Exploring nanosystems for biomedical applications focusing on photodynamic therapy and drug delivery

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DOCTORAL THESIS

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Exploring nanosystems for biomedical applications focusing on photodynamic therapy and drug delivery.

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ABSTRACT

The increasing incidences of cancer and related deaths call for the development of new and improved treatment modalities. Photodynamic therapy (PDT) today is an alternative to conventional treatments, but has limitations. This thesis explores different nanosystems with aim to improve PDT focusing on spectroscopic and *ex vivo* studies.

Nanosystems capable of efficient photodynamic action in anaerobic or hypoxic conditions are gaining much attention. Constructs of cyclodextrin polymer encapsulating anthracene-nitroaniline conjugates, that can release nitric oxide (NO) radicals upon irradiation, were investigated in this thesis. It was demonstrated that concomitant increase of fluorescence can be used for dosimetry of NO release. Pulsed near-infrared laser light can be used for NO photorelease by two-photon excitation process that along with high phototoxicity (observed cell mortality >90%) make this nanosystem a promising technique in PDT (paper I).

A multimodal nanosystem consisting of a cyclodextrin polymer, adamantyl-nitroaniline, and zinc phthalocyanine tetrasulfonate was evaluated (paper II). Multiphoton microscopy showed cytosolic distribution of the nanosystem in *in vitro* cells and the ability of the nanosystem to penetrate into *ex vivo* skin. In addition, the combinatorial phototoxic effect elicited by singlet oxygen and NO (cell mortality >90%), indicates high potential of this multimodal nanosystem in PDT.

Herein, it is demonstrated that conjugation of water non-soluble photosensitizer (*m*THPP) to cyclodextrin can enhance its aqueous solubility and monomerization, thereby leading to improved photophysical properties in aqueous environment (paper III).It was also shown that conjugation facilitates skin penetration *ex vivo*. Fluorescence lifetime imaging demonstrated accumulation of the monomeric conjugate in the cytoplasm *in vitro* cells.

It has been suggested that PDT enhancement can be achieved by a combination of photosensitizer and gold nanoparticles; however, the investigations in this thesis demonstrate a lack of the effect using protoporphyrin IX and PEGylated goldnanorods (paper IV). Cell viability studies were combined with spectroscopic measurements confirming a lack of energy transfer between nanoparticles and photosensitizer. Incubation of cells combining aminolevulinic acid and gold nanorods showed a slightly elevated PDT efficiency, however this effect is most likely attributed to an enhanced delivery of aminolevulinic acid rather than the energy transfer.

Finally, a nanosystem consisting of gold nanoparticle labelled with lactose moieties was explored for tumour-specific delivery (Paper V). Multiphoton microscopy was used to visualise the multiphoton-induced luminescence from the particles loaded to epithelial cancer cells and keratinocytes. The study demonstrates that tumour-specific uptake can be obtained by targeting galecin-3, known to be overexpressed in tumour cells.

Taken together, the work in this thesis presents several promising nanosystems to improve PDT. Of particular interest are the NO photoreleasing nanosystems for hypoxic conditions. Furthermore, improved biodistribution and targeted delivery can be obtained by clever design of the systems, presenting interesting approaches to aid in restraining the acute problem of increasing worldwide occurrence of cancer.

Keywords: nitric oxide, photodynamic therapy, NO-based PDT, PDT enhancement, *m*THPP, cyclodextrin, CD-*m*THPP conjugate, PpIX AuNP combination, targeted drug delivery, galectin-3, two-photon microscopy, FLIM, cell phototoxicity, *ex vivo* skin.

LIST OF PUBLICATIONS

This thesis is based on the following scientific publications, referred to by Roman numerals in the text. The papers are appended at the end of the thesis.

PAPER I **A polymer-based nanodevice for the photoregulated release of NO with two-photon fluorescence reporting in skin carcinoma cells**, Kirejev, V.; Kandoth, N.; Gref, R.; Ericson, M. B.; Sortino, S., J. Mater. Chem. B, 2014, 2, 1190-1195

PAPER II **Two-photon-fluorescence Imaging and bimodal phototherapy of epidermal cancer cells with biocompatible self-assembled polymer nanoparticles,** Kandoth N., Kirejev V., Monti S., Gref R., Ericson MB., Sortino S. (Submitted to Biomacromolecules).

PAPER III **A spectroscopic investigation on *meso*-tetra(*m*-hydroxyphenyl)porphyrin-β-cyclodextrin conjugate focusing on topical delivery**, Kirejev V., Gonçalves AR., Aggelidou C., Manet I., Mårtensson J., Yannakopoulou K., and Ericson MB. (Submitted to Photochem. Photobiol. Sci.).

PAPER IV **Investigative report on the lack of enhancement of photodynamic therapy by combining endogenous or exogenous PpIX with PEGylated gold nanoparticles**, Kirejev V., Manet I., Bauer B., Ericson MB., (Submitted to Scientific Reports).

PAPER V **Galectin-3 targeted multifunctional gold nanoparticles visualized by multiphoton microscopy**, Kirejev V., Aykaç A., Vargas-Berenguel A., Ericson MB., (In manuscript)

CONTRIBUTION REPORT

The contributions from the author (Kirejev V.) to the appended papers have been as follows:

PAPER I Planned and performed the biological and microscopy experiments. Analyzed and compiled data. Contributed to paper writing.

PAPER II Planned and performed the biological and microscopy experiments. Analyzed and compiled data. Contributed to paper writing.

PAPER III Planned and performed the study. Analyzed and compiled data. Drafted the manuscript. Corresponding author.

PAPER IV Planned and performed the study. Analyzed and compiled data. Drafted the manuscript. Corresponding author.

PAPER V Planned the study together with Marica B. Ericson. Performed most of the work. Data analysis was carried out jointly with Marica B. Ericson. Drafted the manuscript. Corresponding author.

PUBLICATIONS NOT INCLUDED IN THE THESIS

Kirejev, V.; Guldbrand, S.; Bauer, B.; Smedh, M.; Ericson, M. B. In Novel nanocarriers for topical drug delivery: investigating delivery efficiency and distribution in skin using two-photon microscopy, *Proc. SPIE* 7903, Multiphoton Microscopy in the Biomedical Sciences XI, 2011, 79032S.

Guldbrand, S.; Kirejev, V.; Simmons, C.; Goksör, M.; Maria, S.; Ericson, M. B., Two-photon fluorescence correlation spectroscopy as a tool for measuring molecular diffusion within human skin. Eur. J. Pharm. Biopharm., 2013, 84, (2), 430-6.

Kirejev, V.; Guldbrand, S.; Borglin, J.; Simonsson, C.; Ericson, M. B., Multiphoton microscopy – a powerful tool in skin research and topical drug delivery science. J. Drug. Deliv. Sci. Tech., 2012, 22, (3), 250-259.

Guldbrand, S.; Evenbratt, H.; Borglin, J.; Kirejev, V.; Ericson, M. B., Multiphoton-induced luminescence from 10 nm gold nanoparticles – the effect of interparticle distance and aggregation. (To be submitted to Nanoletters)

ABBREVIATIONS

1PE	One-photon excitation
ALA	Aminolevulinic acid
AuNP	Gold nanoparticle
AuNR	Gold nanorod
BCC	Basal cell carcinoma
CD	Cyclodextrin
CSLM	Confocal laser scanning microscopy
DDS	Drug delivery system
DMSO	Dimethyl sulfoxide
FLIM	Fluorescence-lifetime imaging microscopy
MIL	Multiphoton-induced luminescence
MPM	Multiphoton microscopy
NIR	Near-infrared light
NMSC	Non-melanoma skin cancer
NO	Nitric oxide
NP	Nanoparticle
PDT	Photodynamic therapy
PEG	Polyethylene glycol
PpIX	Protoporphyrin IX
PS	Photosensitizer
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
TPE	Two-photon excitation
TPM	Two-photon microscopy

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1. INTRODUCTION

The worldwide increasing incidence of cancer is an acute problem requiring particular attention. According to Globcan2012, the number of new cancer cases and related deaths in 2012 has reached 14.1 and 8.2 million, respectively [1]. Compared to other diseases, cancer has the most devastating economic impact, estimated to be as high as 895 billion US dollars [2]. These numbers highlight the importance of cancer research for development of new drugs and treatment strategies or improvement of existing ones.

The difficulty involved in cancer research and treatment is that there exist numerous types of cancers that occur, grow, spread, and respond to treatment differently and require different therapeutic approaches [3]. For most of the cancers, surgical intervention and chemo- and radiotherapy are currently the major treatment strategies, but these have certain drawbacks that warrant the development of alternative means for cancer treatment. One of these alternative methods is photodynamic therapy (PDT), an emerging but already medically approved modality, which has been proven to be successfully applied in neoplastic and non-malignant diseases [4]. PDT is suitable mainly for superficial cancers located on or just under the skin and on the lining of the internal organs and cavities, but many studies have focused on the feasibility of using PDT as a mainstream cancer treatment technique for various types of cancers.

Compared to other treatment techniques, PDT has a number of benefits. For example, the incidences of tissue toxicity and adverse systemic effects are low for PDT treatment. PDT also prolongs survival and improves the quality of life of patients with inoperable cancers. Moreover, PDT gives excellent cosmetic outcome, especially valuable for patients with skin cancer. Furthermore, no intrinsic or acquired resistance mechanisms against PDT have been detected yet [4]. PDT can also be combined with other treatment techniques without compromising the therapeutic effects of either modality involved.

Despite the above benefits, PDT is still considered an alternative therapeutic procedure with several drawbacks, e.g. efficiency of approved photosensitizers (PSs), PS delivery to action site, oxygen depletion during PDT, and efficiency in hypoxic conditions. Intensive studies are required to look into ways for enhancing the treatment efficiency of PDT, e.g. whether combination with other treatment modalities, modification of the PS, enhancement of singlet oxygen generation, or improvement of accumulation of PS at the action site increases the efficiency of PDT.

The abovementioned methods are meant for direct efficiency enhancement; however, indirect methods for increasing PDT efficiency are also available, such as precise dosimetry of generated cytotoxic substances and targeted and traced PS delivery. This thesis focuses on exploring different nanosystems[1] with the aim to improve PDT from several perspectives and ultimately positively influence PDT as a treatment modality for cancer therapy.

[1] In the scope of the thesis, nanosystems are considered to be nanoscale constructs (polymeric nanoparticles, conjugates, or combination of molecules and particles) designed for specific functions.

2. PHOTODYNAMIC THERAPY

PDT is a clinically approved and usually non-invasive therapeutic technique that is the preferred treatment method for a number of diseases such as malignant and premalignant nonmelanomas, basal cell carcinoma, actinic keratosis, etc. [4-8]. PDT involves three main elements: light, PS and oxygen. None of these components are toxic by themselves, but together they initiate photochemical reactions that lead to the production of cytotoxic agents inducing cell death (figure 1) [9, 10]. PDT efficiency depends on several aspects: concentration of the PS at the action site, photophysical and physicochemical properties of the PS, oxygen concentration, and efficiency of excitation light delivery to the PS. These aspects will be discussed in the following sections.

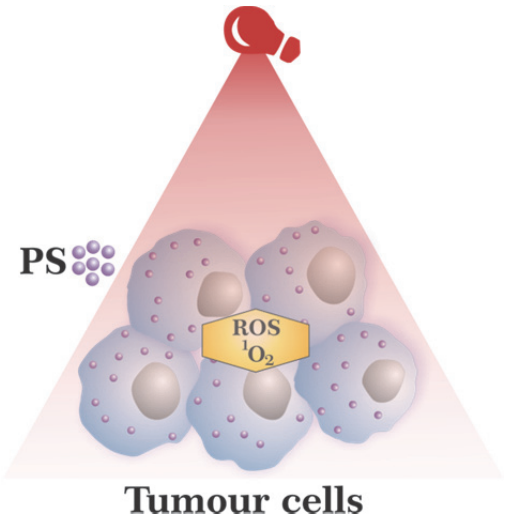

Figure 1. Schematic representation of PDT illustrating the main components: PS accumulating in the cells, excitation light, and cytotoxic species inducing cell death.

2.1. PDT IN ONCOLOGY

Initially, PDT application was limited to skin-related diseases owing to the ease of application and accessibility to light. Although new developments allow light delivery via optical fibres to most of the body cavities, still, PDT is usually administered to patients with skin-related disorders, particularly in oncologic diseases [11, 12].

In many countries, skin cancer is one of the diseases with the highest incidence [13-15]; therefore, even slight enhancement in PDT efficiency will help many people struggling with this disease. Skin cancers are divided in two broad groups: melanoma and non-melanoma skin cancer (NMSC). Melanoma is a cancer of the pigment-producing cells (melanocytes) that are located in the basal layer of the epidermis. [16]. In many countries, melanoma is ranked among

the top 5 cancer types by incidence, accounting for up to 5% of all cancer cases [17]. Melanoma is the most dangerous type of skin cancer and is responsible for a high proportion (up to 75%) of skin cancer-related deaths [13, 17]. Unfortunately, melanoma is generally considered to be resistant to PDT treatment because of the optical interference of highly pigmented melanin and its antioxidant effects, sequestration of the PS inside the melanosomes, and defects in apoptotic pathways [18]. Efforts are being made to overcome the resistivity of melanoma to PDT.

