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Evaluation of photodynamic activity of chlorine-type photosensitizers with β-cyclodextrins nanovectors

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par **Igor YANKOVSKY**

Evaluation de l'activité photodynamique des photosensibilisants de type chlorine avec les nanovecteurs cyclodextrines-β

Evaluation of photodynamic activity of chlorin-type photosensitizers with β-cyclodextrins nanovectors

Le 30 Novembre 2016

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ABBREVIATIONS

CD – cyclodextrin

DDS – drug delivery system

DLI – drug-light interval

DMSO – dimethyl sulfoxide

HDL – high-density lipoproteins

Hp-β-CD – hydroxypropyl-β-cyclodextrin

HpD – hematoporphyrin derivative

IV – intravenous

LDL – low-density lipoprotein

M-β-CD – methyl-β-cyclodextrin

mTHPBC – meta-tetra(hydroxyphenyl)-bacteriochlorin

mTHPC – meta-tetra(hydroxyphenyl)chlorin

mTHPP – meta-tetra(hydroxyphenyl)porphyrin

NMR – nuclear magnetic resonance

PBS – phosphate-buffered saline

PDT – photodynamic therapy

PEG – polyethylene glycol

PS – photosensitizer

QY – quantum yield

ROS – reactive oxygen species

SBE-β-CD – sulfobutylether-β-cyclodextrin sodium salt

TM-β-CD – trimethyl-β-cyclodextrin

$TPPS_4$ – tetrakis(4-sulfonatophenyl)porphyrin

VD – volume of distribution

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GENERAL INTRODUCTION

Photodynamic therapy (PDT) is a minimally invasive photochemical-based treatment with a promising clinical track record for oncological and other diseases. PDT involves three main components (light, photosensitizer, oxygen) to damage the target tissue by generating reactive molecular species. Tetrapyrrole compounds such as meta-tetra(hydroxyphenyl)-chlorin (mTHPC) are dominate photosensitizers (PSs) employed in PDT, but several difficulties associated with high hydrophobicity of these PSs leading to poor water solubility and aggregation within the vasculature after intravenous injection hampers its successful clinical application. Aggregation of PSs results in a decreased photodynamic efficacy, unfavorable biodistribution, moderate selectivity and prolonged skin photosensitivity.

To abolish these problems, special pharmacological forms such as liposomes, polymeric nanoparticles, bioconjugates are proposed for PS administration. Among the biodegradable and nontoxic compounds that can be used for drug delivery, cyclodextrins (CDs) are very promising. CDs are a family of cyclic oligosaccharides with a hydrophobic internal cavity and a hydrophilic outer surface. They are water-soluble, biocompatible in nature and can form stable inclusion (host-guest) complexes with a very wide range of solid, liquid and gaseous compounds. Inclusion of a hydrophobic drug into CD cavity usually increased the PSs solubility, physical and chemical stability, circulation time thus enhancing their bioavailability. Besides, CDs are used in the design of immediate as well as delayed release and targeted drug delivery systems.

mTHPC, the potent second-generation PS, which is currently under clinical trial for the palliative treatment of head and neck cancer, appears as a promising PS exhibiting a high therapeutic ratio. Previously, it was shown that mTHPC forms effectively inclusion complexes with derivatives of β-CD resulting in an increase of the PS solubility and improvement of its photophysical properties in aqueous solution. However, there is no available data on the use of mTHPC-CDs complexes *in vitro* and *in vivo* models so far.

The main objective of present work was to evaluate the effect of different CDs on mTHPC behavior at various stages of its distribution *in vitro* and *in vivo* and estimate the PS release from the carriers. We have also developed new approaches for analyzing mTHPC release from CDs. These approaches were based on the changing of fluorescence polarization degree and variability of mTHPC Soret band in function of PS microinvirement.

CHAPTER I. INTRODUCTION

1. Photodynamic therapy

1.1. An introduction to the photodynamic therapy

The combination of dyes and sunlight for treatment of several skin disorders dates back to ancient Egyptians, Greeks and Indians (Spikes et al., 1985). However, the emergence of the concept of photodynamic therapy (PDT) can be attributed to early observations made by O. Raab and H. von Tappeiner. O. Raab under the direction of Professor Dr. Hermann von Tappeiner observed that low concentration of acridine and some other dyes such as eosin, that had no effect in the dark, provoked the rapid killing of paramecia in the presence of light (Raab, 1900). The first mention of the necessity of the presence of oxygen for photodynamic action was reported in 1902 by C. Ledoux-Lebards. He observed that eosin killed paramecia more efficiently in open flask than in a closed bottle (Ledoux-Lebards, 1902). In 1903, von Tappeiner along with a dermatologist named Jesionek reported the tumoricidal effect of eosin associated with exposure to the white light on skin tumors (Von Tappeiner & Jesionek, 1903).

Despite this early success, PDT did not achieve enough impact and was lost for nearly 50 years when the photodynamic reaction was rediscovered by Lipson and Schwartz. Samuel Schwartz demonstrated that a mixture of hematoporphyrin derivative (HpD) was far more effective anti-tumor agent than hematoporphyrin (Schwartz et al., 1955). Lipson and his colleagues observed that injection of crude preparations of hematoporphyrin led to fluorescence of tumor lesions that could be visualized during surgery (Lipson et al., 1961; Lipson & Baldes, 1960). However, dissemination and application of PDT in clinical practice was associated with the work of Thomas Dougherty and his colleagues at Roswell Park Cancer Institute (University of Buffalo, USA). In contrast to previous studies, Dougherty created a commercially suitable photosensitizing drug, reliable light sources (Dougherty, 1974) and showed long-term HpD-PDT efficacy in animal models and humans (Dougherty et al., 1975, 1978). Chromatographic isolation of HpD led to the design of the photosensitizer (PS) Photofrin®, which was first approved for the treatment of bladder cancer in Canada in 1993. To date, Photofrin® is approved in the US, Europe and Japan for the treatment of advanced and early stage lung cancer, oesophageal adenocarcinoma, cervical cancer, superficial gastric cancer and bladder cancer (Dougherty, 2002; O'Connor et al., 2009).

Treatment by PDT is as follows (Fig. 1.1). PDT uses the combination of a photosensitizing drug (photosensitizers, PSs) and light to cause selective damage to the target tissue. In PDT, PSs are applied topically, locally or systemically (e.g., intravenously, IV). Firstly, PS is administered into the patient and it begins to redistribute into the body. After certain period, when PS accumulation in the tumor becomes greater than in normal tissue, the localized illumination of tumor region is carried out with a light source with an appropriate emission wavelength. Absorption of this light by tumor-localized sensitizer leads to generation of toxic free radicals and finally to the destruction of tumor tissue (Agostinis et al., 2011). Tumor destruction can be realized from three interconnected mechanisms: direct killing of tumor cells, damage to the tumor vasculature, and induction of an inflammatory reaction that can lead to the immune response. The selectivity of PDT is achieved by both the preferential uptake of the PS by the tumor (Jori, 1996) and directed illumination of treated tissue while sparing potentially healthy tissue.

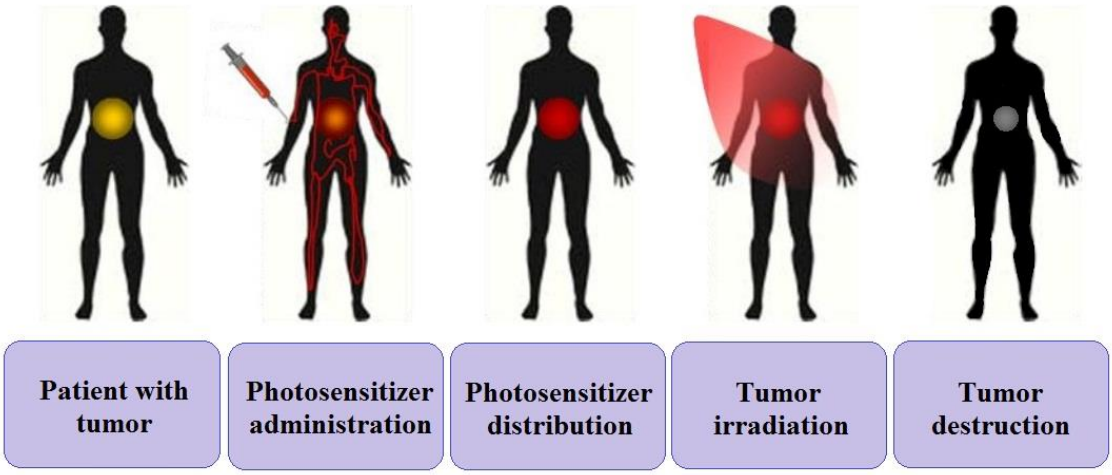


Figure 1.1 Schematic illustration of the treatment process of PDT

So far, about 250 randomized clinical trials have been officially reported for PDT of tumors, and essentially all types of solid tumors with the exception of melanotic melanoma have been found to be positively responsive to the photodynamic treatment (Rapozzi & Jori, 2015). The earliest known tumor approved for treatment was refractory superficial bladder cancer (Nseyo et al., 1998). To date, PDT is also approved for the treatment of obstructive and early-stage bronchial cancers (Usuda et al., 2006), esophageal dysplasia and carcinoma *in situ* (Fayter et al., 2010), and unresectable cholangiocarcinoma. The progress in PDT-protocols and synthesis of second-generation PSs led to efficient clinical treatment of head and neck tumors: widespread and unresectable or recurrent tumors (Biel,

2006), early stage oral cancers (Hopper et al., 2004) and nasopharyngeal tumors (Nyst et al., 2007). PDT has been shown to have high efficacy for basal cell carcinoma, including extensive or recurrent lesions. Prostate cancer treatment is under clinical trials and is performed as a primary therapy for focal tumors (Eggener et al., 2007), as well as the treatment of the whole prostate in patients who have recurred locally following radiation therapy (Trachtenberg et al., 2007). Excellent cosmetic outcomes make PDT suitable for patients with skin cancers (Fayter et al., 2010; Gao et al., 2010). However, the effectiveness of the treatment of many cancer types with PDT remains yet to be proven due to the lack of well-designed clinical trials (Agostinis et al., 2011).

An increasing number of PDT-related studies led to a better understanding of the factors controlling PDT and also extends the field of application of this method. Today PDT finds new applications not only for nononcologic dermatoses but also in the field of otorhinolaryngology, ophthalmology, neurology, gastroenterology and urology (Darlenski & Fluhr, 2013). PDT has indications for non-oncological diseases including psoriasis (Smits et al. 2006), ocular macular degeneration (Ziemssen & Heimann, 2012), chronic wounds (Morley et al., 2013), rheumatoid arthritis (Hansch et al., 2008), acne vulgaris (Pollock et al. 2004), periodontitis (Alwaeli et al., 2015) and PDT of certain bacterial, fungal and viral infections (Rajesh et al. 2011; Reinhard et al., 2015a; Yin & Hamblin, 2015).

1.2. Basic principles of photodynamic therapy

The action of PDT consists in the interaction of three principal components: (i) a nontoxic drug or dye known as a PS, (ii) the light of a specific wavelength and (iii) tissue/molecular oxygen. The lack of any of these components results in the absence of PDT effect. Therefore, to better understand the mechanisms of PDT action, it is important to understand the particular role of each of these components and how they interact. This section provides an overview of PDT with brief descriptions of the photophysical, photochemical and biological aspects of the treatment.

1.2.1. Photophysical and photochemical processes

Most PSs in their ground (usually singlet) state have 2 electrons with opposite spins located in an energetically most favorable molecular orbital (Castano et al., 2004). The biological effects of PDT are a consequence of a basic photochemical reaction involving PS and oxygen, which can be illustrated using modified Jablonski diagram (Figure 1.2). Absorption of light (initial step in all photoreactions) by PS leads to a transfer of one electron

from their ground state (S_0) to a higher energy orbital (S_n). A molecule with a high vibrational level of the excited state S_n (n depending on the PS and excitation wavelength used) will quickly fall to the lowest vibrational level of this state in a process called vibrational relaxation. Also, a molecule in a higher excited state S_n will finally fall to the first excited singlet state S_1 by internal conversion. Then, the singlet state S_1 can rapidly return to the ground state level S_0 by two mechanisms, a radiative process (fluorescence), or a non-radiative process (internal conversion). During this internal conversion, the excess of energy of the singlet state is released as heat, which dissipates usually into the tissue or the solvent. As for the radiative process, a photon is emitted with the energy equal to the energy gap between the S_0 and the S_1 levels, implying that the fluorescence does not depend on the excitation wavelength. Fluorescence is used very widely for PS detection in photodiagnostic applications (Mallidi et al., 2015).

Alternatively, an excited PS molecule may undergo an intersystem crossing forming a more stable triplet state (T_1) with inverted spin of electrons. The lifetime of the triplet state is much longer ($\tau > 10^{-7}$ s) than the lifetime of the singlet state ($\tau \sim 10^{-9}$ s), which greatly increases the probability of a reaction with a neighboring molecules, and the biologically relevant photochemistry is often mediated by this state (Lakowicz, 2006). There are several pathways for the triplet state T_1 to return to S_0 including the emission of a photon (phosphorescence) and intersystem crossing followed by vibrational relaxation.

Generally, only the S_1 and T_1 can be considered as likely candidates for the initiation of photochemical and photophysical reactions. This is due to the fact that higher orders of electronic states ($n \geq 2$) undergo very rapid internal conversion from S_n to S_1 and from T_n to T_1. However, under the special conditions of multiphoton absorption (short pulse, high intensities of irradiation), the upper excited states may be filled and complex photophysical and photochemical processes can occur (Shea et al., 1990; Smith et al., 1994).

The PS in triplet state can also initiate photochemical reactions directly upon interaction with a substrate, giving rise to reactive free radicals (type I reaction), or transfer its energy to molecular oxygen ($^3O_2$), which is unique in being a triplet in its ground state (type II reaction) (Foote, 1968; Sharman et al., 2000). The relatively longer lifetimes for the triplet excited states make the collisional transfer of energy to surrounding oxygen molecules possible. A Type I process can occur whereby the PS reacts directly with an organic molecule in a cellular microenvironment, acquiring a hydrogen atom or electron to form a radical (Henderson & Dougherty, 1992). Both the excited PS and the ground state substrate can act as hydrogen donors. The resulting radical species from type I primary processes can

subsequently participate in different kinds of reactions. In the presence of oxygen, for example, oxidized forms of the PS or of the substrate readily react with oxygen to give peroxyl radicals, thus initiating a radical chain auto-oxidation. Semi-reduced forms of the PS or of the substrate also interact efficiently with oxygen, and the electron transfer generates superoxide anion radical ($O_2^{\bullet-}$). Dismutation or one-electron reduction of $O_2^{\bullet-}$ gives hydrogen peroxide (H_2O_2), which in turn can undergo one-electron reduction to a powerful and virtually indiscriminate oxidant hydroxyl-radical ($HO^{\bullet}$) (Henderson & Dougherty, 1992).

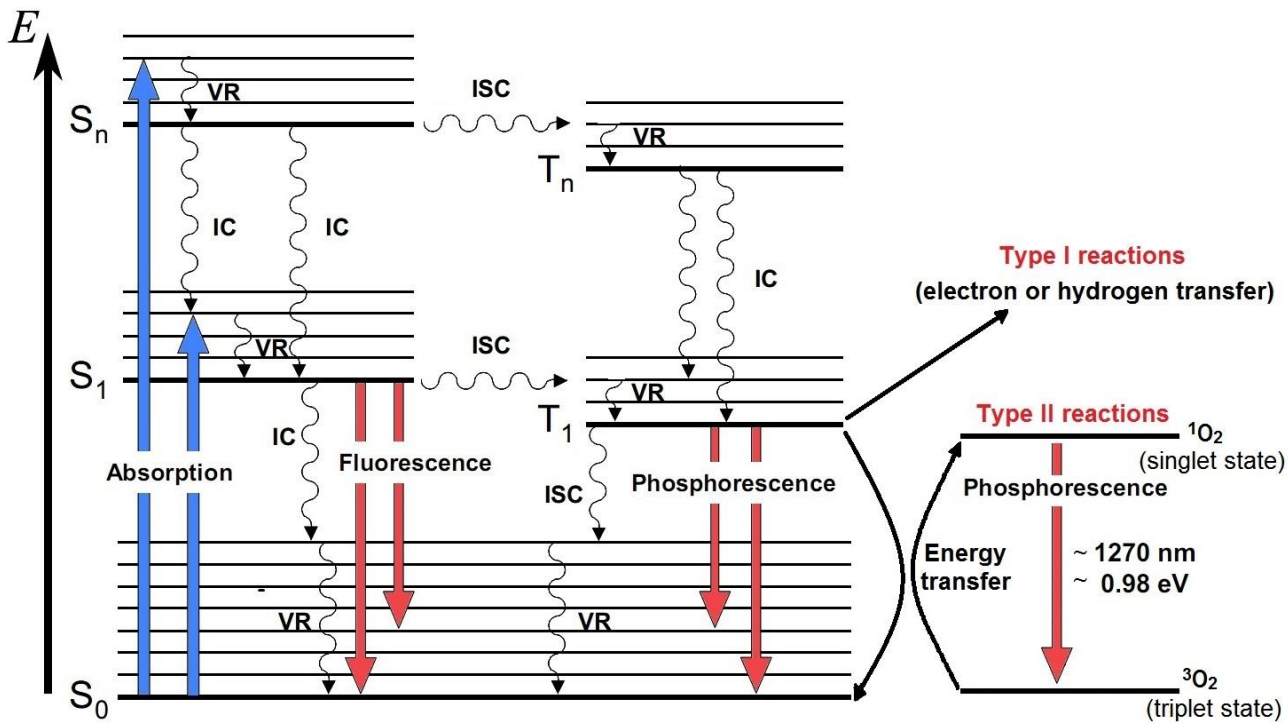


Figure 1.2 Modified Jablonski energy diagram for PS. VR – vibrational relaxation, IC – internal conversion, ISC – intersystem crossing

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 (Aveline et al., 1998; Plaetzer et al., 2009). However, reactive oxygen species (ROS) generation via Type II chemistry is much simpler than via Type I, and most PSs are believed to operate via a Type II rather than Type I mechanism. In type II process, the reaction proceeds via energy transfer from the excited triplet-state PS to the molecular oxygen in its triplet state. Only PSs that possess an energy gap between the ground state and the excited triplet state higher than the energy needed to

excite oxygen into its excited singlet state (94 kJ/mol according to van Lier & Spikes, 1989) can generate singlet oxygen. Theoretically all molecules absorbing light at a wavelength < 1260 nm can mediate generation of $^1O_2$. However, photons with wavelengths longer than 800 nm have insufficient energy to initiate a photodynamic reaction (Juzeniene et al., 2006).

It is worth noting that singlet oxygen is a very reactive species, much more electrophilic than its ground state, and can oxidize biomolecules very rapidly. Lifetime of singlet oxygen varies from about 4 µs in water to 25-100 µs in non-polar organic solutions and greatly decreases (about 10-330 ns) in biological systems due to the presence of various quenchers (Baker & Kanofsky, 1992). This short lifetime allows the diffusion of singlet oxygen to a distance from 10 to 55 nm at the sub-cellular level (Dysart & Patterson, 2005; Moan & Berg, 1991), thus limiting the PDT effect to the immediate intracellular localization of the PS (Moan et al., 1989).

1.2.2. Mechanisms of photodynamic action *in vivo*

PDT destroys tumors by three inter-related mechanisms: direct cytotoxic effects on tumor cells, damage to the tumor vasculature, and processes involving the immune system. These are illustrated in Fig. 1.3. In the first case, the ROS that are generated by PDT can directly destroy tumor cells by apoptosis, necrosis and/or autophagy-associated cell death. PDT also damages the tumor-associated vasculature with impairment of the blood supply to the area via platelet aggregation and, thus, vascular occlusion. Finally, PDT can induce a robust inflammatory reaction that can activate an immune response against tumor. This section will describe the mechanisms of PDT action conditionally separated as cellular effects, vascular effects and systemic immune effects of the treatment.

1.2.2.1. Direct tumor cell death effects

Direct cell kill is an important contributor to PDT mediated tumor destruction *in vivo.* Henderson et al. (1985) showed that exposure of tumors to PDT *in vivo* leads to decrease of the number of clonogenic tumor cells through direct cells photodamage. Direct cell kill can occur through necrosis, apoptosis and/or autophagy-associated cell death. Mechanism of cell death depends on cell and PS type, treatment protocol, and photodynamic dose (Agostinis et al., 2011; Castano et al., 2004, 2005a, 2005b).

