stable for 28 d, but were photobleached upon irradiation with visible light and were therefore protected from light at all times. The lyophilisates dissolved rapidly upon contact with water to produce supersaturated solutions of variable stability. The solid dispersions containing curcumin:MβCD in the molar ratio 1:3 produced the most stable supersaturated solutions. Complete eradication of *E. faecalis* and *E. coli* was achieved after exposure to 0.25 μM curcumin (11 J/cm^2 blue light) and 2.5 μM curcumin (32 J/cm^2 blue light), respectively.

Paper II

The aim of the second study was to investigate NADES as solvents for a poorly water-soluble, neutral PS, the porphyrin THPP. This group of solvents was assumed to be advantageous over the previously investigated solid dispersions because the PS would not be encapsulated in any carrier that would prolong the release time from the vehicle. THPP was soluble in four of the 15 NADES investigated. Two of these NADES preparations (i.e. citric acid:sucrose at 1:1 molar ratio, CS; malic acid:glucose at 1:1 molar ratio, MG) were investigated further in terms of polarity, physical stability of THPP upon dilution of the preparations, photostability of THPP, and antibacterial phototoxicity of diluted THPP-NADES. The NADES that solubilised THPP were more polar (184-200 kJ/mol (44-48 kcal/mol)) than water (201.7 kJ/mol (48.2 kcal/mol) as determined by UV-Vis spectroscopy using Nile Red as a solvatochromic probe)[96, 97] and showed < 0.01 absorbance above 350-370 nm. The study on physical stability of diluted THPP-NADES showed that phosphate buffer reduced the physical stability of THPP in solution. Precipitation of THPP in NADES diluted with MilliQ water was less pronounced. Studies on photostability showed that undiluted THPP-CS and THPP-MG were more photostable than an equal concentration of THPP in methanol (1 mM). However, the photostability was reversed after dilution of THPP-NADES 1:50 with MilliQ water. Both *E. faecalis* and *E. coli* were more sensitive to the photodynamic treatment with diluted (1:200) THPP-CS and THPP-MG in the exponential phase than in the stationary phase of growth. In the exponential phase of growth, *E. faecalis* and *E. coli* were completely eradicated after exposure to 0.25 nM (11 J/cm^2 blue light) and 0.5 nM THPP (32 J/cm^2 blue light), respectively. In the stationary phase of growth, *E. faecalis* and *E. coli* were completely eradicated after exposure to 2.5 nM THPP (11 and 32 J/cm^2 blue light, respectively). It was suggested that the NADES (containing organic acids) could donate H atoms to the pyrroles of THPP, thus inducing closer interactions between the *in situ* formed, positively charged THPP and the negatively charged bacterial surface.

Paper III

To further investigate NADES as solvents for poorly water-soluble PSs, the third study focused on NADES as solvents for curcumin. Curcumin showed superior solubility (>100 μM)

in two of the 19 NADES prepared (i.e. glucose:sucrose 1:1, GS; maleic acid:choline chloride 1:3, MC3), resulting in a colourless solution when dissolved in GS and a yellow solution when dissolved in MC3. Both these NADES were more polar (~200 kJ/mol (47.7-47.9 kcal/mol)) than water (201.7 kJ/mol (48.2 kcal/mol) as determined by UV-Vis spectroscopy using Nile Red as a solvatochromic probe)[96, 97]. The colourless preparation of curcumin-GS could be explained by curcumin being locked in its diketo conformer in this NADES. Curcumin-GS was more photostable than curcumin-MC3, with a photodegradation half-life (Suntest CPS+, 1.2×10^6 lux · h (400-800 nm)) of 4.3 h and 0.8 h, respectively, according to first-order kinetics. Both curcumin-NADES preparations dissolved rapidly in water and produced supersaturated solutions of curcumin. These supersaturated solutions were, however, fairly unstable and considerable amounts of curcumin precipitated within 1 h after dilution. The hydrolytic degradation of curcumin in NADES diluted with buffer depended on the resulting pH; curcumin in GS diluted 1:10 with phosphate buffer pH 8.0 (final pH 8.0) decomposed twelve times faster than curcumin in MC3 diluted with buffer (final pH 1.8). Being in its diketo conformer in both undiluted GS and GS diluted with phosphate buffered saline (PBS) or MilliQ water, curcumin-GS was not phototoxic to *E. coli* (2.6 μM curcumin and 27 J/cm^2 blue light) due to minimal absorption above 400 nm. Curcumin-MC3 (diluted 1:200) induced complete eradication of *E. coli* at 1.25 μM curcumin concentration combined with 27 J/cm^2 blue light, thus being equally (or more) phototoxic as the previously investigated curcumin solid dispersions (which required 2.5 μM curcumin and 32 J/cm^2 blue light).

Paper IV

In the fourth study, the anionic, poorly water-soluble porphyrin TCPP dissolved in NADES was investigated. Anionic PSs have repeatedly been reported to be inefficient in *in vitro* aPDT of Gram-negative bacteria.[30, 36, 64] The aim was therefore to investigate if NADES could increase the phototoxicity of TCPP towards both Gram-positive and Gram-negative bacteria. TCPP was soluble in 13 of the 23 NADES investigated, both in NADES with or without any organic acids, and with polarities ranging from lower (186.2 kJ/mol (44.5 kcal/mol)) to higher (207.5 kJ/mol (49.6 kcal/mol)) than water (201.7 kJ/mol (48.2 kcal/mol) as determined by UV-Vis spectroscopy using Nile Red as a solvatochromic probe) [96, 97]. TCPP

appeared to be in the cationic form in the three NADES MFG (malic acid:fructose:glucose, 1:1:1), ChX (choline chloride:xylitol, 5:2) and CS (cf. Paper II), even though their apparent pH ranged from 0.3-4.3 and TCPP has the (calculated) isoelectric point at pH 3.5.[66] The photodegradation half-life of TCPP according to zero-order kinetics was shorter when dissolved in CS (3.2 h) and MFG (6.9 h) than in methanol (~9 h). This indicated intermolecular interactions between the NADES network and the monomeric form of TCPP, which is more prone to photodegradation than aggregates. The photo- and physical stability of TCPP decreased upon dilution of TCPP-CS and TCPP-MFG with MilliQ water. Upon dilution with PBS, the absorption spectrum of TCPP broadened in CS, MFG and in a citric acid solution, and was blue-shifted in ChX and in PBS (containing 0.5% (v/v) ethanol), which suggested interactions between TCPP and the ions in the buffer. TCPP was investigated at 1-40 nM in CS (diluted 1:400), MFG (diluted 1:400), PBS and a citric acid solution, and 1 μM in ChX (diluted 1:200) and in PBS, combined with 10-27 J/cm^2 blue light. MFG (pH ~5.5) increased the apparent phototoxicity of TCPP towards *E. coli* and *Staphylococcus aureus* compared to a citric acid solution (pH ~4.0) and a pH-neutral reference solution of TCPP, even though TCPP should have appeared negatively charged in diluted MFG. It was observed that CS and MFG increased the dark- and phototoxicity of TCPP towards *E. coli, E. faecalis* and *S. aureus* by unknown mechanisms.

Paper V

Having investigated organic acid-containing NADES as solvents for three different PSs in the previous studies, the aims of the fifth study were to investigate the effect of dilution of the acid-containing NADES network, the antimicrobial activity of the NADES CS and MFG (at 1:200 dilution) on various microorganisms, the phototoxic effect of 1 nM THPP dissolved in the diluted NADES compared to a pH neutral reference solution, and how the NADES might affect the microorganisms adversely. The eutectic network appeared to remain upon dilution ≤1:200. One or both NADES (1:200) induced a significant (p<0.05) reduction in viable colony forming units (CFU)/ml of *Staphylococcus epidermidis* (Gram-positive bacterium), *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Gram-negative bacteria), but not *E. coli* (Gram-negative bacterium) or *Candida albicans* (fungus) in the absence of light. CS (1:200) was

significantly less toxic than an equal concentration of its component citric acid (14.7 mM) to *E. coli, K. pneumoniae* and *S. epidermidis* ($p<0.05$). THPP combined with blue light (27 J/cm^2) induced a larger reduction in survival of *E. coli* when prepared in NADES than in PBS ($p<0.05$). THPP-NADES combined with blue light (27 J/cm^2) induced complete eradication (i.e. >6 log reductions in CFU/ml) of *S. epidermidis*. The antibacterial effect of the organic acid-containing NADES CS and MFG was hypothesised to be due to their low apparent pH after dilution (1:200) with PBS (pH 3.8) and/or an ability to chelate divalent cations in the bacterial membrane of *E. coli*, thus disrupting the membrane integrity. However, no conclusion could be made on the mechanism leading to the observed bacterial toxicity of the NADES.

4. General experimental conditions

A general description of main materials and methods used in Papers I-V is given below. Further details can be found in the individual papers. The investigation of viscosity of the NADES (section 4.3.3) is only described in the thesis.

4.1. Materials

The curcumin used in the present work had been synthesised according to Pabon's method,[51] and was investigated as PS in solid dispersions (Paper I) and in NADES (Paper III). The porphyrin THPP (Frontier Scientific Inc., Logan, UT, USA) was investigated in Papers II and V, and TCPP (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) was investigated in Paper IV, dissolved in various NADES. The porphyrin TMP (Sigma-Aldrich Co. LLC) was briefly investigated for its ability for dissolve in various NADES (only described in the thesis). An overview of general properties of the PSs investigated and some of the excipients used is presented in Table 2. MβCD, HA and HPMC were used in the production of curcumin solid dispersions (see Table 3 for their composition). Table 4 lists the NADES investigated in Papers II-V. See Papers I-V for further details and description of the other materials used.

Table 2 – Overview of general properties of the photosensitisers investigated and some of the excipients used in Papers I-V. The values are based on measurements (reported in the papers), product information sheets, and on calculations from http://chemicalize.org. T_m: melting point. T_g: glass transition temperature.

Photosensitisers:	**Properties:**	
Curcumin	M_w: 368.38 g/mol	Solubility in water: Practically insoluble
	Strongest acidic pKa: 9.1	T_m: 183 °C
	LogD at pH 7.4: 4.1	
THPP	M_w: 678.74 g/mol	Solubility in water: Practically insoluble
	Strongest acidic pKa: 8.6	LogD at pH 7.4: 4.8
	Strongest basic pKa: 9.2	T_m: >300 °C
TCPP	M_w: 790.77 g/mol	Solubility in water: Practically insoluble (Paper IV)
	Strongest acidic pKa: 3.4	
	Strongest basic pKa: 5.0	LogD at pH 7.4: -2.8
		T_m: >300 °C

Excipients:	**Properties:**	
Methyl-β-cyclodextrin	M_w: ~1310 g/mol	Solubility in water: Soluble
	Strongest acidic pKa: 12.1	T_g: ~164 °C (Paper I)
	LogD at pH 7.4: -3.4	
Hyaluronic acid	Proturon™ LV-C	Solubility in water: Soluble
	M_w: 0.05-0.1 × 10^6 g/mol	T_m / T_g: Unknown
Hydroxypropyl	Grade: 603	Solubility in water: Sparingly soluble
methylcellulose	Substitution type: 2910	T_g: ~153 °C (Paper I)
	Labelled viscosity: 3 mPa · s	
Choline chloride	M_w: 139.62 g/mol	Solubility in water: Freely soluble
	Strongest acidic pKa: 14.0	T_m: 302-305 °C
	LogD at pH 7.4: -4.7	
Fructose	M_w: 180.16 g/mol	Solubility in water: Soluble
	Strongest acidic pKa: 10.3	T_m: 119-122 °C
	LogD at pH 7.4: -2.8	
Glucose	M_w: 180.16 g/mol	Solubility in water: Soluble
	Strongest acidic pKa: 11.3	T_m: 150-152 °C
	LogD at pH 7.4: -2.9	
Sucrose	M_w: 342.30 g/mol	Solubility in water: Freely soluble
	Strongest acidic pKa: 11.8	T_m: 185-187 °C
	LogD at pH 7.4: -4.5	
Xylitol	M_w: 152.15 g/mol	Solubility in water: Soluble
	Strongest acidic pKa: 12.8	T_m: 94-97 °C
	LogD at pH 7.4: -3.1	
Citric acid	M_w: 192.12 g/mol	Solubility in water: Freely soluble
	Acidic pKa: 3.1, 4.8, 6.4	T_m: 153-159 °C
	LogD at pH 7.4: -9.5	
Maleic acid	M_w: 116.07 g/mol	Solubility in water: Freely soluble
	Acidic pKa: 3.1, 5.9	T_m: 137-140 °C
	LogD at pH 7.4: -5.0	
Malic acid	M_w: 134.09 g/mol	Solubility in water: Freely soluble
	Acidic pKa: 3.2, 5.1	T_m: 131-133 °C
	LogD at pH 7.4: -6.8	

4.2. Curcumin solid dispersions

4.2.1. Complexation between curcumin and MβCD

Curcumin is practically insoluble in water but may form water-soluble complexes with CDs, thus improving its applicability as a PS in topical aPDT.[53] MβCD has been investigated as a solubiliser of curcumin to produce supersaturated solutions in aqueous media with high bacterial phototoxicity (10 μM curcumin in the presence of 0.01% (w/v) MβCD combined with 29 J/cm^2 blue light induced ~4.2 ± 1.1 log reductions in viable *E. coli* CFU/ml).[48] This CD was therefore selected as solubiliser of curcumin in solid dispersions. The selected amount of MβCD to be used in the preparation of the solid dispersions was based on the results from a phase solubility study in the range 0-10% (w/v) MβCD. It has been reported that temporal stabilisation of a supersaturated solution of curcumin produces high and predictable photoreactivity.[48] Therefore, HPMC and HA were used as potential stabilisers of the supersaturated preparations of curcumin in the presence of MβCD. These polymers were chosen for the following reasons: HPMC had previously been shown to stabilise supersaturated solutions of curcumin.[49, 98] HA has hydrophilic and hydrophobic patches which were thought to be able to interact favourably with both MβCD and curcumin to stabilise the supersaturated solution.[99] The aqueous solubility of curcumin was therefore also investigated with increasing amounts of MβCD and the presence of 0.1% (w/v) polymer(s) in the phase solubility study. These samples were included to evaluate the complexation ratio of curcumin to CD, and whether the polymers affected the complexes in solution. The curcumin concentration was quantified by high-performance liquid chromatography (HPLC). The details are given in Paper I.

