Formulation of photoreactive drug substances: The role of excipients and type of preparation

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Contents

Acknowledgements ................................................................................................................... iii

List of publications .................................................................................................................. 1

Abbreviations .......................................................................................................................... 2

1. Aim of the project ............................................................................................................... 3

2. Definitions ........................................................................................................................... 4

3. Introduction ......................................................................................................................... 5

3.1 Photoreactivity of the drug substance ............................................................................. 7

3.1.1 Direct and indirect photoreactions ............................................................................... 8

3.1.2 Outcome of the photoreactivity of the drug substance .................................................. 9

3.2 Formulation of photoreactive drug substances ............................................................... 15

3.2.1 Influence of the formulation on the photostability ....................................................... 15

3.2.2 Formulation of products intended for aPDT ............................................................... 19

3.2.3 Influence of excipients and type of preparation on protein-PDS interaction ............... 23

3.3 Model photoreactive drug substances ............................................................................. 24

3.3.1 Riboflavin as model of a photolabile drug substance .................................................. 24

3.3.2 Curcumin as the model of a photosensitizer for aPDT ................................................ 26

4. General experimental conditions ....................................................................................... 33

4.1 Materials .......................................................................................................................... 33

4.2 Preparation of samples .................................................................................................... 33

4.3 Methods ........................................................................................................................... 34

5. Results and discussion ....................................................................................................... 35

5.1 The physical state of the drug substance in the solid preparation .................................. 35

5.1.1 The physical state of the riboflavin as a bulk substance applied in tablets ............... 35
5.1.2 Formulation of curcumin as a solid dispersion .......................................................... 35

5.2 Photoreactivity of the drug substance ........................................................................... 40

5.2.1 Photoreactivity of riboflavin in the solid state ......................................................... 40

5.2.2 Photoreactivity of curcumin in solution ................................................................. 43

5.3 Physical stability of the curcumin in solution ............................................................... 45

5.3.1 Supersaturated solutions of curcumin prepared from the solid dispersions ............ 45

5.3.2 Precipitation inhibition of curcumin in a supersaturated solution ......................... 45

5.4 Preparations in biorelevant media: bacterial phototoxicity of curcumin and interaction with human serum albumin .......................................................................................... 47

5.4.1 Phototoxicity of curcumin towards Gram-positive and Gram-negative bacteria ...... 48

5.4.2 Formulation dependent binding of curcumin to human serum albumin................. 50

6. Conclusion ..................................................................................................................... 52

References ......................................................................................................................... 53
List of publications

The present work is based on the following papers, which are referred to by their Roman numerals:

Paper I

Vukicevic M, Randeberg LL, Boschker JE, Tybell T, Tønnesen HH (2014), Influence of crystal modification on the photoinduced color change in riboflavin, Die Pharmazie 69, 117-124

Paper II

Hegge AB, Vukicevic M, Bruzell E, Kristensen S, Tønnesen HH (2013), Solid dispersions for preparation of phototoxic supersaturated solutions for antimicrobial photodynamic therapy (aPDT) Studies on curcumin and curcuminoides L, Eur J Pharm Biopharm, 83, 1, 95-105

Paper III


Paper IV

Vukicevic M, Tønnesen HH (2015), Interaction between curcumin and human serum albumin in the presence of excipients and the effect of binding on curcumin photostability Studies on curcumin and curcuminoides LV, Pharm Dev Technol, accepted for publication, available online, doi:10.3109/10837450.2015.1016618
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPDT</td>
<td>Antimicrobial photodynamic therapy</td>
</tr>
<tr>
<td>C. albicans</td>
<td>Candida albicans</td>
</tr>
<tr>
<td>CD</td>
<td>Cyclodextrin</td>
</tr>
<tr>
<td>D-D reaction</td>
<td>Dye-Dye reaction</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>Enterococcus faecalis</td>
</tr>
<tr>
<td>ESIPT</td>
<td>Excited state intramolecular proton transfer</td>
</tr>
<tr>
<td>Ex</td>
<td>Excipients</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>G-</td>
<td>Gram-negative</td>
</tr>
<tr>
<td>G+</td>
<td>Gram-positive</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HME</td>
<td>Hot melt extrusion</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HPMC</td>
<td>Hydroxypropyl methylcellulose</td>
</tr>
<tr>
<td>HP-β-CD</td>
<td>Hydroxypropyl-β-cyclodextrin</td>
</tr>
<tr>
<td>HP-γ-CD</td>
<td>Hydroxypropyl-γ-cyclodextrin</td>
</tr>
<tr>
<td>PDS</td>
<td>Photoreactive drug substance</td>
</tr>
<tr>
<td>PI</td>
<td>Precipitation inhibitor</td>
</tr>
<tr>
<td>PP127</td>
<td>Pluronic® F127</td>
</tr>
<tr>
<td>PF68</td>
<td>Pluronic® F68</td>
</tr>
<tr>
<td>PF127</td>
<td>Pluronic® F127</td>
</tr>
<tr>
<td>PI</td>
<td>Precipitation inhibitor</td>
</tr>
<tr>
<td>PP123</td>
<td>Pluronic® P123</td>
</tr>
<tr>
<td>PS</td>
<td>Photosensitizer</td>
</tr>
<tr>
<td>PSt</td>
<td>Photostability</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RFbs</td>
<td>Riboflavin biosynthetic</td>
</tr>
<tr>
<td>RFdh</td>
<td>Riboflavin dihydrate</td>
</tr>
<tr>
<td>RFs</td>
<td>Riboflavin synthetic</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Solid dispersion</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SS</td>
<td>Solid state</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Tg</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet–visible</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffractometry</td>
</tr>
</tbody>
</table>
1. Aim of the project

The overall aim of the project was to investigate how the preparation and formulation process influence the photostability and phototoxicity of respectively a photolabile drug molecule in the solid state and a photosensitizer.

The specific aims of the work were to:

- investigate the influence of the solid state properties (crystal modification) on the photoreactivity of the photolabile drug substance riboflavin (Paper I)

- evaluate the effect of the type of preparation and excipients on the photodynamic effect of the photosensitizer curcumin (Papers II and III)

- study the effect of the excipients on the interaction of the photosensitizer curcumin with a biomolecule (human serum albumin) and the photoreactivity of the photosensitizer in biorelevant media (Paper IV).
2. Definitions

A **drug substance** is an active ingredient contained in a formulated preparation intended to provide therapeutic effect directly or indirectly.

Pharmaceutical **formulation** is the process of combining different chemical substances, including the drug substance (active ingredient) in an appropriate fashion, to produce a final preparation.

A formulated **preparation**, typically a product of a drug substance and excipients (non-drug substances), is made to administrate a drug substance to the body. This term is used interchangeably with **product**, **final product**, and **dosage form** throughout this work.

A **photosensitizer** (PS) is a light-activated drug substance which, upon absorption of light, induces a chemical or physical alteration of another chemical entity. Some photosensitizers are employed therapeutically such as in photodynamic therapy of cancer (PDT) or in the treatment of bacterial infections (aPDT).

The **photostability** of drug substances and products is understood not only as the degradation caused by exposure to radiation, but also processes such as radical formation, energy transfer and luminescence, within this work. The term is used interchangeable with **photoreactivity** throughout this work.
3. Introduction

The general aim of pharmaceutical formulation work is to achieve safe, efficient, reproducible and convenient administration of the drug substance to the body. Excipients are added in order to e.g., solubilize, stabilize, suspend, thicken, preserve, emulsify, modify dissolution, improve the compressibility or the flavor of the drug substance (1). Excipients, however, need to be compatible with the drug substance for the final product to be safe, stable and efficient.

Most drug substances are white or slightly colored, i.e., they absorb radiation in the UV or visible part of the electromagnetic spectrum (2). The photoreactivity of such drug substances can be either beneficial or undesirable. In some situations, photoreactivity can be used in photoactivated therapeutic systems. Conversely, many drugs are degraded due to exposure to visible and UV radiation. We therefore need to control the photoreactivity of drug substances. This creates additional complexity during the formulation work.

The type of preparation and the selected excipients can alter the photostability of the photoreactive drug substance (PDS) and affect the efficiency and safety of the final product (3-5). Photostability is recognized as an integral part of the stability studies during the drug development; photostability testing of the new drug substances and products are addressed in the ICH Q1B document of ICH Harmonised Tripartite Guideline (ICH stands for International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) (6).

Therapies based on light-activated substances have been established, such as photodynamic therapy, PUVA therapy (psoralen based UVA therapy) or drug release via light-sensitive drug delivery systems. To maximize the efficiency and safety of such substances or systems, novel preparations tailored for (a) the type of condition that is treated, (b) the site of application and (c) the therapy protocol, need to be developed.

The different aspects of the formulation of photoreactive drug substances that will be addressed in this work are the photostability of the drug substance in both solid and liquid form, preparations specifically designed to combat microbials (aPDT therapy) and formulation dependent bacterial phototoxicity, and interaction with human serum albumin (HSA). The structure of the thesis in relation to these issues is presented in Figure 1.
Riboflavin was selected as the model substance for examining the influence of the formulation on the photostability of a solid state PDS. This substance is usually included in vitamin dietetic supplements and its photoreactivity poses a great challenge for the formulation of the final product (7). Curcumin was studied as a model of a small, lipophilic photosensitizer (PS). The photodynamic antimicrobial activity of curcumin has been demonstrated in vitro, however its application is limited by unfavorable physico-chemical properties of the compound (8). Tailor-made pharmaceutical formulations could overcome these obstacles and facilitate clinical use of this PS. Human serum albumin was selected as a model protein to study interaction with the PS (curcumin) and the influence of excipients on this interaction.
3.1 Photoreactivity of the drug substance

No photochemical or subsequent photobiological reaction can occur unless electromagnetic radiation is absorbed, according to the First law of photochemistry postulated by Grotthus and Draper (9). The ability of a drug substance to absorb radiation is the first indication that a substance may be capable of participating in photochemical and photobiological processes. The most important chemical outcome of the absorption of UV-Vis radiation is the transition

**Figure 2.** The routes to dissipation of excitation energy. D is the drug substance, D* is the drug substance in an electronically excited state and hν is electromagnetic radiation (hν’ and hν” are photons emitted via fluorescence and phosphorescence, respectively), M is an acceptor molecule and M† is an acceptor molecule in an electronically excited state. The symbols *, ** and † indicate excited states, but not the specific quantum state.
of an electron from an orbital in the ground state into a higher energy orbital resulting in an electronically excited-state. The molecule in its excited state can exhibit a dramatically different reactivity from the ground state, not only because it possesses excess energy but also as a result of the new electronic arrangement (10). The energy associated with radiation in the UV-Vis region is of the same magnitude as certain bond energies and thus electronic excitation can cause photochemical degradation of the compound. Furthermore, the absorbed energy can be transferred to other, non-absorptive molecules (e.g., oxygen) as in photodynamic therapy. The resulting products are usually chemically distinct from the thermal degradation products of the drug molecule. Excess energy can also be lost by several other radiative or non-radiative routes (Fig. 2). As a result of intersystem crossing and intermolecular energy transfer, new excited electronic states can be formed that can take part in the processes shown in the diagram (Fig. 2).

3.1.1 Direct and indirect photoreactions

*Direct photoreactions*

Absorption of UV-Vis radiation may lead to chemical changes of the drug substance, i.e., reactions in which the reactant and products differ in chemical identity rather than in their state of excitation. This process is referred to as a direct photoreaction.

The reactivity of the molecule in an excited state is governed by the excess energy it possesses, the intrinsic reactivity of the electronic arrangement and the relative efficiencies of the different competing pathways for loss of the excess energy (9, 11). Therefore, even if the drug substance absorbs radiation strongly, a direct photoreaction is less likely to occur if it fluoresces or efficiently transfers the energy to another molecule present in the preparation. The excited state of the molecule can exhibit markedly altered chemistry compared to the molecule in the ground state, as mentioned above. Bond lengths, bond orders and bond angles in excited state molecules may be considerably different from their corresponding ground states as a consequence of the redistribution of the electron density (10).