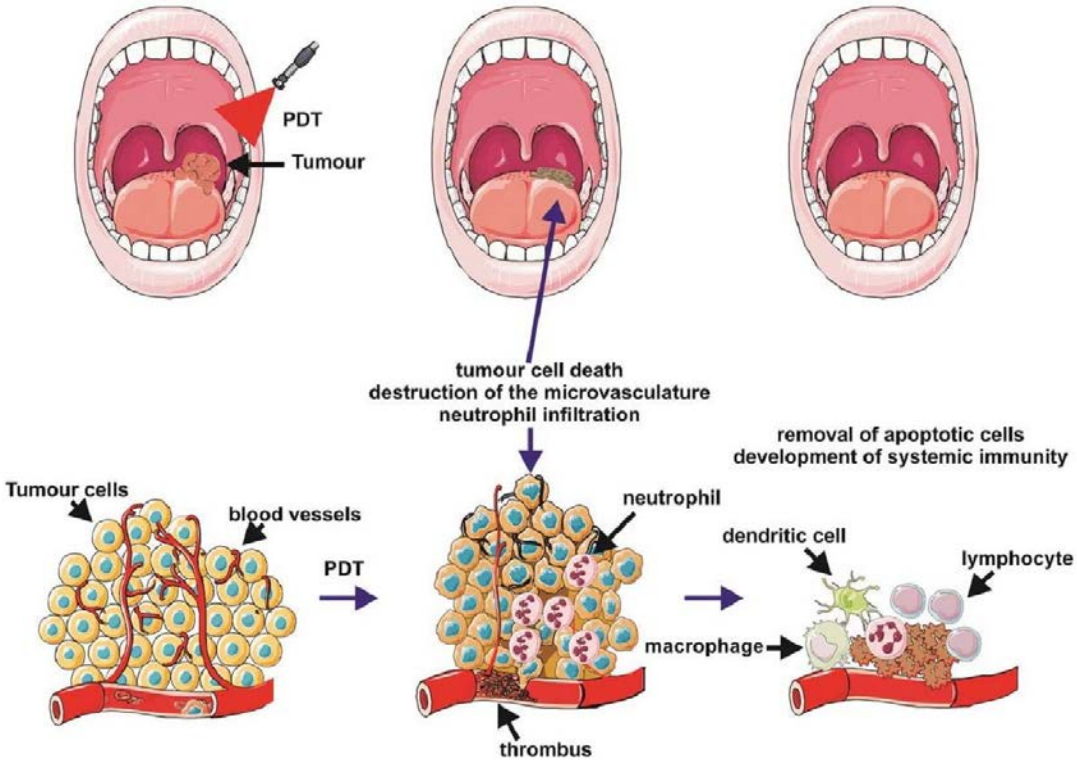


Figure 1.3 Mechanisms of PDT tumor photoeradication (from Agostinis et al. 2011)

Apoptosis is the most studied and generally major cell death mechanism responding to PDT. Apoptosis or programmed cell death is identified in single cells, and morphologically characterized by cell shrinkage, chromatin condensation, cleavage of chromosomal DNA into internucleosomal fragments, blebbing of the plasma membrane, and the formation of apoptotic bodies without plasma membrane breakdown (Agostinis et al., 2004; Almeida et al,. 2004). *In vivo*, apoptotic cells release the "find me" and "eat me" signals required for the clearance of the remaining apoptotic bodies by phagocytes that usually prevents inflammation response. The most important role in the initiation of apoptosis among all subcellular structures is played by mitochondria (Castano et al., 2005a; Kessel & Luo, 1999). The damage to mitochondria after PDT results in a cascade of reactions including the rapid release of mitochondrial cytochrome C into the cytosol followed by activation of the apoptosome and procaspase 3 that eventually cause the apoptosis (Oleinick et al., 2002). However, localization of the PS in the lysosomes may also activate apoptotic cascade (Bonneau & Vever-Bizeta, 2008). Woodburn et al. (1997) demonstrated that lutetium texaphyrin, which is mostly localized in the lysosomes of EMT6 cells *in vitro*, induces apoptosis in EMT6 tumors *in vivo.*

Necrosis is a rapid form of death affecting extensive cell populations, characterized by cytoplasm swelling, destruction of organelles and disruption of the plasma membrane,

resulting in an inflammatory reaction due to the release of cellular contents and pro-inflammatory molecules. Necrosis has been associated with incidental cell death, caused by physical or chemical damage and has generally been considered an unprogrammed process. During necrosis, decomposition of cell is principally mediated by proteolytic activity and inhibition or genetic deficiency of caspases in the cell signaling (Castano et al., 2005a; Kessel, 2002).

Another mechanism of cell death have been also described: mitotic cell death (Castedo et al., 2004), cathepsin-mediated lysosomal death pathway (Leist & Jäättelä, 2001), programmed necrosis (Bizik et al., 2004; Vanlangenakker et al., 2008) and autophagic cell death (Yu et al., 2004). The last (autophagy) is characterized by a massive vacuolization of the cytoplasm and can be stimulated by various stress signals including oxidative stress (Dewaele et al., 2010). In autophagic cell death processes, normal function used to degrade components of the cytoplasm is involved and characterized by appearance autophagosomes, autolysosomes, electron-dense membranous autophagic vacuoles, myelin whorls, multivesicular bodies, as well as engulfment of entire organelles (Castano et al., 2005a). Recent studies describe autophagy as a mechanism to preserve cell viability after PDT treatment (Reiners et al., 2010). On the other hand, photodamage of lysosomal compartment by the PS may compromise completion of the autophagic process.

In rare cases, complete tumor destruction is achieved only through direct damage of tumor cells. Indeed, non-homogenous distribution of PS within the tumor, insufficient depth of light penetration into the tumor or distance of tumor cells from the vessels (Korbelik & Krosl, 1994) as well as availability of oxygen (Tromberg et al., 1990) may hamper the tumoricidal effect. Therefore, other mechanisms involved in the destruction of the tumor during PDT will be discussed in next chapters.

1.2.2.2. Antivascular effects of PDT

The importance of vascular damage for tumor destruction is well recognized. PDT damage of tumor microvasculature leads to severe and persistent tumor hypoxia and restricts nutrient supply to the proliferating tumor cells. Numerous studies revealed that blood vessel of tumors are fundamentally different from normal vasculature (Ausprunk & Folkman, 1977; Eberhard et al., 2000; Siemann, 2011). Tumor vasculature is typified by aberrant structural dynamics and vessels that are immature in nature with poorly developed, chaotic growing architecture and discontinuous endothelial linings (Eberhard et al., 2000). Besides, the

endothelial cells lining tumor vessels have an irregular, disorganized morphology that result in enhanced permeability (Siemann, 2011).

Targeting the tumor vasculature is a promising approach to cancer treatment. Destruction of endothelial cells during PDT treatment seems to be the origin of modifications observed in vasculature (Fingar et al., 2000); therewith endothelial cells were shown to be more sensitive to PDT compared to muscle cells, together with increased PS uptake (West et al., 1990). PDT incites modifications of organization of the proteins of cytoskeleton of endothelial human cells with consecutive induction of a signal to the platelets and neutrophils activation which adhere on the vessel wall (Senge & Radomski, 2013). In the region of injury the following changes are observed: initial blanching and vasoconstriction of the tumor vessels, followed by heterogeneous responses including eventual complete blood flow stasis, hemorrhage, and, in some larger vessels, the formation of platelet aggregates (Krammer, 2001). The combination of vessel constriction, thrombus formation, and increased interstitial pressure leads to tissue ischemia followed by necrosis (Fingar et al., 2000; Henderson et al., 1985). Moreover, vascular destruction after PDT may be accompanied by inflammatory response like after tissue injury (Korbelik, 1996).

It is worth noting that different PSs do not produce the same type of vascular response. PDT with monoaspartyl chlorin e_6 (MACE®) produce blood stasis mainly due to platelets aggregated on the artery walls (McMahon et al., 1994) while certain phthalocyanine derivatives cause primarily vascular leakage (Fingar et al., 1993b). At the same time, using of Photofrin® leads to vessel constriction, macromolecular vessel leakage, leukocyte adhesion and thrombus formation corresponding to platelet activation and release of thromboxane (Fingar et al., 1993a).

1.2.2.3. PDT and the immune response

Nowadays it is shown that PDT can affect both innate immunity and adaptive immunity. The influence of PDT on the immune response is quite complicated and defines by many factors, such as the type of PS and its formulation, pharmacokinetic and location within tumor and surrounding cells (especially immune cells), the area treated and treatment regimen (e.g., drug-light interval (DLI) and settings of irradiation) (Kousis et al., 2007; Reinhard et al., 2015a).

PDT-induced oxidative stress frequently incites a strong acute inflammatory reaction observed as localized edema at the targeted site (Dougherty, 2002). The main task of inflammatory response consists in the disruption of homeostasis and the removal of damaged

cells, thus promoting local healing with the restoration of normal tissue function. The inflammatory signaling after PDT initiates a massive regulated infiltration of matures dendritic cells and other cellular components of both the innate and adaptive immune systems (Castano et al., 2006). Korbelik & Krosl (1994) showed that macrophages demonstrated preferential cytotoxicity to tumor cells after PDT treatment. Furthermore, upon photostimulation macrophages may regulate the release of pro- or anti-inflammatory cytokines (Korbelik & Krosl, 1994; Lynch et al., 1989). Besides, PDT-generated lysates are able to activate dendritic cells and increase ability to stimulate T-cells, which is critical to the development of a cellular immune response (Gollnick et al., 2002). Removal of inflammatory cells or inhibition of their activity after PDT demonstrated significant reducing of therapeutic effect (Agostinis et al., 2011).

It has been showed that PDT can influence the adaptive immune response in different ways, resulted both in potentiation of adaptive immunity and immunosuppression (Castano et al., 2006; Hunt & Levy, 1998). Long-term effect of PDT depends on the induction of antitumor immunity. Canti et al. (1994) showed that resistance to MS-2 fibrosarcomas rechallenge was evident only in normal surviving animals treated by PDT, whereas immunosuppressed surviving animals and animals cured by surgery died after tumor recurrence. PDT treatment of murine EMT6 mammary sarcoma using Photofrin® indicate that whereas the direct effects of PDT can destroy the bulk of the tumor, the immune response is required to eliminate the surviving cells (Korbelik et al., 1996).

It should be noted that all these mechanisms occur as interrelated with each other and the predominance of one or another mechanism of tumor destruction depends on many factors, such as the type of PS, light and PS dose, DLI. Therefore, determination of optimal PDT conditions requires a coordinated interdisciplinary effort.

1.2.3. Photosensitizers

Photosensitizers are one of the main components of PDT. To date, a large number of photosensitizing agents have been studied in PDT. The majority of PSs used both clinically and experimentally has a tetrapyrrole structure, such as porphyrins, chlorins, bacteriochlorins and phthalocyanines (Josefsen & Boyle, 2012; O'Connor et al., 2009). However, other classes of compounds including the synthetic dyes (e.g., phenothiazinium, squaraine) transition metal complexes, and natural products (e.g., hypericin, riboflavin, curcumin) have been investigated (Abrahamse & Hamblin, 2016).

1.2.3.1. General properties of photosensitizers

Photosensitizers used both clinically and experimentally, differ widely in their solubility, photophysical properties, cellular and tissue distribution that requires a detailed understanding of the most important properties of these drugs. At the same time, regardless of the origin of the PS, it must possess certain properties to be considered as a PDT efficient drug.

From a chemistry and manufacturing point of view, synthesis of the PS should be relatively easy and low coast; the PS should be a pure and stable compound. Other important features of an efficient PS are photophysical and photochemical properties, such as optimal excitation wavelength and high singlet oxygen generation yield. PSs should absorb light in the red or far-red wavelengths (600-800 nm) for maximum light penetration into the tissue. Due to light scattering and absorption of tissue chromophores, shorter wavelengths (<600 nm) have less tissue penetration and are mostly absorbed, resulting in high skin photosensitivity (Castano et al., 2004). As mentioned earlier (see chapter 1.2.1), absorption at longer wavelengths (>800 nm) is not sufficient for PS triplet state to transfer energy for singlet oxygen generation. PSs should possess high ROS generation quantum yield (QY) for high photodynamic efficiency (implying high triplet state yield). For diagnostic use, the fluorescence QY needs to be sufficient.

Although certain PS features can be simply modified (e.g., wavelength absorption), other aspects such as manipulation of PS biodistribition and pharmacokinetic profile are not as easily regulated (O'Connor et al., 2009). In this regard, it is important that the medication had optimal absorption, distribution, metabolism and excretion properties for relevant application. PDT drugs should have selective uptake in target tissues and sufficient penetration depth to induce cytotoxic effects, as well as microlocalization to sensitive cellular/subcellular targets (e.g., mitochondria). The substance should have little or no dark toxicity, low incidence of administrative toxicity (e.g., allergic reaction), absence of metabolic creation of toxic byproduct and do not trigger mutagenic effects (regardless of the presence of illumination) (Allison & Sibata, 2010). Besides, PDT drugs should have relatively rapid clearance from normal tissues (ideally, measured in hours or few days), thereby minimizing phototoxic side effects. From clinical point of view PS should be commercially available, reliable and pain-free upon activation, acceptable for outpatient treatment with versatile and easy administration, depending on the clinical situation.

1.2.3.2. Photosensitizers pharmacokinetics and distribution in the body

As discussed in Chapter 1.2.2, treatment protocol and the mechanism of PDT damage to the target tissue is largely determined by the pharmacokinetics and distribution of PS in the body. It is well recognized that the distribution of parenterally administrated PS molecules during PDT of solid tumors may be defined by three main stages: i) PS interactions with the various blood components after injection and transport in bloodstream, ii) PS transfer from the bloodstream to the extravascular tissue and iii) PS interaction with intracellular targets within the tumor environment.

Firstly, after injection into the bloodstream the PS must come to equilibrium with the blood components. Introduced PSs are mostly associated with plasma proteins (Lang et al., 2004); although in cases of relatively hydrophobic PSs a significant part of the administrated drugs can bind to the blood cells (Maugain et al., 2004). The distribution of the PS in blood allows defining three classes of PSs:

- relatively hydrophilic compounds such as chloroaluminum phthalocyanine and sulfonated tetraphenylporphyrin derivatives are bound to albumin and possibly globulins fractions;
- asymmetric and amphiphilic PSs such as monoaspartyl chlorin e_6, the adjacent disulfonates and benzoporphyrin derivative monoacid are primarily partitioned between albumin and high-density lipoproteins (HDL);
- hydrophobic PSs such as unsubstituted phthalocyanines, tin-etiopurpurin and meso-tetra(hydroxyphenyl)porphyrins can be incorporated into the inner lipid core of lipoproteins particularly low-density lipoprotein (LDL) (but also HDL and very low-density lipoprotein)

Plasma protein binding pattern is an important factor governing the *in vivo* pharmacokinetics and biodistribution of PSs, thereby affecting its photosensitizing efficiency during PDT (Castano et al., 2005b). Based on numerous studies it was established that the type of protein-carrier governs the delivery of sensitizer to the tumor and may lead to different mechanisms of tumor destruction. Indeed, the PS associated with albumin and globulins are delivered mainly to the vascular stroma of tumors (Jori, 1989; Peters, 1995), HDL delivers PS to cells via a non-specific exchange with the plasma membrane, LDL probably delivers a large fraction of the PS via an active receptor-mediated pathway (Jori & Reddi, 1993). It has been showed that most effective PS preferentially binds to LDL (better tumor localizers) due to upregulated LDL receptors on tumor cells (Kessel, 1992), but this is not always the case (Korbelik, 1992).

After penetration through the wall of the blood vessels the PSs accumulate in healthy and tumor tissues. It is difficult to compare the distribution of different PSs for a number of reasons (delivery system, route of administration, different animals and tumor types). However, there are common features of the PS organ distribution in experimental animals, which is inherent for most of the studied PSs. In most cases the highest concentration of PS was detected in the liver of investigated animals that is due to its detoxifying role in the body (particularly excretion of organic molecules such as PS from the blood) (Ocakoglu et al., 2015; Qian et al., 1987; Woodburn et al., 1992). Large concentrations of PSs are also found in kidney and spleen, which (like the liver) strongly support the major role of tissue vascularity and are part of the reticuloendothelial system (Jori, 1989). Sulphonated derivatives such as tetrakis(4-sulfonatophenyl)porphyrin ($TPPS_4$) have the greatest accumulation in the kidneys and were found to be neurotoxic on systemic administration (Winkelman & Collins, 1987). Chan et al. (1990) showed on the model of sulfonated phthalocyanines that increase in the degree of sulfonation (increasing of PS hydrophilicity) correlated with the increase of PSs accumulation in the kidney and simultaneous decrease of its uptake by the liver. At the same time, the accumulation of PSs in the spleen varies greatly depending on the PS structure (hydrophobicity and charge) and form of administration (dosage, solution or delivery system) (Egorin et al., 1999; Qian et al., 1987; Woodburn et al., 1992). It is shown that shortly after administration of PSs at a high dosage (generally relates to hydrophobic compounds), the PS's aggregates could accumulate in large quantities in the fine capillary network of the lungs resulting in pulmonary hemorrhage and acute interstitial pneumonia (Egorin et al., 1999). Organs such as the heart, brain, eyes, skeletal muscles and bones, which are relatively impermeable to the blood supply, accumulate the lowest concentrations of PS (Castano et al., 2005b). The organs of the digestive system (stomach, large and small intestines), as a rule, accumulate intermediate amount of PS. It was not revealed that skin accumulates a large amount of PSs (usually not considerably different from PS uptake by a tumor); however, even a small residual concentration of drugs may cause significant cutaneous photosensitivity (Moriwaki et al., 2001).

With the aim of improving the effectiveness of PDT, the ratio between the tumor and normal tissue (peritumoral/distant muscle or skin) should be maximal at the time of tumor irradiation. It is useful to make a difference between selective accumulation and selective retention of PS. As a rule, the tumor localizing ability of the PS with the faster pharmacokinetics is probably due to selective accumulation in the tumor, while the localization of PS with slower pharmacokinetics is likely due to selective retention (Castano

et al., 2005b). The properties of tissues also play an important role in the selective drug accumulation. The selective accumulation may be facilitated by a large number of LDL-receptors (e.g., accumulation of PS bound to LDL) (Bonneau et al., 2004) and/or low interstitial pH of targeted tissues (e.g., accumulation of anionic PS) (Tannock & Rotin, 1989; Pottier & Kennedy, 1990). The correlation between the PS accumulation in tumors and their structure (e.g., lipophilicity, the symmetry of the chemical structure including the distribution of PS polar and hydrophobic chains around the macrocycle and the electric charges of these chains) has been established (Boyle & Dolphin, 1996). Furthermore, each step of distribution and clearance of the photosensitizer from the body is determined by characteristic times, which considerably influence treatment protocol. An important parameter LDI in clinical PDT is determined by preferentially localization of PS in tumors. For example, tumor localization of verteporfin changed from the tumor vasculature at 15 min after injection, to tumor parenchyma at 3 h after injection (Chen et al., 2005). This feature of the PS distribution allows combining both damage of tumor vasculature and parenchyma by two intervals of treatment and leads to enhancement of PDT efficacy. The selective retention of PS in tumors may be favored by retention of protein-bound PS in the tumor extravascular space due to poorly developed lymphatic drainage (Roberts & Hasan 1992) and by macrophages (accumulate up to 13 times the amount of PS compared to cancer cells) infiltration into solid tumors (Korbelik & Krosl, 1994).

The last step of PSs transport is elimination from the tissue and the body. In the case of non-metabolic organ, elimination will be probably by lymphatic drainage and back into the circulation via the thoracic duct. For the majority of PS in clinical and preclinical use, the clearance route being almost exclusively via liver and bile even if a PS mainly accumulates in the kidney (Richter et al., 1990). There has been a wide variation in pharmacokinetics reported for different PSs with significant differences in PS retention into the tumor and its elimination pathways. The study showed that the pharmacokinetics of Photofrin® in patients with carcinoma of the lung or skin is described by a triexponential three-compartment pharmacokinetic model with half-lives of approximately 16 h, 7.5 days, and 155.5 days (Bellnier & Dougherty, 1996). On the other hand, padeliporfin (PS based on the bacteriochlorin macrocycle) has an elimination half-live in the range of minutes (Brandis et al., 2005) that is common to photosensitizers derived from natural bacteriochlorophylls or bacteriochlorins (Dąbrowski & Arnaut, 2015).

It is worth noting that incorporation of PSs into a delivery vehicle may considerably change the plasma behavior and the cellular localization of the PS, as well as its the

pharmacokinetics and biodistribution (Konan et al., 2002; Lucky et al., 2015). For example, Richter et al. (1993) showed that liposomal formulation of the benzoporphyrin derivative monoacid ring A demonstrates faster PS accumulation in the tumor (maximal level reaches at 15 min vs 3 h for dimethyl sulfoxide (DMSO) or phosphate-buffered saline (PBS) injection form of PS) and the highest tumor/normal tissue ratios at 24 h (19.2 vs 12.6 for DMSO or PBS form of PS injection).

1.2.3.3. Classification of PDT photosensitizers

To date, there are a huge number of compounds that are either experimentally studied or have already been approved for clinical use in PDT. Accordingly to their chemical structure, these PDT drugs are generally classified as porphyrinoids (tetrapyrrolic compounds) and non-porphyrinoids (O'Connor et al., 2009). The class of nonporhyrinoid PSs includes several types of molecules of different chemical structure (common is only the presence of multiple conjugated π bounds) and origin (natural compounds, such as hypericin and curcumin, and synthetic dyes, such as phenothiazinium and cyanines). Although the sensitizers of this type are of interest for anticancer therapy, at the moment none of such PSs are approved clinically (Abrahamse & Hamblin, 2016). Some of the nonporhyrinoid PSs such as phenothiazinium salt and hypericin are in the early stages of clinical trials (mostly in the area of antimicrobial PDT or for samples sterilization) (Abrahamse & Hamblin, 2016; O'Connor et al., 2009).