4.2.2. Preparation of curcumin solid dispersions

The curcumin solid dispersions described in Paper I were prepared in three steps: First, co-precipitates of curcumin and MβCD were formed by dissolving the two compounds in methanol followed by vacuum evaporation. Based on the results of the phase solubility study (cf. *4.2.1. Complexation between curcumin and MβCD*), curcumin samples were prepared with curcumin:MβCD at 1:1 and 1:3 molar ratios for optimal solubilisation of curcumin in aqueous media. Too low concentration of CD would result in less than desired amounts of

solubilised curcumin, while too high amounts of CD could lower the phototoxic potential of curcumin due to favourable interactions between curcumin and the CD instead of the bacterial envelope.[48]

Secondly, the co-precipitates were dissolved in MilliQ water to obtain supersaturated solutions of curcumin. The aqueous solutions were shaken for 1 h, based on a preliminary study on the time needed to produce the highest concentration of solubilised curcumin (0.5-24 h). After shaking, the solutions were turbid which indicated the presence of large curcumin-CD aggregates or dispersed curcumin. These were removed by filtration (0.45 μm) to increase the likelihood of obtaining homogenous solid dispersions as the end product. HPMC and/or HA were added to the filtrated supersaturated solutions to temporarily stabilise the curcumin-MβCD complexes. The resulting preparations were named 1:1-HA, 1:3-HA, 1:1-HPMC, 1:3-HPMC, 1:1-CHA and 1:3-CHA based on the molar ratio between curcumin and MβCD and the type of polymer added to the preparation (Table 3). "CHA" indicates that the solid dispersion contained both polymers (Table 3). It was desirable to use small amounts of polymer(s): Too high amounts of polymer(s) might inhibit curcumin-CD complex formation due to steric blockage, as observed in the phase solubility study (see *5.1.1. Curcumin complexation with cyclodextrin*).

Finally, after addition of the polymer(s), the supersaturated solutions were freeze dried to yield the final product. As a lyophilisate, curcumin was locked in a supersaturated state. For more details on the preparation procedure, see Paper I.

Table 3 – Composition of the solid dispersions produced in Paper I.

Solid dispersion	Curcumin:MβCD molar ratio	MβCD (mol)	HA (% (w/v))	HPMC (% (w/v))
1:1-HA	1:1	7.64×10^{-5}	0.1	-
1:3-HA	1:3	2.29×10^{-4}	0.1	-
1:1-HPMC	1:1	7.64×10^{-5}	-	0.1
1:3-HPMC	1:3	2.29×10^{-4}	-	0.1
1:1-CHA	1:1	7.64×10^{-5}	0.04	0.06
1:3-CHA	1:3	2.29×10^{-4}	0.04	0.06

4.2.3. Solid state characterisation of curcumin solid dispersions

The curcumin drug load (% (w/w)) and water content of the lyophilised solid dispersions were quantified by HPLC and Karl Fischer titration, respectively. These properties were used to calculate the amount of curcumin in a weighed sample of lyophilisate. Lyophilised products are hygroscopic and will rapidly attract water from the air which could affect the appearance, hydration time in aqueous solution and shelf-life of the products. The hydration time of the lyophilisates after equilibration at ambient conditions (22 °C, 41% relative humidity) was therefore recorded. A rapid dissolution in aqueous media was desirable if the lyophilised foams were to be applied directly on moist infection sites.

DSC was used to evaluate the thermal stability of the lyophilised solid dispersions, as well as interactions between curcumin and the excipients. The thermograms of curcumin, MβCD, HPMC and HA were compared with the thermograms of the lyophilised solid dispersions of curcumin. From the thermal analysis, an appropriate temperature for accelerated physical stability testing was chosen (40 °C) to evaluate if the solid dispersions were suited for short-term storage at room temperature (~22 °C) protected from light. Thermograms were also recorded of the lyophilised solid dispersions after the accelerated physical stability test (28 d at 40 °C) and after photobleaching in a solar simulator (Suntest CPS+, Atlas, Linsengericht, Germany) according to ICH Guideline Q1B Option 1 (only described in the thesis).[100] For further details, see Paper I.

4.2.4. Characterisation of the dissolved lyophilisates

A supersaturated solution of curcumin was formed upon dissolution of the lyophilisates in water or PBS pH 6.1. The curcumin concentration 0-168 h after dissolution in PBS pH 6.1 was quantified by HPLC (after centrifugation and filtration (0.45 μm) to remove large aggregates, and a second filtration step (0.45 μm) to remove the polymers). The pH was adjusted to 6.1 as it is recommended to use aqueous solutions of pH <7 to improve the hydrolytic stability of curcumin.[39] This buffer would also be compatible with the *in vitro* bacterial phototoxicity studies. For further details, see Paper I.

4.3. NADES as solvents for PSs

4.3.1. Preparation of PS-NADES

The NADES were prepared by the solvent evaporation method: The components of each NADES (Table 4) were dissolved in MilliQ water in a round flask, followed by vacuum evaporation of the water with a rotary evaporator. The evaporation method would rapidly produce NADES with a fairly reproducible volumetric outcome. The remaining water bound in the NADES structure after evaporation lowered the viscosity and made the NADES easier to handle compared to lyophilised samples. A specified amount of PS was added to the NADES and dissolved by magnetic stirring protected from light. The dissolution process was followed by visual inspection. For details on the different PSs, see Papers II-IV.

4.3.2. Characterisation of the pure NADES

The pure NADES were characterised in terms of water content with Karl Fischer titration. Use of a drying method was considered, where the water content of each NADES would correspond to the weight loss. However, the water remaining after preparation of the NADES may be tightly incorporated in the NADES network.[80, 81] A drying method would therefore not give an accurate value of the exact water content. By Karl Fischer titration, a drop of NADES of known mass is dissolved in a solvent (methanol based), thus releasing the bound water which can be quantified.

The osmolality of the NADES was measured to investigate how dilution affected the NADES network. The measurements were based on freezing point depression and the expectation that the freezing point is directly proportional with the osmolality of a solution.[101] The following samples were measured (n=3): Four different NADES (CS, GS, MC3, MG and MFG; cf. Table 4), their individual components, and the components combined without the formation of eutectics at concentrations similar to that in the undiluted or diluted NADES. Dilutions were 1:1, 1:10, 1:50 and 1:100 in MilliQ water, as well as 1:100 and 1:200 in PBS. If the calculated osmolality of a diluted NADES was similar to that of the individual NADES components and/or the components combined without the formation of eutectics, this could indicate the disruption of the NADES supramolecular structure upon dilution. Because it is unknown how the presence of a eutectic would affect the freezing point of an aqueous solution,

Table 4 – Constituents of the natural deep eutectic solvents investigated in Papers II-V, and the acronyms used. *) Suboptimal physical stability; white, solid precipitate was observed during storage for ≤ 21 d.

Component 1	Component 2	Component 3	Molar ratio	Acronym
Citric acid	D-(-)-fructose		1:1	
Citric acid	Sucrose		1:1	CS
Citric acid	D-(+)-glucose		1:1*	
Citric acid	D-(+)-glucose		2:1	
Citric acid	D-(+)-trehalose		2:1*	
Citric acid	Choline chloride		1:1	
Citric acid	Choline chloride		1:2	ChC
Citric acid	Choline chloride		1:3	
Citric acid	Xylitol		1:1	CX
Choline chloride	D-(-)-fructose		1:1	
Choline chloride	D-(-)-fructose		2:3	
Choline chloride	D-(-)-fructose		5:2	
Choline chloride	D-(+)-glucose		1:1*	
Choline chloride	D-(+)-glucose		5:2	
Choline chloride	DL-malic acid		1:1	
Choline chloride	DL-malic acid		1:3	
Choline chloride	Maleic acid		1:1	MC
Choline chloride	Maleic acid		2:1	ChM
Choline chloride	Maleic acid		1:3	MC3
Choline chloride	Glycerol		1:1	GC
Choline chloride	Glycerol		3:2	CG
Choline chloride	Sucrose		1:1*	
Choline chloride	Sucrose		4:1	
Choline chloride	Xylitol		5:2	ChX
Choline chloride	Xylitol	DL-malic acid	1:1:1*	
D-(+)-glucose	Sucrose		1:1*	GS
D-(+)-glucose	D-(-)-fructose		1:1*	GF
D-(+)-glucose	DL-malic acid		1:1*	MG
D-(+)-glucose	Maleic acid		1:4	
Sucrose	D-(-)-fructose		1:1*	
Sucrose	D-(-)-fructose	D-(+)-glucose	1:1:1*	SFG
Sucrose	DL-malic acid		1:1	
Sucrose	Maleic acid		1:1	
DL-malic acid	D-(-)-fructose		1:1	
DL-malic acid	D-(-)-fructose	D-(+)-glucose	1:1:1	MFG

and thus the calculated osmolality, a higher or lower apparent osmolality of the NADES compared to the solutions of the individual components could indicate the presence of a eutectic. For further details, see Paper V. The results of the osmolality measurements of MC3, GS, MG and their individual components are only reported in the thesis.

Further, absorption spectra (190-700 nm) were recorded of undiluted and diluted NADES in MilliQ water or PBS to see if the solvent would interfere with the excitation wavelengths of the PSs. Absorption spectra were also employed to calculate the polarity of the undiluted NADES, by using the absorption maximum of Nile Red (NR, λ_{max}) dissolved in the different NADES in the equation:

$$E_{NR} = hcN_A/\lambda_{max} \qquad \text{Eq. 3}$$

where E_{NR} is the polarity expressed as energy per mol (kJ/mol), h is Planck's constant, c is the velocity of light and N_A is Avogadro's constant.[96, 97] For further details, see Papers II-IV.

4.3.3. Viscosity measurements of NADES (only reported in the thesis)

The viscosity of the NADES and their individual components was measured on a Brookfield viscometer DV2T (Middleboro, MA, USA) equipped with a Grant LTD6G (Grant Instruments, Cambridge, UK) temperature element (25 °C). A CPA-40Z or CPA-52Z cone was used for the samples with expected low or high (>10 mPa · s) viscosity, respectively. The viscometer was calibrated with MilliQ water and PEG 400. To verify whether the viscosity of the NADES was due to the formation of eutectics, the combined components were dissolved in MilliQ water and measured as reference samples. As the combined components were not soluble in the small amount of water representing undiluted NADES, these samples were not investigated.

4.3.4. Solubility of PSs in NADES

The solubility of THPP (Paper II), curcumin (Paper III) and TCPP (Paper IV) in various NADES was investigated by addition of excess amounts of PS to each NADES. The samples were kept protected from light, stirred or shaken for a given amount of time and

centrifuged (6918*g*). Centrifugation was employed instead of filtration to remove any undissolved particles of PS as most NADES were too viscous to be filtered (0.45 μm). A small amount of the supernatant was then diluted with methanol and the HPLC mobile phase prior to quantification by HPLC. For further details, see Papers II-IV.

4.3.5. Characterisation of PS-NADES samples

The PS-NADES samples were characterised by UV-Vis and fluorescence spectroscopy and compared to other solutions of the PSs (e.g. dissolved in organic solvent, in an acidic solution or in a basic solution). Differences in the spectra of the PSs dissolved in the various solvents could indicate ionisation, aggregation or changes in molecular conformation. The spectral shift upon dilution of the PS-NADES (with MilliQ water, buffer or ethanol) was also investigated. In studies on physical stability, UV-Vis and fluorescence spectra were recorded to observe changes in the effective PS concentration upon dilution and storage of the diluted PS-NADES. These changes could include shifts in the absorption or fluorescence maxima, or a decrease in intensity. The hydrolytic stability of curcumin in NADES was investigated upon dilution with phosphate buffer pH 8.0. For further details, see Papers II-IV.

4.4. Bacterial (photo-) toxicity studies

The bacterial phototoxicity and toxicity in the absence of light of dissolved/diluted curcumin solid dispersions and PS-NADES (Papers I-IV) were investigated on planktonic model Gram-positive (*E. faecalis* and/or *S. aureus*) and/or Gram-negative (*E. coli*) bacteria. The protocol involved a pre-incubation phase, an irradiation phase and a post-incubation phase. The pre-incubation phase (37 °C) was included to increase the likelihood of favourable interactions between the PS and the bacterial surface. Close interactions between the PS and the bacteria would imply short distances between the photoproducts produced upon irradiation of the PS and the target bacteria.[28, 48] During the irradiation phase, the PS is excited by the light and may produce toxic photoproducts. These photoproducts may initiate chain reactions which may continue in the dark, hence the post-incubation phase. The post-incubation phase (37 °C) allowed the chain reactions to continue before the samples were plated, which resulted in enhanced eradication of the bacteria.[46] The optimal pre- and post-incubation times and light

doses were investigated for curcumin and THPP (both dissolved in ethanol and diluted with PBS to a final concentration of 0.5% ethanol) in *E. faecalis*, *S. aureus* and *E. coli*. Different combinations of times (0-60 min) were investigated, which resulted in an optimal protocol of 10 min pre- and post-incubation and 10 min blue light irradiation (~10 J/cm^2) for the Gram-positive bacteria and 30 min pre-incubation and irradiation (~30 J/cm^2) and 30 or 60 min post-incubation for *E. coli* (Paper II; unpublished results).

Bacteria were grown in nutrient broth to the stationary phase of growth prior to use (37 °C). For studies in the exponential phase of growth, bacteria in the stationary phase were re-cultured and grown to mid-exponential phase (37 °C). The broth was then replaced with PBS and the bacteria diluted to 10^6-10^7 CFU/ml, corresponding to an optical density at 600 nm (OD_{600}) of 0.02-0.04, depending on the size and pigmentation of the bacteria. This amount of planktonic bacteria was chosen to represent a high bacterial load without the PS being shielded by a large amount of bacteria absorbing or scattering most of the irradiation. Due to operator and bacterial variance, all bacterial studies were performed with 4 parallels repeated on at least two separate days. For further details, see Papers I-IV.