NMSC is one of the most widespread cancer types, with more than 3.5 million NMSC cases registered in the US every year [19]. Fortunately, NMSC is associated with low morbidity and mortality [20] and is responsive to PDT treatment. The two most common cancer types within NMSC are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [21]. BCCs rarely metastasize and rarely cause death. However, if left untreated, BCC can erode the skin and invade the bones and muscles. BCC is usually localised to the head and neck [21], where the cosmetic perspective of treatment becomes an important issue. SCCs, on the other hand, are less common than BCCs, but more aggressive and more likely to invade the underlying skin layers and metastasize [22]. SCC is more likely to develop in the sites of chronic inflammation, on mucous membranes, and on the lips. Keratoacanthoma is a third type of skin tumour in the NMSC group that is commonly found in skin areas exposed to the sun [23]. Keratoacanthoma is sometimes viewed as an aborted SCC which, in rare instances, can evolve into proper SCC. There are other types of skin cancers that are classified as NMSC, e.g. Merkel cell carcinoma, cutaneous (skin) lymphoma, Kaposi sarcoma, skin adnexal tumour, and sarcoma, but all of these together account for ~1% of all NMSC cases.

Areas of PDT application in oncologic diseases in dermatology are presented in table 1. But, currently, PDT is also used to treat many non-oncologic diseases, such as skin abnormalities, inflammations, viral and bacterial infections, etc.

Table 1. Examples of PDT application in oncologic diseases in dermatology. Modified from [12].

Malignant and premalignant conditions	
Malignant melanoma	Bowen's disease
Superficial BCC	Cutaneous T-cell lymphoma
Superficial SCC	Kaposi's sarcoma
Keratoacanthoma	Gorlin syndrome (multiple nevoid BCC)
Actinic keratosis	Penile and vulvar intraepithelial neoplasia
Actinic cheilitis	Langerhans cell histiocytosis
Field cancerization of the skin	Skin metastases
Barrett esophagus	

2.2. PDT PHOTOCHEMISTRY

PDT involves the administration of PS or prodrug and accumulation of PS at the treatment site. Next, the treated area is exposed to irradiation at wavelengths preferably matching the absorption maximum of the PS, for increased phototoxic effect. During irradiation, the PS undergoes transition to the excited singlet state (^{1}PS*) (figure 2, right); subsequently, the energy-enriched PS returns to the ground state via non-radiative decay or by emitting a fluorescence photon. Some of the ^{1}PS* molecules undergo intersystem crossing and form relatively long-lived excited triplet state (^{3}PS*). The ^{3}PS* returns to the ground singlet state by emitting phosphorescence photon or via non-radiative decay. However, the important aspect of PDT is that the ^{3}PS* can react with a substrate or solvent (type I reaction) or transfer energy to molecular oxygen (O_2) (type II reaction) (figure 2, left) [24]. In type I reactions, the ^{3}PS*, via hydrogen atom extraction or electron transfer to a biomolecule, solvent, or oxygen molecule, produces reactive oxygen species (ROS) like superoxide anion radicals, hydrogen peroxides, or hydroxyl radicals. In type II reactions, energy from the ^{3}PS* is transferred directly to ground-state molecular oxygen, resulting in the production of highly reactive singlet oxygen ($^1O_2$). Both type I and II reactions occur simultaneously during PDT, but the ratio of the frequency of the reactions depends on the type of PS, surrounding substrates, and concentration of molecular oxygen in the environment [25, 26].

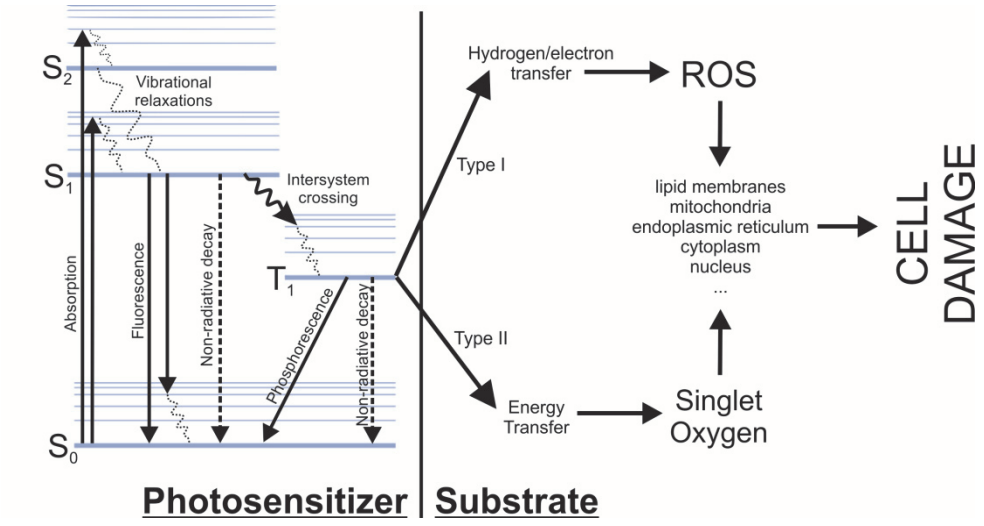

Figure 2. Simplified Jablonski diagram for PS (left) and ways of generating cyctotoxic species during PDT (right). S_1 and S_2 indicate excited-singlet states of PS (or ^{1}PS*). T_1 indicates excited-triplet state of PS (or ^{3}PS*).

Singlet oxygen and most of the ROS generated during the PDT are short-lived compounds with a short free-diffusion path [27]. For example, the lifetime of singlet oxygen is 10–100 μs in organic solvents [28] and ~3.5 μs in water [29]. In the cellular environment, which is abundant in biomolecules, the lifetime of the singlet oxygen can be as short as 0.2 μs [11, 30]. Because of

the high reactivity and short lifetime, the free-diffusion path of the singlet oxygen in the cell is ~10–100 nm since the generation point [31, 32]. Therefore, the PS needs to be located near the vital cellular organelles (nucleus, mitochondria, lipid membranes, endoplasmic reticulum, or cytoplasm) to be able to cause enough damage to the lipids, proteins, and DNA for initiating cell death via apoptosis, necrosis, or autophagy [33].

2.3. PHOTOSENSITIZERS

PSs are one of the main components of PDT. They can be created artificially or produced via inherent cell biochemical cycles. A good example of a natural PS is endogenous protoporphyrin IX (PpIX) whose production in the cell mitochondria can be boosted by addition of aminolevulinic acid (ALA) or its analogues [34-37]. The most common synthetic PSs are usually derivatives of porphyrins, chlorines, or phthalocyanines. Currently, several PSs and their precursors are approved or already in the trial phase (table 2).

Table 2. Photosensitizers or their precursors that are approved for PDT or are currently in clinical trials.

STRUCTURE	PHOTOSENSITIZER, *Trademark*	Approved	In trials	Referen
Porphyrins	*Photofrin/Photosan-3*	Worldwide		[38-41]
Porphyrin precursors	ALA: *Levulan*,	Worldwide		[42-45]
	MAL: *Metvix, Metvixia, Visonac*	US	US	
	HAL: *Hexvix, Cysview*	US	US, DE, CZ, SK, NO	
Chlorines	*Talaporfin*		US	[39, 46-51]
	Visudyne/Verteporfin	US	UK	
	Foscan	Europe	US	
	Purlytin		US	
	Fotolon		BY, RU	
Phthalocyanines	Pc4		US	[11, 52]
	Photosens	RU		
Other:	Texaphyrins: *Lutrin, Optrin, Antrin*		US	[53-61]
	Pheophorbides: *Photochlor*		US	
	Bacteriopheophorbides: *Tookad/ Stakel*		US, CA, FR	
Pc4: silicon phthalocyanine, ALA: 5-Aminolevulinic acid, MAL: Methyl aminolevulinate, HAL:				

Regardless of the origin of the PS, it must possess certain properties to be considered an efficient PS that can be applied in modern PDT. Some of the important features of an efficient PS are photophysical properties such as optimal excitation wavelength, efficient triplet state formation, long fluorescence lifetime, and high singlet oxygen generation yield. The absorption maximum should coincide with the optical window of the tissues, i.e. 600–1300 nm. However, it has been shown, that upper absorption wavelength cannot exceed 850 nm for efficient singlet oxygen production during PDT [62], so the range of optimal PS excitation is limited to 600–850 nm.

Efficient intersystem crossing as well as long-lasting dwell time in the triplet state can ensure high level production of cytotoxic agents by PS. Many synthetic PS systems are specially designed to produce high yield of excited triplet molecular species to increase the probability of generating cytotoxic species [63, 64].

Solubility of PS is also an important aspect in PDT. Solubility affects both biodistribution of the PS as well as triplet state formation efficiency [65]. Modification of the PS structure can increase its aqueous solubility, thereby enhancing intersystem crossing [66]. Further, self-aggregation of many hydrophobic PSs leads to fast decay to the ground state, resulting in reduced intersystem crossing, singlet oxygen generation, and PDT efficiency [67]. An interesting way of reducing self-aggregation of hydrophobic porphyrins was examined in paper III of this thesis, where porphyrin was conjugated with water-soluble cyclodextrin. It was found that the aggregation in aqueous solutions was highly reduced, thereby enhancing absorption, emission, and fluorescence lifetime. The enhancement of photophysical properties can result in the increase in efficiency of intersystem crossing and singlet oxygen generation. This conjugate can be considered a multimodal drug[2] delivery nanosystem, where porphyrin acts as an efficient PS and cyclodextrin acts as a fluorescently labelled drug nanocarrier.

An ideal PS should be non-toxic without illumination within the applied concentration range. Moreover, the PS should not trigger mutagenic effects, regardless of the presence of illumination or localization in the cell [68]. It is also important to consider the fact that during PDT, a PS can be chemically modified or destroyed because of photodegradation, interaction with ROS and singlet oxygen, and metabolisation and biological elimination [31, 69]. Therefore, the cytotoxicity of the photoproducts of PS and its metabolites should also be taken into account when deciding on an appropriate PDT strategy. Elimination of the PS from the body after PDT should preferably be rapid to reduce the photosensitivity period [25].

Another important characteristic of PS is the ability to accumulate specifically in the cancer tissue, thereby reducing photodamage to healthy cells. Selectivity can be based on different cancer tissue properties, e.g. 1) high vascular network and vascular permeability, and reduced lymphatic drainage in the tumour area [70, 71], 2) low pH values in the tumour area [72, 73], or 3) presence of the unique receptors or overexpression of common receptors on the cancer cells [74-77]. In paper V of this study, the aspect of PS selectivity is implicitly examined with respect to cancer cell-specific accumulation of a drug-delivery nanosystem where PS can be loaded. It was found that differences between normal and cancer cells can be used for targeted delivery, especially the difference in carbohydrate-specific human galectin-3 receptor expression between the cells.

[2] In the context of the thesis, drug is considered as active pharmaceutical ingredient.

2.4. NITRIC OXIDE BASED PDT

Some recent trends in PDT research include PDT in anaerobic conditions and overcoming the problem of oxygen depletion during PDT treatment, as it can be a limiting factor for PDT efficiency [78]. One of the options for dealing with PDT dependence on oxygen is the use of nitric oxide (NO)-based phototherapy, also termed as NO photorelease or photoinduced NO release. NO is a small inorganic charge free liposoluble free radical with a half-life of ~5 s and free diffusion path of 40–200 μm. Because of these characteristics, NO plays an important role in many biological regulatory processes like neurotransmission, hormone secretion, vasodilatation, etc.[79-81]. Recent studies have shown that NO acts as an anticancer and antimicrobial agent by inhibiting key metabolic pathways of cellular growth or directly damaging cancer cells and infective microorganisms [82, 83]. Therefore, NO can have beneficial or harmful biological effects, depending on the site of accumulation and local NO concentration [84, 85]. Hence, it is very important to accumulate enough NO radicals within the cell during NO-based PDT to elicit significant phototoxic effects.

Photoinduced NO release is an effective method for yielding high release of NO radicals at the action site (figure 3). Light allows for non-invasive, rapid, and precise spatiotemporal control over NO release. Light is also environment- and bio-friendly and causes no substantial impact on physiological parameters such as pH and temperature. NO-based PDT is similar to "classical" PDT, where light of appropriate wavelength is used to excite the NO photodonor. The absorbed energy is used to break the bond between the carrier and NO moiety, leading to the release of NO radical. However, as opposed to "classical" PDT, the cytotoxic effect of NO-based PDT does not depend on the environmental conditions, e.g. oxygen concentration in the PS vicinity. NO radicals are initially part of the photodonor molecule, and on release of the radical, the photodonor molecule becomes inert, and the source of NO generation gets depleted. In other words, the phototoxic effect depends on the local concentration of the NO photodonor within or around the cell.