*Indirect photoreactions (photosensitized reactions)*

In the case of an indirect or sensitized photoreaction there is a transfer of excitation energy to a molecule other than the compound which initially absorbs the radiation. The compound that absorbs the radiation, the photosensitizer, is initially transformed from its ground state (singlet
state) into a relatively long-lived excited triplet state via a short-lived excited singlet state (Fig. 2, Intersystem crossing; Fig. 3). The excited triplet state can mediate a photosensitized reaction due to the long lifetime and bi-radical nature with unpaired electron spins (11). In the type I photosensitized reactions the sensitizer can transfer an electron, including simultaneous transfer of a proton corresponding to the transfer of a hydrogen atom, to the molecules in its vicinity resulting in a free radical reaction. Energy transfer from the excited triplet state of sensitizer to the spin-matched oxygen in the ground state leads to the formation of excited singlet oxygen. This process is regarded as type II photosensitized reaction (Fig. 2, intermolecular energy transfer; Fig. 3) (12). During the energy transfer process the PS is simultaneously brought back to its singlet ground state where it in principle can take part in further sensitization cycles (13).

3.1.2 Outcome of the photoreactivity of the drug substance

*Outcome of the photoreactivity on in vitro stability and adverse drug reactions in vivo*

The photodecomposition of a large number of drug molecules in both the solution (14) and the solid state (15) has been reported. Loss of the potency can occur as a result of *in vitro* instability, leading to inactive products. Furthermore, PDS degradation may lead to adverse drug reactions due to formation of degradation products during storage (2). However, the inherent photoreactivity of the drug substance is not the only factor determining the storage stability of the final product. The substance will only degrade if it comes in contact with radiation of an appropriate wavelength. The overlap of the absorption spectrum of the drug substance with the spectral output of the incident light is described as the overlap integral, and it determines the rate of the photoreaction (11, 16). Basic understanding of photoreactivity of the compound is required to provide information for handling, packaging, labeling and use of the drug substance and the final product. In most of the cases suitable packaging provides adequate protection for PDS. However, sometimes a modification of the preparation must be considered.

The *in vivo* light-induced interactions of the drug substance with endogenous substrates can lead to adverse photosensitivity effects. Phototoxicity, one type of photosensitivity effect, is defined as an alteration of cell function by an interaction between the phototoxic compound and nonionizing radiation. A phototoxic effect can only occur if the drug substance or the phototoxic metabolite is distributed near the body surface (e.g., the eye, skin, hair or
outermost capillaries of the skin) and the absorption spectrum of the drug overlaps with the transmission spectrum of light through the tissue (17).

**Therapeutic aspects of the drug substance photoreactivity**

The ability of some compounds to cause phototoxicity *in vivo* is utilized in photodynamic therapy (PDT). This is a treatment modality that combines a PS and radiation of appropriate wavelength to treat a number of conditions e.g., cancer, age-related macular degeneration, actinic keratosis, psoriasis, and localized inflammations (especially rheumatoid arthritis) (12). PS can be applied parenterally (e.g., therapy of cancer and age-related macular degeneration) or locally (e.g., therapy of actinic keratosis, psoriasis, localized inflammations and infectious diseases). Upon absorption of radiation, the PS can undergo a type I or type II photosensitized reaction. Following the type I reaction it can react directly with endogenous substrates forming radicals. These radicals can react further with oxygen to produce reactive oxygen species (ROS). Alternatively, the type II reaction involves a direct interaction between the excited PS and the ground state oxygen resulting in singlet oxygen formation. Type I and type II reactions occur simultaneously, and the ratio between these processes depends on the type

![Figure 3. Simplified Jablonski diagram of the pathways leading to photosensitization following application of radiation to a PS. When a PS absorbs radiation it may undergo an electronic transition from the ground state ($^1$PS$_0$) to the first singlet excited state ($^1$PS$_1$). Some of the molecules are transferred to the triplet state ($^3$PS$_1$) via intersystem crossing. The triplet state is lower in energy than the singlet state, and some energy is lost in the process. The charge (type I reaction), or energy (type II reaction) may be transferred from PS to a substrate or to molecular oxygen (in a triplet state, $^3$O$_2$), to generate reactive oxygen species (ROS) or singlet oxygen ($^1$O$_2$) respectively. Adapted from (18).](image-url)
of sensitizer used, the concentrations of substrate and oxygen, as well as the binding affinity of the sensitizer for the substrate (12). The ROS and singlet state oxygen can kill microbial or cancer cells by inflicting damage on biomolecules such as proteins, unsaturated lipids, steroids and nucleic acids (Fig. 3).

Antimicrobial photodynamic therapy

PDT applied in the treatment of microbial infections is termed antimicrobial photodynamic therapy (aPDT). The delivery of the radiation to living tissue is a localized process and therefore aPDT is limited to localized as opposed to systemic infections (19). Furthermore, due to the limited penetration depth of radiation through the tissue, aPDT is mostly applied at areas of the body where radiation can be easily delivered (20). However, with the progress in optical fibre technology even deep-seated infections could potentially be treated by aPDT (21). The therapy is gaining increasing attention owing to the rise of microbial resistance to the major families of antibiotic compounds (18). Many antibiotic-resistant microbial strains have shown to be susceptible to aPDT due to the substantially different pathways of inactivation compared to that of the antibiotic and chemotherapeutic agents. No selection of the photoresistant species occurred even after multiple treatments (18, 19, 21, 22). A comparison of some of the main advantages and disadvantages of the treatment with traditional antimicrobial drugs and aPDT are presented in Table 1.
Table 1. Comparison of treatment with traditional antimicrobial drugs and antimicrobial photodynamic therapy (aPDT) (18, 19, 21-24).

<table>
<thead>
<tr>
<th></th>
<th>Traditional antimicrobial drugs</th>
<th>aPDT</th>
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<tbody>
<tr>
<td><strong>Targets</strong></td>
<td>Typically one type of biomolecule</td>
<td>Large variety of biomolecules (multi-targeted process)</td>
</tr>
<tr>
<td><strong>Resistance</strong></td>
<td>Occurs and is not limited to targeted pathogens</td>
<td>Not likely to develop due to the multi-targeted nature of the process</td>
</tr>
<tr>
<td><strong>Spectrum of action</strong></td>
<td>Typically efficient against one or two pathogen types</td>
<td>Typically broad spectrum of action: bacteria, fungi, yeasts and parasitic protozoa</td>
</tr>
<tr>
<td><strong>Effect on virulence factors</strong></td>
<td>Typically no effect on virulence factors</td>
<td>Reduction of virulence potential of bacteria</td>
</tr>
<tr>
<td><strong>Routes of application</strong></td>
<td>Oral and topical; applicable for localized and systemic infections</td>
<td>Topical; mostly applied for localized infections</td>
</tr>
<tr>
<td><strong>Side effects</strong></td>
<td>Many, including allergies and intestinal disorders</td>
<td>Limited side effects (due to a dual selectivity: PS can be targeted to microbial cells, radiation can be spatially directed to the lesion)</td>
</tr>
</tbody>
</table>

Effect of protein binding on therapeutically applied photoreactions and phototoxicity

Binding of the drug substance to blood and tissue proteins can influence its therapeutic and toxic action. Protein binding may strongly affect the biodistribution of the drug substance. The complex with protein acts as a transporter system to carry the drug substance to the site of action; this transport is extremely important for substances with low solubility in the aqueous portion of the plasma (25). The bound molecules however, cannot readily leave the
capillaries. Only the unbound molecules can be distributed to tissues and exhibit pharmacological activity as well as toxic effects. Thus, depending on the drug substance and the target, the high affinity for plasma proteins may be either beneficial or a drawback for efficacy (26). Binding to plasma proteins also affects metabolism and elimination, since both hepatic uptake and glomerular filtration are directly proportional to the free drug molecule fraction present in the plasma (25, 26).

The factors that can affect binding of drug substance to plasma proteins are concurrent administration of other drugs, excipients in the preparation, pathological conditions, age, and sex (26).

Phototoxicity is dependent upon the concentration of the sensitizer and the overlap integral between the sensitizer and the incident radiation at the site of action. Therefore, a prerequisite for the phototoxic reaction to occur is that the drug substance with sensitizing properties is distributed to the tissues that are exposed to radiation (17). Drug substances highly bound to plasma proteins usually have low apparent volume of distribution (Vd) because they are confined to the vascular space. Conversely, substances that are largely free in plasma are generally available for distribution to tissues. However, some substances that are highly bound to plasma proteins are bound with greater affinity to tissue proteins, resulting in a large Vd value. The distribution of a drug substance is a function of both plasma protein binding and tissue protein binding (26). Prolonged phototoxicity can be expected as the result of delayed elimination of the drug substance with photosensitizing properties if the substance is highly bound to plasma proteins.

The effect of the PS affinity for plasma proteins on the efficiency of photodynamic therapy depends upon the route of administration and the target tissue. Following parenteral administration PS needs to be delivered to the target tissue prior to exposure to radiation. Pharmacokinetics and biodistribution of PS can vary greatly among the different types of photosensitizers (27). These parameters can be influenced by the plasma protein binding as discussed above. Moderate binding to the plasma proteins can promote distribution of PS in the body whereas a high binding affinity may result in prolonged skin phototoxicity (28). The interval between parenteral PS administration and radiation exposure needs to be adjusted according to pharmacokinetic properties of PS to secure PDT efficacy (27).
When the PS reaches the target tissue it can be further influenced by the interaction with plasma proteins. Binding to the plasma proteins have been correlated to both increased (29) and decreased (30) uptake by the target cells. The PS-protein interaction often affects the equilibrium between monomeric and aggregated species which is particularly important for porphyrins and phthalocyanines. The interaction can promote monomerization or support self-assembling (31). Binding-induced aggregation is not a desirable process since it leads to decrease in photodynamic efficiency. Binding can alter the photophysical and photochemical properties of PS. Generally, type I reactions are more likely to occur in the presence of proteins and many PS are photodegraded much faster in medium rich in proteins or amino acids (32). The absorption spectrum of PS (porphyrines) bound to proteins can significantly deviate from the corresponding monomer in an aqueous solution. The spectral effects are due to the altered microenvironment of PS – the polarity of the protein environment is lower than that of water (31). The lifetime of the triplet state of PS bound to proteins recorded in the absence of oxygen is much longer than the corresponding lifetime of the free molecules in solution. However, production of singlet oxygen and ROS competes with other photophysical and photochemical processes in the PS-protein complex (31). It has been suggested that human serum albumin removes singlet oxygen from the sensitizing system. Sole binding of the PS should not affect the generation of ROS under the condition that the PS does not aggregate or is not bound close to protein constituents that quench the excited states (electron/energy transfer processes) (31). The photostability of PS generally decreases in the presence of proteins (33) and amino acids (34) compared to pure buffer.

The influence of physiological conditions on aPDT efficiency has been studied in vitro using biological materials and ex vivo (19, 35). Proteins present in the medium typically protect microorganisms towards aPDT (36-38). However, the actual impact of the protein rich media on the aPDT effect was shown to be dependent both upon the type of PS and microorganism (38, 39).

The following factors have been suggested as the cause of the observed effect of the proteins on the photokilling of microorganisms (37):

- proteins may absorb light and reduce the number of photons available to interact with PS molecules thereby reducing the production of ROS
proteins may compete with the microorganisms for PS, decreasing the number of PS molecules available to interact with target microorganisms

ROS have short lifetimes and, unless generated in close proximity of the cells would be unlikely to produce any cytotoxic effect. Accordingly, proteins may protect microorganisms from cytotoxic species generated in the supernatant. The culture medium has also been reported to quench singlet oxygen and free radicals produced by PS bound to protein is therefore trapped and unavailable to interact with microorganisms while the ROS produced by exposure to irradiation may more easily be quenched by proteins complexed with PS. Moreover, direct reaction of plasma proteins with the photosensitizer methylene blue was proposed as a reason for bleaching of the PS, low singlet oxygen yield, and consequently the low photoinactivation of bacteria in plasma (40). On the other hand, some of the products formed in photodynamic protein oxidation can sustain chain reactions and therefore be highly detrimental to cells (28). This mechanism could explain the recently reported improved aPDT effect of toluidine blue in serum and human serum albumin solution compared to the buffer medium (41).