Porphyrinoids PSs are most widely used in cancer PDT. Porphyrinoid PSs have in common a carbon heterocyclic macrocycle (four pyrrolic sub-units) comprising 18 to 22 electrons in conjugated π bounds (Josefsen & Boyle, 2012). In addition, this class also includes prodrugs (a metabolic precursor of PS). For example, aminolevulinic acid (ALA) is metabolized to photoactive protoporphyrin IX, which is the endogenous photactivating agent. Porphyrinoids PSs that have received clinical approval or are currently in trials are summarized in the Table 1.1. PDT drugs are commonly classified as first, second or third generation PSs. First generation PSs include HpD and their commercial form (Photofrin®, Photogem®) that have been for a very long time the only PSs used in clinical PDT. However, non-optimal photophysical properties (absorption maximum at 630 nm with molar extinction coefficient 1170 $M^{-1}cm^{-1}$), lack of tumor selectivity, poor bioavailability, and unfavorable biodistribution, as well as prolonged photosensitivity of HpD were major reasons for a search and development of new photosensitizing drugs named second generation PSs. They are pure chemical substances of synthetic (e.g., phthalocyanines, naphthalocyanines and purpurins) or

Table 1.1 Photosensitizers approved for clinics or undergoing clinical trials (from Agostinis et al., 2011; Josefsen & Boyle, 2012; Lucky et al., 2015)

Sensitizer	Trade name	Structure	Current status	Potential indications
HpD (partially purified),porfimer sodium	Photofrin, Photogem	porphyrins	Worldwide approved	Endobronchial lung cancer, esophageal cancers, bladder cancer, gastric cancer and cervical cancer
5-Aminolevulinic Acid (5- ALA)	Levulan	Porphyrin precursors	Worldwide approved	Treatment of actinic keratosis, basal-cell carcinoma, head and neck and gynecological tumors; diagnosis of bladder, brain, head & neck cancers
5-ALA-hexylester/ H-ALA	Hexvix, Cysview		Approved / USA	Diagnosis of bladder tumors
5-ALA-methylester/ MALA	Metvix, Metvixia, Visonac		Approved / USA, EU, New Zealand & Australia	Actinic keratosis, Bowen's disease and basal cell carcinoma
Benzoporphyrin derivative monoacid, ring/ Verteporfin	Visudyne	Chlorines	Approved / USA, EU, Canada	Treatment for wet age-related macular degeneration, pathologic myopia, histoplasmosis
meta-tetra-(hydroxyphenyl) chlorin /Temoporfin	Foscan		Approved / EU	Head and neck, prostate and pancreatic tumors
Mono-L-aspartyl chlorin e_6 (Talaporfin,NPe6, LS11 or MACE)	Aptocine		Approved / Japan	Lung cancer and solid tumors from diverse origins
	Laserphyrin			
chlorin e_6 polyvinypyrrolidone /chlorin e_6 derivatives	Fotolon		Approved / Belarus, Russia, Ukraine	Nasopharyngeal, sarcoma, brain, skin cancer;
	Radachlorin, Photodithazine			
Sulfonated aluminium phthalocyanines ($A1PCS_4$)	Photosens	phthalocyanines	Approved / Russia	Age-related macular degeneration, Various cancers
Silicon phthalocyanines	Pc4		Clinical trials / USA	Actinic keratosis, Bowen's disease, T-cell non-Hodgkin lymphoma and skin cancers
Zinc phthalocyanine (ZnPc)	CGP55847		Clinical trials / USA	Actinic keratosis, Bowen's Disease, skin cancer; squamous carcinoma of upper aerodigestive tract
Lutetium texaphyrin	Lutex	texafirins	Approved / USA	Breast cancer and malignant melanomas
Motexafin 1utetium hydrate	Antrin		Clinical trials /USA	Prostate cancer and photoangioplasty
2-[l-hexyloxyethyl]-2-devinyl pyropheophorbide *a*	Photochlor	pheophorbide	Clinical trials / USA	Early esophageal cancers, non- small cell lung cancer
Palladium-bacteriopheophorbide	Tookad (WST09)		Clinical trials / EU	Recurrent prostate cancer
Tin etiopurpurin ($SnET_2$)/ Purlytin/ Rostaporfin	Photrex	purpurins	Clinical trials / USA	Cutaneous metastatic breast cancer, basal-cell carcinoma, Kaposi's sarcoma, and prostate cancer
9-Acetoxy-2,7,12,17-tetrakis-(β-methoxyethyl)-porphycene	ATMPn	porphycene	Approved / Germany	Psoriasis and non-melanoma skin cancer

natural (e.g., pheophorbides and bacteriochlorins) origin with improved photophysical characteristics. However, the second generations PSs are still poorly selective for tumors and very hydrophobic thus requiring the application of special delivery systems. The first and second generation PSs covalently attached to various biological modifiers like oligosaccharides, lipoproteins, antibodies and amino acids, or formulated into drug delivery systems like liposomes, polymeric nanoparticles, cyclodextrins, and emulsions with the aim to improve the photosensitizing and pharmacokinetic properties of the drugs are classified as third generation PSs. It is noteworthy that new generation of drugs are not always superior to older ones (Allison & Sibata, 2010; Lucky et al., 2015) and in each case it is necessary to compare the properties of different PSs and their forms to each other, both in pre-clinical and clinical trials.

Of special interest for this study is mTHPC, which is an extremely potent PS (approximately 100 times more photoactive than Photofrin®) (Mitra & Foster, 2005; Senge & Brandt, 2011). The properties of mTHPC and some of its formulation will be described in detail in the next chapter.

2. Meta-tetra(hydroxyphenyl)chlorin (mTHPC)

2.1. General properties and usage

5,10,15,20-Tetra(*m*-hydroxyphenyl)chlorin (mTHPC, temoporfin) is one of the most potent second generation PS. The discovery and the chemical synthesis of PSs of tetra(hydroxyphenyl)porphyrinoid series (Fig. 2.1) was performed by the group of professor Raymond Bonnett (Berenbaum et al., 1986; Bonnett et al., 1989). The *ortho*, *meta* and *para* isomers (Fig. 2.1) of these compounds have been tested and after thorough comparison the *meta* isomers of the PSs demonstrated the greatest efficiency *in vitro* and *in vivo*. They are namely meta-tetra(hydroxyphenyl)porphyrin (mTHPP), mTHPC and meta-tetra(hydroxyphenyl)-bacteriochlorin (mTHPBC). These PSs have attractive photophysical characteristics compared with the first generation PSs (Table 2.1). All three compounds have a high triplet state QY formation ranging between 0.69-0.89 and a good QY of singlet oxygen formation (0.43-0.45) in aerated methanol (Bonnett et al., 1999). Besides, they are characterized by a strong absorption in the far red region: 3400 $M^{-1}cm^{-1}$ at 644 nm for mTHPP, 29600 $M^{-1}cm^{-1}$ at 650 nm for mTHPC and 91000 M^{-1} cm^{-1} at 735 nm for mTHPBC. Although the photophysical characteristics of mTHPBC from PDT point of view look more attractive compared to mTHPC, the rapid degradation of mTHPBC precludes a practical use in PDT (Grahn et al., 1997; Lassalle et al., 2005).

mTHPC use in PDT is characterized by a very low drug dose (order of 0.1 $mg \cdot kg^{-1}$), light intensity (order of 10 $J \cdot cm^{-2}$) and total PDT doses (light dose x PS dose) more than 100 times higher compared to HpD (Savary et al., 1997, 1998), which explains its popularity in PDT. Along with Photofrin, mTHPC is the only other PS approved for use in systemic cancer therapy. In 2001 mTHPC was approved in the EU and used as a solvent-based formulation (Foscan®; Biolitec Research GmbH, Jena, Germany) for the palliative treatment of head and neck cancers (Senge & Brandt, 2011). In addition, mTHPC has been successfully used for the treatment of early squamous cell carcinoma (de Visscher et al., 2013; Jerjes et al., 2011), basal cell carcinoma (Betz et al., 2012), biliary tract carcinoma (Wagner et al., 2015), prostate (Swartling et al., 2010, 2016), pancreatic cancer (Huggett et al., 2014) and nonmelanoma skin cancers (Horlings et al., 2015). Moreover, mTHPC finds application in areas other than cancer treatment. For examples, there are studies demonstrating the prospects of mTHPC application for antibacterial therapy (Ossmann et al., 2015), rheumatoid arthritis (Hansch et

al., 2008), and for relieving colitis and preventing colitis-associated carcinogenesis (Reinhard et al., 2015b).

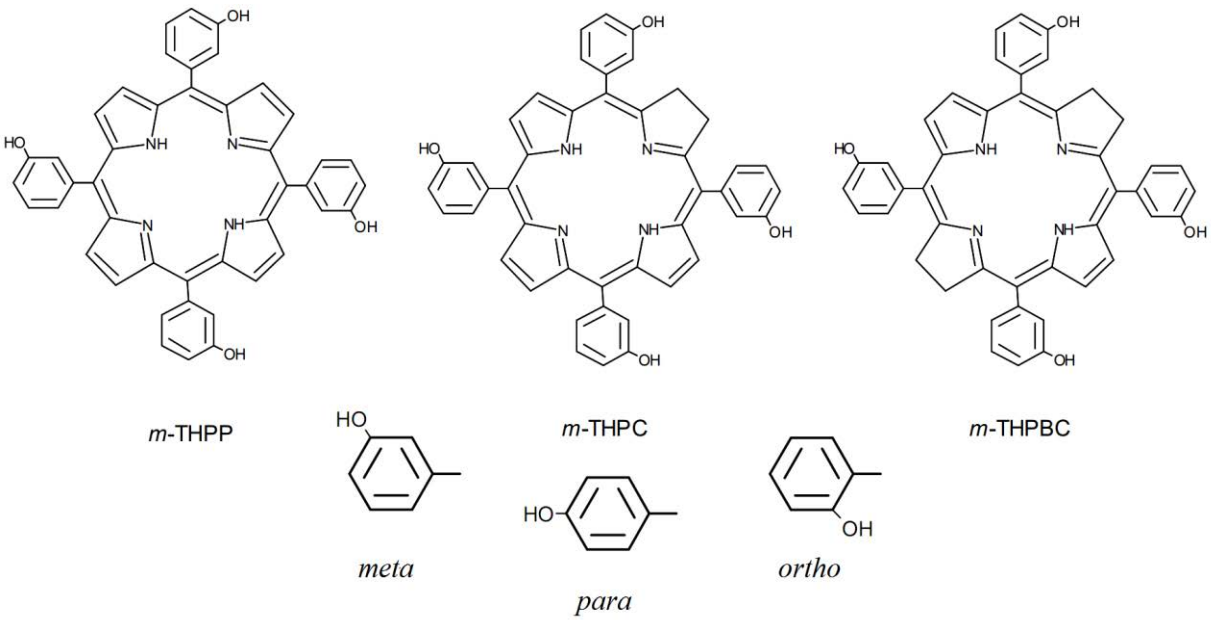


Figure 2.1 Molecular structures of mTHPP, mTHPC and mTHPBC and *m*, *p* and *o* isomers of the hydroxyphenyl substituent

Table 2.1 Photophysical properties of mTHPP, mTHPC and mTHPBC in methanol (Bonnett et al., 1999; Senge & Brandt, 2011)

Property	**Value**		
Compound	mTHPP	mTHPC	mTHPBC
Absorption maximum, nm	644	650	735
Extinction coefficient, M^{-1} cm^{-1}	3400	29600	91000
Fluorescence maximum, nm	649	652	746
Fluorescence quantum yield (Q.Y.)	0.12	0.09	0.11
Triplet Q.Y.	0.69	0.89	0.83
Singlet oxygen generation Q.Y. (air-saturated)	0.46	0.43	0.43
Singlet oxygen generation Q.Y. (oxygen-saturated)	0.59	0.59	0.62

2.2. Biological behavior of mTHPC

The high PDT-efficiency of mTHPC is usually associated with the features of its biological behavior. In many papers it is shown that temoporfin forms large aggregates in aqueous medium (Kruijt et al., 2009; Tikhomirov et al., 2009). As a rule, mTHPC is IV-introduced to patients and the PS's hydrophobic nature defines its pharmacokinetics and biodistribution behavior. Plasma protein binding pattern is an important factor governing mTHPC biological behavior. Previous studies have revealed two main features of mTHPC interaction with plasma proteins. First, about 95% of the sensitizer in plasma is associated with HDL and LDL, and the binding with HDL is twice higher than that with LDL (Reshetov et al., 2012; Triesscheijn et al., 2007). Secondly, as compared with other non-polar drugs, mTHPC releases from lipoprotein carriers very slowly and proceeds on a timescale of hours (Sasnouski et al., 2005, 2006). High affinity to cell membranes and plasma proteins and the presence of PS aggregates modifies the interaction of mTHPC with biological objects, as will be more fully described hereinafter.

Interaction with cells in vitro

Time-dependent mTHPC accumulation demonstrates a continuous increase in PS cellular uptake within the first 20 h, reaching a plateau at 24 h (Berlanda et al., 2010). Temoporfin is rigidly fixed in model membranes (Kachatkou et al., 2009) and is strongly retained in cells *in vitro* (Ball et al., 1999; Berlanda et al., 2010). Cellular uptake of mTHPC is affected by many factors. Temoporfin seems to be taken up by cells in its aggregated form, followed by slow monomerization (Rezzoug et al., 1998) attested by fluorescence lifetime imaging microscopy data (Lassalle et al., 2008). The uptake of PS into cells appears to be pH independent in the rage 6.8-7.8; however, photosensitivity of cells increased with decreasing pH value (Ma et al., 1999). As discussed above, due to the high affinity of mTHPC to lipoproteins, the PS uptake may be mediated by LDL. However, HDL-mediated endocytosis was proposed as the main mode of temoporfin transport into the cells (Sasnouski et al., 2006).

Primary research reported that mTHPC is localized generally within cellular organelles, but not in the nucleus (Melnikova et al., 1999). It has been demonstrated that the Golgi apparatus and endoplasmic reticulum are preferential sites of mTHPC accumulation in MCF-7 human adenocarcinoma cells (Teiten et al., 2003a) and the latter two were the sites of primary PDT damage (Teiten et al., 2003b). On the other side, confocal fluorescence microscopy study showed only weak localization in lysosomes and mitochondria. A study by Kessel (1999) showed mitochondrial damage, release of cytochrome *c* and activation of

caspase-3 resulting in an apoptotic response after mTHPC-PDT *in vitro*. Mitochondrial damage and cytochrome *c* release has been described for various cancer cells (Marchal et al., 2004; Yow et al., 2000). However, investigation of the relationship between mTHPC subcellular localization and post-PDT intrinsic apoptotic pathway demonstrated that temoporfin localization in endoplasmic reticulum improves the photoactivation of the caspase-7 apoptotic pathway, which is poorly related to photoinduced mitochondrial damage (Marchal et al., 2007). Thus, although mitochondria are not affected directly they will significantly contribute to late and indirect apoptotic effects originating in Endoplasmic reticulum and/or Golgi apparatus.

Biodistribution and pharmacokinetic properties

The pharmacokinetics of mTHPC depends on using of animal models (Senge & Brandt, 2011). Early studies showed that mTHPC displays an unusual plasma pharmacokinetic behavior in humans and rabbits, with a secondary peak at about 10 h and 6 h after injection, respectively (Glanzmann et al., 1998; Ronn et al., 1996). These phenomena were interpreted by the initial retention of the PS in the liver or PS aggregates in the vasculature, with subsequent disaggregation, binding to lipoproteins and mTHPC release from the depot. Depending on the study and animal models a half-life of the drug of 26–45 h was established (Campbell et al., 2002; Glanzmann et al., 1998; Ronn et al., 1996; Triesscheijn et al., 2007). It was shown that mean values for plasma drug concentrations were in good agreement with the injected dose of mTHPC. Pharmacokinetic study of radiolabeled mTHPC in tumor-bearing rats demonstrated a tri-exponential model with half-lives of 0.46, 6.91 and 82.5 h, respectively (Jones et al., 2003). However, a detailed study comparing the pharmacokinetics in mice and humans showed the different pharmacokinetics between rodents and human (Triesscheijn et al., 2007). The initial (5 min) plasma drug levels in humans were on average 86% of the maximal plasma concentration, which occurred at about 5 h after injection. On the contrary plasma pharmacokinetics in mice was characterized by a standard bi-exponential decline of the drug concentration. Besides, the drug distribution over the lipoproteins and the metabolism of the lipoproteins had no impact on the plasma pharmacokinetics.

Temoporfin biodistribution studies have shown that the time course of the uptake differs from organ to organ. Highly perfused organs (liver, spleen, kidney and lung) contained a large amount of the drug and the less perfused organs contain less of the PS (Campbell et al., 2002; Whelpton et al., 1996). However, the maximum level of mTHPC can be different

for different animal models and doses of administration (Ronn et al., 1996, 1997). The intratumoral distribution of mTHPC depends on the time of circulation and the distance to blood vessels. Peng et al. (1995) showed that mTHPC was distributed in the vascular interstitial and neoplastic cells of the breast cancer implanted in mice. Mitra et al. (2005) used high-resolution confocal fluorescence imaging to simultaneously map microscopic intratumoral mTHPC localization with respect to perfused vasculature as a function of time after injection. Three hours after injection, maximal mTHPC fluorescence was detected in the periluminal structures, but after 24 h, mTHPC was mainly localized in parenchyma of tumor. All these aspects have clear clinical applications, which will be discussed below.

A study of mTHPC excretion in a murine model showed that nearly 40% of the drug was excreted in the faeces during the first day, and less than 0.2% of the dose was recovered from the urine (Whelpton et al., 1996). Furthermore, *in vitro* and *in vivo* experiments, using HPLC and electrospray mass spectrometry, have confirmed that mTHPC is not metabolized and is excreted unchanged via biliary excretion in the faeces (Cai et al., 1999a, 1999b).

Mechanism of action

Early pre-clinical studies have demonstrated that in PC6 tumor bearing mice the depth of necrosis was 3.79 ± 0.28 mm after mTHPC administration (Bonnett et al., 1989). Melnikova et al. (1999) showed that mTHPC mediates cell photodamage, principally through singlet oxygen formation and its efficacy is sensitive to oxygenation conditions (Coutier et al., 2002). Moreover, in the case of mTHPC-PDT, singlet oxygen dose to the tumor volume does not track even qualitatively tumor response, so in this case any PDT dose metric that is proportional to singlet oxygen creation and/or deposition would fail to predict the tumor response (Wang et al., 2008)

mTHPC has a small initial volume of distribution with important retention in the vasculature together with two peaks of PDT efficacy (2 and 24 h) in rats (Jones et al., 2003). Thus, PDT at early time of irradiation resulted mainly in the destruction of the microvasculature of the tumor while the latter one affected both the vascular walls and the tumor cells. Features of such intratumoral distribution were used in mTHPC-PDT of mice bearing EMT6 tumors (Garrier et al., 2010). Indeed, a fractionated double injection (3 and 24 h prior to PDT) was superior to any single dose of administration of the drug at 3 h, 6 h and 24 h of DLI. The absence of correlation between the mTHPC concentration in tumor and PDT efficiency was observed (Garrier et al., 2010; Ris et al., 1998; Veenhuizen et al., 1997), while accumulation of PS in leukocytes exhibited a good correlation with PDT efficacy (Maugain et

al., 2004). Thus, photodynamic effect will depend on the treatment protocol (i.e. photodynamic dose and DLI). *In fine*, although direct cells damage is a predominated in tumor destruction by mTHPC-PDT, other mechanisms play an important role for the complete tumor destruction and prolonged PDT-effect.

Side effects

mTHPC is a highly active PS with a significant clinical efficacy, however the skin photosensitivity still remains a major issue in clinical management. For example, there is the evidence indicating a mild to moderate pain in the treated area (Allison & Sibata, 2004). The main side effect of mTHPC is its photosensitivity. Although several studies have indicated that mTHPC is less photosensitivity than Photofrin® (van Geel et al., 1995; Wagnieres et al., 1998), skin photosensitivity persists for up to 6 weeks (usually 2-3 weeks) post-mTHPC administration (Allison & Sibata, 2004). Usually, Foscan-based PDT resulted in mild to moderate skin photosensitivity and caused neither functional nor cosmetic impairments after treatment of early oral squamous cell carcinoma (O'Connor et al., 2009). There are also other side effects, but they are very rare and specific (http://sideeffects.embl.de/drugs/60751/pt). Moreover, as noted earlier, mTHPC molecule is hydrophobic, leading to its poor water solubility and aggregation within the vasculature requiring the use of special systems suitable for clinical applications.

2.3. Strategies for improving delivery and efficacy of photodynamic therapy with mTHPC

The side effects of mTHPC clearly accentuate the need to improve the treatment protocols by optimizing the DLI, increasing bioavailability and selectivity of PS accumulation thus reducing the damage to healthy tissues. Various solutions were proposed over the past decades, including mTHPC conjugation with different molecules and using special formulations, such as liposomes, polymer solutions and nanoparticles (Senge, 2012; Senge & Brandt, 2011). The first attempt to improve the pharmacokinetic properties of mTHPC is related to the conjugation with polyethylene glycol (PEG) (Westermann et al., 1995; Morlet et al., 1997). Although mTHPC-PEG conjugate showed increased water solubility and selective retention of the drug in tumor tissue, PDT-effect of unconjugated form of mTHPC demonstrated the same or superior efficiency (Kübler et al., 2001; Westermann et al., 1999). Besides, various glucoconjugated analogues of mTHPC were investigated (Bautista-Sanchez et al., 2005; Laville et al., 2003), along with folic acid conjugate (Gravier et al., 2008).