4.4.1. The light source

The irradiation chamber (Polylux PT, Dreve, Unna, Germany) used in the phototoxicity studies (Papers I-V) is a polymerisation chamber used in dentistry for light-curing of materials. The chamber was equipped with three fluorescent tubes (Ralutec 9 W/71) emitting blue light mainly in the 400-550 nm range (see e.g. Paper II for the emission spectrum). The irradiance was monitored at regular intervals with a UDT 371 radiometer (United Detector Technology, San Diego, CA, USA). The radiometer was equipped with a probe sensitive to the blue light wavelength range (268 BLU).[102] The measurements were performed at 9 different points within the irradiation chamber, which resulted in a mean irradiance of 18 mW/cm^2 (± 10%) in the bacterial studies included in Paper I and II, and 15 mW/cm^2 (± 10%) after 30 min of irradiation or 16 mW/cm^2 (± 10%) after 10 min of irradiation in the studies included in Papers III-V.

4.5. Investigation of the antimicrobial effect of NADES

Papers II-IV suggested a probable antibacterial effect of the pure (diluted) NADES containing organic acids. This antibacterial effect could be the reason for the apparent increase in phototoxic outcome after bacterial exposure to diluted PS-NADES and blue light, if the NADES weakened the bacteria. Due to the composition of the most toxic NADES (containing citric acid) and the structure of the Gram-negative bacterial cell wall (containing divalent cations), it was suggested that the antibacterial effect of such acid-containing NADES was due to i) the low pH of the NADES, ii) the acid component of the NADES, and/or iii) chelation of divalent cations in the bacterial cell wall, thus disrupting it. The latter might explain the observed increase in phototoxicity of the PSs dissolved in NADES compared to pH neutral or acidic reference solutions of the PSs. A disruption of the bacterial cell wall can result in interlocation of the PS and production of photoproducts in close proximity to vital bacterial components during irradiation. These hypotheses were investigated on the Gram-negative bacterium *E. coli*. Additionally, the susceptibility of various bacteria (*E. coli*, *S. epidermidis*, *K. pneumoniae*, *P. aeruginosa*) and a fungus (*C. albicans*) to the same dark (1.5 h) and light (27 J/cm^2 blue light) exposures to pure NADES (diluted 1:200) and 1 nM THPP-NADES (diluted 1:200) was investigated. A combination of Gram-negative bacteria, a Gram-positive bacterium and a fungus was chosen based on the reported higher sensitivity of Gram-negative bacteria towards acid-based DES, and the higher sensitivity of Gram-positive bacteria towards photoinactivation.[9, 15, 26-31, 90] Additionally, these microorganisms are prevalent in infected wounds, sepsis following contaminated implants, and in oral cavity infections.[103-105] An OD_{600} of 0.045 was chosen for all the bacterial solutions diluted in PBS and OD_{600} of 0.10 for *C. albicans*, as the microbial susceptibility to *in vitro* aPDT is affected by cell density, quantity and size.[28] The NADES dilutions, PS concentration and light dose were chosen based on the previous studies on *E. coli*. For further details, see Paper V.

5. Main results and discussion

5.1. Preparations of curcumin

5.1.1. Curcumin complexation with cyclodextrin

A phase solubility study was performed with curcumin and MβCD in MilliQ water with and without the presence of 0.1% (w/v) HPMC, 0.1% (w/v) HA or 0.04% (w/v) HA and 0.06% (w/v) HPMC (Paper I). The concentrations of the polymers were the same as in the curcumin solid dispersions to be prepared (Table 3). A pronounced effect of MβCD on the solubilisation of curcumin was seen, which was linear with respect to the MβCD concentration (Figure 8). A linear relationship between the curcumin concentration in water and MβCD concentration indicated a 1:1 complex formation between curcumin and MβCD, i.e. one curcumin molecule was solubilised by one MβCD molecule. This solubilisation of curcumin was probably due to inclusion of one of the aromatic parts of curcumin in the MβCD cavity, sterically hindering the complexation of the other half of the curcumin molecule with another CD cavity.[106] Formation of inclusion complexes were confirmed by DSC (Paper I). No attempt was made to estimate the association constant of the curcumin-MβCD complexes from the phase solubility study, as the solubility of curcumin in water in the absence of MβCD was below the limit of detection by UV-Vis absorption spectroscopy or reversed-phase HPLC. It was observed that the affinity of curcumin for the MβCD cavity was reduced upon addition of polymers (Paper I). The reduced affinity of curcumin for MβCD upon addition of HA and/or HPMC was not expected as water-soluble polymers are reported to increase the solubilising effect of CDs on various hydrophobic drugs in aqueous solutions.[69]

At MβCD concentrations ≤ 3% (w/v), which was relevant for the preparation of the curcumin solid dispersions, the affinity of curcumin for the CD cavity was lowest in the presence of HPMC, intermediate and similar in the presence of HA or HPMC and HA, and highest without the presence of polymers (Figure 8). This indicated that the polymers introduced steric blockage of the CD cavity, either by interaction with MβCD, curcumin or both. At an MβCD concentration of 3% (w/v), which is comparable to the curcumin solid dispersions containing the most MβCD (Table 3), the aqueous solubility of curcumin was 257 ± 56 μM without polymers, 51 ± 5 μM in the presence of HA, 48 ± 7 μM in the presence of both HA and HPMC, and 36 ± 1 μM in the presence of just HPMC (Figure 8). As the affinity

of curcumin for the CD cavity should not be too high for optimal antibacterial phototoxic effect (cf. *2.5. Cyclodextrins as solubility enhancers*), the effect of the polymers on curcumin-MβCD complexation might be desirable, as the free fraction of curcumin is more suited to interact closely with the target microorganisms and produce a high phototoxic effect.[47, 48] The interactions between curcumin, MβCD and polymers might be affected by the presence of ions, cytokines, enzymes and proteins, which could be present at *in vivo* target sites. However, the influence of such compounds was not investigated in the present thesis.

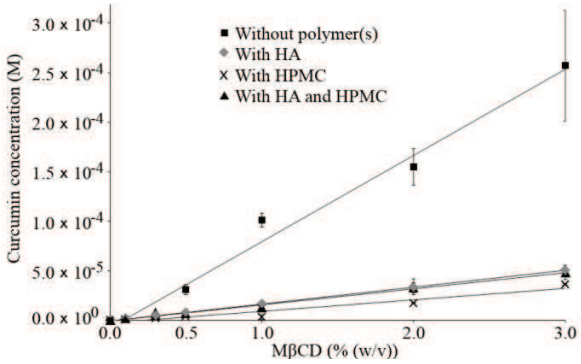

Figure 8 – Curcumin concentration (M) as a function of MβCD concentration with and without the presence of HA, HPMC or both polymers.

5.1.2. Characterisation of curcumin solid dispersions

The lyophilised curcumin solid dispersions appeared bright yellow (at 1:1 molar ratio between curcumin and MβCD) or orange (at 1:3 molar ratio), porous, dry and hygroscopic (Figure 9). The amount of CD present obviously affected the amount of curcumin solubilised in the first preparation step and, thus, the curcumin drug load in the final lyophilisates (ranging from 1.2-5.8% (w/w)). All the lyophilisates appeared homogenous, at least at the macro level, as drug load and thermal analysis by DSC were reproducible. No crystalline curcumin was present in the samples, as indicated by the absence of the melting point of curcumin in the thermograms (Paper I). The lyophilisates displayed one T_g at 160 °C or 162 °C, neither of which overlapped with the T_g of the pure excipients. Only one T_g of the lyophilisates and no overlaps with the excipients indicated the formation of amorphous solid dispersions with interactions between curcumin and the excipients where curcumin either was present in an amorphous form or dispersed at the molecular level (i.e. a solid solution).

Figure 9 – Lyophilised curcumin solid dispersion (1:3-CHA).

HPMC is a polymer commonly used in the preparation of solid dispersions, and has shown to stabilise curcumin supersaturated solutions.[49, 92, 98, 107] HPMC forms minor H-bond interactions with curcumin in solid dispersions, but is a strong crystallisation inhibitor.[95] Due to the strong intramolecular interactions within the curcumin molecule, it has been suggested that polymers interacting only through H-bond formation are poor inhibitors of curcumin crystallisation.[95] HA is a naturally occurring polymer with hydrophilic and hydrophobic patches.[99] Therefore, it was suggested that HA could interact with the CD, complexed curcumin and free curcumin through both H-bonds and hydrophobic interactions. Based on a proposed direct-application of the solid dispersions at a moist infection site, a HA quality with intermediate molecular weight was chosen (Table 2). A very high molecular weight of the polymer(s) used in solid dispersions could reduce the drug release rate due to reduced aqueous solubility and high viscosity of the polymer.[107] The results from the phase solubility study (see *5.1.1. Curcumin complexation with cyclodextrin*) and the thermal analysis (Paper I) suggested the presence of interactions between the polymers and curcumin or MβCD. The high T_g of all the solid dispersions (160-162 °C) further suggested high kinetic stability of the solid dispersions, as molecular mobility is considered to be minor at temperatures ≥50 °C below the T_g.[108] An acceptable thermal stability was confirmed by storage of the lyophilisates, tightly sealed in glass containers with stopper and Parafilm, at 40 °C for 28 d: No substantial change in curcumin drug load was detected (Paper I), and no changes in the DSC thermograms were observed (unpublished results). Nor did the lyophilisates display any changes in their

thermograms after irradiation in a Suntest CPS+ at 765 W/m^2 (310-800 nm) to the endpoint corresponding to 1.2×10^6 lux · h (unpublished results).

5.1.3. Curcumin solubilisation in NADES

Curcumin was attempted solubilised in 19 different NADES, resulting in visible dissolution in five NADES (see Table 4 for acronyms), namely SFG (~14 μM), CS (~18 μM), GF (~46 μM), GS (~141 μM) and MC3 (~181 μM; Paper III). There was no apparent relation between the solubilising potential of the NADES and pH, water content or polarity (Paper III). SFG, GF and GS were composed of non-ionic components, but the resulting solubilisation potential was widely different. Curcumin dissolved in GS formed a colourless solution, while the other four NADES produced yellow solutions (an example in shown in Figure 10). It has previously been confirmed that solubilisation of drugs in NADES is a consequence of intermolecular interactions between the unique solvent and the solute, and not simply due to the individual NADES components' ability to solubilise the drug.[70] The solubilising ability of NADES is attributed to their extensive H-bond network, and the formation of new intermolecular H-bonds and hydrophobic interactions upon addition of a solute. The number of possible interactions with a solute and the spatial structure of interacting groups might affect the ability of NADES to dissolve a drug. Thus, a highly polar NADES (e.g. GF of 186.6 kJ/mol (44.6 kcal/mol)) may solubilise even a practically water-insoluble compound like curcumin (Paper III). The intermolecular interactions between NADES and the PSs applied in this work have not yet been investigated in detail (e.g. by NMR).

The extensive H-bond network of NADES may result in lower mobility of solutes.[71] In the case of curcumin and GS, the NADES appeared to lock curcumin in its colourless diketo conformer.[109, 110] Formation of the diketo conformer of curcumin (Figure 3, left) leads to a substantial reduction in conjugation, hence a blue shift in the absorption maximum compared to the enol form (Figure 3, right) which was present in other NADES. The diketo conformer of curcumin has a calculated absorption maximum at 373 nm, with a fairly low molar absorptivity.[54] The absorption spectrum obtained of curcumin-GS showed two absorption maxima at 370 nm and 436 nm, respectively (Paper III). The first was ascribed to the diketo

form of curcumin and the latter, being the same as the absorption maximum of curcumin-MC3, was ascribed to trace amounts of the enol form of curcumin in GS.

Figure 10 – Solutions of 1) pure GS, 2) saturated curcumin-GS, and 3) saturated curcumin-MC3.

The solubilising capacity of SFG, CS, GF, GS and MC3 was comparable to that of 3% (w/v) MβCD with and without the presence of polymers (*5.1.1. Curcumin complexation with cyclodextrin*). As preliminary bacterial toxicity studies showed a high antibacterial effect of undiluted NADES, the NADES that had the highest solubilisation potential of curcumin (i.e. GS and MC3), were chosen for further studies. For *in vitro* bacterial phototoxicity studies, the curcumin-NADES were diluted prior to use to observe the genuine phototoxicity of curcumin without an additional reduction in bacterial survival induced by the NADES. Dilution of curcumin-NADES was also suggested to produce a supersaturated solution of curcumin with high phototoxic potential. However, as reported in Paper III and in section 5.1.6 (*In vitro bacterial phototoxicity of curcumin preparations*), particularly MC3 (diluted with PBS) may have influenced the results of curcumin phototoxicity, even at dilutions where the pure NADES was not bactericidal, due to a potential weakening effect on the bacteria.

5.1.4. Curcumin drug delivery systems dissolved in aqueous solution

The curcumin solid dispersions dissolved rapidly in water (in less than 1 min) to produce supersaturated solutions of curcumin (Paper I). However, the theoretical concentration of 25 µM curcumin was not obtained after dissolution and filtration (0.45 µm) of the samples prior to quantification by HPLC. This indicated that aggregates containing curcumin were formed upon dissolution of the lyophilisates and/or that curcumin adsorbed to the plastic syringe and/or the filter used for the filtration of the samples (0.45 µm, Spartan 13/0.45 RC filter, Schleider & Schull, Dassel, Germany). The supersaturation study confirmed the formation of supersaturated solutions of curcumin in samples containing both "spring" and "parachute" functions (cf. Figure 7). According to the results on supersaturation after 0-4 h (unpublished results) and after 8-168 h (Paper I), the solid dispersions containing curcumin and MβCD at 1:3 molar ratio appeared to produce the most physically stable supersaturated curcumin solutions. No significant reduction in curcumin concentration 0-4 h after dissolution of 1:3-HA, 1:3-HPMC or 1:3-CHA was observed (p>0.05; unpublished results).