3.2 Formulation of photoreactive drug substances

Formulation of the PDS can be viewed from the two main points:

- the influence of the formulation on the photostability of the PDS
- the effect of the formulation on the efficiency of the light-activated drug substance

3.2.1 Influence of the formulation on the photostability

Most photochemical reactions are affected by the immediate environment of the reacting molecule. Therefore, both the excipients and the type of preparation could influence the photoreactivity of the drug substance.

Solid preparations

In the solid state (e.g., tablets, capsules, powder) the photochemical process takes place at the surface of the dosage form. In most cases the interior of the preparation will be unaffected,
independent of exposure time (3). Therefore, photostability of PDS in a solid dosage form is not only dependent on the photochemistry of the substance in the given environment, but also on factors that will influence the depth of the light penetration, i.e., change in the absorption and reflection at the surface (e.g., particle size, shape and surface properties, color, thickness of powder bed, coating of the individual particles or dosage form) (42). Furthermore, if the photoreaction of the active ingredient results in products that are strong absorbers, only a limited overall reaction will occur (4, 15).

Crystal modification and lyophilization

The photodegradation rate of the substance as a dry powder, in addition to the above listed factors, can be affected by the physical form of the substance (crystalline or amorphous form) and the presence of impurities (3, 4). Common practice in drug development is to prepare several different crystal modifications and amorphous forms and identify the one with the most suitable properties, mainly related to bioavailability and stability. Photostability of the different crystal forms have been shown to vary greatly (43, 44). The factors that can govern photoreactivity of a substance in the solid state are specific inter- and intramolecular bonds (characteristic for each crystalline form), differences in diffusability (crystalline vs. amorphous form) and differences in water content (crystal water, adsorbed water) (3).

The factors that can influence the photostability of freeze dried products are the physical state of the substance, porosity, surface properties and the presence of residue solvent. Although both poor (45) and satisfactory (46) photostability of PDS in the freeze dried state have been reported, no comparison to the non-lyophilized drug substance was provided and therefore it is hard to determine actual influence of the lyophilization. Chongprasert et al. have shown that different crystalline forms of the drug substance were obtained depending upon lyophilization parameters which resulted in different photostability of the products (44).

Tablets (compressed dosage forms)

Aman and Thoma (4) used tablets containing the highly photosensitive drug substances nifedipine or molsidomine to investigate the influence of the formulation and tableting processes on the photostability of the products. Granulation can induce destabilization of the PDS if the substance is soluble in the granulation fluid. A residue of the granulation fluid present in tablets can cause partial dissolution of the substance and lead to increased
degradation (4). Even if the degradation is not increased, the dissolution and recrystallization of the active ingredient can lead to altered photoreactivity. Therefore, the authors suggested use of the direct compression method if the drug substance is soluble in the granulation fluid. Furthermore, high porosity caused by low compression forces did not promote photodegradation in the selected drug substances. Conversely, a relative increase in active ingredient content in the surface regions caused by high compression forces, led to more drug substance being exposed and degraded. The authors proposed that high compression forces should be avoided (4).

The physical state of the drug substance can influence the photostability of the tablets as discussed above. The production method of the bulk substance may therefore determine the photostability of the final product. Furthermore, any change in the physical state of the drug substance introduced by formulation can lead to altered photoreactivity of the final product. The particle size of the bulk substance has been shown to have significant influence on the photostability of the drug powders themselves but not of the tablets (4).

Formulation methods commonly employed to modify solubility and dissolution rate of active ingredients can interfere with the overall photostability of the product. Micronization of raw materials using milling techniques can cause a change in crystalline form or amorphization of the substance, which may alter its photoreactivity (3). Dispersions of drug substance in a carrier can have either a stabilizing or destabilizing effect depending upon the level of dispersion of active ingredient (as molecules or particles), physical state of the compound, transparency and sensitizing properties of the carrier, and mobility of the active ingredient in the solid dispersion (3).

The excipients, due to dilution and other possible shielding effects, usually decrease the effect of radiation. However, some excipients may act as photosensitizers and decrease photostability (15). Conversely, addition of a quencher (substance that can react with any photochemical intermediates ideally to produce harmless products) can stabilize PDS against the radiation (9).

Cyclodextrins (CDs) are cone shaped oligosaccharides, used for increasing stability and water solubility of drug substances by forming water-soluble complexes (47). Although there are reports of increased photostability of the drug substances by CDs in the solid state, it is not always clear whether the inclusion of the molecules in the CD cavity or simply dilution in a
physical mixture brings upon photostabilization (15). The photochemistry and photophysics within the CD cavity can be quite different from those of the uncomplexed substances. The interior of the cavity represents an isolated environment where the included substrates are usually present as single molecules restricting the photochemistry to intramolecular events. In some cases complexation with CDs can increase photodegradation. This would be likely if the guest molecule was only partially included in the CD cavity with the photosensitive region of the molecule exposed (15).

Photodegradation, one of the most obvious outcomes of the molecules’ photoreactivity, may lead to the loss of potency or adverse effects caused by decomposition products. Interaction with light may also result in the modification of physico-chemical properties, usually fading or discoloration of solid state preparations. The change in appearance is not only of aesthetic concern. Even if this is not correlated to decomposition and loss of activity it can lead to reduced compliance and discarding of qualitatively sound products.

**Liquid preparations**

Photoreactivity of the substances in solution may largely differ from that observed in the solid state and is generally more pronounced (4, 15). Secondary reactions of primary photoproducts with the solvent (typically water) can result in the formation of species that are not possible in the solid state (15). In samples containing the drug substance at a high concentration (i.e., high absorbance) the drug molecules in the inner volume of a sample will be protected from irradiation due to absorption of most of the radiation by outer layers of the solution (close to the sample surface). As a result of this process, termed inner filter effect, concentrated solutions of PDS will apparently undergo less photodegradation than diluted samples of the same compound. This may cause severe problems in formulation and application of parenteral preparations which typically contain the drug substance in a low concentration (42).

Buffers, tonicity adjusters, preservatives, bulking agents and protectants commonly present in parenteral preparations can influence photostability of the active ingredient by a number of mechanisms (5). Buffers and tonicity adjusters can influence solvation of the molecular ground or excited state leading to altered absorption of radiation or altered reactivity. Additionally, some salts are able to complex the drug substance. Interaction between primaquine and citrates is suspected to be the reason for photostabilization of the substance in citrate buffer (5). The amino acid histidine is used as a bulking agent in lyophilization and can
serve as part of the buffer system and stabilizer. Histidine is a quencher of singlet oxygen and a scavenger of hydroxyl radicals and can therefore contribute to the overall photostability of PDS in solution (5). Methyl paraben, one of the commonly used antimicrobial preservatives, has been shown to decrease the photodegradation of riboflavin phosphate in solution, probably acting like a radical scavenger (48). Antioxidants can be included in the preparations to protect the active ingredient or excipients from oxidation, if other means cannot be applied (e.g., purging of the preparation by inert gas) (5). Metabisulfite is an antioxidant used in aqueous parenteral preparations. Photochemical decomposition of epinephrine was found to be accelerated by metabisulfite (5). The reaction has a complex pathway and includes the \textit{in situ} formation of a photosensitizer by thermal (dark) reaction in the epinephrine infusion solutions (5).

Cosolvents and surfactants affect the photoreactivity of the active ingredient by changing the polarity of the reaction medium or increasing the solubility of the drug substance and thus changing the absorption properties of the compound or preparation (42).

Complexation with cyclodextrins and encapsulation in micelles are some of the common approaches for solubilization of drug substances suffering from poor water solubility. The steric constriction and microenvironment of molecules included in the cavity of CDs (e.g., polarity, specific interactions, presence of oxygen) will influence the excited state and deactivation pathways (3). Photostabilization of active ingredients by complexation with CDs has been reported in many cases. However, a destabilizing effect can also occur (3). Micellar systems consist of agglomerates of amphiphilic macromolecules with a hydrophobic core and a hydrophilic corona. Hydrophobic compounds can be solubilized in aqueous media by incorporation in the micellar core. Micelles can alter the photoreactivity of the drug substances due to changes in microenvironment (e.g., polarity, viscosity), molecular orientation as well as charge and redox properties. Furthermore, upconcentration of reactant within a small volume occurs within the micellar core. This can lead to stabilization or destabilization of the drug molecules (3).

3.2.2 Formulation of products intended for aPDT

The ideal drug delivery system for aPDT should provide selective accumulation of the PS in the target tissue, and more specifically in the target cells, with little or no uptake by non-target cells. The carrier should not cause loss or alternation of PS activity, and it should preferably
be biodegradable and have little or no immunogenicity. Most PSs suffer from high hydrophobicity and thus the delivery system should facilitate interaction with the aqueous environment. Further, the PS should be delivered in a monomeric state as aggregation of the molecules can decrease ROS production and thereby PDT effect (49). In oncological PDT the PS or its precursor is administrated intravenously and accumulated in target tissues owing to disordered metabolism and blood flow peculiar to neoplastic tissue. Targeting of the PS to sites of infection cannot be achieved by the same mechanism and the PS must be applied topically (50). The uptake by microbial cells occurs in a nonspecific manner (i.e., not mediated by a photosensitizer-specific uptake mechanism). Selectivity of aPDT therefore mainly relies on a more rapid uptake of PS by microbials than by the human cells (18). The combination of a short incubation time, low PS concentration and a low irradiation dose is desirable since it allows a selective killing of microbial pathogens under conditions in which human cells (e.g., fibroblasts or keratinocytes) are spared (51). Delivery systems with a fast release of the active ingredient immediately available for interaction with the pathogens are required to allow for a short incubation time. Furthermore, the site of action may pose specific demands on the formulation of the PS. Preparations aimed to be used in the oral cavity should be able to withstand an aqueous environment, proteins including enzymes and mechanical stress without degradation or removal. For the delivery to dry wounds a moist environment is essential for the PS to reach the target. In some cases the temperature and pH may also affect the release of the PS from the preparation (20). The protein content depends upon the site of action (e.g., wounds vs oral cavity), the state of infection (chronic vs acute infection) and type of microorganisms. Although proteins typically protect microorganisms towards aPDT, this effect may be moderated by selection of appropriate excipients in the preparation as discussed in section 3.2.3. Finally, stability of the preparation and the ease of application are important for clinical use of PS. The preparation should not influence the penetration depth/intensity of the radiation due to scattering. If this is the case the drug delivery system must be removed prior to irradiation (50).

**Supersaturated solutions**

Only PS molecules which are bound to the cells are effective in promoting phototoxic effect due to the short life time and high reactivity of ROS (52). Therefore, the delivery system for antimicrobial PS should provide sufficient interaction between the microbial cell wall or
plasma membrane and the PS. Passive diffusion of the molecule can be assessed in the context of Fick’s First low (53)

\[ J = - D \frac{dc}{dx} \]  

(1)

where \( J \) is the flux of the molecule across a plane of unit area, \( D \) is the diffusion coefficient of the molecule and \( \frac{dc}{dx} \) is the concentration gradient. The rate of transfer, for example uptake of PS in the microbial membrane per unit area and time is proportional to the concentration gradient at the membrane surface. For a poorly water soluble PS a low concentration at the membrane surface can limit the uptake. However, the concentration of the substance does not have to be limited by its equilibrium solubility. Solutions containing the drug substance in a concentration exceeding its equilibrium solubility are known as supersaturated solutions. Higuchi has first recognized the potential of supersaturation as a mean of enhancing the transport of a drug substance across a biological membrane (54). Compared to conventional solubilizing strategies, e.g., incorporation of the drug molecule into colloidal species or complexing agents, a supersaturated solution possesses a higher free drug substance concentration which may create an enhanced driving force for the uptake (55).

A higher energy form of the drug substance (compared to crystalline form) is required in order to generate a supersaturated solution. Some of the approaches include cosolvent systems and lipid-based formulation for the delivery of the substance in the solution or production of high energy solid forms (e.g., amorphous forms, crystalline salts, co-crystals). Supersaturation is a thermodynamically unstable state and therefore prone to precipitation. Supersaturated solutions need to be kinetically stabilized to be useful, i.e., precipitation needs to be temporarily inhibited.