More significant advances were made through the use of nanocarriers for mTHPC development. In order to improve its bioavailability and PDT-efficacy and to reduce side effects of mTHPC, various nanosized vehicles such as nanoemulsion (Primo et al., 2008), lipid vesicle systems (liposomes, invasosome, ethosomes) (Bovis et al., 2012; Chen et al., 2011; Reshetov et al., 2013; Xie et al., 2015), nanoparticles (mainly biodegradable) (Navarro et al., 2014; Rojnik et al., 2012; Silva et al., 2015) were proposed. A study of mTHPC encapsulated in polyethylene glycol (PEG) poly-(D,L-lactide-co-glycolide) nanoparticles showed significant decrease of mTHPC dark cytotoxicity, but also significantly reduced mTHPC cellular uptake, whereas phototoxicity was similar to that of Foscan® (Rojnik et al., 2012). Silva et al. (2015) demonstrated that using mTHPC-loaded magnetic iron oxide microvesicles, the uptake of mTHPC by cancer cells could be kinetically modulated and spatially controlled under magnetic field and that photoinduced cell death was enhanced by magnetic targeting. Another study compared vesicular systems (liposomes, invasosome and ethosomes) with non-vesicular systems on the skin penetration of mTHPC. Both vesicular and non-vesicular formulations provided drug localization predominantly in the superficial skin layer (Chen et al., 2011).

Two mTHPC liposomal formulations (Foslip® and Fospeg®; Biolitec Research GmbH) are the most studied and widely used in experimental research. Foslip® is a formulation of mTHPC embedded in conventional (non-pegylated) liposomes. Fospeg® is a sterically stabilized form of Foslip®, containing a small amount of PEG-phosphatidylethanolamine layer on the surface. Compared to the standard solution of mTHPC (Foscan®), liposomal formulations Foslip® and Fospeg® provide better bioavailability of the PS, higher tumor/tissue ratios and result in better PDT-efficacy (Bovis et al., 2012; Buchholz et al., 2005; Svensson et al., 2007). PDT efficiency with Foslip® in EMT6-grafted mice, studied by Lassalle et al. (2009), was maximal at the DLI 6 h, indicating the presence of both direct and vascular PDT effects. Reshetov et al. (2013) showed that Fospeg®-PDT of HT29 human colon adenocarcinoma bearing mice was more effective than Foslip® treatment at almost all DLIs, except 24 h. Its higher efficiency can be achieved by combination of enhanced permeability and retention-based tumor accumulation, stability in the circulation, and release properties of Fospeg® compared to Foslip® (Reshetov et al., 2013; Xie et al., 2015).

These different nanocarriers of mTHPC, in particular Foslip has now closely approached the clinical stage. Further development of PDT in general and mTHPC-PDT in particular is associated with nanotechnology-based drug delivery systems.

3. Nanotechnology-based drug delivery systems

The application of many drugs is hampered by their non-optimal pharmacological properties, such as low aqueous solubility, irritating nature, lack of stability, rapid metabolism, and non-selective drug distribution. Nanocarriers were developed to palliate these problems by improving drug delivery, opening the era of nanomedicine in oncology including PDT (Calixto et al., 2016; Marchal et al., 2015). Significant efforts have been made toward this goal by developing nanoparticle drug delivery systems (DDSs), having particle diameters up to 200 nm (Khodabandehloo et al., 2016). Nanocarriers can deliver drugs in a spatiotemporally controlled manner that potentially increase therapeutic efficacy of the drugs, reduce their systemic side effects, and improve patient's adherence to regimen by reducing the dose and administration frequency (Lee & Yeo, 2015).

3.1. A rational design and advantages of drug delivery systems

It is well known that drugs should ideally be released at the target sites in a controlled manner to enhance their therapeutic efficiency and reduce the side effects. The design of an efficient DDS requires the knowledge of the drug physicochemical properties, specific intended therapeutic application of the drug and the characteristics of interaction of the DDS with the biological structures (Khodabandehloo et al., 2016).

Smart DDSs are defined as the process when the drugs are not released before reaching target tissues/organs (or with extremely slow rate), and are released only at the sites of action (Liu et al., 2016). Although the drug molecules themselves sometimes can be smart components, here we primarily discuss smart DDSs by means of nanotechnology. Stimuli-responsive DDS can respond to endogenous and/or exogenous stimulus. The endogenous triggers such as pH variations, hormone level, enzyme concentration, small bio-molecules, glucose or redox gradient (Mura et al., 2013; Kelley et al., 2013) are related to the disease pathological characteristics. While the exogenous triggers, including temperature, magnetic field, ultrasound, light, electric pulse/ high energy radiation, can also be used to trigger or enhance the drug release at diseased areas.

For the drug that is already in clinical application, the advantages of a particular DDS shall be compared to a free drug. Besides, toxicity profile (not degree) of the free drug is generally similar to its DDS-entrapped form (Allen & Cullis, 2004), thus facilitating the estimation of the side effects. Hence, in many cases the formulation of the already-approved

drug into the DDS is more efficient than the development of entirely new drugs and their DDS formulation. The mechanism of action of a drug also dictates its suitability for delivery in a particular DDS. If release from the DDS is required, a question arises as to whether the DDS leads to appropriate rates and levels of drug bioavailability. Bioavailability will depend on the release rate. Thus, the methods to measure drug release rates should also be integrated into the development of DDSs, not only the measurements of the total drug level (e.g., in plasma).

Although final properties will depend on the particular design of the DDS, there are several general advantages of using DDS for cancer therapy:

- DDSs can carry a large payload of drug molecules, protect them from degradation and increase drug water solubility.
- Changes in the biodistribution of the DDS generally occur through passive or active targeting. Passive targeting exploits the characteristics of tumor biology that allow nanocarriers to accumulate in the tumor by the enhanced permeability and retention effect (Peer et al., 2007). Chauhan et al. (2012) findings emphasize the importance of size in nanomedicine design by demonstrating that 12 nm particles penetrate tumors better than larger particles. Active approaches achieve this by conjugating nanocarriers containing chemotherapeutics with molecules that bind to overexpressed antigens or receptors on the target cells.
- The alteration of pharmacokinetics and biodistribution compared to a free drug must match the current use of medications. Surface modifications of the DDS such as PEGylation may dramatically change the circulation time, as discussed in the next section. Surface characteristics contribute to the DDS solubility, aggregation tendency, ability to traverse biological barriers (such as the cell wall), biocompatibility, and targeting ability (Mrsny et al., 2006).
- Nanocarriers composed of biocompatible materials are safe alternatives to existing vehicles such as Cremophor® that may cause severe adverse effects (Fang & Al-Suwayeh, 2012). Biodegradability implies that the unloaded carrier is degraded or metabolized into nontoxic components and cleared from the circulation (Petros & DeSimone, 2010). Thus, toxicities associated with the carrier molecules *per se* tend to be mild.

3.2. Types of nanosized antitumor drug delivery systems

To increase the effectiveness of treatment and reduce side effects, the incorporation of nanotechnology-based DDS such as polymeric nanoparticles, solid lipid nanoparticles, nanostructured lipid carriers, gold nanoparticles, protein-based nanoparticles, cyclodextrin complexes and host–guest supramolecular nanosystems represents an interesting option for drug delivery, as demonstrated in Fig. 3.1. These structures are different in the way of drug incorporation (i.e., conjugation, non-covalent inclusion, enzyme binding), have distinct structural features (the composition, surface labeling) and size (1-400 nm), as well as different mechanisms of cellular and tissue targeting (active, passive). Examples of nanotherapeutics approved for clinical use or subjected to advanced clinical trials are listed in Table 3.1.

Polymeric carriers

Important range of application of polymers is its conjugation with the drug molecules. Several polymer-drug conjugates have entered clinical trials and some of them, like Zinostatin stimalamer® and Oncaspar®, are approved at the market (Table 3.1). They are especially useful to increase water solubility and to target blood vessels in tumors. Apart from polymers, there are several ways to functionalize drugs, including conjugation to peptides, saccharides and proteins, which leads to improved bioavailability and delivery specificity (Liu et al., 2016). However, polymer–drug conjugates require chemical modification of the existing drugs; as a consequence, their production could cost more, and further purification is needed. Moreover, the drug-polymer conjugates are usually considered as new chemical entities owing to a pharmacokinetic profile distinct from that of the parent drugs (Bamrungsap et al., 2012).

Micelles

Micelles are spherical colloidal amphiphilic systems, having a particle diameter within the range of 10-100 nm. They consist of self-assembled amphiphilic block copolymers with a hydrophobic core and hydrophilic shell. Among drug carrier systems, micelles provide considerable advantages, since they can solubilize and increase the bioavailability of poorly soluble drugs, and offer long blood circulation (Biswas et al., 2013). Drug release from micelles is dependent on the interactions between drugs and micellar cores (e.g., hydrogen bonding) and, therefore, is determined by molecular weight and hydrophobicity of amphiphilic block copolymers (Lee & Yeo, 2015). To date, several polymeric micelle anticancer drug-targeting systems are at different stages of clinical studies.

Table 3.1 Nanotherapeutics approved for clinical use or being evaluated in clinical trials (adapted from Egusquiaguirre et al, 2012; Gidwani & Vyas, 2015; Marchal et al., 2015)

Nanocarrier	Name	Formulation	Drug	Indications	Status
Liposomes	Doxil®	PEGylated liposome	Doxorubicin	Karposi's sarcoma Ovarian cancer Multiple myeloma	Market
	Myocet®	conventional liposome		Breast cancer (cyclophosphamide)	Market
	Caelyx®	PEGylated liposome		Karposi's sarcoma, multiple myeloma, breast and ovarian cancer	Market
	ThermoDox®	Heat-activated PEGylated liposome		Hepatocellular carcinoma	Phase III
	DaunoXome®	conventional liposome	Daunorubicin	Karposi's sarcoma	Market
	DepoCyt®	conventional liposome	Cytarabine	Lymphomatous meningitis, leukaemia,	Market
	Marqibo®	sulfate liposome	Vincristine	acute lymphoblastic leukemia	FDA 2012
Polymeric and Polymer-drug conjugate nanoparticles	Zinostatin stimalamer®	conjugate protein or copolymer	styrene-maleic acid	Hepatocellular carcinoma	Market
	Oncaspar®	PEG-drug	L-Asparaginase	acute lymphoblastic leukemia	Market
	Docetaxel-PNP	Polymeric nanoparticle	Docetaxel	Various solid malignancies	Phase I
	BIND-014	PEG-PLGA nanoparticle		Various solid malignancies	Phase II
	Abraxane® (ABI-007)	Albumin-bound nanoparticle	Paclitaxel	Breast cancer, non-small cell lung cancer, Pancreatic cancer	Market
	Opaxio® (Xyotax)	Polyglutamic acid-drug		Lung cancer, ovarian cancer	FDA 2012
	XMT-1001	Fleximer®-drug	Camptothecin	Gastric cancer, lung cancer	Phase I
	NKTR-102	PEG-drug	Irinotecan	Breast cancer	Phase III
Host–Guest Supramolecular Nanosystems	CRLX101	CD-PEG nanoparticle	Camptothecin	Various malignancies	Phase II
	CALAA-01	CD-PEG-transferrin-nanoparticle	Anti-RRM2 siRNA	Various solid malignancies	Phase I
	Vfend	SBE-β-CD/drug solution for IV injection	voriconazole	Invasive Fungal Infection Hematological Malignancy	Phase III
Inorganic nanoparticles	NanoTherm®	nanoparticle	Iron oxide	Glioblastoma	Market
Polymeric micelles	Paclical®	Polymeric micelle	Paclitaxel	Ovarian cancer	Phase III
	Genexol-PM®	PEG-PLA micelle		Breast cancer	Market
	Livatag®	Polymeric micelle	Doxorubicin	Hepatocellular carcinoma	FDA 2014
Other drug-conjugated based nanoparticles	Rexin-G	Retroviral-vector-dnG1 plasmid DNA	G1 gene	Various solid malignancies	Market/ Phase III
	Kadcyla®	Antibody-drug	Emtansine/trastuzumab	Breast cancer	Market
	Adcetris®	Antibody-drug	Brentuximab vedotin	CD30+ lymphomas	Market

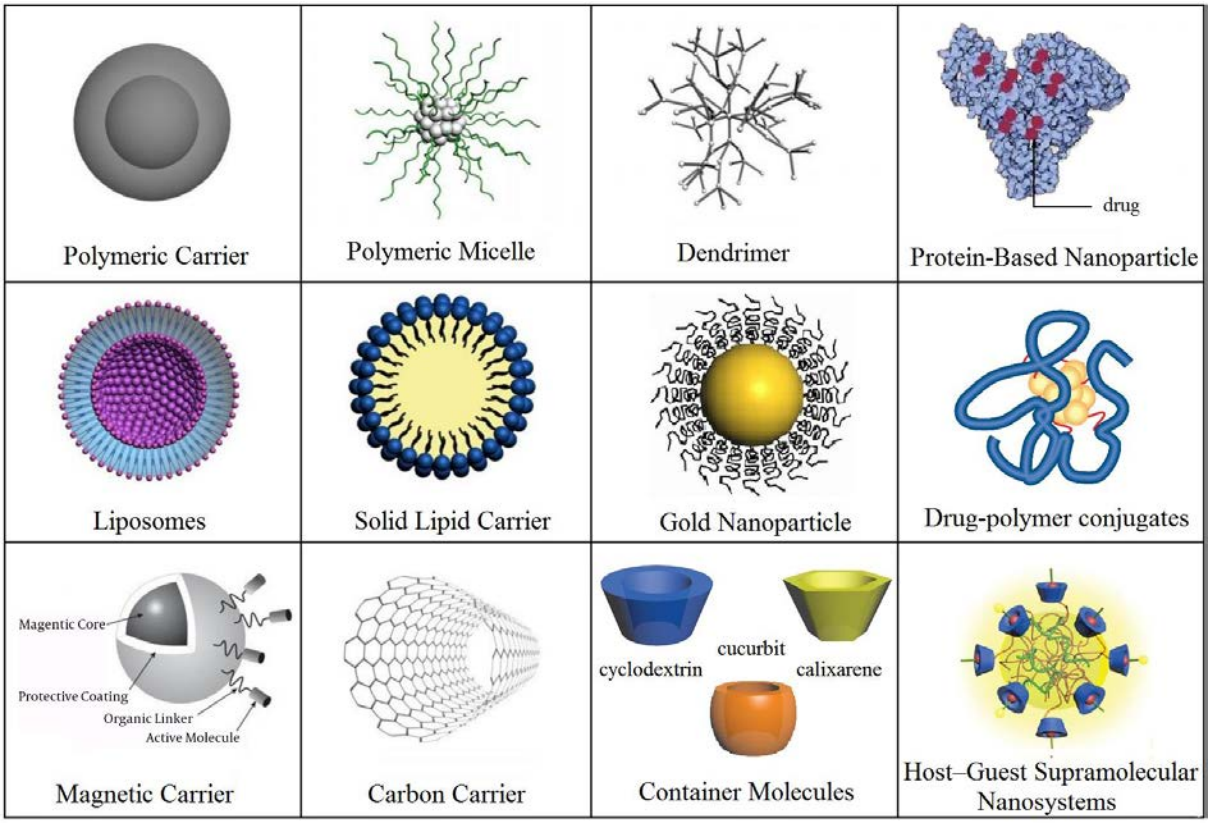


Figure 3.1 Different types of nanocarriers have used for drug delivery (from Bamrungsap et al., 2012; Khodabandehloo et al., 2016; Wang et al., 2013)

Liposomes and solid lipid carriers

Constituted by an aqueous core surrounded by one or several phospholipid bilayers, liposomes are biocompatible and biodegradable entities able to entrap hydrophilic drugs into their cavity, while allowing water insoluble drugs to be inserted into the lipid bilayers (Marchal et al., 2015). PEGylation of liposomes can increase circulation time and protect the cargo during the circulation in the body. Liposomes, together with drug-polymer conjugates, are the most widely used for DDS. Some liposome formulations have been approved for commercial use as shown in Table 3.1. Except for liposomes on the market, a number of lipidic nanoparticles are in the pipeline from concept to clinical application (Liu et al., 2016).

Solid lipid carriers, also referred as lipospheres or solid lipid nanospheres are solid at human physiological temperature and had a diameter of 50-100 nm. The solid lipid carriers could be made from diverse range of lipids including mono-, di- and triacylglycerols, fatty acids, waxes and combination of these materials. The solid lipid carriers could be formed by replacing oil with solid lipid in oil and water emulsion. Also, the solid lipid carriers were biodegradable and biocompatible and could be used in humans because of their reduced cytotoxicity (Wong et al., 2007). These carriers have formed a lipophilic matrix which

enabled the drug to load onto it. The important factors affecting drugs loading into the solid lipid carriers matrix were solubility of drug in lipid (drug must be lipophilic), physical and chemical properties of lipid or mixture of lipids, crystalline properties of lipids in biological temperature, and polymorphic form of the lipids. Solid lipid carriers have been investigated for delivery of various anticancer drugs with promising results in mouse preclinical models (Khodabandehloo et al., 2016).

Dendrimers

Dendrimers are natural and synthetic polymers with a central structure labeled as "the core", from which the branches of other groups called "dendrons" grow through various reactions (Lee & Yeo, 2015). The unique surface characteristics of dendrimers enable them to be simultaneously conjugated with several molecules, such as imaging contrast agents, ligands or therapeutic drugs, and hence have created a multifunctional drug delivery system (Khodabandehloo et al., 2016). Drug molecules may be covalently conjugated to the peripheral groups of a dendrimer or included inside the core via hydrogen bonding, hydrophobic or electro-static interactions. In regards of biomedical applications, the biological properties of dendrimers are important. For example, cationic dendrimers were usually hemolytic and cytotoxic. On the other hand, anionic dendrimers had longer circulation time and higher bioavailability. Dendrimers clearance rate was generation-dependent, and lower generation would have longer circulation time (Jain & Tekade, 2013). Our laboratory together with the laboratory of photobiophysics (Berlin, German) demonstrated that dendrimer formulations of chlorin e_6 may offer effective biocompatible nanoparticles for improved PDT in a preclinical tumor model (Bastien et al., 2015; Pfitzner et al., 2016)

Host–guest supramolecular nanosystems

The typical supramolecular hosts (i.e., cyclodextrins, calixarenes, and cucurbiturils) and their nanosystems have a promising application in cancer diagnostics and therapeutics (Gidwani & Vyas, 2015; Wang et al., 2013). A few examples have been reported where the supramolecular nanosystems could serve as cancer imaging probes and/or drug delivery vehicles in clinical trials (Davis, 2009; Davis et al., 2010). The use of DDSs, based on cyclodextrins or its colloidal silica nanoparticles, represents a promising and innovative strategy that enables effective therapy with minimum side effects. The focus of the following sections will be on cyclodextrins as one of the most promising delivery systems.

4. Cyclodextrins for drug delivery

The inclusion complexes of cyclodextrins (CDs) with wide variety of organic and non-organic compounds refer to a class of the simplest supramolecular systems extensively studied for the last decades. The inclusion complexes based on CDs are of interest from both a theoretical and a practical point of view. On the one hand, their study facilitates understanding of the mechanisms of interaction of molecules and molecular recognition, on the other hand the unique properties of CDs enables using them as additives to improve the properties of various substances (Challa et al., 2005). As a result CDs have been explored as additives in the food, cosmetic and pharmaceutical industries to improve product stability and solubility (Loftsson & Brewster, 2010). Applications of CDs in the pharmaceutical industry are constantly expanding. To date there are above 40 products or formulations containing various CDs, especially β-CD and its derivative (Gidwani & Vyas, 2015). Moreover, CDs are promising delivery systems for PDT purpose (Kryjewski et al., 2015; Mazzaglia, 2012).

4.1. Cyclodextrins: classification, structure and basic properties

The cyclodextrins (CDs) of biomedical and pharmaceutical interest are a family of cyclic oligosaccharides with a hydrophobic internal cavity and a hydrophilic outer surface consisting of six α-cyclodextrin (α-CD), seven β-cyclodextrin (β-CD), eight γ-cyclodextrin (γ-CD) D-glucopyranose units linked by α -(1,4) bonds. The general properties of the native CDs are summarized in the Table 4.1. These so-called "parent CDs" have been used in food (e.g., β-CD approved as a food additive E459) and pharmaceutical products (including anticancer drugs) for many years (Gidwani & Vyas, 2015). CDs are also known by other names (cycloamyloses, cellulosine, cyclomaltoses, etc.) (Loftsson & Duchêne, 2007). Usually CDs are formed as a result of intermolecular transglycosylation reaction (Szejtli, 1998). Due to the steric hindrances CDs with fewer than 6 units cannot be formed. On the other hand, the higher than eight glucopyranose units do exist but are of limited interest as drug solubilizers and stabilizers because they are rapidly reduced to smaller products (Loftsson & Brewster, 1996).