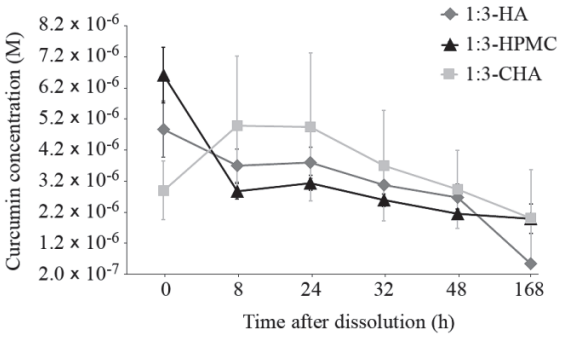

Figure 11 – Supersaturation concentrations (M) of curcumin 0-168 h after dissolution of the amorphous solid dispersions 1:3-HA, 1:3-HPMC and 1:3-CHA containing HA, HPMC or both polymers, respectively (n = 3).

Due to large standard deviations, the curcumin concentrations in the aqueous solutions of the different 1:3-solid dispersions were not significantly different at 8 h after dissolution (p>0.05; Figure 11). However, the reduction in percent of the initial curcumin concentration (at t_0) after 48 h was significantly more pronounced for dissolved 1:3-HPMC, with 33 ± 6% remaining of the curcumin concentration, than for 1:3-HA or 1:3-CHA, with 55 ± 17% and 101 ± 43% (77 ± 13% excluding one parallel with 150% curcumin concentration) remaining, respectively. The overall reduction in curcumin concentration indicated some significant

influence of HA on the metastable supersaturated state of curcumin. The results reflected the interactions between curcumin and MβCD, and between curcumin, MβCD and the polymers. More physically stable supersaturated solutions of curcumin in the presence of a three-fold increase in CD concentration demonstrated the solubilising properties of MβCD on curcumin. The presence of a slight surplus of MβCD may have shifted the inclusion complex equilibrium (Eq. 1) towards complex formation. HA behaves as an unusually stiff polymer in solution with the presence of repeating hydrophobic patches of contiguous CH groups.[99] These hydrophobic regions might have interacted with curcumin in solution, sterically hindered aggregate formation and prolonged the period before uncomplexed curcumin precipitated.

Upon dilution of curcumin-NADES solutions, no "parachute" was present to delay aggregation and precipitation of curcumin in the aqueous medium (Paper III). The supersaturated state of curcumin in solution was therefore dependent on interactions with the remaining NADES network after dilution to retain a metastable supersaturated state (cf. Figure 7). Curcumin-GS diluted 1:10 (i.e. 9.5 μM curcumin) or 1:50 (i.e. 1.8 μM curcumin) in water showed rapid precipitation of curcumin and no dissolved curcumin could be detected after 1 h (Paper III). Curcumin-MC3 appeared more robust upon dilution 1:10 (i.e. 16 μM curcumin) or 1:50 (i.e. 3.3 μM curcumin) with water, with a reduction in curcumin concentration of 82-84% after 30 min and no further reduction after 1 h. The pH of MC3 (pH 0.6) was lower than GS (pH 5.7), and both remained below pH 6 when the NADES were diluted in MilliQ water. Curcumin in solution at pH 1-6 is in the neutral form, the water solubility is generally poor, and the hydrolytic degradation rate is slow ($t_{1/2}$ > 175 d).[39, 53] Therefore, the difference in solubilising properties of diluted GS and MC3 was not due to ionisation or hydrolytic degradation of curcumin (after storage 0-1 h), but rather the intermolecular interactions between curcumin and the NADES. The main difference of the curcumin structure in diluted GS and MC3 was the presence of the diketo and enol forms, respectively. The slower precipitation of curcumin in diluted MC3 was probably due to stronger interactions between MC3 and the enol form of curcumin than between diluted GS and the diketo form of curcumin.

Changes in the Stokes' shifts were observed upon dilution of curcumin-NADES (GS and/or MC3) with water, buffers or ethanol (Paper III). The Stokes' shift of curcumin-GS was reduced (by 72 nm) upon addition of 20% ethanol. This observation was ascribed to the

perturbation of the intermolecular interactions between curcumin and GS and change from the curcumin diketo form to the enol form in the presence of increasing concentrations of ethanol (Paper III). Absorption and fluorescence spectra of curcumin-GS diluted with water or buffer could not be obtained due to the low absorbance of curcumin in diluted (≥1:10) GS. The spectroscopic characteristics of curcumin-MC3 indicated a more gradual decrease in intermolecular interactions between curcumin and MC3 upon dilution with water, buffers or ethanol (Paper III).

A rapid decrease in the supersaturation concentration of curcumin is inconvenient in aPDT unless expulsion from a diluted NADES network or CD cavity facilitates molecular interactions between the PS and the target microorganism (Figure 12; cf. Fick's first law (Eq. 2)). High antibacterial effect of curcumin in a "spring" form without a "parachute" has been reported, which suggests that a supersaturated solution of a PS may facilitate improved PS-target cell interactions compared to a crystalline form of the PS.[48] Hegge *et al.* (2012) reported a high phototoxic effect of 10 μM curcumin (29 J/cm^2 blue light) in the presence of 0.01% (w/v) MβCD, i.e. a "spring" form of curcumin.[48] However, similar results on phototoxicity were obtained with temporarily stabilised supersaturated solutions of curcumin when prepared with HPβCD as a solubiliser and HPMC as a "parachute" (~4 log reductions in viable *E. coli* after exposure to 10 μM curcumin and 28 J/cm^2 blue light).[49] For the highest bactericidal effect, the formulation should keep the PS concentration stable for a certain amount of time to delay aggregation of the PS and increase the potential for interactions between the PS and the target microorganism (Figure 12). The 1:3-solid dispersions of curcumin investigated in Paper I met this criterion. Diluted curcumin-GS and curcumin-MC3 produced supersaturated solutions of curcumin with suboptimal stability in the metastable state (Paper III).

Other NADES not investigated in this thesis might have better stabilising properties on curcumin after dilution in aqueous media. After all, at least 10^8 different NADES have been estimated to exist.[83] However, NADES with limited solubilising properties of a PS may be applied in the undiluted form (which may or may not represent a supersaturated state), assumed that close contact between the PS and the target is obtained and that the NADES is non-toxic to non-target eukaryotic cells. For optimal physical stability of curcumin in the antibacterial phototoxicity studies (see. *5.1.6. In vitro bacterial phototoxicity of curcumin*

preparations), the curcumin solid dispersions and curcumin-NADES samples were dissolved/diluted immediately before use.

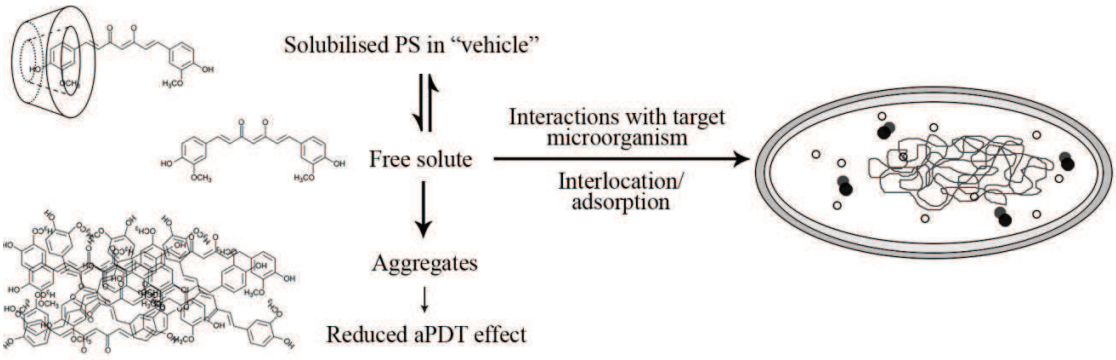

Figure 12 – Proposed equilibrium between a solubilised PS (e.g. curcumin) in an aqueous preparation (e.g. containing CD) and the free, unbound solute (PS). A hydrophobic, free PS may aggregate, leading to a reduced aPDT effect, or interact with the surface of a target microorganism, resulting in interlocation or adsorption.

5.1.5. Hydrolytic stability of curcumin in diluted NADES

Curcumin is susceptible to hydrolytic degradation, which rapidly increases with increasing pH, especially above pH 7.[39] The hydrolytic stability of curcumin dissolved in GS and MC3 followed by dilution 1:10 with phosphate buffer pH 8.0 was investigated (Paper III). Due to the organic acid content of MC3, the resulting pH of curcumin-MC3 diluted 1:10 in phosphate buffer pH 8.0 was 1.8, whereas dilution of curcumin-GS resulted in pH 8.0. The pH values obtained after dilution with phosphate buffer affected the results on hydrolytic stability, as expected since the hydrolytic stability of curcumin is much higher at pH 1.8 ($t_{1/2}$ ~208 d) than at pH 8.0 ($t_{1/2}$ 2.1 min).[39] The degradation half-life according to first-order kinetics was calculated to be 3.4 ± 0.9 h in diluted GS and 40.8 ± 8.8 h in diluted MC3 (Paper III). Thus, the hydrolytic stability of curcumin in GS diluted 1:10 in phosphate buffer at pH 8.0 was ~100 times higher than in pure buffer, while the hydrolytic stability of curcumin in diluted (1:10) MC3 was ~300 times lower than in pure buffer of similar pH. Although not fully understood, hydrolysis of curcumin is believed to occur by nucleophilic attack by OH^- ions at the carbonyl carbon in the keto-enol moiety.[111] As the *cis* enol form of curcumin is planar while the diketo

form is more twisted (i.e. more sterically hindered), this could explain the higher reactivity of curcumin towards nucleophiles in pure phosphate buffer compared to in diluted GS.[54]

The results indicated that the NADES' effect on the hydrolytic stability of curcumin after dilution with phosphate buffer pH 8.0 was dependent on the intermolecular interactions between curcumin and the NADES. While some NADES might increase the hydrolytic stability of curcumin, others might have the opposite effect. For application of curcumin-NADES samples against microbial infections, the environmental pH is usually below 7.4 (except in the intestine), and hydrolysis of curcumin *in vivo* during short-term treatment should not be a major problem.

5.1.6. *In vitro* bacterial phototoxicity of curcumin preparations

The bacterial phototoxicity of supersaturated curcumin preparations based on solid dispersions (1:1-HPMC, 1:3-HPMC, 1:3-HA and 1:3-CHA) or NADES (i.e. diluted curcumin-GS and curcumin-MC3) was investigated. The solid dispersions were selected based on the results from the study on supersaturation, which had shown that the solid dispersions containing curcumin and MβCD in the molar ratio 1:3 produced the most stable supersaturated solutions of curcumin (cf. *5.1.4. Curcumin drug delivery systems dissolved in aqueous solution*). The solid dispersions dissolved in PBS pH 6.1 to final concentrations of ≥0.25 μM curcumin combined with 11 J/cm^2 blue light induced complete eradication of the Gram-positive bacterium *E. faecalis* (Paper I). The Gram-negative bacterium *E. coli* is well known to be more tolerant to photodynamic treatment with curcumin than *E. faecalis*.[42, 46, 47] It was therefore of interest to investigate the phototoxic potential of the curcumin preparations on this bacterium as well. The most phototoxic curcumin solid dispersions (dissolved in PBS pH 6.1) combined with 32 J/cm^2 blue light towards *E. coli* were the HPMC-lyophilisates and 1:3-CHA, which induced complete eradication of the bacteria at 2.5 μM curcumin (Paper I). Addition of polymers (i.e. "parachutes") and the preparation of solid dispersions appeared to increase the phototoxic potential of curcumin compared to the simple supersaturated solution of curcumin containing only 0.01% (w/v) MβCD.[48] Additionally, solubilisation of curcumin by MβCD seemed to result in superior phototoxic properties of curcumin compared to a previously investigated curcumin solid dispersion containing HPβCD and HPMC towards the same

bacterial strain (*E. coli* ATCC 25922).[49] The solid dispersion containing HA as the only polymer (1:3-HA) produced less phototoxic preparations of curcumin, possibly due to a lack of interactions between the PS and the target bacterium due to interference by the polymer. Although it was a different formulation principle, a similar effect was seen with preparations of curcumin in PEG 400 aqueous solutions: The highest phototoxic effect of curcumin was obtained in the presence of the lowest amounts of PEG 400, which also composed the least physically stable preparations.[42] A reduced phototoxic effect was seen with higher PEG 400 concentrations (5-10%) due to sterical trapping of curcumin in PEG 400 aggregates or favourable interactions with the polymer over the bacterial membrane.[42]

Curcumin appeared even more phototoxic towards *E. coli* when dissolved in MC3 diluted in PBS (1:200) to a final concentration of 1.25 μM curcumin combined with 27 J/cm^2 blue light. This exposure induced complete eradication of *E. coli* (>6 log reductions in CFU/ml) and no toxicity in the absence of blue light (Paper III). Curcumin-GS (≤ 2.6 μM curcumin), on the other hand, showed neither dark toxicity nor phototoxicity (irradiated with 27 J/cm^2 blue light) towards *E. coli* after dilution with PBS in an attempt to break the strong interactions between curcumin and GS. Upon dilution of curcumin-NADES with PBS, rapid aggregation of curcumin was expected, as seen in the study on physical stability after dilution (cf. *5.1.4. Curcumin drug delivery systems dissolved in aqueous solution*). Therefore, smaller dilution steps of curcumin-NADES samples with PBS were initially employed. MC3 was highly toxic in the absence of light at less than 1:200 dilution (Paper III). The high toxicity of MC3 could be due to the low pH of the solutions (pH <2.8), a weakening of the bacterial outer membrane due to the solubilising ability of the NADES, or other factors involved (see *5.3. Investigation of the antimicrobial effect of NADES*). Therefore, in an attempt to observe the genuine phototoxic effect of curcumin on *E. coli*, curcumin-MC3 was diluted 1:200 with PBS (resulting in pH 2.8) to eliminate the toxicity of MC3. The seemingly enhanced phototoxic effect of curcumin in MC3 (diluted 1:200) compared to the dissolved curcumin solid dispersions (and the supersaturated curcumin reference solution in PBS containing 0.5% ethanol) could be due to weakening of the bacteria induced by MC3 – e.g. due to the low pH or solubilisation of membrane components – followed by a lethal attack of reactive photoproducts. Whether pure MC3 at 1:200 dilution reduced the bacterial survival when

combined with 27 J/cm^2 blue light was not investigated in this thesis, and cannot be excepted based on observations reported in Paper V.