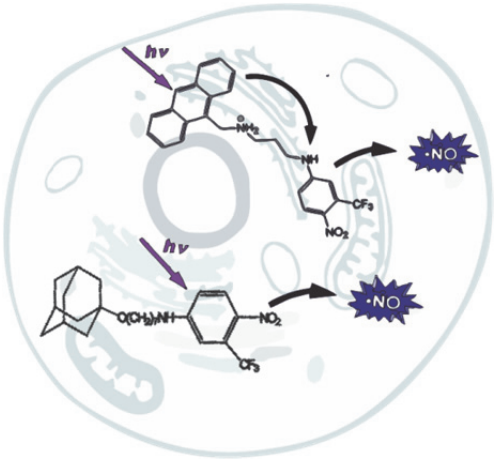

Figure 3 Schematic illustration of NO-based PDT (nanosystems from paper I and II).

2.5. PDT ENHANCEMENT TECHNIQUES

PDT efficiency can be enhanced in many ways. These methods can be divided into the following broad groups: 1) Modification of the PS, 2) enhancement of PS delivery to the action site, 3) combination of PDT with other treatment types, and 4) other means of enhancement, such as increasing the efficiency of singlet oxygen generation without modifying the PS.

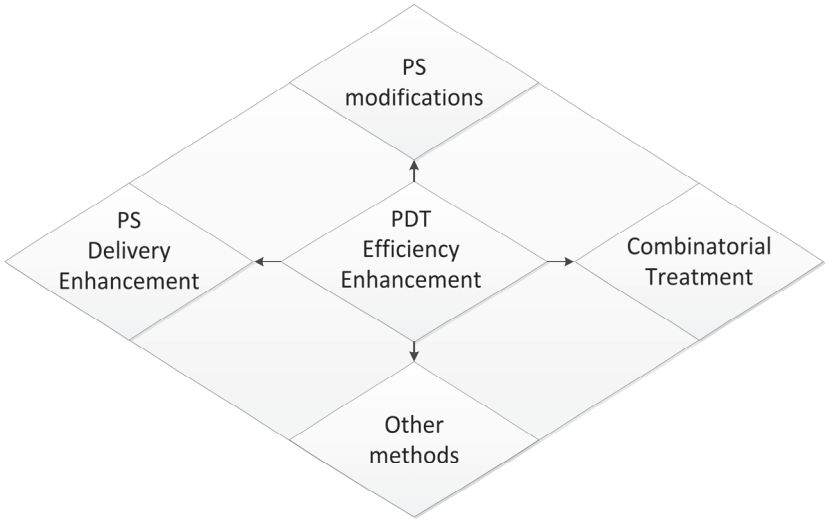

Figure 4. Strategies for PDT efficiency enhancement.

Modification of the chemical structure of the PS is a favourable method that has also led to the synthesis of a vast number of new PSs or modification of conventional ones. Molecules are designed such that they have the desired absorption range, enhanced intersystem crossing, efficient singlet oxygen generation quantum yield, and desired water solubility. A good overview of PS modification and the resulting effect is presented by Dumoulin in “Design and Conception of Photosensitisers” [63]. Various modifications directly influence the physicochemical properties of a PS, thereby enhancing PDT efficiency. Modification of a PS and subsequent changes in its photophysical and photochemical properties were analyzed in paper III of this thesis.

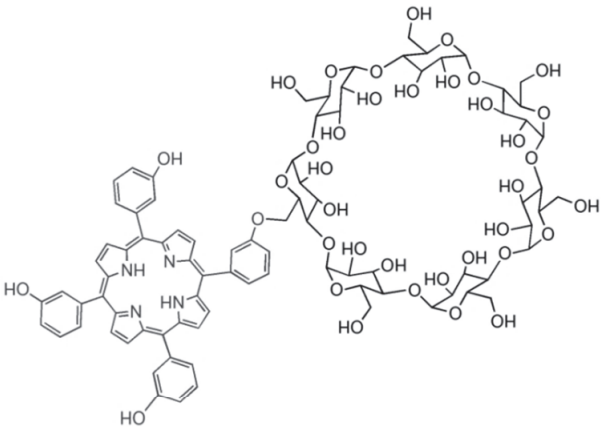

Figure 5. Example of modification of the chemical structure of the PS through conjugation with water-soluble β-cylodextrin (paper III).

Paper III also focuses on enhanced passive delivery of PS to the action site. Change in the solubility of PS can affect its ability to penetrate the biological barriers such as the cellular bilipid membrane [63] or skin [86]. That was observed in the paper III, where *m*THPP conjugated to CD due to enhanced aqueous solubility accumulated in the cell cytoplasm and more efficiently penetrated into *ex vivo* skin, in comparison to unconjugated *m*THPP.

Still, passive delivery results in drug accumulation in the normal cells as well, whereas active targeted drug delivery is aimed specifically at cancer cells. Active transport of PS can be achieved by the introduction of a targeting moiety either by direct conjugation with the PS or with the delivery system used to carry the drug [87]. The types of targeted delivery and their benefits will be discussed in detail in section 3.2.2. Also paper V is focused on a targeted drug-delivery (Figure 6) where selectivity towards cancer cells and detection methods were examined. The indirect connection between the presented system and PDT efficiency enhancement is that PS can be loaded into the DDS and specifically delivered to the active site in concentrated form.

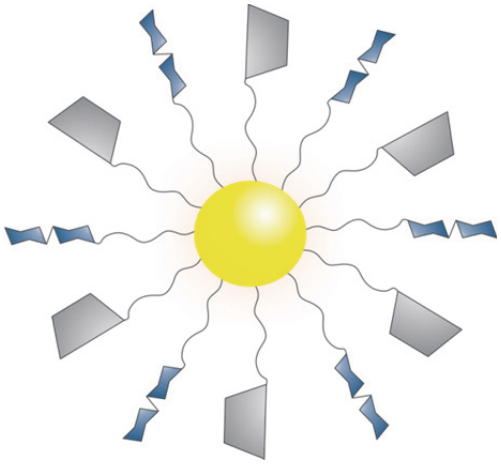

Figure 6. Example of cancer cell-targeted drug delivery system, based on gold nanoparticle bearing simultaneously multiple copies of β-Cyclodextrin for drug incorporation and β-D-lactose for targeting human GAL-3 receptor (paper V).

Studies on PDT enhancement via a combination of several treatment modalities acting simultaneously have become quite popular in recent years. For example, combination of PDT with chemotherapy (PS + anticancer agents) [88] or photothermal therapy (PS + gold nanoparticles (AuNPs)) [89] have shown promising treatment outcomes. The advantage of this kind of systems is that they are able to cause simultaneous cytotoxic effects via various independent routes, thereby reducing the probability of survival of the treated cells. In paper II, a nanosystem combining modalities for PDT and NO radical-based PDT (PS + NO radical photodonor) is presented (figure 7). The cytotoxic singlet oxygen and NO radicals act synergistically and can be used in hypoxic environments.

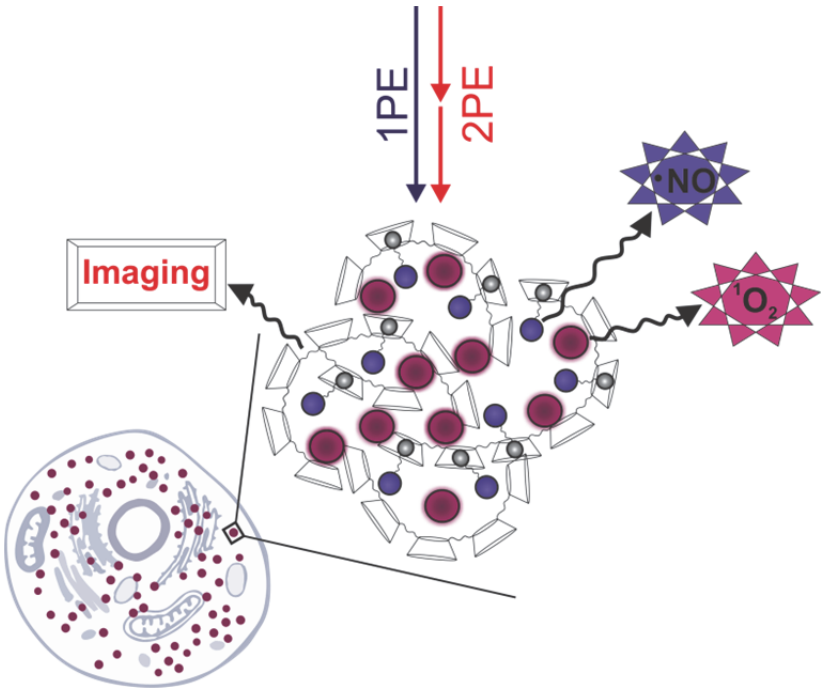

Figure 7. Multimodal system combining PDT and NO-based PDT for cancer treatment (paper II).

Some studies on PDT efficiency enhancement have focused on means other than those mentioned above. For example, it is possible to create an additional source of PS excitation energy via combination with AuNPs. The surface plasmon resonance field occurring around plasmonic nanoparticles can be used for energy transfer to PS molecules [90, 91]. This phenomenon is discussed later (paragraph 4.3.), and our attempt at using this phenomenon for PDT enhancement is presented in paper IV of this study.

3. DRUG DELIVERY

Drug delivery to the action site is a multifaceted issue involving many influencing factors, e.g. bioavailability, pharmacokinetics and pharmacodynamics. A drug has to pass many biological barriers before reaching the target site of action and eliciting a therapeutic effect. Depending on the administration route and properties of the drug, the barriers faced by drug molecule could be the vascular endothelium or gastrointestinal epithelial cell layer, stratum corneum of the epidermis, extracellular matrix barrier, and cell and subcellular organelle membranes. These barriers can highly limit the application and efficiency of many perspective compounds.

Drug delivery to cancer site can be achieved via passive or active targeting (figure 8) [92]. Enhanced permeability and retention (EPR) effect is the major cause of drug accumulation in the cancer site during oral and intravenous drug administration routes [93]. However, in recent years, many studies have focused on finding alternative ways of drug delivery. Topical drug application in some cases could be a feasible option, for oral and intravenous routes [94]. This method allows the drug to bypass the hepatic barrier, binding to the blood components, wide range of pH, biochemical modifications induced by different enzymes in the gastrointestinal tract, and achieve, if needed, a quite localized drug effect [95].

However, EPR and local drug application also usually cause accumulation of the drug in non-cancer cells. On the other hand, active targeting is aimed at drug accumulation only in tumour. Full specificity is not always possible, so non-critical drug accumulation can be observed in some cases. Active targeting uses the differences between normal and cancer cells, aiming for receptors, antibodies, and carbohydrates that are unique to or overexpressed on cancerous cells [96].

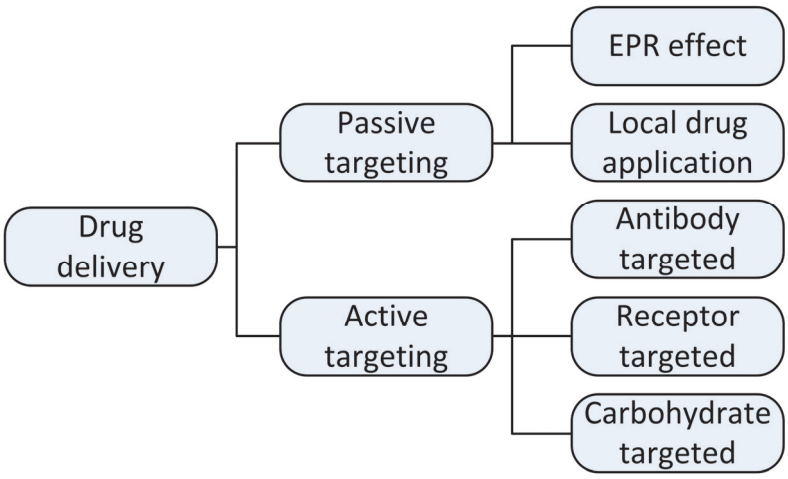

Figure 8. Means of active and passive drug delivery used in cancer-related drug delivery.

3.1. TOPICAL DRUG DELIVERY[3]

PDT and related techniques are mainly aimed at topical diseases because the main requirement for efficient treatment is illumination of the treated area. Although light can be delivered within the body via optical fibres nowadays, the use of PDT is still mostly limited to skin diseases. However, delivering PDT in cases of skin diseases is not a straightforward task owing to the high structural complexity and efficient barrier properties of the skin. Therefore, delivery of drugs and PSs into the skin is still a problematic area.