**Precipitation inhibition**

Precipitation from supersaturated solutions consists of two steps - nucleation and crystal growth (56). Although precipitation is a thermodynamically preferred process, the nucleation step requires activation energy. As long as this energy barrier is not overcome, the metastable state of supersaturation is maintained (57). Once the nucleation is initiated, nuclei can grow to macroscopic crystals. The crystal growth follows two stages: the diffusion of molecules from the supersaturated solution to the crystal interface and integration into the crystal lattice which
is accompanied with desolvation. Precipitation inhibitors (PI) are excipients that can interfere with the nucleation and/or crystal growth and delay the precipitation from the supersaturated solution. PI may act by one or more of the following mechanisms (57):

- increasing the solubility and thus reducing the degree of supersaturation (decreasing both nucleation and crystal growth)
- increasing the viscosity (decreasing both nucleation and crystal growth)
- increasing nucleus-liquid interfacial energy (decreasing nucleation)
- changing the adsorption layer at the crystal-medium interface (e.g., by adsorbing onto the crystal surface) and hindering crystal growth
- changing the level of solvation at the crystal-liquid interface and affecting the integration of molecules into the crystal lattice

The capacity of PI to inhibit precipitation of the drug substance depends on the properties of the inhibitor, the drug substance and the medium and typically needs to be assessed for each system.

*Solid dispersion*

A supersaturated state of the substance can be achieved by dissolving a solid dispersion (SD) of the substance in aqueous medium. SDs aim to generate a supersaturated solution of poorly soluble substances by increasing apparent solubility and/or dissolution properties (57). The increase in dissolution rate and apparent solubility are achieved through reduction of the particle size, improved wetting, reduced agglomeration, changes in the physical state of the substance and possible dispersion at the molecular level (58). Change of the physical state i.e., conversion of the crystalline form to the amorphous form enhances the release of the drug substance because no energy is required to break up the crystal lattice during the dissolution process (59).

The carriers commonly used in the formulation of SDs are polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), polyvinyl alcohol, hydroxypropylmethylcellulose (HPMC), hydroxypropylcellulose, carboxymethylethylcellulose, polyacrylates, polymethacrylates, urea, sugars and their derivatives, emulsifiers and others (58, 60).
### 3.2.3 Influence of excipients and type of preparation on protein-PDS interaction

The binding affinity of a drug molecule to plasma protein (bovine serum albumin-BSA) has previously been found both to increase or decrease in the presence of micelles, depending on the model drug substance, when the substances were delivered from micellar form of the surfactant (61). The same study showed that the micellar solution did not affect the integrity of the binding site. In another study it was shown that increased protein concentration resulted in decreased stability of polymeric micelles, although micellar stability could not be linked to protein adsorption to micelles (62).

Published in vivo studies have indicated that formation of drug substance-CD complexes have negligible effect on drug pharmacokinetics (63-65). Kurkov et al. (66) have studied competitive binding of drug substances between CDs and HSA in vitro and concluded that the molecules with high affinity for both HSA and CD are likely to be affected by parenterally administered CD. Application of CDs was suggested as a mean for photostabilization (both in vitro and in vivo) and the decrease of phototoxicity of drug substances due to the observed altered photoreactivity of the PDS-CD complexes compared to uncomplexed molecules (67). There is the clear evidence that CDs do suppress the photosensitizing power of some PDS (68). However, Partyka et al. (69) have shown in an appropriate in vitro model that due to the displacement of the drug substance from CD complexes by plasma proteins, considerably weaker complexation with CDs can be expected in vivo. Reduction in phototoxicity cannot be attributed solely to the ability of the CD to complex the drug molecules. The inhibition of phototoxicity may be due to CD complexation of toxic photoproducts and trapping of radical species formed during photolysis (69).

Delivery vehicle can affect both the distribution of the substance and the availability at the site of action (i.e., bound to plasma proteins or to the delivery vehicle). The overall outcome depends on the model substance, the excipients and the type of preparation (e.g., monomeric surfactants vs micellar solution). Understanding the effect of the delivery system on the protein-drug substance interaction, combined with information about the outcome of the protein binding on the final effect (e.g., distribution in the body, target cell uptake, photoreactivity of the molecule in the bound state etc.) can help to create a delivery system with improved efficiency and decreased toxicity.
3.3 Model photoreactive drug substances

3.3.1 Riboflavin as model of a photolabile drug substance

Riboflavin (vitamin B2) is part of the vitamin B group and a frequent ingredient of dietary supplements. It belongs to the group of compounds known as flavins, based on nitrogen heterocycle 7,8-dimethylisoalloxazine (Fig. 4). Riboflavin is a yellow-orange powder that can be produced either synthetically or by a fermentation process.

Biological roles of flavins and therapeutic application of riboflavin

Flavins play an important role in aerobic metabolism due to their ability to catalyze two-electron dehydrogenation of numerous substrates and to participate in one-electron transfer to various metal centers through their free radical states (70). Flavins are cofactors for a variety of enzyme systems, such as succinate and NADH dehydrogenases, xanthine oxidase/dehydrogenase, cytochrome P450 system and nitric oxide synthase (70).

Riboflavin deficiency studied in animals resulted in reduced growth and a great variety of lesions including changes in the skin, loss of hair, degenerative changes in the nervous system and liver, impaired reproduction, and congenital malformations in offspring (71). Subclinical riboflavin deficiency in humans may be associated with an increased risk of cardiovascular disease, impaired handling of iron and night blindness (72).

The main source of riboflavin in Western diets is milk and dairy products. High concentrations of riboflavin can also be found in cereals, meats and fatty fish, and in certain fruit and vegetables (72). Riboflavin deficiency is endemic in populations who exist on diets lacking dairy products and meat (72). Riboflavin is widely used as vitamin supplement, colorant and additive in food.

Photochemical reactions of flavins

Photoreactivity of riboflavin in pharmaceutical products (73), parenteral nutrition admixtures (74-76) and food products (77), as well as photostabilization of the substance (48, 78, 79) have been the subject of extensive investigation. The focus of the present work was a specific problem which occurred after a change in production method of the riboflavin bulk substance was introduced. A switch between two qualities of bulk material (i.e., from synthetic to
biosynthetic riboflavin) induced a decrease in the photostability of riboflavin tablets. Severe discoloration was observed after inadvertent exposure to light, although the tablets were quantitatively sound (80). Investigations revealed that a particular photoreactivity of the biosynthetic bulk material caused a decrease in photostability of the tablets (81). A thorough understanding of the reaction mechanism was needed in order to reformulate and stabilize tablets containing biosynthetic riboflavin.

The absorption spectrum of riboflavin in aqueous solutions consists of four structureless peaks centered at 446, 375, 265 and 220 nm.

The three main types of photochemical reactions which flavins take part in are photoreduction, photodealkylation and photoaddition (82). Some or all of these photoreactions may occur concurrently, depending upon the structure of the flavin and the reaction conditions.

Photoreduction may occur as an intermolecular or intramolecular reaction. The overall reaction of the intermolecular reduction can be presented as follows (83):

$$\text{Fl}^* + \text{RH} \rightarrow \text{H}_2\text{Fl} + \text{R}-\text{products}$$

or

$$\text{HFlR}$$

Where Fl* is flavin in excited state, RH is a reducing agent, H2Fl is unsubstituted reduced form (1,5-dihydroflavin) and HFIR is a covalent adduct. Most reduced forms of flavins are oxidized by molecular oxygen. The unsubstituted reduced form reacts rapidly with consequent formation of hydrogen peroxide (83):

$$\text{H}_2\text{Fl} + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{Fl}$$

(3)
The mechanism of the reduction is quite complex and involves a two step reduction via a semiquinone free radical intermediate (HFl') \((82, 83)\). Some of the suggested mechanisms of reduction involve a dimeric triplet state, triplet-ground state reaction or triplet-triplet reaction:

\[ 2\text{Fl} \rightleftharpoons \text{Fl}_2 \rightarrow ^3\text{Fl}_2 \rightarrow \text{Fl}^{+} + \text{Fl}^{-} \]  
\[(4a)\]

Where \(^3\text{Fl}_2\) is a dimeric triplet state of flavins, \(\text{Fl}^{+}\) and \(\text{Fl}^{-}\) are flavin radical species. The reaction between the ground (Fl) and triplet states (\(^3\text{Fl}\)) is known as Dye-Dye reaction (D-D reaction).

\[ ^3\text{Fl} + \text{Fl} \rightarrow \text{Fl}^{+} + \text{Fl}^{-} \]  
\[(4b)\]

Semi-reduced flavin (flavosemiquinone radical) and fully reduced flavin (1,5-dihydroflavin or flavohydroquinone) can be formed starting from flavin radical species:

\[ \text{Fl}^{-} + \text{H}^{+} \rightarrow \text{HFl}' \]  
\[(5a)\]

\[ 2\text{HFl}' \rightarrow \text{H}_2\text{Fl} + \text{Fl} \]  
\[(5b)\]

Hemmerich et al. \((84)\) showed formation of flavosemiquinone radicals in solution in the absence of the electron donors (reducing agents).

Intramolecular reduction involves dehydrogenation of the hydroxyalkyl side chain on the N-10 position to yield a variety of ketonic and aldehydic products. Riboflavin intramolecular photoreduction results in a complex mixture of 2’ and 4’ keto-derivatives together with formylmerthyl flavin \((83)\). It has been suggested that for efficient intramolecular hydrogen-transfer to occur, the side chain should be co-planar with the main flavin ring-system \((82)\). Photodealkylation, a strictly intramolecular process, results in the formation of an alloxazine and an alkene. Photoaddition can occur as an intermolecular or intramolecular reaction (addition of cyanide, ammonia or water to benzenoid subnucleus of flavin, or addition of the C-2’ hydroxyl group at the C-9 position, respectively) \((82, 83)\).

### 3.3.2 Curcumin as the model of a photosensitizer for aPDT

Curcumin, a yellow pigment, is a constituent of turmeric or the rhizome of the plant *Curcuma longa L.* Turmeric has been used in the traditional Chinese and Indian medicine for thousands of years \((85)\). Curcumin is an established photosensitizer in aPDT \((45, 86-91)\). Commercially available pure curcumin is a mixture of curcumin, demetoxycurcumin and bisdemetoxycurcumin, in which curcumin represents the main constituent \((\sim 77\%)\) \((92)\).
Curcumin synthetized according to Pabon (93) was used in the present work in order to avoid interference from the two other curcuminoids present in the commercially available product.

**Physico-chemical properties of curcumin**

Curcumin (diferuloylmethane) is composed of two phenyl rings bearing the methoxy group in meta- and OH group in para- positions, symmetrically linked through the β-diketone moiety. It exists as an equilibrium mixture of tautomers (Fig. 5). The compound is considered as a small lipophilic molecule ($M_w = 368.38$, log P = 2.5) (85). It is practically insoluble in water at acidic or neutral pH values ($\sim3 \times 10^{-8}$ M at pH < 7), but soluble in alkali. However it suffers from rapid hydrolytic degradation at pH > 7 (94, 95).

![Figure 5. The two tautomeric forms of curcumin](image)

Each of the two phenolic protons and the enolic proton are ionisable, with $p$Ka values varying in the range 7.7 -10.7 (depending on the experimental conditions). At physiological pH of 7.4 75-90% of curcumin will be in the neutral state (85).

Both keto and enol tautomeric form of curcumin can exist in cis and trans conformation. In solution curcumin is predominantly present in the cis enol form which is characterized by strong intra-molecular hydrogen bond (H-bond) formation. Formation of the intramolecular H-bond leads to total π-system delocalization. In protic solvents inter-molecular H-bonds perturb the intra-molecular H-bond, while in polar aprotic solvents perturbation is likely to occur by a polarity effect (Fig. 6) (96).
Figure 6. Perturbation of intramolecular H-bond of curcumin in protic (upper reaction) and polar aprotic solvent (lower reaction). Adapted from (96).