Neither of the curcumin delivery systems developed for potential use in aPDT were optimal. The curcumin solid dispersions that produced temporarily stabilised supersaturated solutions might form curcumin aggregates upon dilution, which made it difficult to reach the theoretical curcumin concentration (cf. *5.1.4. Curcumin drug delivery systems dissolved in aqueous solution*). The dissolved curcumin solid dispersions were not filtered in the *in vitro* bacterial phototoxicity study, and potential aggregates were therefore not removed. However, aggregates of the PS have limited phototoxic effect due to intermolecular energy transfer and reduced excitation of the aggregated molecules, and thereby less production of photoproducts.[36, 37] Improved physical stabilisation, and thus reduced aggregation of the PS after dissolution in aqueous media, should be an aim if such solid dispersions are to be investigated further, to obtain reproducible and predictable curcumin concentrations after dissolution.

Aggregation of the PS upon dilution with PBS was also a problem for the NADES preparations investigated in Paper III. An issue with the NADES containing organic acids is the low apparent pH of the undiluted solutions (pH <1 in all the NADES containing acids listed in Table 4). Wounds heal the best at slightly acidic pH (~5.5), while pH values lower than ~3.0 might cause protein denaturation and burns.[103] Curcumin-MC3 could reduce the survival of *E. coli* in the presence of blue light, but the low pH of the system might lead to cellular toxicity *in vivo* depending on the dilution factor of the NADES and the duration of the treatment. Further studies are needed to verify the suitability of the various NADES applied at different infection sites (e.g. topical wounds or in the oral cavity).

5.2. Model porphyrin photosensitisers solubilised in NADES

TCPP was soluble in more of the NADES investigated than THPP, probably due to the higher potential for H-bond formation (Papers II and IV). The same NADES were, however, not investigated in Papers II and IV. The solubilising potential of the different NADES on THPP and TCPP are, therefore, not directly comparable. However, the hypothesis of H-

bonding potential in recurrent solubilisation of a porphyrin seem to be valid when comparing the solubility of the hydrophobic, neutral porphyrin TMP in NADES with that of TCPP (Table 5). TMP was soluble in fewer NADES (5/19) than TCPP (13/19).

Table 5 – Visual detection of solubility and resulting colour of TCPP and TMP in various NADES.

NADES	TCPP	TMP
Citric acid:sucrose (1:1)	Yes, green	No
Citric acid:xylitol (1:1)	Yes, green	Yes, green
Citric acid:D-(-)-fructose (1:1)	Yes, green	Yes, green
D-(+)-glucose:sucrose (1:1)	No	No
D-(-)-fructose:sucrose (1:1)	No	No
D-(-)-fructose:D-(+)-glucose (1:1)	No	No
Choline chloride:citric acid (1:1)	Yes, green	Yes, very pale green
Choline chloride:citric acid (2:1)	Yes, green	Yes, very pale green
Choline chloride:maleic acid (1:1)	Yes, green	No
Choline chloride:maleic acid (2:1)	Yes, green	Yes, green
Choline chloride:glycerol (1:1)	Yes, very pale red	No
Choline chloride:glycerol (3:2)	Yes, red	No
Choline chloride:D-(+)-glucose (5:2)	Yes, green	No
Choline chloride:sucrose (4:1)	Yes, very pale green	No
Choline chloride:xylitol (5:2)	Yes, green	No
Choline chloride:D-(-)-fructose (1:1)	No	No
Choline chloride:D-(-)-fructose (2:3)	No	No
Choline chloride:D-(-)-fructose (5:2)	No	No
DL-malic acid:D-(-)-fructose:D-(+)-glucose (1:1:1)	Yes, green	No

Similar to the NADES that solubilised curcumin (Paper III), no obvious relationship was found between the NADES that dissolved the porphyrins and the NADES polarity, water content after vacuum evaporation or apparent pH. All the NADES that solubilised THPP produced light or dark green liquids, independent on the NADES components being sugars, choline chloride or organic acids (Figure 13). The NADES that solubilised TCPP formed either red or green solutions (Figure 13). The colour intensity seemed to correlate with the amount of porphyrin dissolved in the NADES. Red solutions of TCPP were only formed in

NADES containing pH neutral components, while green solutions were formed in both acidic and pH neutral NADES (Paper IV and Table 5). It is currently unknown why some NADES produced red liquids (similar to solutions of the porphyrins dissolved in methanol) and some produced green liquids (similar to solutions of the porphyrins in formic acid) upon dissolution of the porphyrins, although the phenomenon is probably related to ionisation or charge redistribution due to interactions with the solvent. Intermolecular H-bonding potential and the spatial distribution of interacting groups are likely reasons for the observed solubilising effect of some NADES on THPP and TCPP.

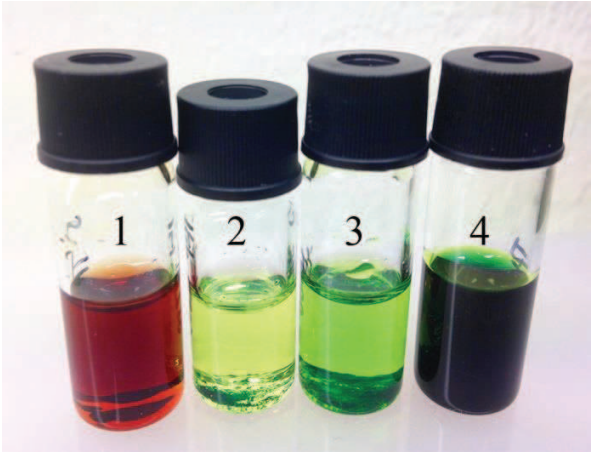

Figure 13 – Solutions of THPP in selected NADES appeared green (e.g. samples no. 2 (THPP-MC) and 4 (THPP-MG)) while samples of TCPP appeared red or green (e.g. samples no. 1 (TCPP-CG) and 3 (TCPP-CS)).

The NADES, being highly polar and miscible with aqueous media, yet having such a wide solubilisation potential (cf. *2.6.2. Natural deep eutectic solvents as solvents for photoreactive drugs intended for aPDT*), may enable the use of several PSs or other pharmaceutically active ingredients that have previously been rejected for clinical use due to poor water-solubility. Poor aqueous solubility of THPP and TCPP (and their lack of a positive charge) has been a major drawback for their use as PSs in aPDT (cf. *2.3. Formulation of products intended for topical aPDT*). Solubilisation of these porphyrins in NADES could be promising for their continued investigation as PSs intended for aPDT, especially if the porphyrins are solubilised in a monomeric state. However, the porphyrins should also remain

in the monomeric form after application where the NADES may be diluted by bodily fluids. The effect of dilution on THPP/TCPP-NADES systems is discussed in *5.2.2. Solubilisation of model porphyrins in diluted NADES*.

5.2.1. Characterisation of porphyrins solubilised in NADES

The absorption spectra of THPP and TCPP were red-shifted in 50% formic acid and in NADES compared to methanol (by 23-28 nm; Figure 14). The similarities between the absorption spectra of the porphyrins in acid and in NADES suggested the formation of the protonated porphyrin cores in both solvents.[67] The presence of J-aggregates, i.e. head-to-tail stacking of the porphyrin, also results in a red-shift of the Soret band.[112] Guo (2008) explained the red-shift of the absorption maximum of THPP in acidic environment as the formation of J-aggregates.[113] However, as observed by De Luca *et al.* (2006), the Soret band of THPP is red-shifted (23 nm) upon addition of small amounts of hydrochloric acid, corresponding to the formation of the monomeric diacid derivative ($H_2THPP(Cl)_2$), whereas larger amounts of acid lead to extensive aggregation and further red-shifts.[114] The fluorescence pattern of THPP in CS and MG did not indicate the formation of J-aggregates, due to the occurrence of a fluorescence peak at a similar wavelength (~734 nm) as H_2THPP^{2+} (formed in the presence of trifluoroacetic acid) and a fairly large Stokes' shift (Paper II).[114] THPP may form aggregates upon addition of increasing amounts of other acids (e.g. hydrochloric acid) due to intermolecular interactions between the protonated porphyrin core, a counterion and the hydroxyl group of an adjacent THPP molecule.[114] The broad Soret band of THPP and TCPP in organic acid-containing NADES diluted with PBS or 0.9% (w/v) NaCl (Papers II and IV) could be an indication of such aggregates.

TCPP molecules are more likely to aggregate than THPP due to their zwitterionic nature. The zwitterions (present in slightly acidic solutions) are likely to form aggregates through intermolecular ionic interactions between the negatively charged peripheral groups and the positively charged porphyrin core. At lower pH (~ pH 3-5), the carboxyl groups and the porphyrin core are protonated (cf. Table 2), which may result in similar J-aggregate formation as described for THPP. The apparent pH of undiluted CS, MFG and ChX were 0.3, 0.8 and 4.3, respectively, which should correspond to TCPP being positively charged

(H_2TCPP^{2+}) in CS and MFG, and the zwitterionic form in ChX (i.e. one or two protonated pyrroles and two to four deprotonated carboxyl groups; Paper IV). The absorption spectra of TCPP-CS and TCPP-MFG support this hypothesis, as they were similar to the spectrum of TCPP in 50% formic acid (Figure 14b). The peak at 443 nm of TCPP-ChX indicated that positively charged TCPP was present also in ChX, even though the apparent pH of this NADES suggested that stacking zwitterions should be dominating. This observation emphasises the importance of the intermolecular network formed in NADES.

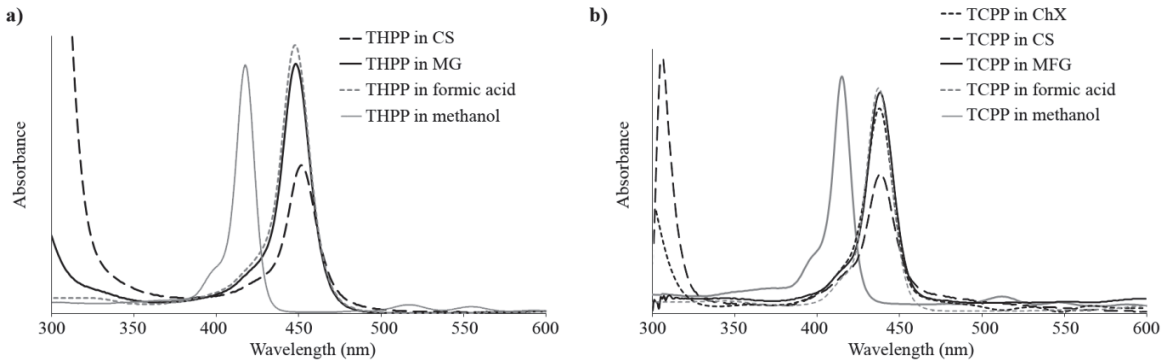

Figure 14 – Examples of absorption spectra of a) THPP and b) TCPP dissolved in various NADES (undiluted), 50% (v/v) formic acid in MilliQ water, and methanol.

5.2.2. Solubilisation of model porphyrins in diluted NADES

It has been reported that the intermolecular H-bonds of the NADES become weaker upon dilution.[85, 86] As pointed out for curcumin, it is important that porphyrins dissolved in NADES do not precipitate immediately upon contact with aqueous media that are present in the *in vitro* toxicity studies or *in vivo*. Investigation of aggregation of the porphyrins dissolved in NADES upon dilution was performed in MilliQ water (both THPP and TCPP) and in PBS (only THPP).

The absorbance at the Soret band (445 nm) remained unchanged for 7 d upon dilution of THPP-NADES (CS and MG) 1:100 with MilliQ water (Paper II). An extended study, which included quantification with HPLC after dilution with water and storage for 42 d, showed that

small amounts of THPP eventually precipitated in the NADES diluted 1:100 (Paper II). The wavelength of the Soret band of THPP solubilised in diluted (1:50-1:200) CS and MG remained unchanged (445 nm) and the reduction in absorbance was linear during storage for 42 d (20±18% reduction for the pooled samples and parallels (n=3). The reduction in absorbance during storage was not significantly different in the various diluted THPP-NADES samples (p>0.05)). Quantification of the THPP concentration in diluted THPP-NADES samples at t_0 and t_{42d} showed that the THPP concentration decreased more in the diluted THPP-CS samples (e.g. ~30% reduction in samples diluted 1:100) than in the diluted THPP-MG samples (e.g. ~5% reduction in samples diluted 1:100). The HPLC chromatograms of the stored THPP-NADES samples diluted 1:50-1:200 with MilliQ water did not display any sign of THPP degradation products. This indicated that the reduction in THPP concentration detected in the diluted THPP-NADES samples during storage was due to precipitation and not degradation. Additionally, the supersaturated state of THPP in the diluted NADES was maintained for several days, which is advantageous for the clinical use of such preparations.

However, the absorbance of THPP (at the Soret band) decreased more rapidly upon dilution (1:100) of THPP-NADES (CS and MG) with PBS (~30% reduction after 20 h; Paper II). The initial absorbance at the Soret band (at 424 nm after dilution with PBS) was reduced compared to the dilutions in water (Soret band at 445 nm). The results suggested that protonated THPP dissolved in NADES interacted with anions present in PBS. Such interactions may lead to formation of THPP aggregates which precipitate during storage (cf. *5.2.1. Characterisation of porphyrins solubilised in NADES*), as observed by the reduction in THPP absorbance. However, a reduction in absorbance of ~30% after 20 h storage of NADES preparations diluted with PBS was acceptable for *in vitro* bacterial phototoxicity studies, as these studies were completed within 2 h after dilution of the NADES preparations. If diluted NADES preparations are to be used *in vivo*, the dilution medium should be chosen *inter alia* based on the resulting osmolality of the preparation, as described in section 5.4.1 (*Osmolality of diluted NADES*). Dilution with sterile water may produce nearly isotonic preparations, and additionally result in more physically stable supersaturated solutions of a dissolved PS compared to samples diluted in buffer.