3.1.1. SKIN STRUCTURE

The skin is one of the largest organs in the human body, and it has many vital functions, e.g. sensory and tactile perception and temperature and water balance regulation [97]. Another vital function of the skin is acting as a barrier between the organism and the surrounding environment. The complex structure of the skin makes it an efficient physical (preventing water loss, protection against UV-radiation, and preventing penetration of exogenous particles and substances), biochemical (hydrolytic enzymes, antibacterial fatty acids, and antimicrobial peptides produced by the skin protect the body against microorganisms and viruses), and immunological barrier (cells of immune system present in the skin) [98, 99].

The physical structure and biochemical composition of the skin is highly complex. The skin has two layers: the epidermis and dermis. The epidermis is avascular stratified squamous epithelium mainly composed of keratinocytes. According to the level of differentiation of the keratinocytes, the epidermis can be divided into four layers: the stratum corneum, stratum granulosum, stratum spinosum, and stratum basale (figure 9) [100, 101]. The outermost layer, approximately 20 μm thick, is called the stratum corneum, and it is the first and main physical barrier that any substance has to pass to penetrate the skin [100]. This layer consists of dead keratinocytes (or corneocytes), embedded in a matrix of lamellar lipid bilayers forming a brick-and-mortar-like structure [100]. Corneocytes are firmly interlinked by intercellular bridges (i.e. desmosomes). A matrix of polar lipids that contains sterols and several hydrolytic enzymes, e.g. lipases, glycosidases, and acid phosphatase [102-105], provides a structurally effective epidermal barrier to permeability.

Below the epidermis lies the dermis, with the basal membrane between the two layers. The dermis is a connective tissue with a large proportion of collagen and elastin fibres in a polysaccharide matrix providing strength and flexibility. The dermis contains blood and lymph

[3] Topical drug delivery is used to describe the delivery of drugs through body surfaces such as skin or mucus membranes; however in the scope of the thesis, topical delivery will be discussing mainly from dermal drug delivery perspective, i.e. delivery of drugs into the skin.

vessels, nerves, smooth muscles, and epithelial structures of adnexa. The dermis is attached to the hypodermis through which it is connected to the internal body structures, e.g. the muscles [106, 107].

The complex structure of the skin makes the skin an efficient, versatile barrier that offers multilayer protection (physical, immunological, and biochemical) against, often aggressive, environmental factors.

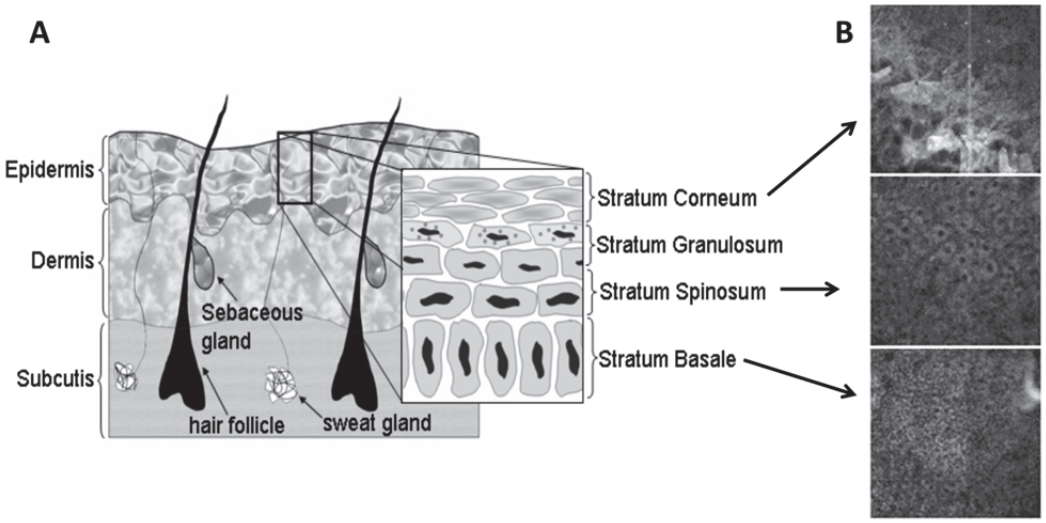

Figure 9. A) Schematic illustration of the epidermis. Modified with permission from [108]. B) Multiphoton images of stratum corneum, stratum spinosum, and stratum basale [109].

3.1.2. SKIN PENETRATION PATHWAYS

Efficient dermal drug application involves concentration of the drug at the action site and ability of the drug to cross the physical skin barrier. There are three possible penetration pathways: intercellular, transcellular, and appendageal (via sweat glands or hair follicles) (figure 10). In the case of the human skin, the appendageal pathway is not considered significant owing to the small surface area of the appendages [110, 111]. The transcellular pathway is highly complicated because of the presence of the cornified cell envelope and high level of keratinization of the corneocytes. In addition, very few molecules are able to pass both the lipid bilayer of cell membrane as well as the aqueous intracellular environment because of their physicochemical properties. Thus, the intercellular pathway, via the lipid matrix, is considered the main road for drug diffusion through the stratum corneum. In this case, the lipophilic molecules travel via the lipid matrix and the hydrophilic ones, via water channels that are present in extracellular space. Eventually, it is the sum of the skin properties (structural and biochemical composition of healthy or diseased skin) as well as physicochemical properties of the diffusing molecules that define the diffusion route [112].

In papers II and III, drug delivery into the skin as well as drug biodistribution was analysed with the help of multiphoton microscopy. The results of paper II indicate that polymeric cyclodextrin-based nanoparticles of ~35 nm tend to use the extracellular skin penetration pathway. The results of paper III clearly show how the physicochemical properties of the PS affect its skin penetration efficiency. Highly hydrophobic compounds, aggregating in the aqueous solutions, are not able to pass the stratum corneum barrier. Modification of PS towards enhanced water solubility increases the ability of the drug to penetrate the skin, thereby increasing the likelihood of positive outcomes for PDT treatment of skin diseases.

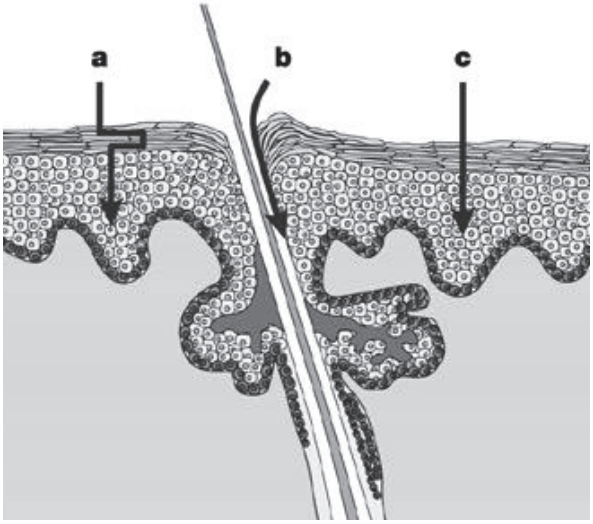

Figure 10. Drug penetration pathways: a) intercellular, b) appendageal, and c) transcellular. Modified and reprinted by permission from [113], copyright (2004).

3.2. DRUG DELIVERY AT THE CELLULAR LEVEL

Cancer tissues and cells have unique properties that can be used for preferential accumulation of therapeutic and imaging agents in cancer lesions and cells. For example, enhanced vascular permeability, low pH, high osmotic pressure, and other abnormalities in the physical and chemical characteristics of cancer cells are some features that distinguish them from normal cells. Moreover, cancer cells contain cancer-specific biomarkers that can be targeted in cancer cell-targeted drug delivery [114, 115].

3.2.1. INTRACELLULAR DRUG DELIVERY

A drug molecule can enter a cell in a number of ways. Depending on physicochemical properties (e.g. lipophilicity, size, and ionization) of the drug and type of cells intracellular delivery can be achieved via passive diffusion, facilitated passive diffusion, active transport, and endocytosis (figure 11).

Passive diffusion follows Fick's first law and mostly depends on the concentration gradient. It is one of the easiest ways of cellular drug delivery, but it is mostly effective only for small uncharged molecules, owning to the negative charge of the lipid membranes of the cells. Some of the endogenous substances and nutrients like vitamins sugars, amino acids and ions are delivered into the cells via facilitated passive diffusion or active transport. These two cellular delivery methods are based on a reversible binding to the carrier protein on the surface of the cell membrane with subsequent transport across the lipid membrane and release [116].

Another pathway of delivery of substances into the cell is endocytosis, involving different mechanisms of internalization of different exogenous substances. Endocytosis can be divided in two major groups: phagocytosis (cell eating), mainly in cases of large particles (>200 nm), and pinocytosis (cell drinking). During phagocytosis, the membrane extends outwards, and the extensions (or pseudopodia) wrap around the target and pull it within the cell, forming a membrane-bound phagosome. Later, the phagosome fuses with a lysosome where the target is exposed to proteolytic enzymes and acidic pH that degrade the contents of the phagolysosome to some extent [117]. Pinocytosis involves four basic mechanisms: clathrin-dependent endocytosis, caveolin-mediated endocytosis, macropinocytosis, and dynamin- and clathrin-independent endocytosis [118]. Endocytosis can be non-selective, when invaginations of the cell membrane non-specifically entrap extracellular fluids and particles. However, in general, endocytosis is initiated by receptor binding (receptor mediated endocytosis), which triggers signalling pathways, leading to reorganization of membrane components [119, 120].During receptor-mediated pinocytosis, the target is recognized by cell surface receptors, triggering membrane invagination. Then, the target molecule is entrapped in a vesicle and transported to various cell organelles or fused with lysosomes for degradation and disposal. Pinocytosis is observed in cases of majority of the cells, whereas phagocytosis is observed only in specific cells like neutrophils, macrophages, monocytes, and endothelial cells [121].

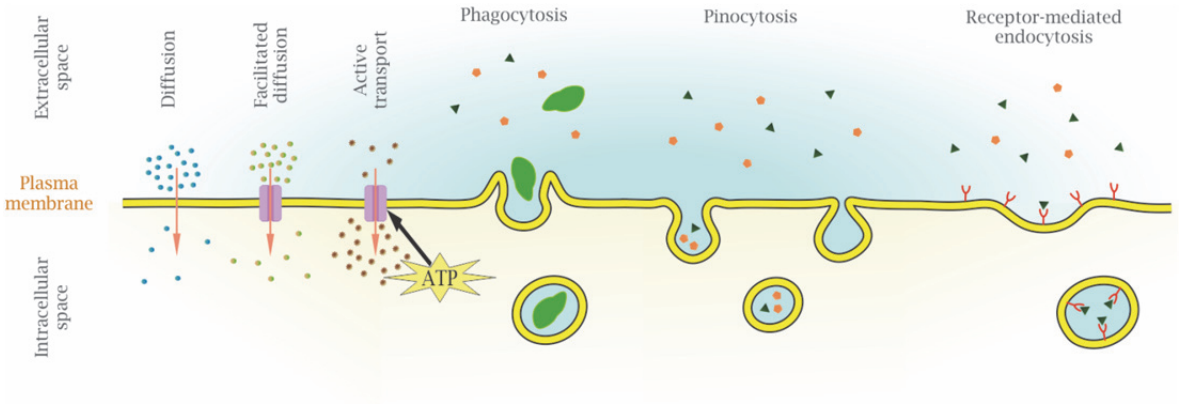

Figure 11. Intracellular transport pathways of molecules and particles such as diffusion, facilitated diffusion, active transport, phagocytosis, pinocytosis, and receptor-mediated endocytosis.

In the context of drugs and DDSs, determining the possible pathways of intracellular delivery and involved cell compartments helps evaluate the bioavailability and pharmacological activity of the drug. In addition, knowledge of the properties of carriers and particles that dominate the internalization pathway can help in the designing of delivery systems with specific transportation routes aimed at particular intracellular targets. In this thesis the routes of intracellular delivery were not specifically investigated, but the intracellular delivery pathways of different nanosystems are of high interest and could be in focus of future studies.

3.2.2. TARGETED DRUG DELIVERY

Cancer cells can be defined as cells that differ from normal owing to the lack of response-to-control mechanisms in these cells [122]. The transformation of normal cells into cancerous is considered to be a multistep process, involving genetic physiologic alterations such as self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [123].

Tumour cells have intrinsic genetic instability [123]. Therefore, carcinogenesis can be disrupted at different stages, resulting in a high level of heterogeneity not only between the tumours but also between cancer cells within the same tumour mass. This highly complicates treatment and targeted drug delivery. In addition, tumour tissues are often less differentiated than normal and are histologically closer to foetal embryonic than to normal adult tissue. Cancer cells often express biomarkers that are of embryonic origin and not expressed by differentiated normal adult tissue. These cancer-specific biomarkers can be tumour-specific glycoproteins and mucins, oncofoetal antigens, etc. Also, cancer cells can have tumour-associated biomarkers, such as abnormally expressed (usually overexpress) carbohydrates, hormones, enzymes, receptors, and growth factors, typically produced by normal tissues at lower concentrations [115]. Most of these tumour-specific and tumour-associated biomarkers can be detected and even quantified by different immunohistochemistry and immunoassays. Of note, these biomarkers can be used as targets in targeted drug-delivery assays for specific accumulation or concentrated accumulation of drugs in cancerous cells. The ultimate goal is to create a targeted system that is able to deliver the PS into specific cellular compartments [124-126].