Curcumin absorbs strongly in the UV-Vis region of the electromagnetic spectrum with the absorption maximum ($\lambda_{\text{Abs}}$) between 408 and 434 nm, depending on the solvent. In aqueous based media $\lambda_{\text{Abs}}$ ranges from 418 to 426 nm (85). The fluorescence spectrum, fluorescence maximum ($\lambda_{\text{Fl}}$) and fluorescence quantum yield ($\Phi_{\text{Fl}}$) have been found to be sensitive to the surrounding medium. The fluorescence spectral profiles are broad and structureless with the exception of spectra recorded in cyclohexane where three emission peaks occur. The central fluorescence peak in cyclohexane is at 470 nm. Redshift of $\lambda_{\text{Fl}}$ occurs in polar aprotic solvents (494-538 nm), and further redshift occurs in H-bond donating and accepting solvents (96). The fluorescence quantum yield ($0.17 \leq \Phi_{\text{Fl}} \leq 0.154$) is found to be lowest in protic media and highest in non-polar media. The non-radiative processes dominate the radiative processes; the major relaxation pathway from the $S_1$ is the excited state intramolecular proton transfer (ESIPT) which is facilitated by the tight intramolecular hydrogen bond.
Curcumin degrades upon exposure to UV-Vis radiation, both in solution and the solid state. Several degradation products are formed including vanillin, vanillic acid, ferulic aldehyde, ferulic acid, and 4-vinylguaiacol (97). Reported photodegradation quantum yields in methanol and acetonitrile are 0.021 and 0.06, respectively (96). The rate of photodegradation depends on the surrounding medium (98-100). Absorption and fluorescence maxima and fluorescence quantum yield of curcumin are affected by the binding to plasma proteins (human and bovine serum albumin) (85).

Curcumin as a photosensitizer with antimicrobial effect

The potential of curcumin as an antimicrobial and anticancer PS has been extensively studied over the past 25 years (8, 86, 88, 89, 101-104). The phototoxicity of curcumin is found to be oxygen dependent (101). The phototoxic effect is attributed to the production of ROS such as singlet oxygen ($^1\text{O}_2$) and superoxide anion ($\text{O}_2^{-}$). However, production of $^1\text{O}_2$ was barely detectable in protic solvents (96, 105) and could not be detected in the biological model involving Gram-negative (G-) bacteria (106). The role of singlet oxygen in curcumin phototoxicity is therefore not yet clear. The superoxide anion was detected upon irradiation of curcumin under both protic and non-protic conditions and may be involved in the photodynamic action of the compound (105). Studies on the uptake of curcumin by bacteria showed that the compound is adhered to G- bacteria Escherichia coli (E. coli). In Gram-positive (G+) bacteria Enterococcus faecalis (E. faecalis) curcumin is either absorbed or adsorbed to the bacterial wall (8, 106). A recent study showed that photoactivated curcumin induced damage of the membrane integrity of the G+ Staphylococcus aureus (S. aureus), leading to bacterial death (107).

Low concentrations of curcumin are sufficient to photoinactivate a broad range of pathogenic species in vitro: G+ bacteria (8, 45, 107) including methicillin resistant S. aureus (88), G- bacteria (45, 91) and fungi (87, 89). Microbial cells were found more susceptible to the phototoxic effect of curcumin than the host cells (88, 89, 108) although significant reduction of the host cells was reported (88, 89). In a recent investigation of oral candidiasis caused by Candida albicans (C. albicans) in a murine model, curcumin mediated aPDT caused significant reduction in C. albicans viability without harming the host tissue of mice. Finally, studies on healthy humans showed that curcumin combined with blue light efficiently reduced the concentration of salivary microorganisms up to 2 h post-treatment (109, 110). No major
adverse effects were reported (110). The main properties of curcumin are compared to the properties of an optimal photosensitizer in Table 2.

**Table 2.** Summary of the main properties of curcumin, compared to the properties of an optimal antimicrobial PS as described by Jori *et al.* (21).

<table>
<thead>
<tr>
<th><strong>Optimal PS</strong></th>
<th><strong>Curcumin</strong></th>
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<tr>
<td>Suitable photophysical characteristics: high extinction coefficient in the deeply tissue-penetrating wavelength regions (mainly the red and far-red spectral interval); for treatment of superficial infections also the intensely absorbed blue light (400–420 nm) can be useful. A long-lived electronically excited triplet state, a high quantum yield for the generation of reactive oxygen species (i.e., singlet oxygen) when exposed to excitation with visible light.</td>
<td>Absorbs strongly in the blue region and is, thus, suitable for superficial infections or presurgical disinfection. The role of singlet oxygen in the microbial phototoxicity is not yet clear, the compound is considered to take part in both type I and type II reactions upon irradiation.</td>
</tr>
<tr>
<td>A large affinity for the broadest possible classes of microbial cells.</td>
<td>Affinity for several classes of microbial cells has been demonstrated.</td>
</tr>
<tr>
<td>Preferential binding of the photosensitizer to the cytoplasmic membrane whose photosensitive constituents (e.g., unsaturated lipids, proteins) will consequently represent the main targets of the photoinactivation process; as a consequence cell death will mainly be caused by membrane damage rather than attack on the genetic material.</td>
<td>The cytoplasmic membrane is hypothesized to be the main target of curcumin mediated aPDT.</td>
</tr>
<tr>
<td>A broad spectrum of action on bacteria, fungi, yeasts and parasitic protozoa in order to achieve a high therapeutic effect also in the treatment of infections which are characterized by the presence of a heterogeneous flora of pathogens.</td>
<td>Action on bacteria (including G+ and G-), fungi and yeasts has been demonstrated.</td>
</tr>
</tbody>
</table>
The research on curcumin as an antimicrobial PS is currently moving from *in vitro* to *in vivo* studies. Although application of curcumin in aPDT is limited by low penetration depth of blue light in tissue (111), the broad antimicrobial spectrum and efficacy of the compound (as shown *in vitro*) and few side effects (reported in the limited number of *in vivo* studies) make it an attractive candidate for treatment of, e.g., superficial wounds and oral infections that are characterized by the presence of a heterogeneous flora of pathogens (21). Additional advantages of curcumin are: cheap and easy production and purification (93), good tolerability when administrated orally (85) and fast elimination *in vivo* after oral administration (112).

*Formulation of curcumin as solid dispersion for ex tempore preparation of supersaturated solutions*

Within this work the possibility of formulating curcumin as a solid dispersion for *ex tempore* preparation of phototoxic supersaturated solutions has been explored. The high bacterial phototoxicity of curcumin in the supersaturated state has already been shown *in vitro* (45, 86-91). However, to be beneficial clinically curcumin needs to be in a preparation which is convenient for storage and application. Therefore, readily dispersible SDs of curcumin able to generate curcumin supersaturated solutions of desired and reproducible concentration were developed. Precipitation inhibitors were included in the preparations in order to prolong the supersaturated state. The overall stability of *ex tempore* prepared supersaturated solutions (i.e., rate of precipitation and hydrolytic and photolytic stability of curcumin in the solutions) was estimated. Finally, phototoxicity against G+ and G- bacteria was tested (Papers II and III).

Solid dispersion is a beneficial formulation approach for increasing apparent solubility and dissolution properties of curcumin. The high melting point of curcumin (183°C) reflects strong crystal lattice energy and therefore a major improvement in apparent solubility can be expected upon disruption of the crystalline structure (113). Indeed, the amorphous form of curcumin possesses higher solubility compared to its crystalline counterpart (114). The carriers selected for the SDs prepared within this work were aimed to meet two requirements:

- to facilitate creation of supersaturated solutions quickly and easily upon dissolution of SDs
- to enable a prolonged metastable supersaturated state.
CDs and polymeric surfactants Pluronics® were selected for this purpose.

The common criticism towards *ex tempore* preparation of solutions aimed for aPDT is that the procedure is not practical in a clinical setting when many people may need immediate treatment. *Ex tempore* preparation can also introduce error in dose if performed incorrectly (20). Suitable dosage forms consisting of premeasured SD of the compound with or without the adequate amount of the dissolution medium may facilitate the use and secure a correct dose. Another disadvantage of solution-based topical preparations is that they are hard to keep at the site of action (20). This may be resolved by addition of a viscosity modifier or mucoadhesive agent either to the dissolution medium or to the SD.

The application of a solid preparation, on the other hand, does not require any manipulation before application and would be easier to keep at the target site. A disadvantage of such a system in this context is the dependence upon some liquid already present at the target site to facilitate dissolution. A solid preparation could also prevent the radiation from reaching the target and might need to be removed prior to irradiation.

The general advantage of solid forms over creams, gels and solutions typically used in aPDT is increased stability and thus longer shelf-life of the product. However, in the case of solid dispersions an additional concern is the physical stability of the active ingredients within the matrix. Due to the possibility of a crystallization of active ingredients resulting in markedly changed bioavailability, the number of SD based products on the market is still very limited (60). The prototypes of solid dispersions for the preparation of supersaturated solutions as presented here, could be further modified according to the particular site of action and type of infection.
4. General experimental conditions

A general description of materials, sample preparation and methods are given below. The specific experimental conditions are described in the individual papers (I-IV).

4.1 Materials

Riboflavin (both biosynthetic and synthetic sample) were generously provided as a gift by Weifa A/S (Oslo, Norway). Riboflavin dehydrate was produced by exposing a biosynthetic riboflavin sample to elevated humidity (Paper I). Curcumin was synthetized according to method described by Pabon (93).

Hydroxypropyl-β-cyclodextrin (HP-β-CD) and HPMC were used for making SDs in Paper II. In Paper III Pluronics® F127, F68 and P123 (PF127, PF68 and PP123) were employed for production of SDs; HPMC, polyethylene glycol 400 (PEG 400) and hyaluronic acid (HA) were tested as the second PI. Within the Paper IV the effect of the following excipients on the photostability and the protein binding of curcumin was studied: hydroxypropyl-γ-cyclodextrin (HP-γ-CD), HP-β-CD, PEG 400 and PF127.

4.2 Preparation of samples

Within Paper II the solid dispersions were prepared in two steps. In the first step, co-precipitates of curcumin and HP-β-CD were prepared by evaporation from methanol solutions with varying CD/curcumin molar ratios (0.5, 0.9, 1.8 and 2.8). Depending on the molar ratio, co-precipitates are referred to as 0.5, 0.9, 1.8 and 2.8. In the second step supersaturated curcumin solutions obtained by dissolving selected co-precipitates were added HPMC and freeze dried to yield the final product. Depending on the co-precipitate used for the production, lyophilizates are referred to as 0.9, 1.8 and 2.8.

The solid dispersions made within Paper III were produced by the solvent evaporation method from methanol or methanol-dichloromethane solutions. The second PI was added to the dissolution medium to evaluate its potential to stabilize the supersaturated solution.

Curcumin was added from a stock solution in methanol to the solutions containing the excipient of interest and/or human serum albumin within the Paper IV.
4.3 Methods

The following methods were applied to identify the crystalline form of riboflavin in Paper I: X-ray diffractometry (XRD), Fourier-Transform Infrared Spectroscopy (FT-IR) and scanning electron microscopy (SEM). The influence of the radiation exposure on the samples was studied by visible (Vis)-, near Infrared (NIR)-, FT-IR and fluorescence reflectance spectroscopy. Qualitative thin layer chromatography (TLC) and HPLC were employed for the detection of amino acids and the degradation products of riboflavin.

The solid dispersions and the co-precipitates of curcumin in Paper II were studied by differential scanning calorimetry (DSC), Carl-Fisher titration and SEM, to evaluate crystallinity of curcumin within SDs, morphology and the moisture content of SDs, respectively. The drug load was determined by HPLC. Solutions of solid dispersions were characterized by UV–Vis spectrophotometry and HPLC to estimate their physical stability and the hydrolytic stability of curcumin, respectively. Solutions of the selected SDs were assessed for potential phototoxicity towards E. coli.

The solid dispersions produced within Paper III were examined by XRD to determine the physical state of curcumin. The dissolved SDs with and without the second PI added to dissolution medium, were studied by UV–Vis spectrophotometry to evaluate the stability of the supersaturated curcumin solutions. HPLC was employed to study hydrolytic and photolytic stability of curcumin in the solutions. Supersaturated solutions of selected SDs were tested for phototoxic potential towards the bacterium E. faecalis.