The consequences of dilution were also investigated for TCPP-CS and TCPP-MFG (Paper IV). The absorbance and fluorescence signals of TCPP-CS diluted ≥1:100 with MilliQ water were low and not possible to quantify, and the absorbance at the Soret band (440 nm) of TCPP-CS diluted 1:1 decreased quite rapidly (~62% decrease after 6 d). TCPP-MFG displayed a similar reduction in absorbance after dilution 1:1 with water and storage for 6 d (~60% decrease). The 1:100 and 1:200 diluted samples of TCPP-MFG, however, appeared to be more stable with regard to absorbance (~25% decrease after 6 d). These results indicated that TCPP was more physically stable in MFG after dilution 1:100 than in samples diluted 1:1. However, this could be ascribed to the TCPP concentration in the diluted TCPP-MFG solutions at t_0, which was 1.3 μM and 25 μM in the samples diluted 1:100 and 1:1, respectively. The decrease in TCPP concentration due to precipitation during storage may be less in samples with TCPP concentration ≤ the saturation concentration than in supersaturated samples. The solubility of TCPP was ~8 μM in MilliQ water, and below the detection limit of the HPLC system in 2.1 M malic acid (Paper IV). Investigation of TCPP precipitation following dilution of the NADES at shorter time intervals was not performed. However, the observed 20-60% reduction in absorbance 6 d after dilution of TCPP-NADES in the dilution range 1:1-1:200 suggested limited precipitation of TCPP within 2 h after dilution. This was the maximum time needed for the *in vitro* bacterial phototoxicity studies to be performed. Precipitation of THPP was more rapid in the presence of buffer, and it would probably be so for TCPP as well given the low solubility of TCPP in PBS ($< 2.82 \times 10^{-9}$ M, Paper IV). However, the results from the *in vitro* bacterial phototoxicity study indicated a high production of toxic photoproducts upon irradiation, even after dilution of TCPP-NADES 1:200-1:400 in PBS (see next section). If TCPP formed aggregates upon dilution of the TCPP-NADES solutions in buffer in the phototoxicity study, the remaining fraction of solubilised, monomeric TCPP still appeared satisfactory to maintain a high phototoxic potential (cf. Figure 12).

Dilution of porphyrin-NADES with water or PBS formed supersaturated solutions which slowly decreased in porphyrin concentration during storage at room temperature (22 °C; Papers II and IV). The partial stabilisation of the porphyrins in the supersaturated state could be attributed to the remaining interactions with the diluted NADES. For some combinations of PS and NADES, the use of an additional "parachute" may, therefore, not be necessary to prolong the supersaturated state for a period of time sufficient for application in topical aPDT.

5.2.3. *In vitro* bacterial phototoxicity of model porphyrins in NADES

Nanomolar concentrations (0.25-40 nM) of TCPP and THPP in certain organic acid-containing NADES (diluted 1:200-1:400) combined with blue light induced a large reduction in bacterial survival (>99.99% reduction in CFU/ml). These results were obtained for the Gram-positive bacteria *E. faecalis* and *S. aureus*, and the Gram-negative bacterium *E. coli* (Papers II and IV) using up to ~30 J/cm^2 blue light. Even though TCPP and THPP in the diluted NADES were in partially aggregated states, the PS molecules available for excitation must have been sufficiently excited to produce reactive photoproducts in close enough proximity to the target bacteria (cf. Figure 12). For comparison, 1 μM THPP or TCPP in PBS (containing 0.5% ethanol) could not reduce the survival of *E. coli* by more than 57% (i.e. ≤ 0.4 log reduction; Papers II and IV), even though the porphyrins were in supersaturated states also in this solvent (i.e. following dilution of their stock solutions in ethanol 1:200 with PBS; unpublished results). The reduction in effective concentration of THPP in diluted NADES was also quite large compared to the 29 μM THPP (151 J/cm^2 light dose from two unfiltered tungsten lamps) required to achieve 3 log reductions in viable *E. coli* (serotype O111/B4), even in the presence of the membrane-disorganising peptide PMNP, as reported by Nitzan *et al.* (1995).[36] Since the protocol and light source used by Nitzan *et al.* were not the same as those employed in the present thesis, the results cannot be directly compared. However, micromolar concentrations of neutral or cationic porphyrins are usually required to induce a high degree of phototoxicity in Gram-positive and Gram-negative bacteria.[26, 30, 33, 36] Indeed, in the investigation of supersaturated THPP in PBS (containing 0.5% (v/v) ethanol), 20 μM THPP induced 2.3 log reductions (corresponding to 99.5% reduction) in viable *E. coli* (Paper II). This result on *E. coli* phototoxicity was comparable to that reported by Nitzan *et al.* (1995) on THPP in the presence of PMNP.[36] That 250-500 picomolar THPP dissolved in CS and MG (1:200 with PBS, pH ~3) combined with blue light induced complete eradication of *E. coli* in its exponential phase of growth (Paper II) was encouraging for continued investigation of PS-NADES systems.

Anionic porphyrins such as TCPP have been reported to be inefficient in the photoinactivation of Gram-negative bacteria due to the permeability barrier function of the overall negatively charged outer membrane.[14, 15, 30, 33, 35, 36] The pH of the solutions of TCPP-CS, TCPP-MFG and TCPP-ChX samples diluted 1:200-1:400 in PBS (pH ~4.5-7.5) suggested

that TCPP carried a negative charge, while THPP in CS and MG diluted 1:200 in PBS (pH ~3.0) should carry a positive charge. The positive charge of THPP in the diluted organic acid-containing NADES may have contributed to the slightly higher phototoxicity observed of THPP-NADES (0.5 nM in CS (1:200) induced ~99.7% reduction in *E. coli* survival; Paper II) compared to TCPP-NADES (1 nM in CS (1:400) induced ~63% reduction in *E. coli*; Paper IV). However, another likely contributor to the observed difference in bacterial survival after exposure to THPP and TCPP dissolved in diluted NADES combined with blue light is the antibacterial effect exerted by the NADES itself. Indeed, as reported in Paper V, the toxicity of the NADES and/or their weakening effect on the bacteria, making them more susceptible to other harmful stimuli (e.g. blue light), affect the overall outcome of the photodynamic treatment. The toxicity of the pure NADES depend on their components, dilution factor and the resulting NADES concentration (Figure 15).[90, 91] CS (1:200) combined with blue light in the absence of THPP induced a 0.4 log reduction (corresponding to ~59% reduction) in CFU/ml of both *E. faecalis* and *E. coli* (Paper II). CS (1:400), however, was not toxic to *E. faecalis, S. aureus* and *E. coli*, neither in the absence nor presence of blue light (Paper IV). Therefore, the bacteria may have been more susceptible to the phototoxic effect of THPP in CS (1:200) than TCPP in CS (1:400). Note that the resulting concentration of CS after dilution reported in Figure 15 was higher (13.5 mM of each CS component after dilution 1:200) than in Paper II (10 mM of each CS component after dilution 1:200), which may have made the bacteria more susceptible to the blue light than in the latter case. These differences may be a result of slightly different water contents in the undiluted NADES used in the different studies, which were produced by the solvent evaporation method. This is discussed in section 5.4 *(Are NADES suitable as solvents for photosensitisers in aPDT?)*. A larger reduction in bacterial survival obtained after exposure to NADES in the presence of blue light compared to in the absence of light was unlikely to be caused by a phototoxic effect of the NADES. The emission from the blue light polymerisation unit was negligible at wavelengths where CS, MG, MFG and ChX diluted ≥1:200 absorbed (i.e. < 315 nm).

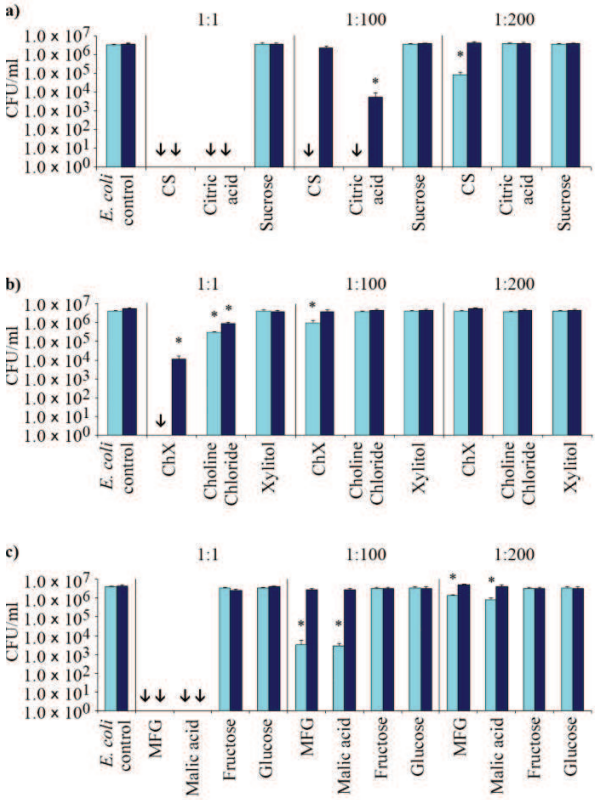

Figure 15 – Viable *E. coli* CFU/ml (+SD) after exposure to diluted a) CS, b) ChX, and c) MFG, or their individual components at equal concentrations as in the diluted NADES (n = 4). Dilutions 1:1, 1:100 and 1:200 were made in PBS. Light and dark columns represent exposure with and without 27 J/cm^2 blue light, respectively. Arrows indicate no viable bacteria detected. *) significant reduction in CFU/ml compared to the untreated control (p<0.05). See ref. 91 or Paper IV for materials and method. Adapted from ref. [91], including unpublished results.

In Paper IV, 1 nM TCPP-CS (1:400, pH ~4.5) combined with 10 J/cm^2 blue light induced 4.2 log reductions in viable *E. faecalis* (>99.99% reduction in bacterial survival). The concentration of TCPP was increased to 40 nM in CS (1:400) to induce complete eradication of *E. coli* in the presence of blue light. Even though this dilution of pure CS was not bactericidal, addition of 40 nM TCPP resulted in a significant reduction in bacterial survival after exposure in the absence of light (0.5 log reduction (corresponding to 72% reduction) in CFU/ml, p<0.05). The reduction in bacterial survival after exposure to TCPP-CS in the absence of light probably contributed to the complete eradication obtained when 27 J/cm^2 blue light was included in the treatment (Paper IV). TCPP-MFG stood out in the study on *E. coli* (Paper IV): Pure MFG diluted 1:400 with PBS did not reduce the bacterial survival after exposure in the absence or presence of light. Dark toxicity was not observed upon addition of 40 nM TCPP, but a combination of 40 nM TCPP-MFG and 27 J/cm^2 blue light induced a 96% reduction in bacterial survival (Paper IV). TCPP-MFG was significantly more phototoxic than TCPP in the acidic reference solution containing 6.8 mM citric acid in PBS (p<0.05), even

though the pH in diluted MFG was higher (pH ~5.5) than the acidic reference solution (pH ~4.0) indicating the presence of a higher fraction of anionic TCPP in diluted MFG. Additionally, the absorption spectra of the solutions were similar, but the absorbance, and thereby the spectral overlap with the emission spectrum of the light source, was higher for the acidic reference solution than for diluted TCPP-MFG (Paper IV). MFG may have increased the bacterial susceptibility to TCPP phototoxicity by increasing adsorption or uptake of the porphyrin, or by weakening the bacteria by solubilising or extracting compounds from the outer membrane. The influence of NADES on the phototoxicity of a PS was further investigated in Paper V.

In Papers II-IV, it was observed that the presence of diluted (1:200-1:400), organic acid-containing NADES could reduce the bacterial survival induced by the PS in the presence, and occasionally in the absence, of blue light compared to conventional preparations in PBS (pH 6.1-7.4). A similar observation was reported in Paper V, where 1 nM THPP prepared in CS or MFG (1:200, pH 3.8) was significantly more phototoxic to *E. coli* than THPP in PBS (Figure 16a, ~1% survival after exposure to THPP-NADES). The higher phototoxicity of THPP in the NADES compared to the pH neutral preparation reported in Paper II and Paper V may be explained by protonation of THPP in acidic solvents and/or the toxicity of the diluted NADES in the absence and/or presence of ~30 J/cm^2 blue light. As reported in Papers II and IV, the porphyrin may gain a positive charge in solvents with low pH (below pH 7), which may induce electrostatic interactions with the negatively charged bacterial surface and thereby increase the phototoxicity of the PS. It was confirmed in Paper V that the acidic NADES (CS and/or MFG) at 1:200 dilution may reduce the bacterial survival after exposure for 1.5 h (observed for *S. epidermidis* (Figure 16b), *K. pneumoniae* (Figure 17), and *P. aeruginosa* (no CFUs detected)). The acidic NADES may make the bacteria more susceptible to the potential harmful effect of the blue light (observed for *E. coli* (Figure 16a), *S. epidermidis* (Figure 16b) and *K. pneumoniae* (Figure 17; Paper V). Additionally, they may increase the dark toxicity of THPP when compared to the toxicity of the pure NADES (observed for *E. coli* (Figure 16a) and *S. epidermidis* (Figure 16b; Paper V). A combined effect of the solvent, the blue light, the dark toxic PS and/or the photoactivated PS may therefore influence the results on antimicrobial activity. The toxic effects of these individual or combined factors may affect various microorganisms differently, including different strains of the same bacterium (e.g. *E.*

coli ATCC 25922 used in Papers I-IV and BW25113 used in Paper V). In order to better understand the influence of NADES as solvents for PSs intended for aPDT, dose-response studies on these factors, including their different combinations, should be carried out.