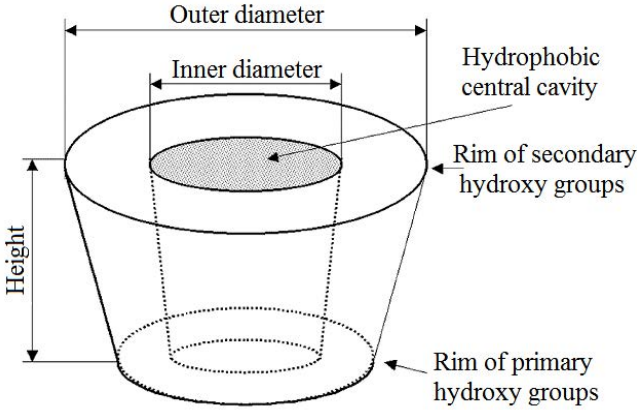
Due to the chair structure of the glucopyranose molecules, CDs have the shape of a truncated cone (Table 4.1). According to X-ray study, the secondary hydroxyl groups (C_2 and C_3) are located on the wider rim and the primary hydroxyl groups (C_6) on the narrow side (Kusmin et al., 2008). It should be noted that the primary hydroxy groups can rotate, while the secondary ones are relatively fixed. The apolar C_3 and C_5 hydrogens are located in the cavity

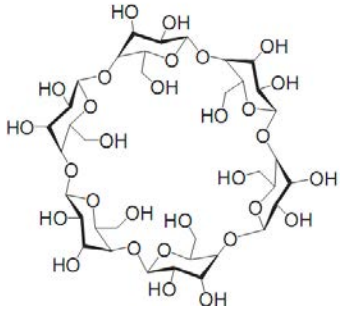
of CDs (Del Valle 2004). As a result CD molecules have a hydrophilic outside ($LgK_{octanol/water}$ between (−13) and (−17)), and an apolar cavity, which provides a hydrophobic matrix comparable to about a 60–70% aqueous ethanolic solution (Connors, 1997; Loftsson & Brewster, 2013). The presence of a hydrophobic interior cavity allows forming inclusion host-guest complexes with a large variety of organic and non-organic compounds. While the height of the CDs torus remains constant, the diameter of the cavity increases considerably with increase in the number glucopyranose units (Table 4.1). It is worth noting that despite the fact that β-CD has been more widely used in the pharmaceutical applications than other native CDs (Rajewski &Stella, 1996), for each specific drug, it is necessary to select the type of CD, depending on the size of the guest molecules or its side substituents. Thus, molecules with aliphatic chains fit better into the small α-CD cavity, whereas molecules containing phenyl groups fit better into the larger cavity of either β- or γ-CD (Duchêne, 2011; Gabelica et al., 2002)

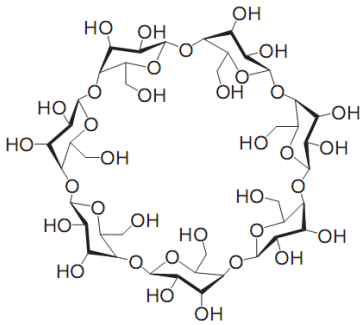
An important characteristic in the pharmaceutical industry is CDs solubility in water. In the case of native CDs solubility changes in the range 18-250 mg/ml (Table 4.1) that is much lower than that of similar acyclic saccharides (Duchêne, 2011). Relatively low water solubility of CDs, especially of β-CD, significantly reduces their applicability. Chemically modified CD derivatives can overcome these problems and extend the physicochemical properties and inclusion capacity of parent CDs. These derivatives usually are produced by aminations, esterifications or etherifications of primary and secondary hydroxyl groups of the CDs (Szente & Szejtli, 1999). Almost all derivatives have a changed hydrophobic cavity volume and can improve solubility, physical and microbiological stability, reduce parenteral toxicity and help to control the chemical activity of guest molecules (Challa at al., 2005; Davis & Brewster, 2004).

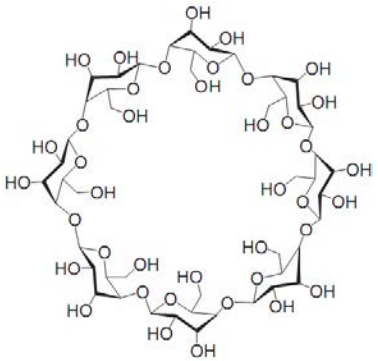
Structural and physicochemical properties of some commercially available CD derivatives of pharmaceutical interest are summarized in the Table 4.2. The physicochemical properties of CDs, including their complexation ability, depend on the structure of the attached substituents but also on their number and location within the CD molecule. The molar degree of substitution (MS) is defined as the average number of substituents *per one* glucopyranose repeat unit (Table 4.2). However, the “degree of substitution” does not uniquely characterize a CD derivative due to the variation of possible locations of substituted groups (e.g., hydroxypropyl) at different positions on the parent CD molecule (Blanchard & Proniuk, 1999).

Table 4.1 Structure and physicochemical properties of native CDs (adapted from Loftsson & Brewster 2013; Del Valle 2004)



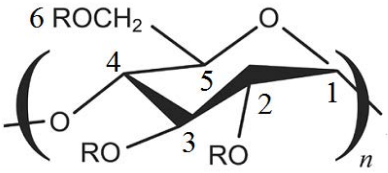






Property	α-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin
Number of glucopyranose units:	6	7	8
Molecular weight of anhydrous compound (Da):	973	1135	1297
Melting temperature range (°C):	255–260	255–265	240–245
Crystal water content (% w/w):	10.2	13.0–15.0	8–18
Height of torus (nm):	0.78	0.78	0.78
Inner diameter (nm):	0.50	0.62	0.80
Outer diameter (nm):	1.46	1.54	1.75
Cavity volume (nm^3)	0.174	0.262	0.427
Solubility in water at 25 °C (mg/ml):	129.5 ± 0.7	18.4 ± 0.2	249.2 ± 0.2
Calculated LgK(octanol/water) at 25 °C:	−13	−14	−17

Table 4.2 Structural and physiochemical properties of some CDs of pharmaceutical interest (adapted from Loftsson & Brewster, 2013; Vyas et al., 2008)



Glycoside α-1,4 bond

Cyclodextrin	n	R	MS[a]	MW[b] (Da)	Solubility[c] (mg/ml)	$LgK_{o/w}$[d]
2-Hydroxypropyl-α-cyclodextrin (HP-α-CD)	6	$-CH_2CHOHCH_3$ or -H	0.65	1199	>500	< -10
2-Hydroxypropyl-β-cyclodextrin (HP-β-CD)	7	$-H_2CHOHCH_3$ or -H	0.65	1400	>600	-11
Sulfobutylether-β-cyclodextrin sodium salt (SBE-β-CD)	7	$-(CH_2)_4SO_2^-Na^+$ or -H	0.9	2163	>500	< -10
Randomly methylated-β-cyclodextrin (M-β-CD)	7	$-CH_3$ or -H	1.8	1312	>600	-6
Maltosyl β-cyclodextrin (Mal-β-CD)	7	Maltaosyl- or -H	0.14	1459	>500	< -10
2-Hydroxypropyl- γ -cyclodextrin(HP-γ-CD)	8	$-H_2CHOHCH_3$ or -H	0.6	1576	>600	-13

[a]Molar degree of Substitution : average number of substitution (R) per glucose repeat unit; derivatives may have differing degrees of substitution on the 2, 3, and 6 positions (inserted figure);
[b]MW, molecular weight;
[c]Solubility in pure water at approx. 25 °C;
[d]Partition coefficient octanol/water;
n, number of glucopyranoside units

In aqueous solutions CDs are chemically stable under neutral and basic conditions and they are more resistant to both enzymatic and non-enzymatic hydrolysis than the linear sugars (Frömming & Szejtli, 1994). The stability of CDs toward acid hydrolysis depends on the temperature and acidity. Under normal experimental conditions (pH higher than 3.5 and temperature below 60 °C), CDs are fairly stable (Hirayama et al., 1992). Besides, CDs are quite resistant to light within the ultraviolet-visible spectrum and infrared ranges (Loftsson & Brewster, 1996).

4.2. Inclusion complex formation and mechanism of drug release

Inclusion complexes

Despite the fact that CDs are able to form non-inclusion complexes and complex aggregates (de Jesus et al., 2012; Loftsson et al., 2004), it is assumed that the most common, as well as representing the greatest pharmaceutical interest are non-covalent inclusion complexes (Duchêne, 2011; Loftsson et al., 2005). The most notable feature of CDs is their ability to form stable inclusion complexes (host – guest complexes) with a very wide range of solid, liquid and gaseous compounds by a molecular complexation (Laza-Knoerr et al., 2010; Del Valle, 2004; Vyas et al., 2008). Inclusion complexation is accomplished by the intermolecular interaction between CDs (host molecule) and guest, which leads to the penetration of the guest molecule partly or completely into the central cavity of the CDs, as illustrated in Figure 4.1 (Song et al., 2009). Besides, depending on their respective size, one CD molecule can bind more than one drug molecule, as a drug molecule may form complexes with several CD molecules (stoichiometry of inclusion complex).

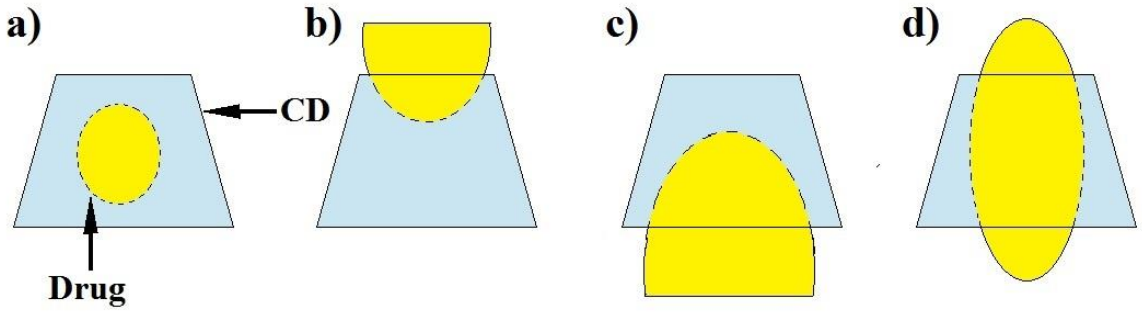


Figure 4.1 Schematic illustration of the association of CD and drug. Topology of inclusion complexes: (a) complete, (b) partial through the narrow side, (c) partial through the wide side, (d) axial

Two most important properties of the complexes are their stoichiometry and the values of their association constants. The stoichiometry of the complex varies depending on the CD-drug combination. The most common stoichiometry is 1:1 (Figure 4.1a) (Tablet et al., 2012), but others like 1:2, 2:1, 2:2, 1:3,…can be encountered as well (Baglole et al., 2005; Sancho et al., 2011). Moreover, often there are systems in which there is a mixture of complexes with different stoichiometry. For example, Tablet et al. (2012) showed that 7-Diethylamino-3-carboxycoumarin with γ-CD formed the mixture of 1:1+1:2 complexes. The

efficiency of complexation is determined by the values of association constants also known as affinity constant, stability constant or binding constant. The value of association constant correlates with the stability of the inclusion complexation (lower probability of dissociation). In the case of Drug/CD 1:1 complexes the values of the binding constants are usually between 10 and 10^3 M^{-1} and values > 10^5 M^{-1} are very rare (Stella & He, 2008). One of the few examples of high value of the binding constant (about 10^7 M^{-1}) is the complexes of rocuronium with sugammadex (modified γ-CD) (Adam et al., 2002).

Inclusion complexation with CDs is a reversible process (dynamic equilibrium), i.e. each molecule can dissociate from the complex. There are two key factors influencing the ability of CDs to form inclusion complex with a guest molecule: steric and thermodynamic. Complex formation is a dimensional fit between host cavity and guest molecule (Muñoz-Botella et al., 1995). It is important that no covalent bonds are broken or formed during formation of the inclusion complex (Schneiderman & Stalcup, 2000). The driving forces for the complex formation include release of enthalpy-rich water molecules from the cavity, electrostatic interactions, van der Waals interactions, hydrophobic interactions, hydrogen bonding, release of conformational strain and charge-transfer interactions (Loftsson et al., 2005; Liu & Guo, 2002).

It is important to note that the physicochemical properties of free drug molecules are different from those bound to the CD molecules. Any changes in the physico-chemical properties of the guest molecules through complexation with CDs allow determining the stoichiometry of the complexes and the value of binding constants (Del Valle, 2004). These modifications include changes in solubility (Higuchi & Connors, 1965), changes in chemical reactivity (Másson at al., 1998), changes in photophysical properties (Lang et al., 2004), nuclear magnetic resonance (NMR) chemical shifts (Pessine et al., 2012), changes in drug retention (e.g., in liquid chromatography), changes in the physicochemical properties of aqueous complexation media (e.g., measurements of conductivity, viscosity), etc. Although there are many techniques, information on the structure of the complexes can be experimentally obtained only from 2D-NMR and induced circular dichroism spectra. Especially interesting is the last one. Circular dichroism is essentially an absorption phenomenon occurring when an optically active molecule absorbs to different degrees the right and left components of a circularly polarized light beam (Berova et al., 2000). Optically active molecules of CDs themselves do not show any significant ultraviolet absorbance and do not fluoresce. Being symmetrical molecules, CDs have no dichroic activity, but they can modify that of guest molecules by perturbation of the microenvironment polarity resulting

from the inclusion (Duchêne, 2011; Ventura et al., 2006). The formation of an inclusion complex between an optically inactive molecule and CDs may result in the appearance of an induced circular dichroism (Desroches et al., 2001; Nablet et al., 2012).

It is worth noting that the efficiency of the formation of inclusion complexes is determined by many factors, including temperature, solvents composition, amount of water (the degree of complexes dilution), complexation techniques. Ionization of the guest, salt formation, formation of metal complexes, application of supercritical fluids and addition of organic co-solvents to the drug/CDs aqueous solution will, if the conditions are correct, lead to enhanced complexation efficiency (e.g., enhanced solubility) (Arun et al., 2008; Loftsson & Duchêne, 2007; Van Hees et al., 1999).

4.3. Effects of cyclodextrins on drug properties in cyclodextrin-based formulations

Inclusion in CDs exerts a profound effect on physicochemical properties of guest molecules as they are temporarily locked or partially caged within the host cavity giving rise to beneficial modification of guest molecule properties. Indeed, CDs increase drug solubility, dissolution, circulation time thus enhancing their bioavailability (Acartürk & Çelebi, 2011).

4.3.1. Effect on drug solubility, dissolution and stability

Drug solubility and dissolution

The most common application of CDs in pharmacy and other fields is related to their ability to increase solubility of poorly water-soluble substances. The increased apparent solubility allows solution-based dosage forms, such as parenteral (IV) and peroral formulation. Besides, improving the apparent solubility of a drug through CDs complexation can increase its dissolution rate and, for compounds whose oral bioavailability is limited by solubility or dissolution rate, can increase oral bioavailability (Acartürk & Çelebi, 2011; Ahuja et al., 2011). For example, the solubility of cortisone acetate increased from 0.039 g/L to 7.382 g/L (almost 200 times) in the presence of HP-β-CD that is far superior to dimethylformamide and ethanol (Kalogeropoulos et al., 2009).

The use of CDs is usually preferred over organic solvents from both a toxicological perspective and solubility mechanism. In the case of organic solvents or co-solvent cocktails, significant drug solubility in aqueous solution is occurred at sufficiently high content of

organic solvent (> 30-40%) and solubility depends in a very nonlinear fashion on water quantity (Stella & He, 2008).

CDs may acting as tablet dissolution enhancers for drugs at high dose (with which use of a drug/CD complex is difficult) e.g., paracetamol (Tasić et al., 1992). Out of some commercially available CDs, methylated CDs with a relatively low molar substitution appear to be the most powerful solubilizers (Challa et al., 2005). The water solubility enhancement factor of melarsoprol was about $7.2 \cdot 10^3$ times greater when the drug was complexed with M-β-CD (Gibaud et al., 2005).

Reduction of drug crystallinity upon complexation with CDs also contributes to the CD increased apparent drug solubility and dissolution rate (Becket et al., 1999; Vyas et al., 2008). For example, during storage carbamazepine strongly crystallizes that significantly reduces predictable dissolution and bioavailability. Introduction of Hp-β-CD, as a carrier, reduced crystalline nature of carbamazepine in solid dispersions and resulted in better and predictable dissolution profiles (Londhe & Nagarsenker, 1999). Furthermore, CDs can significantly accelerate the drug release, for example β-CD and their derivatives enhanced the release rate of poorly soluble naproxen and ketoprofen from inert acrylic resins and hydrophilic swellable (high-viscosity hydroxypropyl methyl cellulose) tableted matrices (Ann et al., 1997; Bettinetti et al., 1992; Sangalli et al., 2001).

Drug stability

CDs can improve both chemical and physical stability of drugs. CDs reduce the rate of drug hydrolysis, oxidation, steric rearrangement, racemization, isomerization and polymerization as well as enzymatic and photo-decomposition of labile drugs thus increasing the shelf life of drugs (Ahuja et al., 2011; Babu & Pandit, 1999; Li et al., 2002; Loftsson & Brewester, 1996; Lutka, 2002). Ansari et al. (2009) demonstrated that dihydroartemisinin/HP-β-CD complexes prepared using commercial (untreated) or recrystallized dihydroartemisinin showed a 40% increase in thermal stability and a 29-fold decrease in hydrolysis rates compared to dihydroartemisinin. It was reported that CD-induced enhancement of drug stability may be a result of inhibition of drug interaction with vehicles and/or inhibition of drug bioconversion at the absorption site (Matsuda & Arima, 1999). By providing a molecular shield, CD complexation encapsulates labile drug molecules at the molecular level and thus insulates them against various degradation processes (Arima et al., 2011). Since the hydrolysis of drugs encapsulated in CDs is slower than that of free drugs (Brewster et al.,

1992), the magnitude of the complex stability constant plays a significant role in determining the extent of protection (Kang et al., 2002; Nagase et al., 2001).

The stabilizing effect of CDs depends on the nature and effect of the included functional group on the drug stability and the nature of the vehicle. The large charged groups on SBE-β-CD showed greater stability enhancement of many chemically unstable drugs than other CDs including the Hp-β-CD and M-β-CD (Ma et al., 2000; Ueda et al., 1998). From the solubility of phenytoin and the kinetic information, the fosphenytoin shelf life was as high as nine years at 25 °C and pH 7.4 in the presence of 60 mM of SBE_{7m}-β-CD, although increasing the pH to 8 could increase the shelf life fosphenytoin even more (Narisawa & Stella, 1998).

However, there are enough examples that CDs catalyze the degradation of some drug molecules (Jarho et al., 2000; Narisawa & Stella, 1998; Tonnesen et al., 2002). The effects of CDs on the rate of drug degradation are pH-sensitive and depend on ionization stage of the drug (Loftsson & Brewster, 2013).

4.3.2. Effect on drug permeation through biological membranes

CDs can enhance the permeation of poorly water-soluble drugs through different biological membranes (Másson et al., 1999). For example, permeation studies of omeprazole indicated a 1.7-fold increase in drug permeation when complexed with M-β-CD (Figueiras et al., 2009). Only relatively lipophilic molecules can penetrate through the biological membranes. Through CD complexation it is possible to increase aqueous solubility of drugs without affecting their intrinsic abilities to penetrate through lipophilic membranes. However, the effect of CDs cannot be explained by only increasing of the drug solubility in the aqueous solution or by assuming that CDs act as classical permeation enhancers (reducing the barrier function of the lipophilic membrane). The CDs allow delivering the drug to the biological membrane via the water phase. In general, the resulting unstirred water layer formed by water molecules in the skin is relatively thin and permeable (Wertz, 2004). On the other hand, mucosal epithelium is covered by the layer of mucus, a gel-like fluid containing mainly water (~95%) and mucin (Bansil & Turner, 2006). Researchers have shown that passive drug diffusion through mucus is up to 100-times slower than through pure water (Khanvilkar et al., 2001).

Complexation of hydrophobic drugs with CDs makes them available at the surface of the biological barrier (e.g., eye cornea, skin). Besides, inclusion complexes permeate into the membrane without disrupting the lipid layers of the barrier. After analyzing the existing

studies (Ahuja et al., 2011; Khanvilkar et al., 2001; Loftsson et al., 2007; Loftsson & Brewster, 2013), the following patterns can be identified:

- introduction of excess amount of CDs, more than is needed to solubilize the drug, leads to decreasing drug permeation. Study by Másson et al. (1999) demonstrated that the flux of hydrocortisone at different M-β-CD and Mal-β-CD concentrations increased sharply with increase of CD concentration and decreased when CDs were in excess;
- only with considerable resistance of unstirred water layer (equal or greater than resistance of the membrane), the use of CDs can improve the delivery of drugs across biological membranes;
- CDs allow better penetration through the skin surface only water-based dermal vehicles, i.e. requires the presence of unstirred water layer at the membrane surface. For example, CDs enhance dermal delivery of hydrocortisone from o/w (oil in water) creams but not w/o creams (Preiss et al., 1995);
- CDs themselves and their inclusion complexes do not penetrate biological membranes. Therefore drug molecules have to be released from the complexes before they can permeate the membranes;
- permeation of labile drugs may be increased through CD-stabilization, i.e. CDs are able to prevent enzymatic degradation of drugs at the exterior aqueous membrane (Majumdar & Srirangam, 2009);
- usually, CDs do not enhance membrane permeation of water-soluble drugs;
- CDs can significantly increase the solubility and enhanced permeation of ionized drugs. For example, the flux of the ionized ketoprofen at 10% w/v HP-β-CD concentration was enhanced approximately 8 times compared to the intrinsic permeability of the nonionized drug (Sridevi & Diwan, 2002).

4.3.3. Effect on drug bioavailability and safety

CDs enhance the bioavailability of wide range of drugs (especially hydrophobic) by increasing the drug solubility, dissolution, and/or drug permeability. Literature data have shown that CDs are used to increase oral (Corti et al., 2008), nasal (Tas et al., 2009), ocular (Cappello et al., 2001), rectal (Değim et al., 2005) and dermal–transdermal (Kear et al., 2008), bioavailability. The mechanisms enhancing bioavailability of the drugs complexed with CDs are summarized as follows (Acartürk & Çelebi, 2011; Ahuja et al., 2011; Davis & Brewster,

2004; Miyake et al., 2000; Rajewski & Stella, 1996; Stella et al., 1999; Vyas et al., 2008; Uekama et. al., 2006; Uekama, 2004):

- increasing the solubility, dissolution rate, and wettability of hydrophobic drugs in inclusion complexes;
- CDs prevent the degradation of chemically unstable drugs, as well as during storage;
- competitive inclusion complexation with endogenous components of blood (e.g., cholesterol) to release the included drug;
- CDs disturb the membranes fluidity lowering their barrier function, which consequently enhances the absorption of medication through the mucosa;
- CDs can also allow reducing drug-related irritation and masking bitter tastes;
- CDs (e.g., DM-β-CD) can affect multidrug efflux pump, P-glycoprotein (P-gp) and multidrug resistant-associated protein 2 enabling oral bioavailability of hydrophobic drugs (e.g., tacrolimus and vinblastine) (Carrier et al., 2007; Yunomae et al., 2003).