In Paper IV the effect of excipients on curcumin-HSA binding was studied using HSA fluorescence quenching. Absorption and fluorescence emission spectra of curcumin in the solutions containing HSA, excipients or both were recorded. HPLC was employed to study the effect of excipients and HSA on curcumin photostability.

In Papers II, III and IV the samples were exposed to the radiation in the wavelength range 350–500 nm (emission maximum at 430 nm) for the photostability studies and bacterial studies.
5. Results and discussion

5.1 The physical state of the drug substance in the solid preparation

The physical state of the drug substance can govern the physico-chemical properties of the substance and the final product such as melting point, solubility, stability, density and hardness (115). Some of these properties, such as solubility and stability, play a key role in the drug formulation and the quality of the final product. As part of this project, the influence of the physical state of PDS on the apparent solubility and dissolution rate, and photostability in the solid state was studied.

5.1.1 The physical state of the riboflavin as a bulk substance applied in tablets

A change in the production method of the active ingredient induced a change in the photostability of riboflavin tablets (section 3.3.1). The two different qualities of riboflavin were examined by means of XRD and FT-IR. Both techniques indicated a difference in the crystalline structure (Paper I). Synthetic riboflavin (RFs) and biosynthetic riboflavin (RFbs) were identified as the previously described forms anhydrate I and monohydrate II respectively, by means of XRD analyses (116). Furthermore, the previously described dihydrate II was prepared by exposing RFbs to elevated humidity and identified by XRD. The FT-IR study indicated the presence of a markedly different H-bonding pattern in the two forms of riboflavin. The morphology of RFs and RFbs crystals observed by SEM and the color of the powder correlated to previously reported morphology and color of anhydrate I and monohydrate II crystals, respectively (117). A clear difference in the shape of the fluorescence emission spectra of RFs and RFbs (in the solid state) could be observed. This was ascribed to the structural differences between the two examined forms of riboflavin.

5.1.2 Formulation of curcumin as a solid dispersion

_in vitro_ studies showed that curcumin can be efficient in photoinactivation of G+ bacteria (E. faecalis) at concentrations as low as 5×10^{-7} M (combined with a radiation dose of 11 J/cm^{2}) and of G– bacteria (E. coli) at concentrations of 5×10^{-6} and 10^{-5} M (combined with a radiation dose of 32 J/cm^{2} and 16 J/cm^{2}, respectively) in an appropriate preparation (45). In a recent animal study in a murine model of oral candidiasis the most promising results of photoinactivation of C. albicans were achieved with the combination of a 8×10^{-5} M curcumin solution and a 37.5 J/cm^{2} radiation dose (90). A concentration of curcumin much higher than
the saturation solubility is required for the successful application of the compound as PS in aPDT in vivo. In Papers II and III the possibility of increasing the apparent solubility and dissolution rate by formulating curcumin as a solid dispersion was explored.

Formulation of solid dispersions of curcumin in HP-β-CD and HPMC was a two step process (Paper II, section 4.2). In the first step the co-precipitates of curcumin and HP-β-CD were prepared and investigated by DSC. A loss of curcumin crystallinity was indicated in all of the samples except for the co-precipitate with CD/curcumin ratio of 0.5. This product was not studied further. Two glass transition temperatures (Tg) were observed for the remaining co-precipitates. Neither could be superimposed on Tgs of the pure HP-β-CD or amorphous curcumin, suggesting the occurrence of inter-molecular interactions between the ingredients. The dissolution of the co-precipitates in water resulted in a supersaturated curcumin solution, as shown by the decrease in curcumin concentration upon prolonged dissolution. The concentration of dissolved curcumin depended on the CD/curcumin molar ratio of the co-precipitates (Paper II). CD-curcumin interactions may dictate the physical state of the SD and thus affect the release of the active ingredient. Once the supersaturated solution is formed, a high concentration of HP-β-CD present in solutions of co-precipitates with a high CD/curcumin ratio may offer more efficient crystallization inhibition than corresponding solutions with a low CD/curcumin ratio (58).

In the second production step lyophilizates 1.8 and 2.8 were selected for further studies based on the DSC investigations. These samples displayed uniform thermal behavior and batch to batch reproducibility. No crystalline curcumin was present in the freeze dried product, as indicated by the absence of the curcumin melting endotherm. Similar to co-precipitates, two Tgs were observed. This may indicate a mixed system comprised of the amorphous drug substance and the solid solution (118). However, the lyophilizates did not display the Tg of the amorphous curcumin. Therefore, two glass transitions may indicate the existence of two types of molecular interactions between curcumin and the carriers within the solid dispersions. Scanning electron microscopy of the lyophilizate 2.8 revealed the porous structure characteristic for freeze dried products.

Dispersion of curcumin within the polymeric carriers was made by evaporation method and investigated for the presence of crystalline curcumin by XRD (Paper III). PF127 and PP123 were combined with Pluronic®/curcumin in 0.5:1 and 1:1 molar ratio, while PF68 and
curcumin were combined in 1:1 and 2:1 molar ratio. SDs based on PP123 could not be studied by XRD for practical reasons. The remaining four SDs showed no presence of crystalline curcumin.

Solid dispersions presented here were made by use of the solvent method which has many advantages over the melting method but is associated with many ecological and economic problems. Because of this the current method of choice for the manufacturing of solid dispersions is hot melt extrusion (HME) (60). The low melting point of Pluronics® makes them suitable carriers for SD production by HME, since the technique requires one or more ‘meltable’ excipients to be included in the carrier system (119, 120). Therefore HME should be considered for scaling up the production of Pluronic® based SDs.

A combination of HP-β-CD and HPMC offered efficient crystallization inhibition of curcumin during freeze drying and amorphous final products were obtained (Paper II). The drug load of the final products was ranging from 0.5 to 1% w/w. In Paper III we show that no crystalline curcumin precipitated during fast evaporation of the common solvent at the given carrier/curcumin ratios, yielding solid dispersions with a drug load in the range 2.1 - 5.5% w/w. Within the same work the crystallinity of curcumin in the solid dispersions with the highest drug loads (i.e., 6.1 and 11.4% w/w, based on PP123) could not be determined by XRD for practical reasons. However, good dissolution properties and stability of the supersaturated solution formed upon dissolution of these SDs suggested the absence of a crystalline compound, as will be discussed later. Thus, the selected formulation methods combined with the selected carriers in the chosen ratios resulted in SDs with curcumin either transformed to an amorphous form or dispersed on a molecular level (i.e., as a solid solution). The physical state of the SDs depends on the physico-chemical properties of the carrier and active ingredient, active ingredient-carrier interactions and the formulation method. When prepared by the solvent method the physical state of SD depends on the temperature, the evaporation rate and the type of solvent (58). Curcumin can be considered an intermediate crystallizer according to the classification system of Van Eerdenbrugh et al. (113, 121). Therefore, it was not possible to obtain the amorphous curcumin by evaporation (Paper II), and curcumin-Pluronic® interactions were essential for the transformation of the physical state of the compound (Paper III). Wegiel et al. (113) have recently studied the physical stability of amorphous curcumin SDs and concluded that, for a compound like curcumin which forms intramolecular H-bonds, formation of H-bonding with the carrier is hindered and thus it is
more difficult to inhibit crystallization. The carrier of choice for the curcumin SDs should allow the formation of interactions other than H-bonds (113). Even though both cyclodextrins and Pluronics® may form H-bonds with curcumin the main types of interactions are inclusion complex formation (CDs) and hydrophobic interactions (Pluronics®) (98, 122). Thus, regarding the production and stability of SDs, as well as inhibition of the formed supersaturated solution upon dissolution, these carriers are good candidates for solid dispersions of curcumin.

Table 3. Main properties of the selected curcumin solid dispersions. Lyophilizate number indicates CD/curcumin molar ratio of co-precipitate used for the production. SD - solid dispersion. Confer Paper III for further details on SD1, SD5 and SD7.

<table>
<thead>
<tr>
<th>Solid dispersion</th>
<th>lyophilizate 1.8</th>
<th>lyophilizate 2.8</th>
<th>SD1</th>
<th>SD5</th>
<th>SD7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Excipients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP-β-CD and HPMC</td>
<td>HP-β-CD and HPMC</td>
<td>Pluronic® F127</td>
<td>Pluronic® P123</td>
<td>Pluronic® F68 and HPMC</td>
<td></td>
</tr>
<tr>
<td><strong>Drug load (% w/w)</strong></td>
<td>0.8</td>
<td>1</td>
<td>5.5</td>
<td>11.4</td>
<td>3.8 *</td>
</tr>
<tr>
<td><strong>Production method</strong></td>
<td>Freeze drying of supersaturated aqueous solution</td>
<td>Fast evaporation of methanol solution</td>
<td>Fast evaporation of methanol-dichloromethane solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dissolution properties</strong></td>
<td>Excellent</td>
<td>Very good</td>
<td>Good</td>
<td>Very good</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hydrated solid dispersion (10⁻⁵ M curcumin solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitation/degradation after 24h</td>
</tr>
<tr>
<td>Hydrolytic stability</td>
</tr>
</tbody>
</table>

* Theoretical drug load, not confirmed by HPLC. (Paper III)

** Decrease in curcumin concentration in the solution ascribed to precipitation and/or agglomeration.

Dissolution of the curcumin solid dispersions

Lyophilizates 1.8 and 2.8 containing HP-β-CD and HPMC (Paper II) could be hydrated within 5 and 3 s, respectively, to yield ~10⁻⁵ M curcumin solutions. Some flaky particles did however, remain at the surface of the solutions after the hydration of lyophilizates. They disappeared within a few seconds when the solutions were gently stirred. The presence of two types of
curcumin-carrier interactions within the SDs as suggested by the presence of two Tgs may explain this behavior.

Dissolution of SDs containing PF127 and PF123 based SDs resulted in $10^{-5}$ M curcumin solutions corresponding to the theoretical amount of the substance in the dissolved state. PF68 based SDs were as easily dissolved but did not yield in the theoretical curcumin concentration (Table 3).

The good dissolution properties of these SDs could be explained by good dissolution properties of the carriers, particle size reduction, change in the physical state of active ingredient and possibly dispersion at the molecular level (58). When the drug substance is dispersed at molecular level, the dissolution rate is determined by the dissolution rate of the carrier (60). Freeze dried SDs additionally benefit from their porous structure and the amorphous state of the carriers (i.e., HP-β-CD and HPMC) and display better dissolution properties than Pluronic® based SDs.

A short incubation time prior to irradiation is favored in aPDT. Thus preparations with a fast drug release are required. The lyophilizates hydrated almost instantaneously, resulting in a clear solution. They were proposed for application on infected, moist wounds (Paper II). SDs based on Pluronics® are suggested to be used after hydration in the form of a supersaturated solution (Paper III). Pluronics® display a complex phase behavior. Depending on the temperature, polymer concentration and the type of polymer (polyethylene oxide/polypropylene oxide composition ratio), unimer, micellar, cubic (gel), hexagonal and lamellar phases can be observed (123). The drug release from each of these states differs. Depending upon viscosity of the preparation the light scattering/absorption could pose the problem if the preparation is not removed prior to exposure to radiation. Neither the amount of dissolution media (wound liquid/saliva) nor the temperature of the infected area can be controlled. Supersaturated solutions containing polymer in a submicellar concentration and prepared immediately before use, should be applied to control the release of curcumin from Pluronics® containing preparations.
5.2 Photoreactivity of the drug substance

5.2.1 Photoreactivity of riboflavin in the solid state

Photoinduced color change of riboflavin was observed for both qualities of the substance and the dihydrate produced for the purpose of the study in Paper I (Fig. 7). However, each riboflavin modification followed different patterns of the color change. RFbs showed the most pronounced initial color change but reversible after storage in the dark. RFs exhibited a less pronounced and irreversible color change. The color change of RFdh was the least pronounced but reversible. The surface color of RFdh continued to change throughout the measurements. This was ascribed to drying of the surface layers and transformation back to the riboflavin modification used for production of the dihydrate (monohydrate II). The color of the bulk material changed towards green upon exposure to light in all cases. No new products were detected by HPLC analyses of the light exposed samples (RFs and RFbs). It was therefore assumed that the change causing the discoloration was reversible by dissolution of the samples in water prior to the HPLC analyses. The green products could therefore only be observed in the solid state. It is also possible that the quantity of the new formed green product was below the detection limit of the HPLC method. The fluorescence intensity at the emission maximum decreased upon exposure to light for all the examined riboflavin samples (RFbs, RFs and RFdh). The fluorescence intensity of RFbs and RFdh was restored after 24 h storage in the dark (Paper I).
Figure 7. The total color change ($\Delta E_a b^*$) of synthetic riboflavin (RFs), biosynthetic riboflavin (RFbs) and riboflavin dihydrate II (RFdh) as a function of the lag-time between light exposure and detection of the surface color. The values for the dark controls are subtracted.