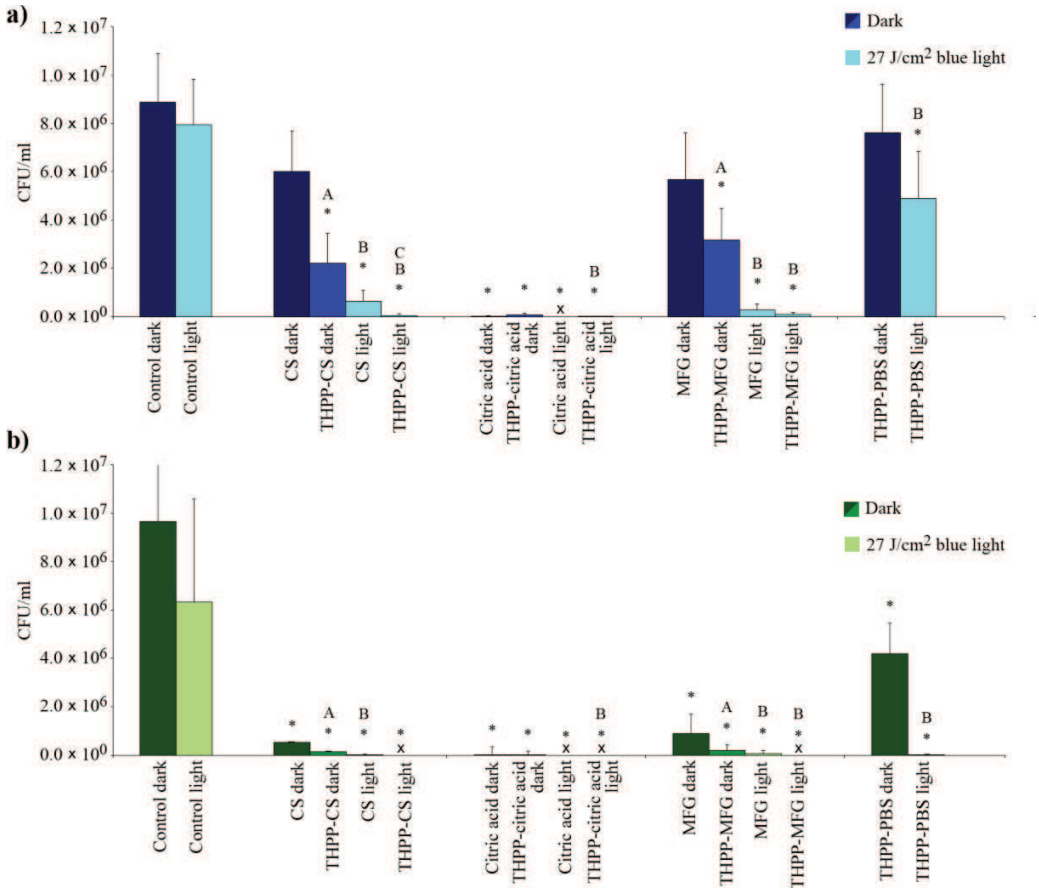

Figure 16 – Bacterial survival expressed as CFU/ml (+SD) after light protected exposure (1.5 h) or light exposure (27 J/cm^2 blue light) of a) *E. coli* and b) *S. epidermidis* to CS (1:200, pH 3.8), citric acid (14.7 mM, pH 2.9) or MFG (1:200, pH 3.8) without and with 1 nM THPP, and 1 nM THPP in PBS (0.5% (v/v) residual ethanol; n = 4×3). *) Significant reduction in CFU/ml compared to the corresponding dark or light control (p<0.05). X: no viable CFUs detected. A: significantly different bacterial survival compared to exposure to the same solvent in the absence of THPP and light (p<0.05). B: significantly different bacterial survival compared to exposure to the same sample in the absence of light (p<0.05). C: significantly different bacterial survival compared to exposure to the same solvent in the presence of light and absence of THPP (p<0.05).

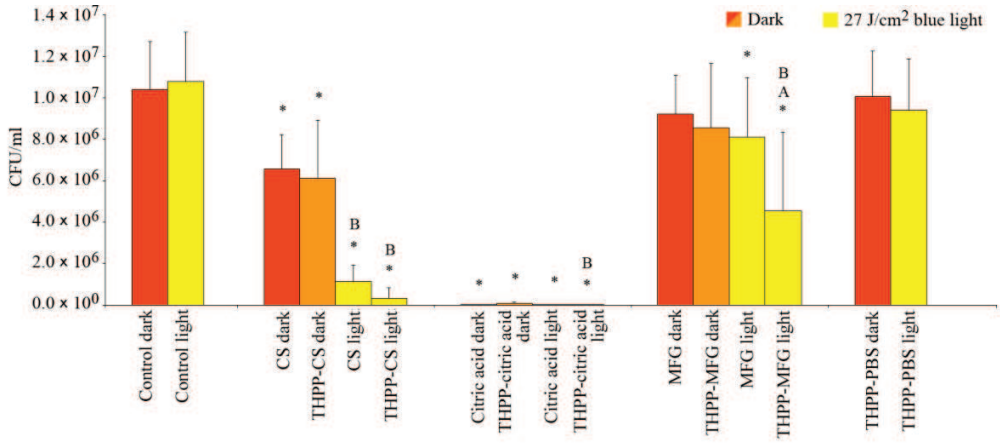

Figure 17 - Bacterial survival expressed as CFU/ml (+SD) after light protected exposure (1.5 h) or light exposure (27 J/cm^2 blue light) of *K. pneumoniae* to CS (1:200, pH 3.8), citric acid (14.7 mM, pH 2.9) or MFG (1:200, pH 3.8) without and with 1 nM THPP, and 1 nM THPP in PBS (0.5% (v/v) residual ethanol; n = 4×3). *) Significant reduction in CFU/ml compared to the corresponding dark or light control (p<0.05). A: significantly different bacterial survival compared to exposure to the same solvent in the presence of light and absence of THPP (p<0.05). B: significantly different bacterial survival compared to exposure to the same sample in the absence of light (p<0.05).

5.3. Investigation of the antimicrobial effect of NADES

The NADES that exhibit antibacterial activity themselves, without being toxic to mammalian cells, may be employed as an antibacterial treatment modality without the presence of a PS and/or visible light. Such NADES may also be valuable as solvents for a PS (undiluted or diluted) in aPDT of resilient infections, where a combined antibacterial effect from the NADES and the photoactivated PS may be seen.

The toxicity of diluted (1:200) CS and MFG in the absence of light was investigated on various Gram-positive and Gram-negative bacteria and the fungus *C. albicans* to evaluate the antimicrobial potential of these samples and differences in microbial susceptibility (Paper V). Exposures to the NADES (1:200, pH 3.8) without THPP for 1.5 h induced complete eradication *P. aeruginosa* (results not shown), reduced the number of viable CFU/ml of *S. epidermidis* by ~90-95% (Figure 16b) and *K. pneumoniae* (only CS; Figure 17) by ~37%. That only *P. aeruginosa*, and not *E. coli* and *K. pneumoniae* (Gram-negative bacteria), were more sensitive than *S. epidermidis* (Gram-positive bacterium) to the pure NADES did not fully

correspond with a previous report: Zhao *et al.* (2015) reported that Gram-negative bacteria were more sensitive than Gram-positive bacteria to acid-based DES.[90] Thus, the susceptibility of various bacteria to acid-based eutectic solvents cannot be generalised across DES and NADES exposures. The reason why *P. aeruginosa* was more sensitive to CS and MFG (1:200) than *E. coli* and *K. pneumoniae* could be related to its higher phosphate content in the outer membrane and, thereby, a dependence upon divalent cations for optimal membrane stability.[115] It was hypothesised that diluted CS and MFG, due to the chelating properties of their acidic components citric acid and malic acid, respectively,[116-119] could chelate divalent cations in the outer membrane of Gram-negative bacteria, and thus destabilise it. Through addition of excess Ca^{2+} to the samples, it was assumed that the chelating ability of the diluted NADES would be saturated, resulting in less chelation of cations in the bacterial membrane and therefore reduced antibacterial effect of both (diluted) pure NADES and THPP-NADES.[116, 118] However, no reduction in toxicity was observed upon addition of increasing amounts of Ca^{2+} to the extracellular environment of *E. coli* (Paper V). No conclusion could be drawn as to whether chelation of membrane-bound divalent cations was a contributing mechanism of action of the diluted organic acid-based NADES on *E. coli*. Chelation may be involved in the antibacterial action of NADES towards other microorganisms (e.g. *P. aeruginosa* due to its dependence upon divalent cations for optimal membrane stability)[115]. Other mechanisms involved in the antimicrobial action of NADES and their potentiating effect on some PSs may be a disruption of the intracellular pH homeostasis or solubilisation of other membrane components, since NADES may solubilise a wide range of small and large, hydrophilic and lipophilic, molecules.[70, 80-84, 88, 120] No reduction in survival of *C. albicans* was observed after exposure to any of the NADES samples with or without THPP and the presence of blue light (Paper V). Higher concentrations of PS and NADES than those intended for bacteria may be needed if fungi are to be included in the application of THPP-NADES in aPDT.[91]

It was suggested that the antibacterial and potentiating effects of certain NADES on the dissolved PSs was due to a low pH which weakened the bacteria and made them more susceptible to other factors, or due to specific actions of the individual NADES components. While a low pH of some NADES probably contributes to the antimicrobial action at low dilutions,[90] it could not be concluded that this was a main mechanism of action for CS and

MFG at 1:200 dilution, which resulted in pH 3.8 (Paper V). Exposure of *E. coli* to PBS pH 3.8 in the absence of light induced significantly less reduction in bacterial survival than CS and MFG ($p<0.05$). However, none of the exposures reduced the bacterial survival significantly compared to the control ($p>0.05$). The individual NADES components (citric and malic acid, sucrose, fructose and glucose) also resulted in bacterial toxicity different from the NADES towards *E. coli* (Paper V). Interestingly, citric acid at equimolar concentration as diluted CS (14.7 mM) was significantly more toxic than CS towards *E. coli* (Figure 16a), *S. epidermidis* (Figure 16b) and *K. pneumoniae* (Figure 17; $p<0.05$; Paper V). A higher toxicity of the acid compared to the eutectic has been ascribed to charge delocalisation of the acid in the eutectic leading to a reduction in the binding affinity between the anions and the cellular membrane.[75] However, charge delocalisation has also been reported to increase the toxicity of a mixture compared to the individual components with localised charges.[90] Therefore, the redistribution of charge of the organic acids upon incorporation in eutectic solvents do not offer a general explanation regarding the toxicity of the samples without considering the other eutectic component(s) or the specific microorganism. As described in Paper V, the difference in pH of CS diluted 1:200 (thus containing 14.7 mM citric acid; pH 3.8) and 14.7 mM citric acid in PBS (pH 2.9) was indicative of the eutectic network of CS remaining even at dilution 1:200 with PBS.

The effects that NADES have on cells have been investigated in more detail than their effect on bacteria at present. It has been reported that some NADES disrupt cell membranes and reduce cell growth, an action which may be ascribed to accumulation and aggregation of NADES on cell surfaces by strong interactions with H-bond donors (mainly) and acceptors, and nonpolar regions.[75, 77, 89] There was, however, no apparent relationship between cell toxicity and the NADES' affinity for phospholipids.[89] Due to their high polarity (Papers II-IV), NADES may solubilise proteins and polar phospholipids in the cell membrane, thus disrupting it. Indeed, an increase in cell membrane porosity and an increase in intracellular ROS concentration have been observed after *in vitro* treatment of various cell lines with diluted NADES.[79] The increase in membrane porosity after exposure to NADES may be a result of the broad solubilising potential of NADES. It is apparent that the details regarding the toxic effect of various NADES towards cells and bacteria are not fully understood at present, but new insight is continuously gained.

5.4. Are NADES suitable as solvents for photosensitisers in aPDT?

The postulated presence of NADES intracellularly in plants (e.g. to assist during drought or germination) and other species suggests their low cytotoxicity and biocompatibility.[80, 81] Based on the toxicity data of the individual NADES components, the solvents have been regarded as pharmaceutically acceptable.[70, 121] However, it is now known that the NADES may be either more or less toxic than their individual components and their individual components combined without the formation of a eutectic (Paper V).[74-79, 91]

Cytotoxicity of (NA)DES has been investigated on a wide range of cells and organisms, including human, fish and mice cell lines, brine shrimp, hydra, garlic cloves and wheat.[73-79, 89] Reports have not indicated a clear trend concerning the cytotoxicity of various eutectics and their constituents, although the (NA)DES containing organic acids appear to reduce cell and bacterial viability the most.[73, 76, 89-91] Recent reports on the cytotoxicity of NADES state that NADES in general seem to be less toxic than their DES counterparts.[79, 89] Regarding the toxic NADES, their toxicity is concentration dependent.[75, 79, 89, 91] This knowledge may be useful in the development of NADES with an acceptable cytotoxicity profile *in vivo* and a desired antibacterial effect, e.g. for pure NADES to be employed as antiseptics. The unique solubilising properties of NADES, antibacterial properties and possibly enhanced intracellular ROS accumulation during NADES exposure[77, 79] make these solvents quite interesting as solvents for PSs in aPDT. A combined antibacterial effect may be obtained through the selection of a NADES with optimal intrinsic antimicrobial activity in addition to a dissolved PS (solubilised or supersaturated). However, the *in vitro* and *in vivo* cytotoxicity of NADES, as well as their biocompatibility and –degradability, must be further evaluated. The toxic effects of NADES on bacteria and mammalian cells at different dilutions have not yet been thoroughly compared. With regard to aPDT, eukaryotic cells may have a higher tolerance than bacteria.[28, 29]

Even though the components of NADES are primary metabolites that are abundant in cells, the eutectics they form may have different properties and degrade at different rates. Not all DES can be classified as readily biodegradable,[75, 76] so this should be investigated for the related NADES as well. If cytotoxicity is an issue, pH neutral NADES may be more applicable than acidic NADES based on the generally observed toxicity of NADES containing

organic acids. Additionally, it is important to notice that *in vitro* bacterial or cell toxicity does not necessarily imply *in vivo* toxicity.[23]

From the studies on diluted NADES in Paper II-V, as well as the examples included in ref. [91], it has been concluded that the solvent evaporation method is not recommended for the production of NADES intended for pharmaceutical or microbiological research. The water content may vary in each production, resulting in NADES with slightly different properties (e.g. viscosity, polarity, bacterial toxicity). In the present work, the citric acid content (10-14.7 mM after dilution 1:200) of diluted CS (Papers II and V, and reference [91]) appeared to influence the bacterial toxicity. Therefore, the heating method described by Dai *et al.* (2013) may be more suited for preparation of NADES with a specified water content and reproducible properties.[81]

5.4.1. Osmolality of diluted NADES

Bacteria may survive in a wide range of environments with dramatic changes in extracellular osmolality.[122] Structural changes in the bacterial membrane are likely to occur following osmotic upshifts or downshifts, e.g. cell shrinkage is expected to follow an osmotic upshift due to cellular dehydration. Such structural changes may affect drug uptake.