Various factors associated with CD complexation affect the bioavailability of drugs: (1) the binding constant, (2) the type of CDs, (3) the properties of the drug, (4) the complexation methods and (5) the composition of ingredients used in formulation (Acartürk & Çelebi, 2011). For example, the high association constant (10^6 M^{-1}) between ozonide antimalarial and SBE_7-β-CD resulted in slow dissociation of the complex after IV administration (Charman et al., 2006). It causes significant alteration of pharmacokinetics of the ozonide expressed as an 8.5-fold decrease in the steady-state blood volume of distribution, a 6.6-fold decrease in the mean residence time and a greater than 200-fold increase in renal clearance of the drug. Moreover, CDs with such a high association constant can alter the pharmacokinetics and bioavailability of the already administered drug through an additional injection of CDs. (Mannila et al., 2012). In addition, CDs also prevent crystallization of active ingredients by complexing individual drug molecules so that they can no longer self-assemble into a crystal lattice (Arima et al., 2001). Thus, CDs can enhance the oral bioavailability of drugs in different ways (Challa et al., 2005).

The increased drug efficacy and potency (i.e., reduction of the dose required for optimum therapeutic activity), caused by CD-increased drug solubility, may reduce drug toxicity by making the drug effective at lower doses. For example, β-CD enhanced the antiviral activity of ganciclovir on human cytomegalovirus clinical strains and the resultant

increase in the drug potency reduced the drug toxicity (Nicolazzi et al., 2002). The toxicities associated with crystallization of poorly water-soluble drugs in parenteral formulations can often be reduced by formation of soluble drug/CD complexes (Blanchard et al., 2000). Finally, inclusion of drugs into CDs cavity at the molecular level prevents their direct contact with biological membranes and thus reduces their side effects and local irritation with no drastic loss of therapeutic benefits (Challa et al., 2005; Uekama et al., 1998).

4.4. Pharmacokinetics, biological activity and toxicology of cyclodextrins

As shown earlier, CDs have a great potential in the pharmaceutical field due to their ability to improve the drug properties. However, application of CDs as drug delivery systems requires knowledge of its pharmacokinetic and pharmacodynamic properties including *in vitro* cellular interactions and *in vivo* safety profiles. Moreover, CDs have ability to interact with various endogenous and exogenous molecules (e.g., cholesterol), and affect cellular processes. We shall further consider the most important properties of the cyclodextrin behavior in biological systems.

Pharmacokinetics and biodistribution

Pharmacokinetics of CDs and their excretion from the body will be defined *inter alia* by route of administration. However, there are general patterns, which will be described, for example, for oral and parenteral routes of CDs administration. Due to the relatively high molecular weight (from 973 to over 2000 Da) and hydrophilic nature only insignificant amounts of intact CDs are absorbed from the gastrointestinal tract, thus, their oral bioavailability is generally below 3% (Loftsson & Brewster, 2011). The absorbed CDs are rapidly excreted with the urine without any significant metabolism. The native α-CD and β-CD are practically resistant to stomach acid and pancreatic enzyme digestion, whereas γ-CD is metabolized partly by amylases in the gastrointestinal tract. The oral bioavailability of Hp-β-CD in humans is between 0.5 to 3.3% depending on the dosage and formulation (Gould & Scott, 2005; Szathmary et al., 1990; Zhou et al., 1998). Besides, more than half of the initial oral dose is excreted intact in the feces (Loftsson & Brewster, 2013). At the same time more lipophilic methylated CDs have a strong absorption in the body. According to Mosher & Thompson (2007) the absorption of DM-β-CD in rats is 6.3% to 9.6% and in the case of M-β-CD can reach 15% (Antlsperger & Schmid, 1996).

Parenteral administration of CDs, on the other hand, can be somewhat more limited. IV-injected CDs eliminate rapidly from systemic circulation and more than 90% of introduced CDs are renally excreted in intact form (Stella & He, 2008). For example, IV-administrated to humans, Hp-β-CD has a relatively small volume of distribution VD ≈ 200-250 mL/kg and short half-life $t_{1/2}$≈1.8 h, and is mainly excreted unchanged with the urine (Szathmary et al., 1990; Zhou et al., 1998). Rapid elimination and a small volume of distribution are inherent for the majority of CDs administered to animals or humans (see Table 4.3). The small VD indicates that systemically absorbed CDs distribute mainly in the extracellular compartments without involving deep compartments or storage pools (Stella & He, 2008). However, due to the ability to interact with components of blood and cellular membranes, the methylated β-CD has a larger volume of distribution (VD ≈ 2.5 L/kg) and longer half-life ($t_{1/2}$ ≈7 h) compared to other CDs derivatives (Thompson, 1997). For parenterally administered Hp-β-CD in humans, a non-saturable excretion mechanism is observed even at high doses. The pharmacokinetics of SBE-β-CD is very similar to that of Hp-β-CD. Indeed, the total plasma clearance for HP-β-CD and SBE-β-CD in all tested species is similar to the glomerular filtration rate (Davies & Morris, 1993; Rajewski & Stella, 1996) and almost all introduced CDs are excreted in the urine within 12 h (Zhou et al., 1998), except methylated β-CD. For example, M-β-CD persists for at least 6 days after a single IV administration (10 mg /kg) and is almost at the same level in the renal cortex (50-70% of all absorbed CD according to radioactivity study) as at 24 h after injection (Antlsperger & Schmid, 1996). In this regard, it is necessary to use CDs with extreme caution in patients with renal failure, because CDs, in this case, will accumulate in the body (Slain et al., 2001; von Mach et al., 2006).

The main distribution areas of CDs after their absorption include the following tissues: kidney, bladder, adrenal glands and liver (Antlsperger & Schmid, 1996; De Bie et al., 1998; Gerloczy et al., 1990; Kubota et al., 1996; Van Ommen et al., 2004). The highest concentration of absorbed CDs is in the kidneys. In regards to biodistribution in tumor-bearing rodents, the results of several studies have showed that a significant amount of methylated CDs is distributed in the tumor tissue, e.g. primary effusion non-Hodgkin lymphoma (Gotoh et al., 2014), and various carcinoma in xenograft tumor bearing mice (Grosse et al., 1998; Onodera et al., 2013a). Grosse et al. (1998) have showed that M-β-CD after 3 hours of parenteral administration in MCF7 xenografts was accumulated mostly in tumor and kidneys and a small quantity in intestine and liver. Concentrations in lungs and muscle were almost undetectable.

Table 4.3 Pharmacokinetic parameters of some CDs IV administered (from Stella & He, 2008)

Cyclodextrin	Species	Dose (mg/kg)	$t_{1/2}$ (min)	VD (mL/kg)	Plasma clearance (mL/min/kg)
β-CD	Rat	50	21.6	201.8	7.53
HP-β-CD	Rat	200	23.9	194	7.2
HP-β-CD	Human	43	102	222	1.57
HP-β-CD	Human	114	114	253	1.54
SBE-β-CD	Rat	600	18	300	9.8
SBE-β-CD	Rabbit	600	30	200	5.2
SBE-β-CD	Dog	240	66	430	4.7
SBE-β-CD	Human	100	84	200	1.9
M-β-CD	Rabbit	200	420	2500	3.8

Toxicology of CDs

To date, there are a huge number of publications, including a detailed review of the toxicological status of CDs and their biological activity (Antlsperger & Schmid, 1996; Arima et al., 2011; Irie & Uekama, 1997; Gould & Scott, 2005; Loftsson & Brewster, 2011; Mosher & Thompson, 2007; Thompson, 1997; Zidovetzki & Levitan, 2007). We shall further briefly review the toxicology issues.

Toxicological studies of various CDs have been conducted following different administration routes in numerous formulations. In the case of oral administration of CDs the following abnormalities were observed: soft feces or diarrhea, cecal enlargement on oral administration, and renal effects subsequent to systemic absorption (Mosher & Thompson, 2007; Thompson, 1997). The major findings following parenteral administration of CDs at high doses or during a long period of application include vacuolation of hepatocytes and urinary bladder and foamy macrophages in the liver, lung, and lymph nodes (Stella & He, 2008). With the exception of α-CD, β-CD, and methylated-β-CD, no evidence of inflammatory responses, cell death, and cell degeneration or regeneration is observed in studies reported to date (Mosher & Thompson, 2007). Furthermore, in the case of γ-CD, Hp-

β-CD, and SBE-β-CD vacuolation is reversible after discontinuation of the CDs (Frijlink et al., 1991a; Rajewski & Stella, 1996).

Since kidneys are the main absorption site of CDs in the body, the renal toxicity should be carefully considered. The main effect of CDs in the kidney is vacuolation of proximal tubular epithelium. Many studies have demonstrated that CDs, especially β-CD and its methylated derivatives cause renal toxicity (Frank et al., 1976; Irie & Uekama, 1997; Thompson, 1997). These changes begin with an increase of apical vacuoles and the appearance of giant lysosomes. Prominent acicular microcrystals are observed in lysosomes. These changes are followed by disruption of mitochondria and other organelles, and eventually, irreversible cell injury and kidney dysfunction occurs. The effective dose of M-β-CD to induce kidney damage is even lower than that of β-CD (Antlsperger & Schmid, 1996; Irie & Uekama, 1997). The renal toxicity may be caused by CDs according to the following scheme: (1) IV-injected cyclodextrin binds endogenous cholesterol and some phospholipids (also CDs may extracted its from the cells); (2) glomerular filtration of the cholesterol-cyclodextrin complex is followed by dissociation of the complex in the ultrafiltrate, resulting in cholesterol accumulation in the proximal tubule cells; (3) due to limited aqueous solubility of the complexes in renal tissue, the complexes crystalize and formed needle-like crystals cause kidney damage (Frijlink et al., 1991a). However, CDs like γ-CD, Hp-β-CD, and SBE-β-CD may be used in high doses without causing any significant effects on the human kidney. For example, IV-introduction of Hp-β-CD in healthy volunteers has been studied at a single dose up to 3 g and was well tolerated (Gould & Scott, 2005). CDs such as Hp-β-CD and SBE-β-CD are widely used in marketed parenteral drug formulations (Kurkov et al., 2012).

It should be noted that at the joint introduction of CDs with drugs, a decrease of toxicological effects of both substances was reported (Arima et al., 2011; Mosher & Thompson, 2007). For example, IV injection of M-β-CD (5 mg/kg) to tumor-bearing mice significantly increased blood urea nitrogen, aspartate aminotransferase and lactate dehydrogenase levels, compared to control, suggesting induction of systemic side effects (Onodera et al., 2013a). On the other hand, co-administration of doxorubicin (5 mg/kg) with M-β-CD (12 mg/kg) significantly reduces systemic side effects of both compounds (in the case of M-β-CD compared to 5 mg/kg dosage) (Motoyama et al., 2013).

Biological activity

Numerous studies have shown that a variety of cellular functions are affected when cells are treated by β-CD and its derivatives. For example, M-β-CD has the high affinity for

cholesterol and is used widely as a lipid raft–disrupting agent (Kilsdonk et al., 1995; Mohammad et al., 2014; Zidovetzki & Levitan, 2007). Due to the highest affinity for inclusion, cholesterol is a primary target of CDs in the cell membrane (Irie et al., 1992; Ohtani et al., 1989; Ohvo & Slotte, 1996). Depletion of membrane cholesterol results in increased membrane fluidity and induces membrane invagination through a loss of bending resistance and as such causes cell lysis (Challa et al., 2005). It has been shown that under certain conditions β-CD and its methylated derivatives induce apoptosis via the activator caspase-8, which subsequently activates the effector caspases-3/-7 (Onodera et al., 2013b; Schönfelder et al., 2006). Removal of cholesterol disrupts integrity of lipid rafts and concurrently enhances the permeability of ions and small non-electrolytes (Chen et al., 1978; Mohammad et al., 2014). On the other hand, membrane cholesterol depletion at high CD concentration (higher than 1-5 mM) inhibits endocytotic processes (O' Neill et al., 2011; Zuhorn et al., 2002) and increases exocytosis (Chen et al., 2010).

Decreasing the concentration of the CDs and/or using shorter incubation time result in moderate depletion effects. On the other hand, depletion of membrane phospholipids (e.g., phosphatidylcholine and sphingomyelin) by CDs results in bilayer imbalance. The removal of lipids may also promote in part the formation of stomatocytes through an inward bending of biological membranes (Figure 4.2). A similar mechanism leads to the lysis of artificial membranes treated by CDs (Hatzi et al., 2007). However, unlike detergents, CDs solubilize membrane components without entering inside cells and, thus, the perturbing effects of CDs can be mild and reversible (Challa et al., 2005; Irie & Uekama, 1997). Besides, CDs may function as a cholesterol shuttle, enhancing cholesterol bidirectional flux without changing the equilibrium cholesterol distribution between cells and medium (Atger et al., 1997). Leventis & Silvius (2001) demonstrate that β-CDs and γ-CDs can dramatically accelerate the rate of cholesterol transfer between lipid vesicles.

At the initial stage after administration, CDs interact with blood cells, thus, erythrocytes are good and simple model for assessing the instinct toxicity of CDs. The *in vitro* hemolytic activity of CDs is reported in the order β-CD >α-CD > Hp-β-CD >γ-CD >> Hp-γ-CD ≥ Hp-α-CD in red blood cells freshly collected from human and P388 murine leukemic cells (Leroy-Lechat et al., 1994; Rajewski & Stella, 1996). Irie & Uekama (1997) reported that hemolytic effects of various CD derivatives decreased in the order dimethyl-β-CD >> trimethyl-β-CD >β-CD > Hp-β-CD ≥ α-CD >>> γ-CD. The concentration of Hp-β-CD less than 5 mg/ml did not cause hemolysis. It is clear that these differences are attributed to the differential solubilization effects of membrane components by each CDs rather than their

intrinsic solubility or surface activity (Arima et al., 2011). This is supported by the fact that a positive correlation arises between the hemolytic activity of several CDs and their capacity to solubilize cholesterol and some phospholipids (Irie & Uekama, 1997; Thompson, 1997). The suggested mechanisms of morphological changes in erythrocytes induced by different CDs are represented in the Figure 4.2.

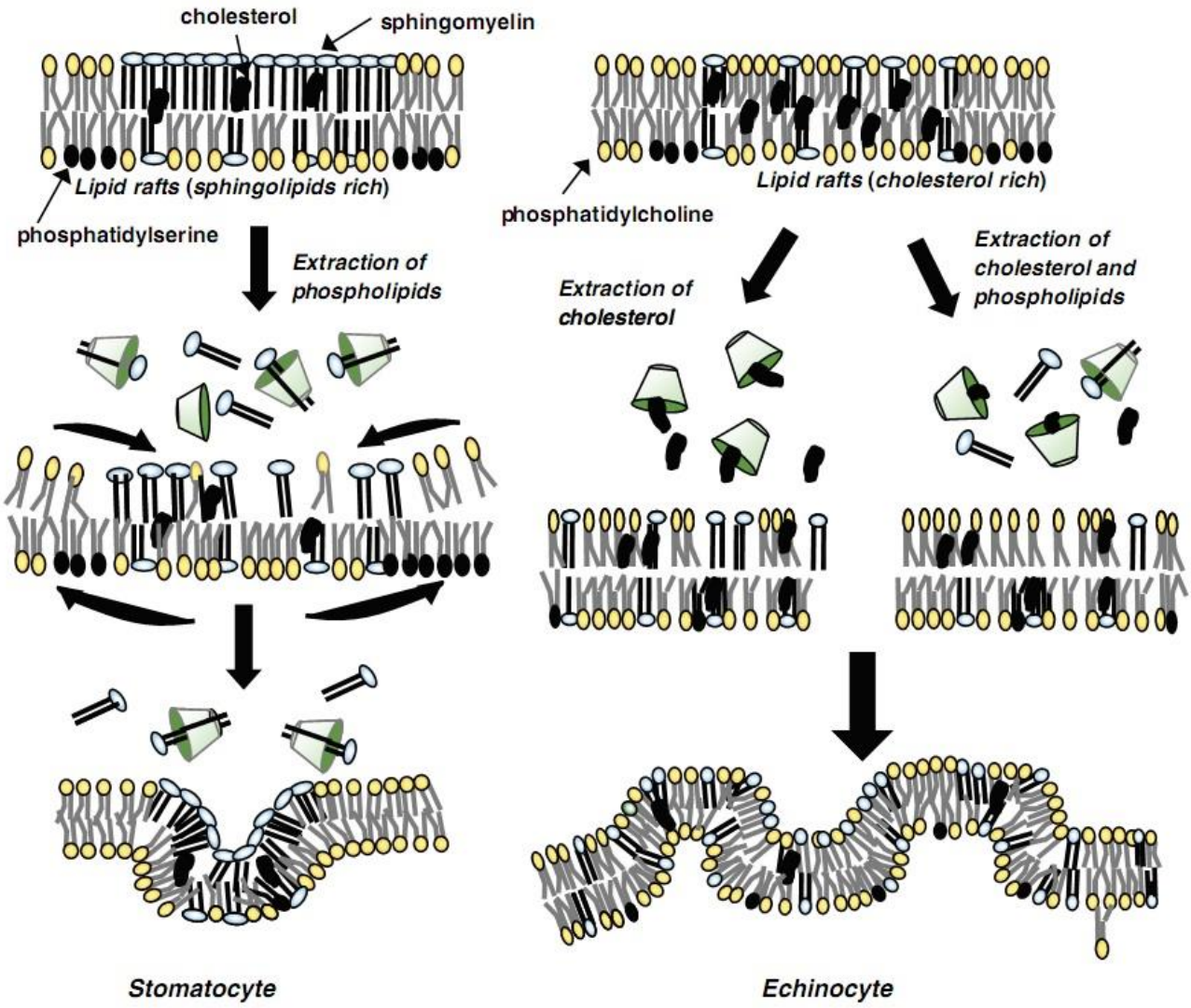


Figure 4.2 Mechanism proposed for morphological changes in red blood cells induced by CDs (from Arima et al., 2011)

4.5. Mechanism of drug release from cyclodextrin complexes

Studies on drug release mechanism provide important information about the behavior and functioning of drug/carrier complex in biological systems. Although it is possible to predict the pharmacokinetics of the drug after administration from the value of drug/CD association constants, physiological environment has to be considered (e.g., plasma proteins competition with CDs for drug binding) (Loftsson et al., 2016). Different mechanisms play an important role in drug release from the drug/CD inclusion complex. Drug/CD complexes are dynamic system continually forming and dissociating with life-times in the range of milliseconds or less (García-Ruiz et al., 2005; Stella et al., 1999). Therefore, it will not be the governing factor in the study of the kinetics of drug release. Even at high affinity the drug is quickly released from the complexes. Despite the fact that the major driving force for dissociation of drug from the complex is related to simple dilution (Stella & He, 2008), other mechanisms have a significant impact, such as competitive displacements of the drug from the complex, binding to protein, uptake by tissues (Stella et al., 1999).

Dilution

Dissociation due to dilution appears to be a major release mechanism for the drug from CD complexes. Indeed, a simple dilution of 1000 times of drug/CD complex with the association constant 610 M^{-1} leads to the release of more than 95% of the drug from the complex (Stella et al., 1999). Only for strongly bound drugs, with association constants of 10^4 M^{-1} or higher, other ways to of release may be considered (Loftsson et al., 2016; Stella & He, 2008). Dilution is minimal when a drug/CD complex is administered by ophthalmic, transdermal and transmucosal routes (Rajewski & Stella, 1996). Peroral administrated complexes also dissociate rapidly upon dilution in the stomach and intestine and it is considered that only the drug is absorbed (not the complex) (Loftsson & Masson, 2001; Tong & Wen, 2008).

Protein binding

Drug binding to plasma proteins is an important mechanism of drug release from a drug/CD complex. Obviously the proteins may effectively compete with CDs for drug binding and thus facilitate the *in vivo* drug release. Frijlink et al. (1991b) studied the effect of Hp-β-CD on the displacement of both naproxen and flurbiprofen from plasma proteins *in vitro*. The complexes of Hp-β-CD with naproxen ($K = 2207$ M^{-1}) or flurbiprofen ($K = 12515$ M^{-1}) characterized by 2207 M^{-1} and 12515 M^{-1} binding constants, respectively. Only in the case of flurbiprofen a significant fraction of the drug associated with the CD in

serum was observed *in vitro*, which is reflected with altered biodistribution and pharmacokinetic of flurbiprofen by Hp-β-CD immediately after IV-injection (10 min) *in vivo*. In the case of complexes with moderate binding constant (less than 10^4 M^{-1}) and relatively high affinity to plasma proteins, binding of drug to plasma proteins will play a major role in the drug release from the compexes and its further distribution (Kurkov et al., 2012). Oppositely, for drugs showing large CD complex binding constant and low protein binding like rocuronium in presence of sugammadex ($K = 2.5 \cdot 10^7$ M^{-1} and 30 % protein binding) (Adam et al., 2002), significant decrease in distribution volume and enhanced excretion of unmetabolized drug are observed (Loftsson et al., 2016).