Photoreduction of the riboflavin molecule may explain the color change towards green upon exposure to light. Recovery of original color in the case of RFbs and RFdh may be due to re-oxidation in the presence of oxygen. The decrease in fluorescence signal indicates the reduction of riboflavin. The reduced riboflavin exhibits weak fluorescence compared to the strong fluorescence from the oxidized riboflavin (124). Furthermore, reduced forms of riboflavin (flavohydroquinone and flavosemi-quinon) are both green and are prone to re-oxidation in the presence of molecular oxygen (82, 125). The green crystals of reduced riboflavin produced by Ebitani et al., slowly oxidized to the yellow riboflavin when stored in air and could not be characterized by HPLC due to immediate conversion to the oxidized form upon dissolution (124).

It was first suspected that an external electron donor, e.g., trace amounts of amino acids that may be present in the biosynthetic sample, could cause the photoreduction and extensive color change of RFbs. However, no evidence supporting this claim was found (Paper I).
Photoreduction in the absence of an external electron donor could proceed via a dimeric triplet state, triplet-triplet or D-D reaction, in the presence of H⁺ (82, 83). Alternatively, intramolecular photoreduction may lead to reduction of the isoalloxazine nucleus and formation of ketonic and aldehydic functions in the ribityl side chain (see section 3.3.1).

Although FT-IR and near infrared spectroscopy were employed to study the structural changes upon exposure to light, no conclusive results were obtained. Spectral changes potentially indicated irreversible intramolecular photoreduction of RFs although a strong evidence for the reduction of RFbs was not found. Our hypothesis on photoreduction was however, supported by the results obtained by Penzkofer (126). He studied the blue-light photoreduction of riboflavin and lumiflavin in solid starch (α-amylose) films and obtained results comparable to our findings. Photo-excitation of the flavin doped starch films caused repeatable, reversible reduction of flavins, as shown by the decrease in absorption, fluorescence and phosphorescence, which recovered in the dark. The authors suggested that starch acted as the reducing donor. The thereby generated oxystarch was the oxidizing agent of reduced flavins. Furthermore, hydrogen abstraction from the ribityl side chain is thought to contribute to the isoalloxazine nucleus reduction in the case of riboflavin in starch and the reason for the incomplete re-oxidation in the dark.

The observed differences between RFs and RFbs in our study are most likely due to a difference in their crystalline structure. Some of the factors controlling solid state reactions are the separation distance, mutual orientation and the space symmetry of reactive functional groups. Furthermore, the conformation of the reacting molecule is dictated by the crystalline structure. In solid state reactions the intrinsic reactivity of a molecule is thought to be less important than the nature of the packaging of the neighboring molecules of the reactant (127).

The course of the photoinduced reaction (e.g., D-D vs intramolecular reduction) can be governed by the mutual orientation of the molecules and inter- and intramolecular bonds in the crystal lattices of RFs and RFbs. A different availability of H⁺ required for formation of flavosemiquinone in a D-D or triplet-triplet reaction, can be expected in RFs (anhydrate) and RFbs (monohydrate).

The crystal modification of riboflavin should be controlled either by controlling the production parameters or by recrystallization of the bulk material during the production of the bulk riboflavin material, in order to control photostability of the final product. Alternatively,
solid dispersions of riboflavin in an appropriate carrier can separate riboflavin molecules and prevent the inter- and intramolecular interactions that may lead to photoreaction.

5.2.2 Photoreactivity of curcumin in solution

The photostability of curcumin in Pluronic® (F127, P123 and F68) solutions obtained from dissolution of SDs was studied within Paper III. The curcumin photostability in Pluronic® (F127), CD (HP-γ-CD) and PEG 400 solution of varying excipient concentration was examined in Paper IV.

Curcumin (10⁻⁵ M) exhibited very low photostability in the presence of submicellar concentrations of PF127 or PP123 (5×10⁻⁶ M) whereas a slightly better photostability was observed in the presence of PF68 (10⁻⁵ M) and HPMC (0.001% w/v) (Paper III). A better photostability in the PF68 solution was correlated to a more rapid agglomeration of the compound observed in this solution compared to PF127 or PP123 solutions.

Poor curcumin photostability was not limited to a low Pluronic® concentration. Curcumin solutions (5×10⁻⁶ M) containing 1.5×10⁻⁴, 2×10⁻³ or 4×10⁻³ M PF127 (i.e., above CMC) were also rapidly degraded upon exposure to radiation (Paper IV).

HP-γ-CD and PEG 400 offered increased protection against photodegradation. Solutions of curcumin (5×10⁻⁶ M) containing HP-γ-CD or PEG 400 in the range 0.05 - 5% w/v exhibited comparable photostability, and considerably better than observed in Pluronic® solutions (Paper IV).

Curcumin has previously been shown to be photochemically more stable in H-bonding media (e.g., alcohols, ethanol/buffer) than in polar non-H-bonding media (chloroform, acetonitrile, ethyl acetate) (94, 96, 97, 100). Solubilization of curcumin in micellar or CD solutions seems to have a destabilizing effect compared to that of the free molecule in alcohol or in alcohol/buffer solution (94, 100).

Photochemical decomposition of curcumin competes with other deactivation routes for the loss of excitation energy, i.e., radiative and non-radiative photophysical processes (Fig. 2). The deactivation pathways intersystem crossing and photodecomposition are not dominant in polar solvents (H-bonding and non-H-bonding) in case of curcumin (96). The ESIPT is the leading non-radiative decay mechanism of S₁ and it is faster when curcumin is in the
intramolecular H-bonded cis enol form. In H-bonding solvents formation of H-bonds between curcumin and solvent molecules results in the perturbation of intramolecular H-bonds and thereby prevents direct ESIPT (Fig. 6). The fast-decaying intramolecular H-bonded enol structure can be formed only after desolvation. However, alternative decay mechanism can be the delivery of excitation energy to the solvent molecules, which is expected to be highly enhanced when intermolecular H-bonds are formed. On the other hand, polar non-H-bonding solvents can also efficiently perturb intramolecular H-bonds and inhibit deactivation through ESIPT (Fig. 6), but their interactions with curcumin do not introduce new decay mechanisms. More pronounced photodecomposition of curcumin in polar non-H-bonding than in H-bonding environments is probably the consequence of the less efficient non-radiative deactivation in the first case.

Very low curcumin photostability in the Pluronic® solutions could be explained by analogy with polar non-H-bonding solvents. Ghosh et al. (129) suggested that the ESIPT mediated decay channel of curcumin is inhibited in Tween® 20, Tween® 80, Pluronics® P123 and F127 micelles. Furthermore, the authors suggested that the decrease of ESIPT rate observed in polymeric micelles compared to micelles of conventional surfactants was a result of the interaction between curcumin and the lone pair oxygen of oxyethilene moiety (128, 129). Addition of cosolvents (PEG 400 or ethanol, 20% v/v) to micellar solutions of Pluronics® (P123, F127 or P85) was correlated to the transfer of curcumin from the core of the micelles to the bulk solvent and resulted in slightly increased photostability of curcumin, as reported by Singh et al. (98).

Increased photostability of curcumin in HP-γ-CD and PEG 400 solutions compared to Pluronic® solutions could be rationalized by a more efficient S1 deactivation pathway through energy transfer to solvent and/or solubilizer molecules. Fluorescence emission studies suggested that curcumin may be in a H-bonding environment in these media (Paper IV). However, other factors can influence the behavior of the excited state since the complexation with HP-γ-CD may determine curcumin conformation and tautomeric form (130).

Although CDs and PEG offer better photostabilization of curcumin compared to Pluronics®, other aspects of the formulation should also be considered such as phototoxicity of curcumin and inhibition of precipitation. Furthermore, the demands regarding the drug photostability depend upon the type of the preparation. Solid dispersions of curcumin as suggested to be
used in aPDT either in the solid state or after rehydration with an aqueous medium, could easily be protected from light exposure during the storage period, both before and after rehydration. Further, as a part of aPDT protocol, the application site could also be protected.

### 5.3 Physical stability of the curcumin in solution

Solid dispersions of curcumin were prepared within Papers II and III in order to increase the apparent solubility and dissolution rate of curcumin as discussed above. Solid dispersions typically yield supersaturated solutions of the active ingredient upon dissolution which are thermodynamically unstable systems prone to precipitation (55, 58). Following administration the drug substance must remain in solution to ensure a high and reproducible interaction with the target such as the bacterial membrane in the case of aPDT. Thus, precipitation of curcumin from hydrated SDs needs to be delayed for the relevant time period.

#### 5.3.1 Supersaturated solutions of curcumin prepared from the solid dispersions

The decrease in curcumin concentration in the solutions of lyophilizates 1.8 and 2.8 (theoretical curcumin concentration of $1 \times 10^{-5}$ and $2.5 \times 10^{-5}$ M) upon storage (up to 168 h) and the decrease in UV-Vis absorbance of the solutions during 24 h storage suggested the agglomeration and precipitation of the substance since no indications of hydrolytic degradation products were found by HPLC (Paper II). Furthermore, the shape of the absorption spectrum of curcumin in dissolved lyophilizate indicated the presence of the free curcumin molecule rather than the curcumin-HP-β-CD complex.

A decrease in curcumin concentration and UV-Vis absorbance upon storage (2, 8 and 24 h) were also observed for dissolved Pluronic®-based SDs (Paper III).

Thus, both the HP-β-CD-HPMC based SDs prepared within Paper II and Pluronic® based SDs prepared in Paper III can generate supersaturated solutions of curcumin.

#### 5.3.2 Precipitation inhibition of curcumin in a supersaturated solution

Solutions of lyophilizates 1.8 and 2.8 equivalent to $10^{-5}$ M curcumin exhibited a decrease in curcumin concentration $\geq 18\%$ within 24 h (Paper II). Solutions of PF127 and PP123 based SDs ($10^{-5}$ M curcumin) showed a decrease in curcumin concentration $\geq 10\%$ during the 24 h storage whereas solutions of PF68 based SDs exhibited much more pronounced curcumin precipitation, comparable to that of a supersaturated solution without PI. The capability of the
second PI (HPMC, PEG 400 and HA) to improve the physical stability of curcumin supersaturation obtained from Pluronic® based SDs was evaluated. HPMC showed the best results and SD based on PF68 was reformulated by addition of this excipient. However, this SD was shown to be hydrolytically unstable in solution and was therefore not studied further (Paper III).

In general, PF127 and PP123 based SDs showed better physical stability in the solution than HP-β-CD-HPMC based SDs, despite a 5-10 times higher drug load in the former (Table 3). Thus, PF127 and PP123 can be considered better precipitation inhibitors for curcumin than the combination of HP-β-CD and HPMC. It is difficult however, to distinguish between the contribution of each of the excipients included in the lyophilizates to the stability of the supersaturated solutions generated from these lyophilizates.