Table 6 displays the osmolality of NADES included in bacterial studies in Papers II-V diluted in MilliQ water. The osmolality of NADES diluted less than 1:10 could not be obtained by the applied method. If the eutectics dissolved completely upon addition of more than 50% water (as suggested by Dai *et al.* (2015)),[85] the osmolality of the NADES and the individual components combined should have been fairly similar. For most of the NADES, this was not the case which indicated that the eutectic network was maintained (to some degree) upon extensive dilution with water (i.e. > 1:1). Indeed, it was found in Paper V that the eutectic network of CS and MFG even appeared to remain upon dilution 1:100 with water or 1:200 with PBS. Due to intermolecular interactions, the osmolality of the combined NADES components without the formation of eutectics were not necessarily the same as the combined osmolality of the individual components (Table 6; Paper V).

Table 6 – Osmolality (mmol/kg) ± SD of CS, GS, MC3, MG and MFG after dilution 1:10, 1:50 or 1:100 with MilliQ water, solutions of the combined NADES components without the formation of eutectics, and the individual components at similar concentrations as in the diluted NADES (n = 3). [a)] Significantly different osmolality from the related NADES at the same dilution (p<0.05). [b)] The combined values of the individual components are significantly different from the related NADES at the same dilution (p<0.05).

Sample	**1:10**	**1:50**	**1:100**
CS	781.8 ± 46.2	92.3 ± 0.6	46.3 ± 12.7
Combined C_1+S	600.3 ± 10.1[a]	104.3 ± 3.2[a]	44.3 ± 15.1
Citric acid (C_1)	376.7 ± 3.2[b]	67.3 ± 0.6[b]	37.0 ± 0.0
Sucrose (S)	246.3 ± 3.2[b]	41.0 ± 0.0[b]	7.7 ± 1.5
GS	335.0 ± 44.2	68.0 ± 1.0	29.7 ± 0.6
Combined G+S	416.7 ± 1.2	77.3 ± 1.5[a]	38.0 ± 1.0[a]
Glucose (G)	233.3 ± 1.5[b]	42.3 ± 1.2[b]	18.0 ± 3.0
Sucrose (S)	246.3 ± 3.2[b]	41.0 ± 0.0[b]	7.7 ± 1.5
MC3	1229.3 ± 39.0	243.3 ± 5.8	119.7 ±3.5
Combined M_1+C_2	1089.3 ± 0.6[a]	223.7 ± 1.5[a]	117.0 ± 1.0
Maleic acid (M_1)	272.0 ± 2.0	57.7 ± 1.5	15.0 ± 3.0[b]
Choline chloride (C_2)	1040.0 ± 21.0	187.3 ± 3.8	46.7 ± 1.2[b]
MG	856.7 ± 38.9	138.0 ± 2.0	82.7 ± 6.1
Combined M_2+G	714.0 ± 36.4[a]	132.0 ± 2.0[a]	68.3 ± 0.6
Malic acid (M_2)	554.0 ± 2.6	57.7 ± 1.5[b]	53.0 ± 1.7
Glucose (G)	233.3 ± 1.5	42.3 ± 1.2[b]	18.0 ± 3.0
MFG	804.0 ± 13.7	133.3 ± 1.5	42.3 ± 7.8
Combined M_2+F+G	1258.7 ± 12.5[a]	217.3 ± 4.9[a]	95.3 ± 0.6[a]
Malic acid (M_2)	554.0 ± 2.6[b]	57.7 ± 1.5[b]	53.0 ± 1.7[b]
Fructose (F)	323.0 ± 1.0[b]	62.0 ± 2.6[b]	30.3 ± 0.6[b]
Glucose (G)	233.3 ± 1.5[b]	42.3 ± 1.2[b]	18.0 ± 3.0[b]
PBS	302.3 ± 1.2 (undiluted)		

The NADES were diluted with PBS rather than water in the bacterial studies. Naturally, this resulted in samples of higher osmolality than similar dilutions in water, as the osmolality of the phosphate buffer used in all the bacterial studies was ~302 mmol/kg (Table 6). Upon dilution of CS and MFG 1:100 or 1:200 with PBS, the final values of osmolality appeared to be between 324-395 mmol/kg (Paper V). The osmolality of NADES diluted less than 1:100 with PBS was not investigated in this thesis. The slight increase in osmolality compared to

pure PBS might affect the bacteria or mammalian cells adversely. Immediately after an osmotic upshift, cells dehydrate and shrink. This phase is considered to last for milliseconds to minutes after the osmotic upshift.[122] After few minutes, rehydration begins, and cell growth and division is eventually resumed.[122] In the timeframe of the *in vitro* phototoxicity studies, the bacteria might have experienced slight initial dehydration followed by rehydration when exposed to NADES diluted ≥1:100 with PBS. The slight increase in osmolality after addition of diluted NADES was assumed to not have a major impact on the outcome of the *in vitro* studies, as only minor changes in *E. coli* growth rate in minimal medium was reported at such external osmolalities.[123] However, other diluted (millimolar concentrations) NADES (composed of choline chloride and fructose or glucose) have been found to shrink cancer cells, resulting in incipient necrosis and death.[79] The morphological changes observed by Mbous *et al.* (2017) were consistent with high solvent osmolality. Thus, the osmolality of an undiluted or diluted NADES may affect its cytotoxic and/or antibacterial properties.

The desired osmolality should be taken into account when choosing a medium for dilution of NADES±PS. As described in section 5.2.2 (*Solubilisation of model porphyrins in diluted NADES*), the dilution medium may affect the physical stability of a dissolved PS in NADES. Water seems to be a more suitable solvent for dilution of PS-NADES than buffers due to potentially less impact on the physical stability of a hydrophobic PS and an osmolality of the preparation closer to isotonic (if diluted ≤1:50). As addition of water or buffer would lead to different intermolecular interactions in the NADES network, the dilution medium might also affect its intrinsic antimicrobial properties. As displayed in Figure 15, the bactericidal effect of different NADES was reduced upon dilution with PBS. Whether a similar result would have been obtained by dilution with water is currently unknown, and is expected to depend on *inter alia* the resulting pH of the solutions and the osmolality.

5.4.2. Viscosity of undiluted and diluted NADES

The viscosity of the NADES (Table 7) is a result of their water content and rigid supramolecular structure reposing on a strong H-bond network.[85, 89] High viscosity may be beneficial after application of a preparation on a topical infection site, as the preparation will remain on-site even if the area is uneven or the patient moves. This applies for the use of

certain undiluted NADES (Table 7). However, high viscosity may lead to some practical problems, including time-consuming application and reduced diffusion rate of a drug through the solvent. As seen in Table 7, the viscosity of NADES is reduced by dilution with water. This has been ascribed to breakage of H-bonds between the NADES components upon addition of water, which has been confirmed by ^{1}H-NMR analysis.[85, 86]

Table 7 – Viscosity (mPa · s) ± SD of undiluted CS, GS, MC3, MG and MFG, and after dilution 1:1, 1:10 or 1:50 with MilliQ water. Included is also the viscosity of solutions of the combined NADES components without the formation of eutectics as well as of the individual NADES components at similar concentrations as in the diluted NADES (n = 3). Samples of component combinations at similar concentration as the undiluted NADES were impossible to prepare.

Sample	**Undiluted**	**1:1**	**1:10**	**1:50**
CS	2829.0 ± 66.8	7.3 ± 0.5	1.3 ± 0.2	1.0 ± 0.1
Combined C_1+S	-	12.6 ± 0.2	1.3 ± 0.1	1.0 ± 0.1
Citric acid (C_1)	6.5 ± 0.2	2.1 ± 0.1	0.8 ± 0.7	1.0 ± 0.1
Sucrose (S)	26.3 ± 3.3	3.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
GS	291.8 ± 24.3	5.2 ± 0.4	1.2 ± 0.2	1.0 ± 0.1
Combined G+S	-	7.8 ± 0.2	1.3 ± 0.1	1.0 ± 0.1
Glucose (G)	3.2 ± 0.1	1.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Sucrose (S)	26.3 ± 3.3	3.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
MC3	72.7 ± 4.4	2.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Combined M_1+C_2	-	2.8 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Maleic acid (M_1)	1.5 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
Choline chloride (C_2)	10.0 ± 0.1	1.8 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
MG	519.1 ± 42.2	5.7 ± 0.2	1.2 ± 0.1	1.0 ± 0.1
Combined M_2+G	-	4.4 ± 0.1	1.2 ± 0.2	1.0 ± 0.1
Malic acid (M_2)	6.4 ± 0.1	2.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Glucose (G)	3.2 ± 0.1	1.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
MFG	457.2 ± 5.9	5.6 ± 0.2	1.2 ± 0.1	1.0 ± 0.2
Combined M_2+F+G	-	18.1 ± 0.3	1.4 ± 0.1	1.0 ± 0.1
Malic acid (M_2)	6.4 ± 0.1	2.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Fructose (F)	6.7 ± 0.1	2.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Glucose (G)	3.2 ± 0.1	1.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.1

Taking the intrinsic water content of the NADES (prepared by solvent evaporation) into account, dilution 1:1 with water corresponded to ~61% water content, 1:10 to ~92% and 1:50 to ~98% water content. Addition of about 60% water to a NADES may produce solutions of intermediate viscosity (Table 7). Thus, specified amounts of water may be added to the NADES during (i.e. heating method) or after preparation to produce solutions with the desired viscosity. However, as discussed in section 5.2.2, addition of water may reduce the physical (and chemical) stability of a PS dissolved in NADES. If an elevated water content results in NADES with less than optimal solubilising properties of a PS, the product may be prepared and stored undiluted and diluted *ex tempore*. To prepare NADES with a low (i.e. < 20% (w/w)) and reproducible water content, the heating method is recommended (cf. *5.4. Are NADES suitable as solvents for photosensitisers in aPDT?*).

If applied to a moist infection site, the PS-NADES will be slightly diluted which may increase the diffusion of the PS from the formulation to the target microorganisms. Addition of small amounts of water prior to use may be beneficial if high solvent viscosity is the limiting factor for successful aPDT.

6. Concluding remarks

In the present thesis, solid dispersions and NADES were investigated as formulation strategies for poorly water-soluble PSs intended for aPDT. Upon dissolution of the curcumin solid dispersions and dilution of the PS-NADES, supersaturated solutions were formed. Whether the PS was present in a solubilised or a supersaturated state in the undiluted NADES is currently unknown. Among the aqueous, supersaturated solutions of curcumin, the solid dispersions stabilised the supersaturated state of curcumin to a larger extent than the diluted NADES. However, the theoretical curcumin concentration was not obtained upon dissolution of the curcumin solid dispersions. Therefore, although both the solid dispersions and curcumin-NADES were highly phototoxic towards the Gram-negative bacterium *E. coli*, neither possessed optimal characteristics for potential use in aPDT without further optimisation. Aggregation and precipitation of curcumin were regarded as the main limitations for the use of this PS in the phototoxicity studies performed in this thesis.

The preparations of THPP and TCPP in NADES were better suited for phototoxicity studies than the curcumin-NADES preparations. The decrease in the porphyrin concentration after dilution of the porphyrin-NADES was acceptable for potential *ex tempore* dilution and use in aPDT, even though no polymer was present to delay precipitation of the supersaturated solutions. The results on curcumin and the porphyrins showed that some NADES may possess both "spring" and "parachute" properties, depending on their composition, the dilution medium and the solute. The eutectic network of certain NADES investigated appeared to remain upon dilution ≤1:200 or ≤1:400 in aqueous media. However, the dilution medium must be selected based on its impact on the preparation osmolality and the physicochemical stability of the PS.

An apparent increase in phototoxic potential of the porphyrins in diluted NADES was ascribed to a supersaturated state of the PS, a positive charge obtained in the organic acid-containing NADES, and an additional weakening effect by the NADES on the bacteria. Preliminary results indicate that the antibacterial mechanism of action of different NADES is complex and cannot be explained by a simple pH effect, the antibacterial effect of the individual NADES components or by chelation of divalent cations in the bacterial membrane (studied in *E. coli*). Nevertheless, an additive or synergistic antibacterial effect may be obtained by using NADES with antibacterial properties as a solvent for a PS intended for aPDT.

An aim of this thesis was to identify the most promising strategy for the formulation of hydrophobic PSs. Overall, based on the possible additive or synergistic antibacterial effect that can be obtained by using NADES as the solvent for a PS, this formulation strategy was considered the most intriguing for further investigations. The physical and chemical stability of the NADES in combination with a dissolved PS should be further investigated to find the optimal formulation.

7. Future perspectives

NADES have great potential as antibacterial agents alone, or as solvents for hydrophobic PSs or other antimicrobial drugs. However, investigation of NADES is currently in its infancy. Only a small fraction of the estimated possible combinations (10^8) of components to produce NADES has been investigated in this thesis.[83] Further studies are required to get an overview of the solubilising potential of various NADES, their cytotoxicity, antimicrobial properties, biocompatibility and biodegradability. The optimal NADES as solvent for a PS intended for aPDT is one with high solubilising capacity, a potential to stabilise supersaturated PS solutions formed (even upon dilution), low cytotoxicity, high antibacterial effect, and with the ability to maximise the phototoxic effect of the PS.

It is of interest to characterise the intermolecular interactions between the NADES and the PS as a function of water content, e.g. by FTIR and/or ^{1}H-NMR. Such knowledge would be important in the selection of a NADES with optimal solubilising properties of a PS even upon dilution with wound exudate *in situ* or other aqueous media before application. Further, for application on topical infection sites, the preparation must be sterile. Investigation of sterilisation methods of the NADES with and without a dissolved PS should be performed.

The antimicrobial and phototoxic properties of the preparations should be investigated on microorganisms and biofilms with different resistance profiles. Such studies could potentially demonstrate the efficacy of PS-NADES in aPDT as an alternative and highly efficient antimicrobial treatment. Based on current knowledge of bacterial and cell toxicity of various (NA)DES, NADES composed of pH neutral components might possess a more acceptable toxicity profile for *in vivo* studies. Further, a dose-response study on bacteria and cells should be performed to identify the optimal combination of NADES, dilution factor, dilution medium, PS concentration and light dose. The incubation times employed, the amount of microorganisms and their cell size would also affect the outcome of the studies.

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