Competitive displacement

Another important mechanism influencing the drug release from the complexes is competitive displacement by endogenous molecules *in vivo*. Frijlink et al. (1991b) study showed that the cholesterol could displace both naproxen and flurbiprofen from CD by competitive binding. On the other hand, the addition of a competing agent allows a controlled release of the drug from the CD complexes (Hirayama & Uekama, 1999). Oral administration of the complex of cinnarizine with β-CD showed decrease in the drug bioavailability due to strong complexation (Tokomura et al., 1986). Co-administration of phenylalanine (competing agent) improved the bioavailability of cinnarizine in comparison to the conventional tablets formulation.

Drug uptake by tissue

In the case of highly bound drugs or when the complex is administered at a site where dilution is minimal (e.g., ocular, nasal, rectal administration) drug uptake by tissues is a potential contributing mechanism for drug release from CD complex. If the lipophilic drug has an access to tissue, which in ite turn is not available to the CD or the complex, the tissue then acts as an absorber causing dissociation of the complex based on simple mass action principles (Stella et al., 1999). The scheme of drug absorption from the CD complex by tissues can be demonstrated on ocular drug administration. Upon ocular administration, the free drug is transported across the cornea competing with elimination of the free CDs and the complex via the nasolacrimal drainage (Loftsson & Stefansson, 1997). The absorption rate will depend on CD concentration (laws of mass action).

Change in ionic strength and temperature

Binding of drug to CD is an exothermic process, i.e. any increase in temperature results in weakening of the complex, and thus increasing the release of drug (Inoue et al., 1993). Most of the drug/CD complexes are usually prepared and stored at/or below room temperature. Normal body tissue temperatures (37°C) may contribute to drug dissociation in physiological environment (Stella et al., 1999). In the case of a weak electrolyte, the strength of binding to CD is determined by the charged state of the drug and, hence, depends on the pH of environment. For most molecules, the ionized or charged form of the molecule has weak affinity to CDs compared to the non-ionized or neutral form of the drug especially when bound to a neutral CD (Patel et al., 2007).

In summary, the relative contribution of all mentioned mechanisms will however depend on the route of administration, volume of distribution of drug and CD, binding strength and concentrations of drug and CD, as well as relative affinity and concentration of competing agent (e.g., proteins, cholesterol).

5. Use of cyclodextrins in photodynamic therapy

In previous chapters it was shown that CDs effectively interact with a large number of compounds improving their properties, hence, it is not surprising that the CDs were tested as delivery systems in PDT. Although the first mention of the use of CDs in the field of PDT dates back to the early 1990s (Kessel et al., 1991; Garbo, 1990), intensive research began only in the last decade (Kryjewski et al., 2015; Lang et al., 2004; Mazzaglia, 2011). Nowadays, over than 150 papers and conference reports have been published on CDs application in PDT. Most of these studies are related to the tetrapyrrolic (porphyrinoid) PSs. At the same time literature data indicate the successful use of CDs with other PSs, such as hypericin (Sattler et al., 1997; Zhang et al., 2013), aminolevulinic acid (Aggelidou et al., 2014), fullerene (Ikeda et al., 2013; Iohara et al., 2012; Zhao et al., 2009), squaraine dyes (Arun et al., 2011), curcumin (Haukvik et al., 2009; Wikene et al., 2014) and phenosafranin (Kundu et al., 2016). However, in this section we will focus exclusively on the porphyrinoid (e.g., porphyrins, chlorins and phthalocyanines) PSs.

The following problems were tried to be solved by means of the use of CDs in PDT: i) enhancement of PSs water solubility, ii) changes of the photophysical characteristics of PSs, including the generation of ROS and iii) improving of PS delivery to targets cells/tissues. The solution to these problems was carried out by various ways, such as non-covalent inclusion complexation, covalent conjugation or formation of nanoassamblies between PSs and CDs (Figure 5.1).

5.1 Inclusion complexes of cyclodextrins with photosensitizers

Host–guest complexes between tetrapyrrole PSs and CDs are usually formed by incorporating a part of the sensitizer (e.g. aryl substitutes) into CD cavity due to relatively large size. The small size of the α-CD cavity does not allow forming inclusion complexes with porphyrinoid PS. The binding modes of aryl substituted tetrapyrroles can be classified into three types (Lang et al., 2004):

- Inclusion through the secondary face (typical for β-CDs). Inclusion complexes of tetrakis(3,5-dibromo-2-hydroxylphenyl)porphyrin with β-CD is an example of this inclusion mode (Zhang et al., 2005).
- Inclusion through the primary face (typical for γ-CD). For example, complexes of tetrakis(4-sulfonatophenyl)porphyrin ($TTPS_4$) with β-CD and γ-CD show

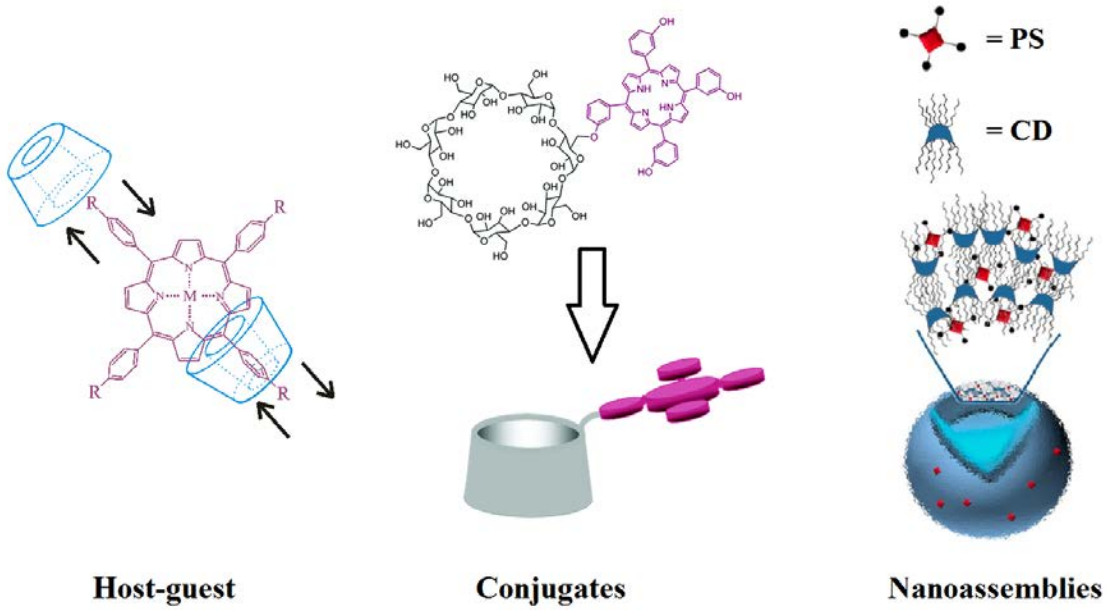


Figure 5.1 The type of CDs binding with tetrapyrrole photosensitizers: host-guest (Lang et al., 2004), conjugate (Kirejev et al. 2014), nanoassemblies (Mazzaglia et al. 2013)

different geometry: insertion of the phenyl substituent through the CD secondary face for β-CD and through the primary face for γ-CD (Ribó et al., 1995).

- Non-specific external binding. This type of binding is of interest in the formation of CD-PS nanoassembly and will be discussed later (chapter 5.3)

Binding of PS to CDs increases water solubility of PS, usually leading to spectro-fluorescence changes and minimally affecting the formation of singlet oxygen compared to monomer form of PS. For example, inclusion complexation between mTHPP and Hp-β-CD results in nearly 300 times enhancement of fluorescence intensity of PS in PBS solution (Guo et al., 2005). It is worth noting that PS:CD 1:2 stoichiometry along with the 1:1 is the most common. Although, computer modeling of interaction between Zn-phthalocyanines and native CDs showed 1:4 stoichiometry in all cases (Lu et al., 2016). The inclusion of PS in the CD does not alter the equilibrium between Type I and Type II pathways in the ROS production (Cellamare et al., 2013; Lang et al., 2004).

The study of Mosinger et al. (2002) showed the influence of different CDs on the photophysical properties of porphyrin PSs ($TPPS_4$, Zn-$TPPS_4$, Pd-$TPPS_4$ and tetrakis(4-carboxyphenyl)porphyrin). Almost all obtained complexes were characterized by 1:1 stoichiometry of binding. The binding constants (10^2–10^5 M^{-1}) depend on the cavity size and the functionalization of the primary and secondary faces of CDs. The highest binding

constants and relevant changes in photophysical characteristics were obtained for Hp-β-CD (~10^5 M^{-1}). While the magnitude of the QY of fluorescence and triplet state are practically not changed, the lifetime of the triplet states of the bound porphyrins in the absence of oxygen is considerably extended (in some cases more than an order of magnitude). This fact is due to the exclusion of water molecules from the solvation shell of porphyrins and reduction of collisional quenching (Lang et al., 2004). Furthermore, in the case of Hp-β-CD complexes the QY of singlet oxygen generation increased by 15-20% compared to free PS, which is associated with an increase in the lifetime of the triplet state. Similar results were obtained for the other porphyrinoid/CD inclusion complexes (Cosma et al., 2006; Guo et al., 2005; Kano et al., 2002, 2005; Wang et al., 2012; Wu et al., 2005; Yang et al., 2001). On the other hand, cationic sensitizer tetrakis(N-methylpyridinium-4-yl)porphyrin binds with the external surface of native CDs and complex formation do not affect the behavior of the PS triplet states (Mosinger et al., 2009). Typically, methylated CDs have the highest affinity for aryl substituted tetrapyrrole PS. Indeed, in the case of $TTPS_4$ the association constants for different CDs keep the following order: M-β-CD> Hp-β-CD> β-CD (Wu et al., 2005).

In addition, the complexes of CDs with PSs such as *chlorophyll a* and *chlorin* e_6 were studied (Cellamare et al., 2013; Cosma et al., 2008; Dentuto et al., 2005, 2007; Paul et al., 2016). Paul et al. (2016) showed that *chlorin* e_6 effectively interacts with Hp-β-CD ($K = 5.6 \cdot 10^3$ M^{-1} at pH 7) with 1:1 stoichiometry. The solubility of *chlorin* e_6 increased by about 14-fold at pH 7. Contrary, due to low affinity of CDs to *chlorophyll a* ($K \sim 10$ M^{-1}) (Dentuto et al., 2005), a complexation with CDs leads to only to a partial dissolution of PS in PBS showing the appearance of a different aggregates (Dentuto et al., 2007; Cosma et al., 2008). Besides, the quantum yield of $^1O_2$ generation of TM-β-CD/*chlorophyll a* complexes is low too (0.076 relative to *chlorophyll a* in CH_3-CN) (Dentuto et al., 2005).

One way to improve the delivery of a PS to target tissues is conjugation of specific molecules (e.g., antibodies, aptamers, and peptides) to the DDS surface or to the sensitizer molecules. Kitagishi et al. (2013, 2015) studied the interaction of octaarginine-conjugated $TPPS_4$ with permethylated-β-CD and $TPPS_4$ with an octaarginine-conjugated permethylated-β-CD aiming to improve tumor cells targeting (octaarginine chain is used as a cell-penetrating peptide). In both cases CDs demonstrated high affinity to sensitizers and formed 1:2 inclusion complexes preventing self-aggregation of PSs. Furthermore, *in vitro* studies demonstrated that conjugation (§ 5.2) of octaarginine to CD was a more effective compared to conjugation of octaarginine to PS.

5.2 Photosensitizer/cyclodextrin conjugates

One of the possible ways to improve the physicochemical properties of the PS is its conjugation with various biomolecules and therefore a covalent conjugation of porphyrinoid molecules to CDs has been explored (Gonzalez et al., 1984; Kano et al., 2003; Kryjewski et al., 2015). In most cases, conjugation of tetrapyrroles with CDs enhances the water solubility of PSs, improves their photophysical characteristics and increases the generation of singlet oxygen (Kryjewski et al., 2015). According to absorption and fluorescence spectra, the conjugation of protoporphyrin IX with β-CD leads to increase of aqueous solubility of PS at biologically relevant concentrations (μM) range, while protoporphyrin IX alone was soluble only in DMSO (Aggelidou et al., 2013). Kirejev et al. (2014) showed that CD/mTHPP conjugate had a significantly improved solubility of CD/mTHPP in aqueous solution compared to unconjugated mTHPP resulting in more than tenfold increase of fluorescence intensity compared to free mTHPP in 4% DMSO-PBS solution (v/v). Conjugation of meso-tetrakis(pentafluorophenyl)-chlorin to four β-CD leads to about 250 times enhancement of PS fluorescence QY in PBS, as well as causes increase of the QY of singlet oxygen generation from less than 0.05 (free PS) to 0.4 (conjugated PS) in PBS (Silva et al., 2008). On the other hand, Lang et al. (2002) demonstrated that the attachment of β-CD to meso-tetrakis(pentafluorophenyl)porphyrin does not affect the spectral properties including quantum yields of the triplet states of the PS. Contrary, 5,10,15-tris(pentafluorophenyl)corrole conjugates with one or two molecules of β-CD demonstrated a 25 % decrease in the fluorescence QY in the case of one β-CD conjugation and 40-50 % decrease in the QY of singlet oxygen generation for both conjugates compared to unconjugated PS in dimethylformamide solution (Barata et al., 2015).

Increasing the amount of CDs conjugated to a porphyrinoid molecules (e.g., silicon(IV) phthalocyanines and tetrakis(pentafluorophenyl)porphyrin) leads to a greater solubility of PS (Králová et al., 2006; Lau et al., 2011a). On the other hand, the water-solubility of such systems primarily depends on the solubility of the cyclodextrin. Indeed, Lourenço et al. (2014) showed that phthalocyanines conjugated with α-CDs and γ-CDs were more water soluble compared to conjugates with β-CDs. In addition, the CD cavity can include (non-covalently) another PS, anticancer or antibiotic compound making possible a multimodal therapy against cancer or pathogens (Ermilov et al., 2011; Králová et al., 2010; Theodossiou et al., 2015). For example, Leng et al. (2007) demonstrated that formation of a stable 1:1 host-guest complex between a silicon(IV) phthalocyanine conjugated axially with

two permethylated beta-cyclodextrin units and a tetrasulfonated porphyrin exhibits a light-harvesting property and works as an efficient photosensitizing system against HT29 human colon adenocarcinoma cells.

5.3. Photosensitizers encapsulated in cyclodextrin nanoassemblies

Chemical modifications of native CDs by grafting substituent groups to different positions (narrow rim, wide rim, or both rims) provides non-ionic or cationic CD derivatives able to form supramolecular nanoassemblies. Nanoassemblies are supramolecular colloidal systems formed by drugs and usually amphiphilic CDs (Zerkoune et al., 2014). In addition, the grafting of small portions of polyethylene glycol at the secondary rim of CD can increase drug bioavailability and potentially decrease the immunogenicity (Mazzaglia, 2011). The size of obtained nanoassemblies is in a range 20 nm- 1000 nm depending on CD nature, preparation method, included drug and so on (Ferro et al., 2009; Mazzaglia et al., 2005, 2006). For example, self-aggregation of natural β-CD leads to the formation of polydispersed stable nanoaggregates with the size of 200–300 nm (Bonini et al., 2006). Mazzaglia et al. (2011) demonstrated that the presence of the dansyl group gives rise to 2-o-amino oligo-ethylene amphiphilic CD aggregates with sizes smaller (50 nm) than those of the CD precursor alone (190 nm). The formation of nanoassemblies between PSs and CDs leads to the enhancement of water solubility of PS, causes spectro-fluorescence changes of PS properties, provides a high yield of singlet oxygen photogeneration and as a result high PDT-efficiency toward tumor cells (Conte et al., 2014; Sortino et al., 2006). Inclusion of 5-[4-(1-dodecanoylpyridinium)]-10,15,20-triphenyl-porphyrinyl chloride in CD nanoagregates at a molar ratio 1:10 causes drastic alterations in their spectroscopic properties such as 4-times increasing of its fluorescence QY and provides a high QY of PS triplet formation (≥ 0.9) and $^1O_2$ generation (≈ 0.9) (Ferro et al., 2009). The physical–chemical properties of PS-CD nanoassemblies such as size, fluorescence lifetimes and QY of singlet oxygen generation are strongly dependent on the PS:CD molar ratio. For example, Sortino et al. (2006) demonstrated that high loading of the nanoassemblies (i.e., 1:1, 1:2) causes aggregation of the PS with the subsequent loss of its photodynamic activity. Increasing the amount of CDs in the nanoassemblies (1:10-1:50) leads to PS monomerization, which is reflected in the increase of the quantum yield of singlet oxygen generation. Moreover, cross-linking CD with polymer molecules allows forming nanoparticles combining two-photon fluorescence imaging and bimodal phototherapeutic activity in a single nanostructure that was achieved through the

appropriate choice of a PS (singlet oxygen) and a nitric oxide (NO) photodonor (Fraix et al., 2013, 2014; Kandoth et al., 2014).

5.4. Influence of cyclodextrins on photosensitizers in biological systems

The effective photophysical characteristics and the high QY of generation of singlet oxygen not always allow achieving high parameters of PDT effectiveness in biological systems. In the case of complexes of CDs and PSs it is important to determine which parameters will have the greatest impact. While the main part of the researches was focused on the study of the photophysical characteristics of such systems and their ability to sensitize singlet oxygen (Cellamare et al., 2013; Lu et al., 2016; Mazzaglia et al., 2006; Silva et al., 2008), there are a number of studies which demonstrated that CDs can be successfully used as carriers in photodynamic anti-cancer therapy (Garbo, 1990; Králová et al., 2006) and photodynamic antimicrobial therapy (Ferro et al., 2009; Hanakova et al., 2014).

Inclusion complexation

Influence of inclusion complexation with CDs on the accumulation of arylporphyrins and some other porphyrin-like sensitizers in cellular systems have been studied by several research groups (Dentuto et al., 2007; Kitagishi et al., 2015; Kolárová et al., 2003, 2004). $TPPS_4$ and Zn-$TPPS_4$ in the inclusion complex with Hp-β-CD represent efficient PSs with a high phototoxicity towards G361 human melanoma cells (Kolárová et al., 2003, 2005). Nevřelová et al. (2005) determined that the generation of ROS and H_2O_2 in photosensitised cells was higher for free Zn-$TPPS_4$ than for the sensitiser bound to Hp-β-CD. In the study of Kolárová et al. (2004) the one hundred excess of Hp-β-CD in relation to the PSs $TTPS_4$ and Pd-$TTPS_4$ leads to a 2 fold increase of $TTPS_4$ and Pd-$TTPS_4$ uptake in melanoma G361 cells with a simultaneous (~1.5 times) increase in phototoxicity. At the same time, Kitagishi et al. (2015) observed that the addition of $TTPS_4$ complexed with 2.4 time excess of trimethyl-β-cyclodextrin led to approximately 30% reduction of PS cellular uptake and consequently cell-photokilling ability in HeLa cells. Similarly, Dentuto et al. (2007) showed that the interaction of *chlorophyll a* with trimethyl-β-cyclodextrin at a ratio 1:10, Hp-β-CD (1:100) or Hp-γ-CD (1:100) resulted in a 2-4 fold decrease in phototoxicity observed in Jurkat cell line. Despite the lower cells accumulation, inclusion complexes of *chlorin* e_6 with Hp-β-CD demonstrated significantly higher phototreatment efficacy of human oral squamous carcinoma compared to unbounded PS (the value IC_{50} decreses 3 times) (Paul et al., 2016).

Surprisingly, to date, there is only one publication on the influence of inclusion complexation on PDT efficacy *in vivo* (Garbo, 1990) and no one study on the effect of CDs on the PS distribution *in vivo.* Garbo (1990) showed that PDT of the bladder tumor transplanted into rats with inclusion complexes of tin etiopurpurin and γ-CD was 3.5 and 5 times more efficient compared to emulsion and liposome delivery systems respectively.

Conjugates

Conjugates of PS-CD demonstrated significant PDT-efficiency *in vitro* and *in vivo*. The results of Kirejev et al. (2014) indicated that CD-porphyrin conjugates exhibit improved skin distribution compared to PS alone using aqueous vehicles. Lau et al. (2011a, 2011b) investigated PDT efficacy of different phthalocyanines/CD conjugates *in vitro* and *in vivo.* In the case of most active permethylated-β-CD bis-substituted phthalocyanines, the values of IC_{50} for phototreated HT29 and HepG2 cells *in vitro* were 0.04 and 0.05 μM respectively. The study of subcellular localization of this conjugate showed that PS targets lysosomes and not mitochondria, and that the major cell death pathway was apoptosis. *In vivo* tests of this PS-CD conjugate in HT29 tumor bearing mice demonstrated that the tumor size in the treated group was less than half of that found in the control group on 15 day post PDT treatment (Lau et al., 2011a). In another study, the phthalocyanine axial conjugated with permethylated-β-CD from one side and galactose unit from other side demonstrated a 2-times improvement of *in vitro* PDT efficacy toward the same cells line compared to describe above PS-bis-CD conjugate, but reduction in tumor growth was similar at the same condition (Lau et al., 2011b). It is worth noting that the amount of conjugated CD molecules has an ambiguous effect on the PDT-efficiency in various models. Králová et al. (2006) demonstrated that mono-CD porphyrin conjugates exhibited stronger *in vitro* phototoxic effect than bis-CD conjugates. Hovewer, bis-analogue was found to be a better PS *in vivo*, exhibiting fast and selective tumor uptake, high PDT-antitumor activity, and fast elimination from body.