Active targeting is achieved by labelling drugs or DDs with target-specific ligands aiming for receptors or antigens that are expressed or overexpressed on the cancer cells [96]. A number of targeted treatments using antibodies are already approved by the FDA (table 3), and many others are in the trial or research stages. For example, antibodies F5 and C1 can be used to target ErbB2 growth factor that is overexpressed in 20–30% of human breast carcinomas and

adenocarcinomas [127]. In addition, folate receptor that is expressed on the surface of many cancer cells can be targeted with Fab/scFv antibodies [128]. It was also found that AuNPs labelled with anti-EGFR can act as a good contrast agents for visualisation of cancer cells by multiphoton microscopy [129].

Table 3. Some targeting ligands approved by the FDA by 2014 [130].

Generic name	Proprietary name	Target	Yea approved	Clinical indication
Rituximab	Rituxin®/ Mabthera®	CD20	1997	NHL, $CD20^+$ CLL, FL, RA
Transtuzumab	Herceptin®	HER-2	1998	$HER\text{-}2^+$ MBC
Alemtuzumab	Campath®/ Mabcampath®	CD52	2001	CLL, T-cell Lymphoma
Tositumomab	Bexxar®	CD20	2003	NHL
Cetuximab	Erbitux®	EGRF, HER-1	2004	$EGRF^+$ MCC
Bevacizumab	Avastin®	VEGF	2004	MCC
Panitumumab	Vectibix™	EGRF, HER-1	2006	MCC
Ofatumumab	Arzerra™	CD20	2009	CLL
Ipilimumab	Yervoy™	CTLA-4	2011	MMel
Pertuzumab	Perjeta™	EGFR2, HER-2	2012	BC
NHL: Non-Hodgkin's Lymphoma, CLL: Chronic Lymphocytic Leukemia, FL: Follicular Leukemia, RA: Rhematoid Arthritis, MBC: Metastatic Breast Cancer, MCC: Metastatic Colorectal Cancer, MMel: Metastatic melanoma, BC: Breast Cancer.				

In paper V of this thesis, a targeted delivery nanosystem (lacto-CD-AuNP[4]) was examined for selectivity towards cancer cells expressing the human Gal-3 receptor. Gal-3 is known to be overexpressed in some types of cancers [131, 132] and plays an important role in tumorigenicity (i.e. cell proliferation, apoptosis, cell invasion, and metastasis) [133-135]. Previous studies have shown that Gal-3 binds to β-D-lactose (targeting moiety of lacto-CD-AuNP) in the cuvette [136]. The results showed that lacto-CD-AuNP is able to selectively bind to cancer cells and can be visualized using the MIL from AuNPs with the help of TPM. This system can be used for targeted delivery of the PS to cancer cells, thereby reducing the applied dose and the likelihood of side effects; moreover, specific accumulation of the drug at the action site might enhance PDT efficiency.

[4] Multimodal drug delivery system based on gold nanoparticle bearing simultaneously multiple copies of β-Cyclodextrin (βCD) for drug incorporation and β-D-lactose for targeting human galectin-3 (Gal-3).

4. NANOSYSTEMS

4.1. NANOSYSTEMS FOR DRUG DELIVERY

DDSs have attracted much attention in recent years, with the advances in biotechnology and biomedical sciences providing opportunities for the development of a number of drug-carrier systems that show enhanced drug delivery to a target location without the need to modify the structure and intrinsic properties of the drug. The purpose of a DDS is to increase the bioavailability and concentration of a drug at the action site as well as prevent or reduce the likelihood of harmful side effects [137]. An efficient DDS should have high drug loading and optimal release properties, a long shelf life, and low toxicity [138]. Conventional DDSs or vehicles consisted of semisolid or liquid drug vehicles, e.g. ointments, creams, gels, lotions, emulsions, and suspensions. They act by solubilising the drug, thereby creating homogenous solutions and changing the partitioning coefficients [139]. Now, depending on the drug, area of application, and the target, it is possible to choose from more sophisticated DDSs, e.g. liposomes, polymeric particles, cyclodextrins, etc. (figure 12 A).

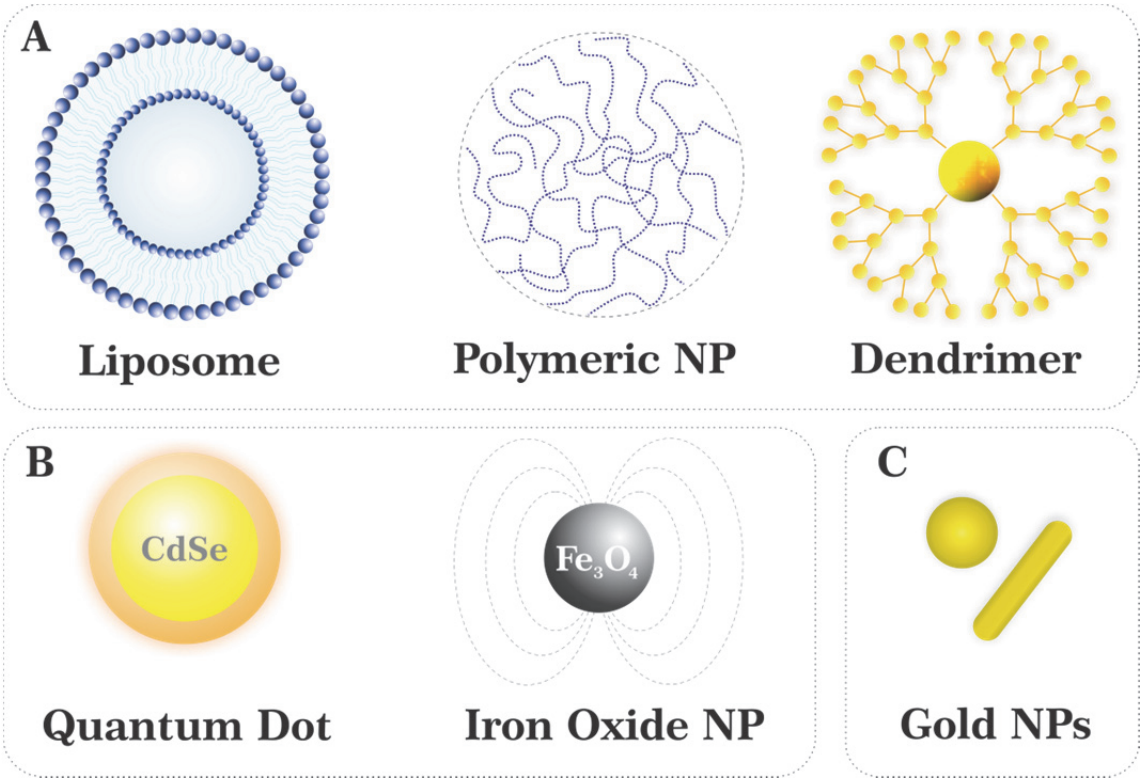

Figure 12. Examples of nanosystems A) for drug delivery; B) for contrast mechanism; C) gold nanoparticles- as multimodal systems.

4.1.1. LIPOSOMES

Liposomes consist of single (unilamellar), few (oligolamellar), or many (multilamellar) concentric phospholipid bilayers capable of encapsulating both hydrophilic and hydrophobic molecules [140]. The hydrophilic molecules are entrapped in the aqueous inner sphere, while the hydrophobic molecules are usually integrated into the phospholipid bilayer [141]. The physicochemical properties of liposomes such as size, charge, and elasticity are defined by their composition and can be fine tuned for specific purposes [142-145].

4.1.2. POLYMERIC PARTICLES

Polymeric particles of various shapes, sizes, and compositions can also be used for drug delivery. Nano- or micro-sized polymer particles usually consist of biodegradable materials such as polylactic (PLA) acid, poly(lactic-co-glycolic) acid (PLGA), or polycyanoacrylate or modified natural products such as chitosan, albumin, or polydextran. Polymeric particles have high loadability [146] and can encapsulate both hydrophilic and hydrophobic drugs as well as proteins and nucleic acids [147]. Polymeric nanoparticles can improve bioavailability and efficacy of the carried drugs [148].

A dendrimer is another type of polymeric nanoparticle. Dendrimers are composed of synthetic or natural components like sugars, amino acids, and nucleotides and have a well-defined dendron-like structure [149]. Dendrimers are capable of incorporating different molecules in their hydrophilic core or lipophilic shell. Drugs can be conjugated to dendrimers for facilitating stimuli-specific drug release [146].

4.1.3. CYCLODEXTRINS

Since the discovery of cyclodextrins in 1891 by Villiers, they have been used in different industries: food, cosmetics, agriculture, and chemical industry [150, 151]. However, only recently, cyclodextrins were introduced in pharmaceutical science as possible drug nanocarriers. Cyclodextrins are truncated, cone-like, naturally occurring oligosaccharides consisting of 6–8 glucopyranoside units (figure 13) [152]. The inner surface of cyclodextrins consists of hydrophobic carbon backbones of glucopyranose units, so the interior of the molecules is relatively hydrophobic. Because most cyclodextrin molecules are hydrophilic, they are efficient solubilizers for poorly water-soluble compounds. Variation in the number of the glucopyranose units in the structure of the cyclodextrins allows for inclusion of various molecules in the cavity of CD [153, 154]. The non-toxicity of cyclodextrins also contributes to their extensive application in the biomedical field [151, 155]. Cyclodextrins are highly interesting as DDSs. By creating host-guest complexes, the solubility [156, 157] and photo- and

chemo-stability [158-160] of inclusion drugs can be increased, thereby influencing their pharmacokinetics and pharmacodynamics. Drug release from the cyclodextrin-drug complexes is usually driven by simple dissolution process. However, other processes like drug-protein interactions, drug partitioning from complex to tissue, and competitive binding can influence drug release.

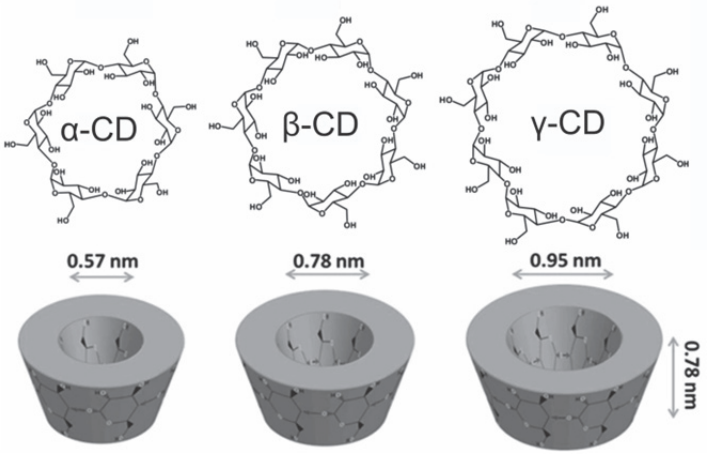

Figure 13. Schematic illustration and molecular structure of α-, β- and γ- cyclodextrins.

In this thesis, cyclodextrins were used in various DDSs. For example, in papers I and II, cyclodextrin polymer was used to efficiently encapsulate and solubilise hydrophobic PS and NO photodonors in aqueous solution. The nanoparticles formed by cyclodextrin polymer and the encapsulated drug were able to penetrate *ex vivo* cells and skin samples, confirming their applicability in the drug delivery field. In paper III, cyclodextrin was conjugated to photosensitizer, enhancing photophysical properties of porphyrin in aqueous solutions by increasing water solubility. Also, conjugation of *m*THPP to CD allows using this nanosystem as a fluorescently labelled DDS. In paper V, cyclodextrin was used as a component of a multimodal-targeted delivery system to perform drug encapsulation and transportation functions.

4.2. NANOSYSTEMS AS CONTRAST AGENTS

4.2.1. QUANTUM DOTS

Quantum dots (QDs) (figure 12 B) are semiconductor nanoparticles with unique size-dependent electronic and optical properties [161], e.g. wide absorption range, narrow emission range, that can be tuned over a wide range, high emission efficiency, and high stability. QDs can be used as contrast agents in fluorescence microscopy, as cell labels, and as biomolecule fluorescent markers [162, 163]. The drawback of QDs is that the core is usually composed of heavy atoms, limiting their biomedical applicability owing to the increased likelihood of toxicity.

4.2.2. IRON OXIDE NANOPARTICLES

These nanoparticles are usually composed of an iron oxide core coated with hydrophilic biocompatible polymers (figure 12 B) [164]. Iron oxide nanoparticles are interesting imaging agents because they are superparamagnetic. On application of an external magnetic field, they become magnetized, and on removal of the field, they exhibit zero net magnetization. They can be used as contrast agents in magnetic resonance imaging [165]. Iron oxide nanoparticles have be proven to be superior to conventional gadolinium-chelate contrast agents owing to their low toxicity and high sensitivity [166].