HP-β-CD and HPMC have been proposed to interact with an active ingredient by establishing H-bonds which increase the activation energy of the crystal nucleation. Furthermore, due to the ability to form H-bonds these excipients can adsorb to the crystal surface and prevent the crystal growth (57, 131, 132). Similarly, Pluronics® are suggested to adsorb to the crystal surface and inhibit crystal nucleation and growth (133-135). However, a hydrophobic reaction between Pluronics® and the active ingredient is expected to be the main driving force behind the precipitation inhibition mechanism (134). HP-β-CD and Pluronics® can improve the solvation of the drug substance in solution and thereby increase the activation energy for desolvation during the crystal growth (55, 133, 136). Finally, HP-β-CD and Pluronics® can solubilize the drug substance and decrease the degree of supersaturation (137, 138). Curcumin is however, not expected to be in the solubilized state in the examined SD solutions and the delay of precipitation is not likely due to solubilization. Dissolved lyophilizates probably contain curcumin in a free form rather than complexed with HP-β-CD, as indicated by the UV-Vis spectra. Pluronic® based SD solutions contain submicellar concentrations of Pluronics® and therefore curcumin is most likely in the molecular state rather than encapsulated in micelles.

The mechanisms suggested to explain precipitation inhibition by HP-β-CD and HPMC on the one side and Pluronics® on the other, largely overlap. However, the main difference seems to be the type of interactions that these excipients form with the substance i.e., H-bonds (HP-β-CD and HPMC) or hydrophobic interaction (Pluronics®). It has been suggested that the
Excipients mainly forming H-bonds with curcumin are not optimal carriers to inhibit crystallization of amorphous curcumin in the solid dispersions due to the strong intramolecular H-bonds in curcumin (113). Curcumin forms intramolecular H-bonds in the solution as well as in the solid state. Therefore Pluronics® F127 and P123 may inhibit precipitation of dissolved curcumin more efficiently than the combination of HP-β-CD and HPMC because the former mainly form hydrophobic bonds with curcumin. PF127 and PP123 were found to be more efficient in stabilizing supersaturation than PF68, likely due to the larger size of hydrophobic polypropylene oxide block of the two former copolymers (Paper III). This emphasizes the importance of the hydrophobic interactions between the excipients and curcumin for the physical stability of a supersaturated solution.

The various supersaturated solutions formed from the SDs may not have the same individual requirements for the physical stability. SDs based on the Pluronics® need to be rehydrated prior to application (as discussed in the section 5.1.2) and thus the curcumin solutions need to be stable both during the short term storage and the in vivo incubation time. Application of the SDs based on combined HP-β-CD-HPMC in the solid state requires only the stabilization of the curcumin supersaturation during the in vivo incubation time.

5.4 Preparations in biorelevant media: bacterial phototoxicity of curcumin and interaction with human serum albumin

Sensitivity of bacteria to photodynamic inactivation by curcumin in vitro was shown to be dependent upon the formulation. A similar effect could be expected under physiological conditions (8, 45, 139). It is therefore essential to optimize the formulation in regard to interaction with bacterial cells prior to in vivo studies. Dai et al. (19) pointed out that for aPDT to be clinically useful a combination of PS and light needs to be able to kill microbial cells in the environment found in actual infections. A number of in vitro studies showed the reduction of the aPDT effect in protein rich media (e.g., serum, saliva, albumin suspensions). The decrease in PS molecules available for interaction with bacteria due to competitive binding to the proteins present in the media was suggested as one of the causes of the described effect (37). It is therefore important to investigate how the formulation will affect the protein-curcumin binding.
5.4.1 Phototoxicity of curcumin towards Gram-positive and Gram-negative bacteria

Encouraging in vitro aPDT results and the recently reported in vivo study emphasize the potential of curcumin as a PS (88-90, 108). The required concentration of curcumin in these studies was achieved by use of DMSO (10%) or ethanol as a cosolvent. DMSO is immediately being absorbed through human tissues and it can carry dissolved substances with it across the membranes. This is undesirable in the case of aPDT where local action is the main aim. Furthermore, high concentration of DMSO can have a damaging effect on the tissue and use of this solvent as an enhancer produces a foul odour on the breath (140). Therefore alternative formulation approaches should be found for the application of curcumin in a clinical setting. A number of formulations with curcumin solubilized by use of cyclodextrins, micelles and polymers were developed to avoid use of DMSO or other organic solvents (8, 102, 141). A high concentration of curcumin was achieved by use of CDs or micelles but the PDT effect was decreased (even completely lost in the case of 5% HP-β-CD and γ-CD) compared to formulations with organic cosolvents (5% DMSO or 1% ethanol) (8, 102). This clearly showed that a high concentration of solubilized curcumin is not sufficient for the phototoxic effect. To be efficient curcumin needs to be in the free molecular form rather than complexed or encapsulated. Application of supersaturation rather than inclusion complexation enhances the free curcumin concentration and facilitates interaction with the microbial outer wall (55, 91).

Supersaturated curcumin solutions cannot be stored for longer periods due to rapid precipitation. Even phototoxicity, shown to be high in vitro, might decrease in vivo if the longer incubation times are required (141). Solid dispersions made within Papers II and III were aimed to generate supersaturated solutions upon hydration without the use of organic cosolvents and to maintain physical stability of supersaturation and hydrolytic stability of curcumin for the sufficient time periods. Phototoxicity of selected hydrated solid dispersions was studied against G- bacteria E. coli (Paper II) and G+ bacteria E. faecalis (Paper III). A phototoxic effect higher than or comparable to the one previously obtained with a 5% DMSO preparation proved that the stabilizing effect by the excipients did not reduce the curcumin efficiency (Table 4).

Further development of the supersaturation concept gave improved results. Wikene et al. (45) have described solid dispersions based on a M-β-CD-HPMC or M-β-CD-HPMC-HA
combination produced by lyophilization. Solutions of these SDs efficiently eradicated *E. coli* and *E. faecalis* at a concentrations $2.5 \times 10^6$ and $5 \times 10^{-7}$ M respectively, and the results corresponded to a reference supersaturated curcumin solution containing 1% ethanol (45). Winter *et al.* described a PVP based solid formulation of curcumin and examined phototoxicity against *S. aureus* and *E. coli* upon dissolution in aqueous media (139). The minimal concentration required for successful inactivation of *S. aureus* was $5 \times 10^{-6}$ M ($\geq 99.99\%$ reduction of viable bacteria), and thus could be compared to the results previously obtained with a 10% DMSO curcumin solution as demonstrated by Ribeiro *et al.* (88). However, this preparation was less efficient in photoinactivation of *E. coli* than 5% DMSO solutions reported by Haukvik *et al.* (8). The curcumin-PVP solution was unstable as shown by the authors, and the adequate concentration may not be maintained long enough for the preparation to interact with the bacteria. This may explain the lower activity of the PVP preparation towards *E. coli* than the reference DMSO containing solution.

Work presented here shows that the supersaturation approach is a realistic alternative to the DMSO preparations typically applied in aPDT with curcumin.
**Table 4.** Phototoxic effect of curcumin preparations: comparison of hydrated solid dispersions (Papers II and III) and preparations with 5% DMSO (Haukvik et al.) (8).

<table>
<thead>
<tr>
<th>Type of preparation</th>
<th>Minimal curcumin concentration [M] and irradiation [J/cm²] required for photoinactivation of bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td>Curcumin concentration</td>
</tr>
<tr>
<td>HP-β-CD-HPMC based SD (lyophilizate 2.8) *</td>
<td>2.5×10⁻⁵</td>
</tr>
<tr>
<td>Pluronic® F127 based SD (SD1) *</td>
<td>10⁻⁶</td>
</tr>
<tr>
<td>Pluronic® P123 based SD (SD5) *</td>
<td>10⁻⁶</td>
</tr>
<tr>
<td>5% DMSO **</td>
<td>2.5×10⁻⁵</td>
</tr>
</tbody>
</table>

* Complete bacterial inactivation, corresponding to a 6 log₁₀ reduction of viable bacteria.

** Less than 0.2% bacterial survival corresponding to a 3 log₁₀ reduction of viable bacteria.

### 5.4.2 Formulation dependent binding of curcumin to human serum albumin

The protein content of saliva ranges from 0.1 to 0.3% while wound fluids which are mainly serum derived, contain between 0.15 (acute) and 0.06% (chronic wounds) proteins (142, 143). Binding of PS molecules to serum proteins is likely to impact the efficiency of aPDT treatment of oral infections or infected wounds. Curcumin has been shown to bind to human serum albumin (HSA) with moderate affinity (144-146). The potential of selected excipients to influence curcumin binding to HSA was examined within Paper IV. HSA-curcumin binding studies were carried out in 3×10⁻⁶ M HSA solution, corresponding to 0.02% w/v. It was found that HP-γ-CD, HP-β-CD and Pluronic® F127 interfere with curcumin-protein binding. Inhibition of HSA fluorescence quenching by curcumin with an increase in excipient concentration was observed in the concentration range 0-0.5% w/v. Fluorescence quenching of HSA by curcumin was ascribed to the formation of a protein-ligand complex. Inhibition of the quenching may indicate a decrease in curcumin binding to HSA (147). It was assumed that protein and excipients (Ex) compete for curcumin (Cur) and with the increase in excipient concentration the equilibrium tends to be shifted from a HSA-curcumin (HSA-Cur) towards a curcumin-excipient (Cur-Ex) complex (Eq 6). At about 0.5% excipient concentration the majority of the curcumin molecules is already associated with the excipients and a further increase in excipient concentration did not largely affect the fluorescence quenching.
This model did not take into account the possible interaction between HSA and excipients. Relying on literature data and the results obtained within the present study it was assumed that excipient-protein interactions are not considerably influencing HSA-curcumin binding. However, it is reasonable to expect excipient-protein interactions, particularly at high excipient concentrations.

The study of curcumin fluorescence spectra showed that an increase from low (0.05% CDs, 0.19% PF127) to high (5% CDs, 5.04% PF127) excipient concentration at constant protein concentration resulted in evident change in the curcumin microenvironment. The change in excipient concentration indicated a shift from the complex with HSA towards the complex with the excipients and thus confirmed the findings of the fluorescence quenching study.

PEG 400 also influenced the HSA fluorescence quenching by curcumin but in the different manner than PF127 and CDs. Constant inhibition of the HSA fluorescence quenching was observed over the whole investigated PEG 400 concentration range (0-5%). The study of curcumin fluorescence spectra showed that even at the highest examined PEG 400 concentration curcumin was still associated with HSA. It has been suggested that the decrease in fluorescence quenching is caused by a PEG 400-HSA interaction.

A considerable impact of proteins on curcumin aPDT effect might be expected if the excipient concentration at the application site is low (e.g., supersaturated solutions) or excipients have low affinity for PS. Formulations leading to high excipient concentration upon application (e.g., curcumin solubilized by complexation with CDs or micellar solutions) with high affinity for PS may be less affected by the presence of proteins.
6. Conclusion

- Any modification of the production of bulk material or the final product that may introduce a change in the physical state of the PDS can alter the photostability of PDS and the final product in the solid state. Therefore, the physical state of the PDS should be assessed after a modification of the production method accompanied by a photostability study of the bulk material/final product according to the ICH Guidelines (6). The type and concentration of excipients can strongly influence the photostability of the PDS in solution. The overall effect is difficult to predict and each combination must be investigated separately.

- A supersaturated solution increases the thermodynamic activity of the photosensitizer curcumin and enhances the delivery to the target bacterial cells thereby enhancing the efficiency of aPDT. Furthermore, solid dispersions which can create the state of supersaturation provide a fast delivery of the PS. This is one of the requirements for the drug delivery systems aimed for aPDT.

- Supersaturated solutions need to be physically stabilized for the time period sufficient for both in vivo interaction and the short in vitro storage period. Although both the HP-β-CD-HPMC combination and Pluronics® F127 and P123 provided satisfactory physical and hydrolytic stability of curcumin in the solution, Pluronics® were shown to be better precipitation inhibitors, emphasizing the importance of hydrophobic interactions between curcumin and the PI.

- The type and concentration of excipients affect curcumin binding to plasma protein (HSA). Cyclodextrins and PF-127 seem to compete with HSA for curcumin, whereas PEG 400 appears to affect binding differently. The complexation of curcumin with plasma proteins can in turn influence aPDT efficiency through e.g., changed uptake of PS by the target cells or modified photoreactivity of the PS.
References


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