4.3. GOLD NANOPARTICLES: MULTIMODAL NANOSYSTEMS

Owing to their unique properties, AuNPs (figure 12 C) have gained much attention in biomedical research recently. In the presence of an oscillating electromagnetic field, free electrons of the nanoparticle begin to collectively and coherently oscillate relative to the positive metallic lattice. These oscillations are resonant to specific frequencies of the light and are termed as localized surface plasmon resonance oscillations. The decay of these oscillations can occur via energy radiation, resulting in light scattering, or via non-radiative pathway, resulting in the conversion of absorbed light to heat [167]. Along with the tunable optical properties of AuNPs, the ease of production and surface modification as well as lack of cytotoxicity make AuNPs interesting and suitable for many biological applications, e.g. use as contrast mechanism for different types of microscopy techniques, as heat generators during photothermal therapies, and as a core for multimodal DDSs (figure 14) [168, 169].

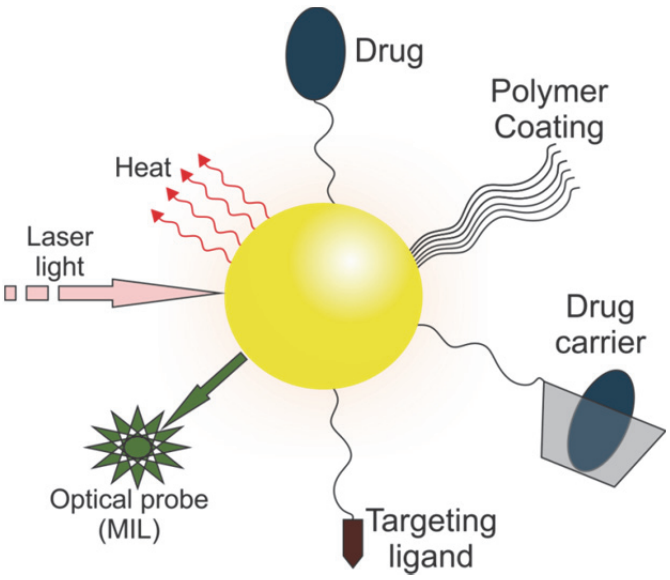

Figure 14. Schematic illustration of gold nanoparticle incorporating receptor, conjugated drug, polymer coating, and drug nanocarrier. On irradiation, the gold core can act as an optical probe and heat generator.

4.3.1. GOLD NANOPARTICLES AS CONTRAST AGENTS

Owing to their optical, physical, and chemical properties, AuNPs are used as contrast agents in many microscopy techniques such as dark-field mode microscopy [170], electron microscopy [171], Raman spectroscopy [170], optical coherence tomography (OCT) [172], photoacoustic tomography (PAT) [173] and Raman spectroscopy [170].

AuNPs are also known for their ability to generate multiphoton-induced luminescence (MIL) that can be utilized in two-photon microscopy [129, 174]. MIL is a process involving sequential absorption of photons and consequent recombination of electrons and holes from sp- and d-bands [175]. MIL signal has been shown to be more than 50 times higher than the two-photon fluorescence signal from fluorescent molecules [176], in addition, the high stability of AuNPs makes them more preferable contrast agents over fluorophores for two-photon microscopy (TPM). The ability of AuNPs to luminesce under two-photon irradiation was utilized in paper V, where a multimodal targeted DDS based on gold nanoparticle was investigated for selectivity towards cancer cells with a help of TPM.

4.3.2. GOLD NANOPARTICLES AS THERAPEUTIC AGENTS

As mentioned before, in the presence of an oscillating electromagnetic field, the electrons create a surface plasmon resonance field around the AuNPs (figure 15). The non-radiative decay of this field is accompanied with an increase in the local temperature damaging biomolecules and melting membranes, resulting in cell death [177, 178]. Conjugation of AuNPs with targeting moieties allows cancer cell-specific accumulation of nanoparticles, resulting in reduction of irradiation laser powers for efficient photothermal cancer cell destruction [179, 180]. Thus, the multimodal-targeted DDS described in paper V also possesses the capability of an additional photothermal modality.

A highly localized plasmonic field can also be used as a source of excitation for fluorophores [181-183]. This phenomenon can be used in PDT to enhance PS excitation and concomitant singlet oxygen generation [184, 185]. The ability of AuNPs to enhance the excitation of PS was examined in paper IV, where PEGylated AuNPs were combined with exogenous and endogenous PSs. With regards to the photophysical perspective, the energy transfer from gold nanoparticles to PS should be reflected in the steady-state and time-resolved spectroscopy measurements. Also, the process of enhanced PS excitation, if it occurs, should be detected in *in vitro* cell phototoxicity studies as an increase in the PDT efficiency of PS.

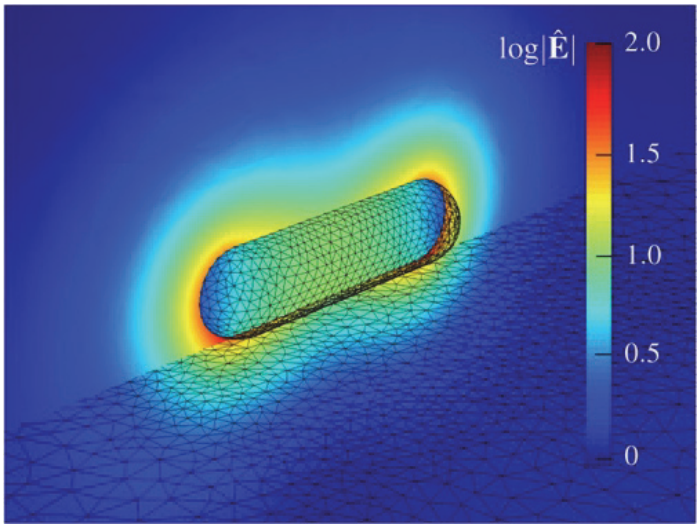

Figure 15. Plasmonic field generated by single gold nanorod. Adapted with permission from [186].

5. METHODOLOGY

Owing to the interdisciplinary nature of this study, the methodology will be presented as conceptual background of the methods used along with specific details. This will help readers from different backgrounds to clearly understand the rationale behind the choice of the methods applied in the thesis.

5.1. FLUORESCENCE BASED METHODS

Use of fluorescence in biomedical research areas has been remarkably growing. Steady-state and time-resolved fluorescence spectroscopy and microscopy techniques have become an essential part in biochemistry, biophysics, biotechnology, cell biology, drug delivery and other areas of science.

Fluorescence occurs when an electron in the excited singlet state, approximately after 10^{-11}–10^{-8} s, returns to the ground state and simultaneously emits a photon (figure 2) [187]. Compared to excitation of light, the emission occurs typically at lower energies and longer wavelengths, and this phenomenon is called the Stokes shift

Fluorescence microscopy and its many variations occupy a large niche in biosciences. Modern fluorescence detection-based microscopy techniques have a resolution of several nanometres and the ability to detect single fluorescent molecules, but everything started with conventional epifluorescence microscope. Epifluorescence microscope uses a white light source for sample irradiation and equipment with special excitation and emission filters that enable tuning for specific fluorophores. In wide-field microscopy, the entire field of view is illuminated, leading to excitation of fluorophores throughout the whole excitation cone (figure 18B), resulting in poor axial resolution and possible photobleaching of fluorophores even outside the areas of interest. The shortcomings of conventional epifluorescence microscopy and the need to enhance the sensitivity, contrast, and resolution led to the development of a number of new fluorescence-based microscopy techniques (figure 16).

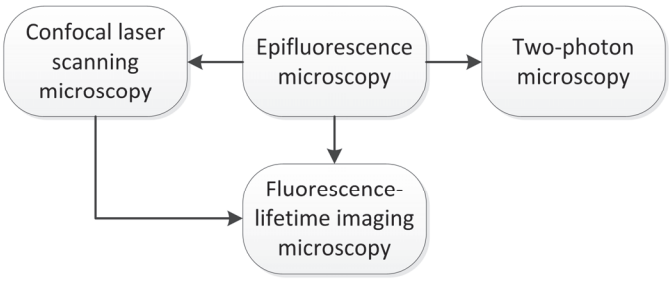

Figure 16. Examples of fluorescence-based microscopy techniques that are descendants of epifluorescence microscopy.

5.1.1. CONFOCAL MICROSCOPY

Confocal laser scanning microscopy (CLSM) features increased contrast and resolution (figure 17 A). CLSM is different from wide-field fluorescence microscopy in the following ways: 1) Laser in combination with raster scanning is used for sample irradiation, thereby partially reducing the photobleaching of the sample as well as allowing 3D image reconstruction; 2) in the configuration of the CLSM optical path, the existing pinholes allow for detection of the fluorescence only from the focal plane, thereby enhancing axial resolution.

CLSM was used in paper III of this study for visualization of porphyrin-cyclodextrin nanosystem distribution *in vitro*. The system was an inverted Nikon Ti-E microscope (Nikon Co., Shinjuku, Japan) equipped with a 405-nm pulsed/CW diode laser (PicoQuant GmbH, Berlin, Germany). Fluorescence was detected using a Nikon PLAN APO VC 60 NA 1.40 oil-immersion objective (Nikon Co.). Filters were set to distinguish between cell autofluorescence (500–550 nm) and porphyrin fluorescence (660–740 nm).

5.1.2. FLUORESCENCE-LIFETIME IMAGING MICROSCOPY

Most fluorescent microscopy techniques are based on the intensity of the fluorescence signal which directly depends on the local concentration of the fluorophore [188]. Fluorescence-lifetime imaging microscopy (FLIM) measures fluorescence lifetime, which is an intrinsic property of the fluorophore in a specific environment (interaction with quenchers, specific molecules, or ions) and does not depend on concentration [189]. FLIM even allows discrimination between fluorophores with identical excitation and emission properties in terms of their intrinsic fluorescence lifetimes characteristics. In FLIM, every pixel of the recorded image contains full time-resolved fluorescence spectra, which is analyzed and usually represented in false colour image, representing the distribution of the lifetimes within the sample (figure 17 B). Figure 17 B clearly shows that on certain occasions, the FLIM images provide more information than intensity-based images acquired by CSLM.

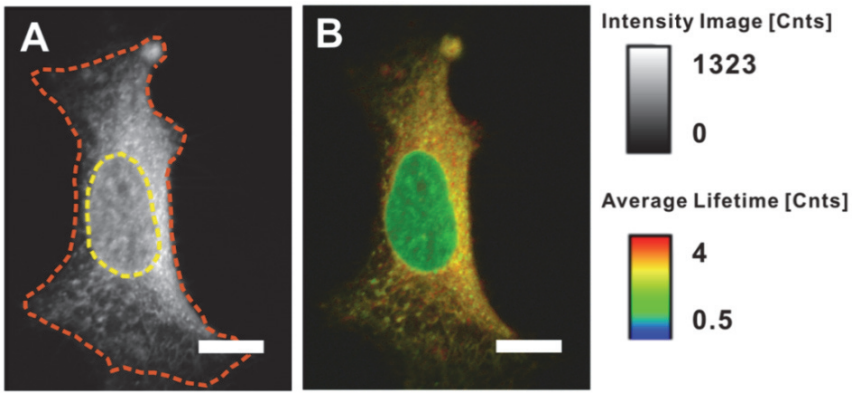

Figure 17. A) Fluorescence intensity and B) fluorescence lifetime images of HeLa cells treated with doxorubicin. Modified from [190].

FLIM imaging was used in paper III in combination with CSLM, as described above. The FLIM system was a Nikon Ti-E microscope (Nikon Co.) equipped with a 405-nm pulsed/CW diode laser operating at 40 MHz (PicoQuant GmbH) and a Nikon PLAN APO VC 60 1.40 NA oil-immersion objective (Nikon Co.). Measurements were taken by an integrated PicoHarp 300 electronics TCSPC system (PicoQuant GmbH). Fluorescence was detected by a single-photon avalanche diode detector equipped with a 625–675-nm band-pass filter. A tail fit was performed on the histogram calculated for a region of interest of the sample image. Fluorescence decay profiles were analyzed with a least-squares method, using multiexponential decay functions and deconvolution of the instrumental response function using the software provided by IBH Consultants Ltd.

5.1.3. MULTIPHOTON MICROSCOPY

The history of multiphoton microscopy (MPM) dates back to 1931 when Maria Göppert-Mayer stated the theory behind the two-photon excitation process (TPE) [191]. 30 years later with the invention of the monochromatic coherent light source i.e. laser [192] technical realization of MPM became possible [193]. Since the first application (W. Denk in 1990 [194]) the technique has significantly advanced and has become a powerful tool in many areas of biomedical research.

TPM[5] involves the excitation of molecules by light of a specific wavelength. Excitation is induced by two photons with lower energy in comparison to single photon excitation process (Figure 18 A). If these photons hit the molecule within a time gap of ~10^{-16} s and total energy of the photons is sufficient, the molecule is excited and the fluorescence photon is emitted when the molecule returns to the ground state [195]. The TPE is a very low-probability process requiring high local photon density. The spatiotemporal photon density compression can be achieved by using high numerical aperture (NA) objectives and pulsed femtosecond lasers (Figure 18 C). TPE is a non-linear process that has quadratic dependency on excitation light intensity that along with the requirement for high photon density leads to excitation occurring mainly in the focal volume of the objective [195] (Figure 18 B). This eliminates the need for pinholes, as in CSLM systems, because the majority of the fluorescence photons that reach the detectors originate from the focal excitation volume (~1 femtolitre).

The advantage of TPM is that wavelengths in the red and near-infrared (NIR) regions of the electromagnetic spectrum can be used for excitation of the fluorophores. These wavelengths match the so-called optical window of the biological tissues (600–1300nm) where the scattering and absorption of light by endogenous biomolecules is the lowest [196], allowing the

[5] Two-photon microscopy (TPM) is subtype of multiphoton microscopy method that specifically uses two photons for excitation of the fluorophores. TPM was utilized in this thesis, so the description of TPM is given here.

effective photon penetration depth to be up to 6 mm [197, 198]. However, in practice, imaging depths hardly exceed 200 µm in the epithelial tissues [199]. This is explained by the possibility of reabsorption of emitted photons, increased scattering of fluorescence light that is blue-shifted relatively to excitation light, and the intensity of emitted light being much weaker than that of excitation light.

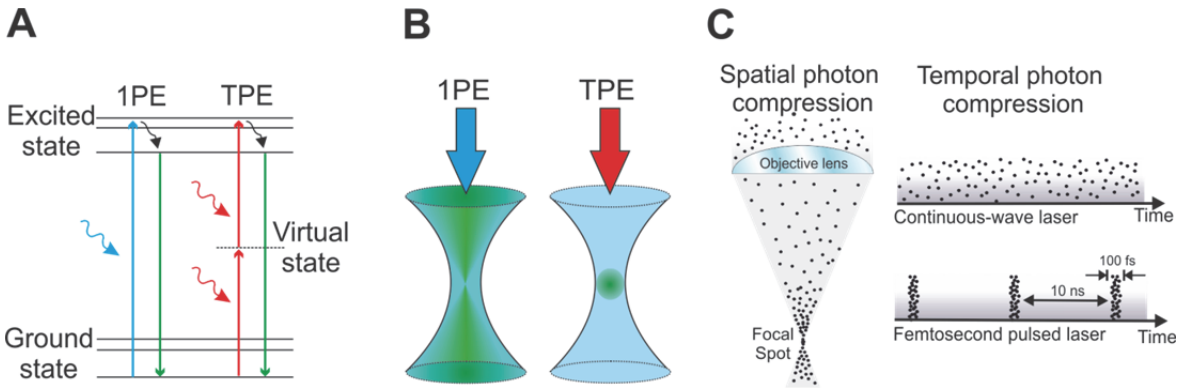

Figure 18. Basics of two-photon microscopy: A) Simplified Jablonski diagram for single (1PE) and two-photon excitation (TPE) processes; B) excitation volume for single and two-photon microscopy; C) spatial and temporal compression of photons.

TPM was utilized for investigation of the distribution and penetrative abilities of nanosystems in *ex vivo* cells (paper I) and skin (paper II). Additionally, in paper I, TPM was used for two-photon-induced NO radical release, simultaneously measuring the increase in the fluorescence concomitant process of NO radical generation. In paper V TPM was employed for detection of MIL signal arising from gold nanopaerticle-based targeted drug delivery systems specifically attached to cancer cells.

TPM was performed using an LSM 710 NLO microscope (Carl Zeiss, Jena, Germany) equipped with near infrared (NIR) mode-locked femtosecond pulsed Mai Tai DeepSee laser and Plan-Apochromat 20× water-immersion objective (NA 1.0) (Carl Zeiss). The applied laser power was not allowed to exceed 20 mW to minimize the likelihood of photodamage to the skin or cell samples. Filters for detection of fluorescence or MIL signal from nanosystems of interest varied and were set to 400–600 nm in paper I, 600–760 nm in paper II, and 590–710 in paper III. In paper V, multispectral imaging was performed, so the applied filters varied depending on the excitation wavelength.

Two-photon-induced NO photorelease was conducted by applying the “photobleaching” function. In brief, one image was recorded before the photobleaching procedure. Next, photobleaching was conducted on the selected region by scanning it for 20 iterations; then, the image was recorded. This procedure was repeated 10 times resulting in 10 images + the image

at time point 0. The images were analysed to evaluate the efficiency of NO release by evaluating the increase in the fluorescence.

Spectral imaging presented in papers I and V was conducted by applying lambda scan, i.e. two-photon-induced emission spectroscopy imaging with a spectral resolution of 10 nm. Spectral imaging allowed us to distinguish between the cell autofluorescence and MIL signal arising from the AuNPs that highly overlapped in the green region (paper V).

5.2. SKIN PERMEATION STUDIES

Many different techniques are used to investigate dermal drug delivery. Often, *in silico* calculations and simulations are used to predict the efficiency of skin penetration taking into account the physicochemical properties of the substance (e.g. molecular weight, partition coefficient (logP), etc.) [200, 201]. However, the results of *in silico* calculations are often vague.

The use of the diffusion cells (figure 19) can be considered a standard method for transdermal delivery studies involving xenobiotic agents. An artificial membrane or *ex vivo* skin specimen is used as a model for mimicking the *in vivo* skin [202]. Sampling the concentration of the analyte in the donor and acceptor compartments (before and after the incubation) allows estimation of dermal and transdermal delivery. However, this method estimates only the amount of penetrated compound and provides no information on the distribution of the compound in the skin. However, the diffusion cell method can be slightly modified and used for incubation of *ex vivo* skin samples with fluorescent substances and subsequent imaging with fluorescent microscopy techniques. This allows for imaging of penetration and distribution pattern of xenobiotics (i.e. haptens, DDSs, etc.) [203-208].

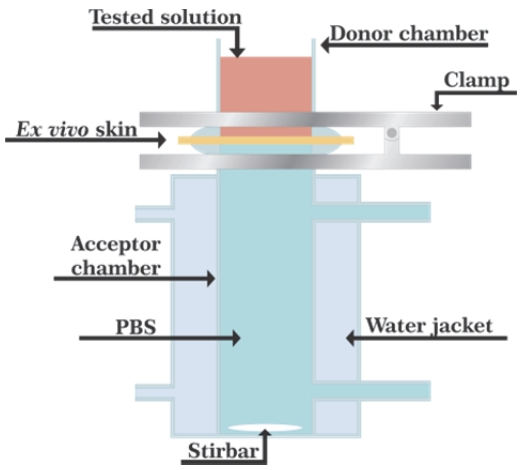

Figure 19. A flow-through Franz diffusion cell used for incubation of skin with different substances for subsequent biodistribution analysis with TPM.

The ability of nanosystems to penetrate *ex vivo* skin samples was examined in papers II and III. In brief, all *ex vivo* skin samples were obtained from Caucasian females; the samples were leftover specimens from breast reduction surgeries. Specimens, measuring 1 × 1 cm, were maintained at a temperature of -70 °C for no longer than 6 months. Prior to the experiments, skin samples were removed from the freezer and thawed. A major part of the subcutis was removed mechanically using a scalpel. Afterwards, full-thickness skin samples were mounted in flow-through diffusion chambers with the stratum corneum facing the donor chamber. The acceptor compartment was filled with phosphate buffered saline (PBS), whereas the donor chamber was used to load the test solutions. Parafilm was used to cover the donor compartment to prevent evaporation. During incubation (20 h), the diffusion cell was maintained at a temperature of 30 °C, covered with aluminium foil in a dark environment.

After exposure, the samples were taken out of the diffusion cell, thoroughly rinsed with PBS, and mounted on custom-made imaging chambers consisting of a No. 1.5 cover slip (0.18-mm, Menzel-Gläser, Saarbrückener, Germany) and a double-sided sticky tape.

5.3. CELL TOXICITY STUDIES

Many *in vitro* toxicity studies are carried out to determine the potential cytotoxicity of a compound, either to prove its safety and non-toxicity, or to highlight its potential cytotoxic effects. *In vivo* experiments can be very expensive and are strictly regulated, so *in vitro* studies are usually preferred as they require less effort and costs than *in vivo* studies to show the proof of principle or select the most suitable substances to undergo further evaluation. It is difficult to recreate the complex pharmacokinetics of a drug (e.g. drug metabolism, tissue penetration, clearance, etc.) in an *in vitro* cellular assay, but these assays are a good starting point for further studies and development.

Depending on the purpose, some assays might require analysis of specific markers, e.g. alteration of gene transcription, cell signalling, etc. [209], whereas studies on anticancer agents may require analysis of *cytocidal effects* (i.e. cell killing). Generally, *in vitro* assays can be divided into several groups: 1) viability, 2) survivability, 3) genotoxicity and transformation, and 4) irritancy. In the scope of this study, only viability and survivability assays will be briefly introduced; information on all of the assays can be found in a book by Freshney [209].

Viability assays measure the number of viable cells after potentially harmful procedures such as cryopreservation, exposure to highly toxic agents, phototoxicity of the PS during PDT, etc. Most of the assays are based on cell membrane disruption, measuring the uptake of dyes that usually are unable to penetrate the membrane (trypan blue, erythrosin, naphthalene black, and propidium iodide [210, 211]) or the release of the substances that are usually absorbed by the cells (diacetyl fluorescein or neutral red [211-213]). The effect of leaky membrane presents

immediately, thus most of these assays are usually quick and easy to perform. However, these tests tend to overestimate the viability of the cells as they provide information about only those cells that are already dead and do not take into account those that suffer severe damage and are in the process of dying.

Survivability assays are used to determine the survival rate of the cells and are based on the proliferative capacity of cells (i.e. plating efficiency or cell counting). These assays can be time consuming, especially when large numbers of samples are to be analysed. Therefore, metabolic activity assays (e.g. MTT, XTT, MTS, and increased amount of DNA or proteins [214-217]) are becoming more popular. Metabolic activity-based assays indirectly evaluate cell death by measuring the continued metabolism processes of living cells. MTT is a common method used for *in vitro* phototoxicity studies. Water-soluble tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced by mitochondrial succinate dehydrogenase into insoluble purple formazan crystals (figure 20 A). This process occurs only in living cells, so the extent of formazan crystal production reflects the number of living cells (or their metabolic activity). Formazan crystals dissolved in inorganic solvents (e.g. DMSO or isopropanol) produce a purple solution (figure 20 B) that can be quantified by measuring the absorption. The advantage of MTT-based assays is that they can be performed in multiwell plates, meaning multiple experiments can be conducted in the same conditions, and the obtained data can be statistically evaluated in a reasonable span of time. MTT assays were shown to provide accurate results, reproducing values yielded by standard clonogenic assays [218].

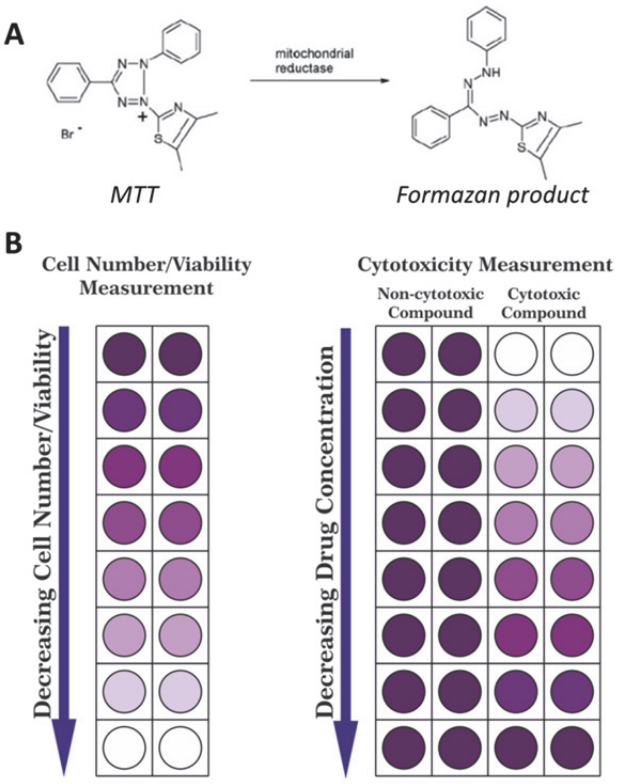

Figure 20. A) Reduction of tetrazolium dye to formazan product by mitochondrial reductase; B) example of application of MTT assay for cell number/viability or drug cytotoxicity experiments.

MTT assay for evaluation of cell viability and survivability was used in papers I, II, and IV for evaluation of NO-based PDT efficiency and of PDT efficiency enhancement. Briefly, 24 h prior the phototoxicity experiments, human squamous carcinoma cells (A431, HPA Cultures, Salisbury, UK) were seeded into a 96-well plate at a concentration of 10^4 cells/well. Several hours before the light exposure procedure (for precise protocols address the article I, II or IV), full-growth media was substituted with either fetal bovine serum-free media or PBS solution containing the nanosystem intended for phototoxicity studies. After incubation, the cells were irradiated with an LED array lamp at spectral position of 405 and/or 630 nm. The second set of cells, exposed to phototoxic nanosystems was kept in the dark outside the incubator as the control plate. Then, the solution was substituted with full-growth media, and the cells were placed in the incubator to recover, either for 24 h (papers I and II) or 30 min (paper IV). After the recovery time, tetrazolium dye was added to each well of the test and control plates and left for incubation for 2–3 h. Thereafter, the solution was substituted with 1% HCl in DMSO to solubilise formazan crystals. A microplate reader, Spectramax M2 (Molecular Devices, Berkshire, UK), was used to read the absorption for subsequent analysis of phototoxicity efficiency.

6. SUMMARY OF PAPERS

6.1. PAPER I

A polymer-based nanodevice for the photoregulated release of NO with two-photon fluorescence reporting in skin carcinoma cells

The nanosystem explored in this study[6] consists of 2 components: anthracene-nitroaniline NO photodonor (AntNO) and βCD-polymer (figure 21 A). Anthracene-nitroaniline is a FRET pair where anthracene is energy donor, whose fluorescence is quenched by nitroaniline moiety. At the acceptance of the energy from the donor, nitroaniline releases the NO and the quenching of anthracene is ceased. Thus, the increase of the fluorescence is an indicator of release NO radical, and can be utilized for precise dosimetry. However, AntNO is water insoluble, thus the role of βCD-polymer is to solubilize the AntNO in aqueous solution and to create nanoparticles of ≈25 nm in diameter.

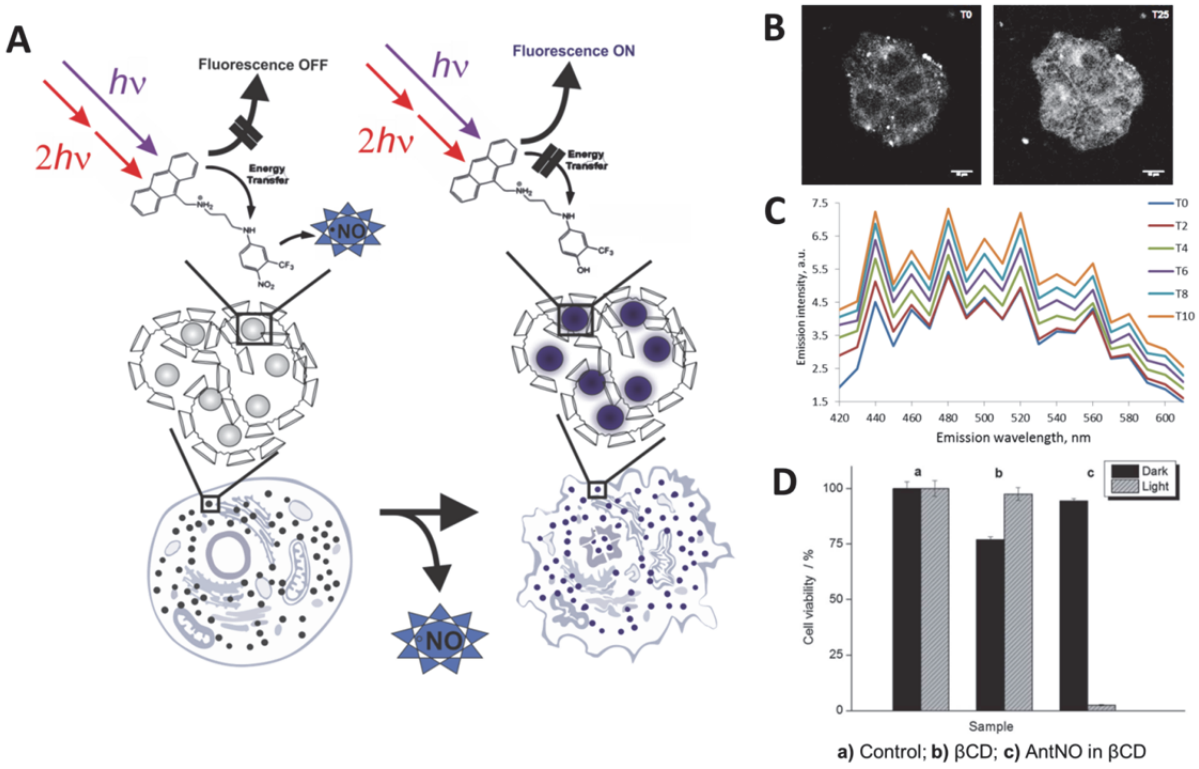

Figure 21. A) Schematic illustration of polymer-based nanodevice capable of NO radical release under one- and two- photon irradiation; b) TPM images of cells incubated with AntNO nanosystems before and after 25 min of irradiation with white light; c) Increase in the fluorescence measured with TPM at different points of irradiation; d) Phototoxicity of the nanosystem investigated in *in vitro* A431 cell culture.

[6] Synthesized by the group of Prof. S. Sortino, University of Catania, Catania, Italy

TPM imaging performed on *ex vivo* A431 cells incubated with the nanosystem, showed that anthracene-nitroaniline was accumulated generally in the cell cytoplasm with the possibility to relocate to the cell nucleus upon irradiation (figure 21 B). The findings of cell viability studies showed, that the nanosystem has high phototoxicity, resulting in cell mortality of above 90% when irradiated with 405 nm light (figure 21 D). However, this wavelength is not optimal for PDT in the biological tissue. Therefore, two-photon-induced NO release from *in vitro* cells at 700 nm was examined. The increase in the fluorescence of anthracene upon sample irradiation confirmed the possibility of application of two-photon excitation for photoinduced NO release (figure 21 C). High phototoxicity along with the possibility of two-photon-induced NO release implies that this nanosystem is a good candidate for further trials and, possibly, future medical application for NO-based PDT.

6.2. PAPER II

Bichromophoric nanoparticles for bimodal imaging and therapy

This chapter has been temporarily removed from the online version of this thesis since the data it is referring to has not yet been published. A new online version of the thesis will be published as soon as the manuscript has been accepted for publication

6.3. PAPER III

A spectroscopic investigation on meso-tetra(m-hydroxyphenyl)porphyrin-β-cyclodextrin conjugate focusing on topical delivery

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6.4. PAPER IV

An investigative report on the lack of enhancement of photodynamic therapy by combining endogenous or exogenous PpIX with PEGylated gold nanoparticles

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6.5. Paper V

Selectivity of β-cyclodextrin bearing gold glyconanoparticles towards Gal-3-overexpressing A431 cells in vitro

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7. CONCLUSIONS

This thesis explored different nanosystems such as Ant-NO in pCD (paper I), ZnPcS4 and AdaNO in pCD (paper II), *m*THPP-βCD conjugate (paper III), PpIX and PEGylated AuNP combination (paper IV) and lacto-CD-AuNP (paper V). The focus was on PDT and drug delivery to identify a means for improving PDT efficiency and the following conclusions were made:

- Nitric oxide photoreleasing nanosystems presented in paper I (AntNO) and paper II (AdaNO) are promising for PDT particularly in hypoxic or oxygen depletion conditions;
- The strategy proposed in paper IV (i.e. combination of PS with PEGylated AuNP) is not a feasible method for improving PDT efficiency;
- The systems studied in paper III (CD-mTHPP) and paper V (lacto-CD-AuNP) have shown promising biodistribution: for uptake (paper III) and targeted delivery (paper V).

To sum up, there are various direct and indirect ways for improving PDT efficiency: modification of PS, creation of multimodal systems for combinatorial treatment, alternative PDT methodologies, targeted delivery, and use of alternative sources of excitation energy for PS. Some methods did show promising results but they require further detailed examination in different conditions, but some other methods did not yield expected results.

Some cancer types such as SCC, cervical cancer and sarcoma are known to create hypoxic conditions within the tumour mass [220-223], or oxygen can be depleted during PDT procedure [224]. In these cases, it might be useful to apply combinatorial PDT approach such as those presented in papers I and II. Considering that NO molecules are transported to the tumour site as a structural part of the drug and are released specifically upon irradiation, the efficiency of PDT treatment, in this case, would not depend on oxygen concentration, thus allowing PDT to be efficiently conducted in hypoxic or oxygen depletion conditions. Thus, it is possible to compensate for low oxygen concentrations by photorelease of NO radicals, an alternative source of cytotoxic agents.

Moreover, the possibility to construct a multimodal DDS using a water-non-soluble PS conjugated to cyclodextrin is shown. The outcome is enhanced solubility, monomerization and fluorescence. The examination of *ex vivo* biodistribution in aqueous solution yielded promising results: cytoplasmic distribution and increased dermal penetration imply the effectiveness of PDT in medical application. The construct facilitates different cancer therapeutic strategies such as combinatorial treatment with PDT in combination with chemotherapy or NO-based PDT depending on the choice of load for cyclodextrin cavity.

Furthermore, mixing of the PS with PEGylated AuNPs did not facilitate energy transfer. The energy from the surface plasmon field around the PEGylated particles cannot be transferred to the PS, probably because of the PEG coating. Therefore, no interaction was detected in spectroscopy studies, and no enhanced PDT efficiency was observed in cell phototoxicity studies, thereby warranting the development of sophisticated systems with PS and AuNPs conjugated at carefully calculated distances. However, the proposed system composed of ALA and PEGylated AuNPs showed partial enhancement of PDT efficiency, supposedly owing to enhanced intracellular delivery of ALA by AuNP. Considering that currently ALA-based PDT is a common treatment modality for many skin diseases, further investigation with a focus on ALA delivery will be valuable.

The targeted multimodal nanosystems that we studied yielded promising results for cancer-specific drug delivery, especially valuable when there is a need to reduce the side effects and undesirable accumulation of the drug in non-cancer cells. It is possible to use the nanosystem with various drugs, for PDT or chemotherapy, in the cavity of cyclodextrins and determine the distribution and accumulation of the nanosystem by TPM. Combination of the nanosystems with TPM, operating in the red-infrared region of the electromagnetic spectrum of light, allows for efficient biomedical application. Furthermore, the system can be used for diagnostic purposes, e.g. identifying and attaching to the cancer cells, with a possibility of detection with TPM.

To conclude, many interesting and promising systems where studied and found to influence PDT in various ways. In many cases proof of principle systems were investigated and additional *in vitro* and *in vivo* studies are necessary for characterising the nanosystems and their actions from all possible perspectives. Even though at the moment some of the presented in the thesis nanosystems look promising for enhanced PDT efficiency and drug delivery, much work is yet to be done.

8. FUTURE OUTLOOK

Many systems presented in this study are in the proof-of-the-concept stage and have to be further investigated if they are to ever reach medical application and join the fight against the growing threat of cancer. For example, the systems presented in papers III (CD-mTHPP) and V (lacto-CD-AuNP) can be combined with components from the systems presented in paper I (AntNO) and II (AdaNO) to provide combinatorial and targeted treatment under hypoxic conditions.

In addition, the system presented in paper IV needs to be modified. Conjugates of PS and AuNPs with precisely defined distance between them need to be developed or research has to be shifted towards ALA AuNR combination. For the former conjugate, energy transfer and PDT efficiency need to be evaluated. For the latter combination, intracellular drug delivery and physical interactions between ALA and AuNP need to be examined.

Next, the nanosystem presented in paper V requires the application of additional techniques, e.g. TPM-based FLIM. Considering that there is a difference in the lifetimes of the fluorescence of some biomolecules [225] and the MIL from AuNPs [226], this method might facilitate the separation of the signals and detection of AuNPs. In addition, electron microscopy techniques (scanning or transmission) might provide additional information on where targeted AuNPs localize after incubation with cells at different durations.

Furthermore, in the lacto-CD-AuNP nanosystem the cavity of CD is located at a distance of ~4 nm from the surface of gold nanoparticle. Placing the PS inside of CD might induce the energy transfer1) from or 2) to the AuNP core. In first case it would be possible to observed enhanced singlet oxygen generation that was expected to be observed in paper IV. And in the second case, the energy transfer from PS to AuNP would lead to quenching of PS fluorescence[227]. The recovery of the fluorescence would indicate the release of the CD load allowing tracking the PS delivery efficiency and intracellular location of the released drug.

We recommend that nanosystems from papers I, II, and III be tested for penetration efficiency in *ex vivo* skin specimens in combination with additional skin penetration enhancers and delivery vehicles [228]. In addition, *in vivo* experiments need to be conducted should the systems show promising results. The experiments on mice such as biodistribution after topical or intravenous application, PDT on mice tumour models, application of different imaging modalities for DDS containing AuNP core, could yield valuable data on perspectives of the systems and possibilities of translating these nanosystems to medical practice.

Therefore, considerable work needs to be done to take small successful steps towards a big win in the battle against cancer.

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