The synergistic therapeutic efficiency of PDT with PS-CD conjugates and chemotherapy drugs incorporated into CD cavity was an object of several works (Aggelidou et al., 2013; Kejík et al., 2011; Králová et al., 2010; Theodossiou et al., 2015). For example, the combination of porphyrin-bis-CD (β-CD or γ-CD) conjugates with the corresponding drug (paclitaxel and doxorubicin, respectively) in mice bearing subcutaneously growing mammary carcinoma 4T1 leads to increased therapeutic efficiency of both PDT (20-30 %) and chemotherapy (40-50 %) (Králová et al., 2010). Moreover, using metalloporphyrin rends possible forming a supramolecular coordination complexes with immunoglobulins that in the

case of PS-CD conjugate allows combined PDT, chemotherapy and immunotherapy (Kejík et al., 2011).

Nanoassemblies

It was shown that encapsulation of PSs into CD *nanoassemblies* improves PS delivery and PDT-efficacy *in vitro* (Mazzaglia, 2011). For example, Sortino et al. (2006) demonstrated that anionic porphyrin binding to nanoassemblies leads to a decrease in singlet oxygen production, but enhances the delivery of PS to the HEP-2 cells resulting in a higher phototoxicity compared to free PS. Different studies suggest that the uptake of the nanoassembly is mediated by endocytosis (Conte et al., 2014; Mazzaglia et al., 2011). Mazzaglia et al. (2013) showed that delivery of poor-water-soluble porphyrin by nanoassemblies based on nonionic and hydrophilic CD increases the internalization efficiency and photocytotoxicity (apoptosis) and, at lower concentrations, changes the morphology of A375 melanoma cells and prevents its proliferation. Besides, the monocationic meso-substituted porphyrin complexed into supramolecular aggregates of cationic amphiphilic CDs demonstrated high antimicrobial activity towards both gram-positive and gram-negative bacterial pathogens (Ferro et al., 2009). Further development of the nanoassembly system was achieved by introducing NO photodonor (Kandoth et al., 2012). Studies on HeLa cells proved that there was a synergistic cytotoxic effect, caused by singlet oxygen and NO. In addition, *in vitro* study of Conte et al. (2016) demonstrated that CD based nanoparticles may be used for dual delivery of zinc-phthalocyanine and docetaxel combining photodynamic and antimitotic effects toward tumor cells. However, to date, there are no studies devoted to the PS-CD nanoassemblies *in vivo*.

5.5. Inclusion complexes of mTHPC with cyclodextrins

The complexes of mTHPC with native CDs, M-β-CD and Hp-β-CD were studied by P. Prognon and A. Kasselouri group from France (Demore et al., 1999; Desroches et al., 2001; Bautista-Sanchez et al., 2005). It was shown that α-CD does not form inclusion complexes with mTHPC while β-CD has limited effect on the PS solubility due to relatively low own water solubility. On the contrary, mTHPC effectively forms inclusion complexes with derivatives of β-CD and γ-CD resulting in the destruction of mTHPC aggregated forms with the restoration of the absorption spectrum of the monomer form (like in methanol) (Demore et al., 1999). The formation of such inclusion complex leads to enhanced mTHPC fluorescence intensity in water solution up to 300-times in the case of M-β-CD. Indeed, M-β-

CD demonstrates a higher affinity to mTHPC molecules as reflected by the high value of the association constant: $1.2 \cdot 10^5$ M^{-1} for 1:1 (Bautista-Sanchez et al., 2005) and $2.8 \cdot 10^{10}$ M^{-2} for 1:2 complexes (Desroches et al., 2001). Besides, in the case of M-β-CD and Hp-β-CD association with mTHPC, the authors observed an increase in the QY fluorescence about 1.4 times compared to monomeric PS (Bautista-Sanchez et al., 2005). Taking into account the molecular size and Van der Waals interactions between mTHPC and CDs, molecular modeling demonstrates that one mTHPC molecule cannot bind more than two CDs molecule. According to Demore et al. (1999) calculations, the most convenient model appears to be the one-step 1:2 mTHPC:CD association. The data on circular dichroism confirm the formation of inclusion complexes (Desroches et al., 2001). Insertion of mTHPC phenyl group into CD cavity causes an appearance of induced circular dichroism spectrum with three main circular dichroic signals: a positive maximum at 416 nm and two negative signals at 432 and 652 nm that well correlate with three main mTHPC absorption bands.

The study of Bautista-Sanchez et al. (2005) showed ambiguous effect of CDs on triplet state characteristics and QY of singlet oxygen generation of mTHPC. On the one hand, calculated quantum yields values of triplet state and singlet oxygen generation of mTHPC were not affected by the presence of M-β-CD, but triplet lifetimes were strongly enhanced (about 5 times). On the other hand, the value of QY of singlet oxygen generation determined from infra-red measurement of $^1O_2$ luminescence was lowered in 1.5 times. The authors suggest that such changes can be related to some protective effect of the CD ring, i.e. the formation of the complex reduces the interaction of mTHPC with molecular quenchers, including oxygen.

The effective formation of mTHPC/β-CD inclusion complex can be obtained in the whole plasma (Desroches et al., 2001). This study demonstrated that competition between lipoprotein and CDs for mTHPC binding occurs in human plasma. Besides, competing displacement of mTHPC from CD cavity by lipid molecules was observed. The addition of M-β-CD (0.01 M) to plasma containing mTHPC leads to an increase of PS fluorescence intensity by a factor of 1.9. Such effect allows exploiting fluorescence measurements for direct mTHPC pharmacokinetic studies with improvement of the sensitivity of mTHPC detection in human plasma (Desroches et al. 2001).

Despite the wealth of data about mTHPC/CD inclusion complexes, there is no available data on the use of CDs as a delivery system of mTHPC on *in vitro* and *in vivo* models so far.

OBJECTIVES

Supramolecular systems of mTHPC with derivatives of β-CD are the general objects of this study. Comprehensive characterization of drug/carrier (PS/CD) complex is essential to achieve an adequate understanding of drug behavior after introduction to biological systems and resulting therapy outcome. To this end, it is necessary to investigate the physicochemical, photophysical and biological properties of the system and how these parameters correlated with each other. An important parameter is the drug release from the DDS and its distribution between components of the biological system. Investigations of the drug release from DDS *in vitro* and *in vivo* require developing of suitable and preferably minimally invasive methods for monitoring and control of these processes.

The objective of the work was to determine the effect of β-CD derivatives on mTHPC properties *in vitro* and *in vivo* and estimate its release from CD vehicles. For this purpose, the following studies were carried out:

- Determination the kinetic and equilibrium distribution of mTHPC between β-CDs and serum.
- Investigation of β-CDs influence on the rate of mTHPC cellular uptake.
- Estimation the effect of β-CD derivatives on mTHPC intracellular distribution and photodynamic efficiency *in vitro.*
- Study of mTHPC/CD complexes distribution processes in tumor bearing mice.

These tasks are addressed in the first research section (Chapter II, section 1).

- Determination of the absorption and fluorescence characteristics of mTHPC in solutions and model biological systems. Identification of differences between the spectral-fluorescent characteristics of the sensitizer bound to CDs and other biological structures (e.g., serum proteins, lipid membranes).
- Development the method based on the variation of mTHPC Soret band to evaluate PS redistribution from CD carriers in biological systems. Evaluation of the possible application fields of this technique.
- Comparison the spectroscopic-based methods suitable for mTHPC release studies from β-CD carriers in biological systems.

These points are addressed in the second research section (Chapter II, section 2).

CHAPITRE II. RÉSULTATS

1. Influence des cyclodextrines sur la distribution et l'activité photodynamique de la mTHPC en milieu biologique

La première partie des résultats est décrite dans l'article « Inclusion complexation with β-cyclodextrin derivatives alters photodynamic activity and biodistribution of meta-tetra(hydroxyphenyl)chlorin » et décrit l'effet des dérivés β-CD sur le comportement de la mTHPC aux différents niveau de sa distribution *in vitro* et *in vivo*.

Les propriétés photophysiques de la mTHPC libre ou complexée aux β-CDs ont été comparées. Il a été démontré que l'association de la mTHPC avec les β-CDs abolissait complétement son agrégation après son introduction dans le sang. La cinétique et l'équilibre de distribution de la mTHPC entre les β-CDs et les protéines du sérum ont été étudié par chromatographie d'exclusion de taille et par mesure de polarisation de fluorescence. Les β-CDs augmentent les mouvements de diffusion de la mTHPC entre les structures biologiques. De plus, il a été démontrés que les β-CDs ont un effet concentrations dépendantes sur le procédé de distribution dans le sérum et d'accumulation dans les cellules de la mTHPC. Les études de photosensibilisation ont montré que les β-CDs affectent la distribution intracellulaire de la mTHPC et améliore sa phototoxicité sur une lignée cellulaire HT29. Les procédés de biodistribution de la mTHPC sur des souris porteuses de tumeur HT29 après l'injection du PS seul ou accompagné des β-CDs ont été comparés. L'étude *in vivo* confirme le fait que l'utilisation des β-CDs permet de modifier la biodistribution de la mTHPC sur des animaux porteurs de tumeur, se traduisant par une diminution du taux d'accumulation du PS dans la peau et dans le muscle accompagné d'une augmentation de son accumulation tumorale.

Cette partie du travail a été publié dans le journal « *European Journal of Pharmaceutical Sciences »* et présenté si après sous sa version publiée.

CHAPTER II. RESULTS

1. Influence of cyclodextrins on mTHPC distribution and photodynamic activity in biological systems

The first part of the results is described in the article "Inclusion complexation with β-cyclodextrin derivatives alters photodynamic activity and biodistribution of meta-tetra(hydroxyphenyl)chlorin" on the effect of β-CD derivatives on mTHPC behavior at various stages of its distribution *in vitro* and *in vivo*.

Comparison of mTHPC photophysical characteristics in the serum and β-CDs solutions was carried out. It was showed that association of mTHPC with the β-CDs completely abolishes its aggregation after introduction into blood. Kinetic and equilibrium distribution of mTHPC between β-CDs and serum were studied by using size-exclusion chromatography and fluorescence polarization measurements. β-CDs increase diffusion movement of mTHPC molecules between biological structures. It was demonstrated that the β-CDs have a concentration-dependent effect on the process of mTHPC distribution in blood serum and accumulation in cellular culture. Photosensitization studies have shown that the β-CDs affect intracellular distribution of mTHPC and enhance its photocytotoxicity in HT29 cultured cells. The processes of mTHPC biodistribution in HT29 tumor bearing mice after intravenous injection of PS alone or with the β-CDs were compared. *In vivo* study confirms the fact that the use of β-CDs allows modifying mTHPC distribution processes in tumor bearing animals that is reflected in the decreased level of PS accumulation in skin and muscles, as well as in the increased PS accumulation in tumor.

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Inclusion complexation with β-cyclodextrin derivatives alters photodynamic activity and biodistribution of meta-tetra(hydroxyphenyl)chlorin

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Biodistribution

ABSTRACT

Application of meta-tetra(hydroxyphenyl)chorin (mTHPC) one of the most effective photosensitizer (PS) in photodynamic therapy of solid tumors encounters several complications resulting from its insolubility in aqueous medium. To improve its solubility and pharmacokinetic properties, two modified β-cyclodextrins (β-CDs) methyl-β-cyclodextrin (*M*-β-CD) and 2-hydroxypropyl-β-cyclodextrin (Hp-β-CD) were proposed. The aim of this work was to evaluate the effect of β-CDs on mTHPC behavior at various stages of its distribution *in vitro* and *in vivo*. For this purpose, we have studied the influence of the β-CDs on mTHPC binding to the serum proteins, its accumulation, distribution and photodynamic efficiency in HT29 cells. In addition, the processes of mTHPC biodistribution in HT29 tumor bearing mice after intravenous injection of PS alone or with the β-CDs were compared. Interaction of mTHPC with studied β-CDs leads to the formation of inclusion complexes that completely abolishes its aggregation after introduction into serum. It was demonstrated that the β-CDs have a concentration-dependent effect on the process of mTHPC distribution in blood serum. At high concentrations, β-CDs can form inclusion complexes with mTHPC in the blood that can have a significant impact on PS distribution out of the vascular system in solid tissues. Besides, the β-CDs increase diffusion movement of mTHPC molecules that can significantly accelerate the delivery of PS to the targets cells and tissues. *In vivo* study confirms the fact that the use of β-CDs allows to modify mTHPC distribution processes in tumor bearing animals that is reflected in the decreased level of PS accumulation in skin and muscles, as well as in the increased PS accumulation in tumor. Further studies are underway to verify the optimal protocols of mTHPC/β-CD formulation for photodynamic therapy.

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1. Introduction

Photodynamic therapy (PDT) is a minimally invasive photochemical-based treatment with a promising clinical track record for oncological and some other diseases (Agostinis et al., 2011; François et al., 2013). PDT uses light-activated drugs (photosensitizers, PSs) to induce cell death mediated by the production of singlet oxygen and oxygen radicals. These reactive oxygen species induce damage in malignant and nonmalignant cells alike, depending on the PS distribution (Castano et al., 2005a). Clinical application of PDT encounters several difficulties due to the high hydrophobicity of most PSs, thus leading to poor water solubility and aggregation within the vasculature after intravenous injection (Josefsen and Boyle, 2012). *In fine*, a decrease in photosensitizing efficiency and extended skin photosensitivity caused by unfavorable biodistribution are considered as major drawbacks of PDT.

The use of biocompatible nanoscale systems for PSs delivery demonstrated a significant improvement in their bioavailability, biodistribution and in treatment efficacy (Marchal et al., 2015). Special delivery forms such as liposomes (Reshetov et al., 2013), polymeric nanoparticle (Ogawara et al., 2016) and dendrimers (Bastien et al., 2015) were extensively studied and showed important progress in drug delivery and PDT. Among the investigated drug delivery systems, cyclodextrins are promising PSs carriers (Kryjewski et al., 2015; Mazzaglia, 2011). Cyclodextrins are a family of cyclic oligosaccharides with a hydrophobic internal

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cavity and a hydrophilic outer surface consisting of six α-cyclodextrin, seven β-cyclodextrin (β-CD), eight γ-cyclodextrin or more glucopyranose units. Due to its appropriate cavity size, β-CD derivatives are most commonly used for inclusion complexation with PSs, especially aryl substituted porphyrins. Sensitizers binding to cyclodextrins usually increased the solubility of highly hydrophobic drugs, their physical and chemical stability (Kryjewski et al., 2015; Lang et al., 2004). Several reports documented that cyclodextrins change PSs delivery into the cells and their photosensitizing activity (Dentuto et al., 2007; Kitagishi et al., 2015; Kolárová et al., 2003, 2004). It should be noted that currently available experimental data on the influence of cyclodextrins on PSs accumulation and photosensitized processes in cells are highly contradictory.

One of the most potent clinically approved PS is meta-tetra(hydroxyphenyl)chlorin (mTHPC). The main limitation of mTHPC application is related to its low water solubility necessitating the use of special delivery systems (Senge and Brandt, 2011). It was shown that this PS forms effectively inclusion complexes with derivatives of β-CD (Demore et al., 1999). Introduction of β-CDs into aqueous solutions of mTHPC causes PS disaggregation with the restoration of the absorption and fluorescence spectra of the monomer form (Bautista-Sanchez et al., 2005; Demore et al., 1999; Desroches et al., 2001). However, there is no available data on the use of cyclodextrins as a delivery system of mTHPC on *in vitro* and *in vivo* models so far. In the present study, we analyzed the influence of methyl-β-cyclodextrin (*M*-β-CD) and 2-hydroxypropyl-β-cyclodextrin (Hp-β-CD) at wide range concentrations on the processes of mTHPC biodistribution and its photosensitizing activity.

The aim of this work was to evaluate the effect of two β-CDs on mTHPC behavior at various stages of its distribution *in vitro* and *in vivo*. For this purpose we have studied the influence of β-CDs on mTHPC binding to the serum proteins, its accumulation, distribution and photodynamic efficiency in HT29 cells. In addition, the processes of mTHPC biodistribution in HT29 tumor bearing mice after intravenous injection of PS alone or with β-CDs were compared.

2. Material and methods

2.1. Chemical and reagent preparation

mTHPC was kindly provided by Biolitec Research GmbH (Germany). Phosphate buffered saline (PBS), phenol-red-free RPMI-1640 medium, penicillin-streptromycin and L-glutamine were supplied by GibcoBRL (Belgium). Fetal bovine serum (FBS) was purchased from PAN™ BIOTECH GmbH (South America). M-β-CD and Hp-β-CD were purchased from AraChem (Netherlands).

mTHPC stock solution (1 mM) was prepared in absolute ethanol (99.6%) and was kept at 4 °C in the dark. The concentration of mTHPC in the solution was estimated by a spectrophotometric method using an mTHPC molar extinction coefficient of 30,000 M^{-1} cm^{-1} at 650 nm in ethanol.

β-CDs and mTHPC complexes were formed using co-precipitation method (Martin Del Valle, 2004). Briefly, β-CDs were dissolved in PBS at the required concentrations with the subsequent addition of mTHPC stock solution. The final content of ethanol in the mTHPC/β-CD solutions did not exceed 0.5%. Solution was thoroughly mixed over 15 min under magnetic stirring.

For *in vitro* cell culture experiments mTHPC stock solution was diluted in RPMI medium, supplemented with 2% FBS to reach unless otherwise indicated the final mTHPC concentration of 1.47 μM.

All other chemicals and solvents used in our experiments were commercial products of the highest grade of purity and its manufacturers will be specified below.

2.2. Spectroscopic measurements

The absorption spectra were measured on PerkinElmer Lambda 35 UV/Vis (PerkinElmer, Waltham, USA) and Solar PV1251 (Solar, Belarus) spectrophotometers using 1 cm optical path quartz cuvettes. Fluorescent measurements were performed on PerkinElmer LS55B (PerkinElmer, USA) and Solar SFL 1211A (Solar, Belarus) fluorescence spectrometers equipped with thermostat cuvette compartments (PTP-1 Peltier temperature controller in the case of LS55B) and magnetic stirring. mTHPC fluorescence lifetime was measured in a pulsed fluorometer PRA-3000 (Photochemical Research Associates, Canada), working in photon counting mode.

Absorption and fluorescent characteristics of mTHPC associated with β-CDs or serum proteins were carried out for samples containing 50 μM M-β-CD, 500 μM Hp-β-CD or 2% FBS (vol/vol) providing complete PS binding with studied structures. All measurements were carried out at room temperature 22–24 °C.

2.3. Fluorescence polarization measurements

Measurements of mTHPC fluorescence polarization degree (***P***) were performed on PerkinElmer LS55B equipped with polarizers. In this case, samples were excited at 435 nm and fluorescence was registered at 652 nm similar to (Reshetov et al., 2011b). Kinetic and equilibrium characteristics of mTHPC distribution between β-CDs and serum proteins were controlled on the base of fluorescence polarization measurements as described in (Rossi and Taylor, 2011). Quantitative characteristics of mTHPC distribution were estimated from ***P*** values taking into account the difference in the fluorescence quantum yields of mTHPC in β-CDs and serum solutions.

Fluorescence polarization technique was also used to estimate the cyclodextrin effect on the molecular movement of mTHPC in blood plasma. For this purpose, different concentrations of mTHPC (0.1–1 μM) were added to 5% FBS solution and incubated for 10 h at room temperature (22–24 °C). Binding of several mTHPC molecules to a molecule of low density lipoproteins (LDL) or high density lipoproteins (HDL) was accompanied with concentration-dependent depolarization of PS fluorescence (effect occurs at mTHPC concentrations above 0.5 μM). Mixing mTHPC loaded serum with an excess of PS-free serum (ratio 1:19) was accompanied by the PS redistribution, expressed as the change in ***P*** value. Final concentration of FBS was kept as 5%.

2.4. Size-exclusion chromatography

mTHPC (1.47 μM) was incubated during 2 h with different M-β-CD concentrations in PBS supplemented with 2% FBS at 37 °C under light protection.

Chromatography experiments and biochemical analysis of the protein fraction were performed as described earlier (Reshetov et al., 2012) except that we used Sigma 1.5 × 35 cm column filled with Sephadex G-200 gel (GE Healthcare, USA), pre-equilibrated with PBS. Concentration of mTHPC in the chromatographic fractions was estimated by fluorescence intensity measured after the addition of 0.2% neutral detergent Triton®X-100 (Sigma–Aldrich, USA) to the samples. Intensities were corrected for fraction volume.

2.5. Cell culture

HT29 human adenocarcinoma cells were obtained from the ATCC® (LGC Standards, France) and regularly controlled for mycoplasma contamination. HT29 cells were maintained in RPMI medium supplemented with 9% (vol/vol) heat-inactivated FBS, penicillin (10,000 IU), streptomycin (10,000 μg/mL), and 1% (vol/vol) 0.1 M glutamine. Cells were kept as a monolayer culture in a humidified incubator (5% CO_2) at 37 °C. Cell culture was reseeded every week to ensure exponential growth.

2.6. Cellular uptake of mTHPC

HT29 cells ($5 \cdot 10^4$ cells per ml) were seeded in the 6-well plate, 48 h later the cells were washed twice with PBS and incubated in RPMI supplemented with 2% FBS, containing 1.47 μM mTHPC alone or complexed with β-CDs at various concentrations. After incubation, the cells were three times consecutively washed with PBS, trypsinized and centrifuged. Cells were counted with a Thoma hemocytometer by using trypan blue exclusion assay in order to determine cell viability. The viability of the control (untreated cells) was regarded as 100%. Then the supernatant was discarded and the methanol was added to the pellet. Afterwards, the suspension was sonicated during 15 min and centrifuged. The mTHPC fluorescence contained in the supernatant was measured with Perkin-Elmer LS 55B spectrometer and mTHPC concentration was evaluated by fluorescence calibration curves constructed by plotting the peak height of PS standard solutions in methanol. The obtained mTHPC concentration was normalized to the number of viable cells.

2.7. Fluorescence microscopy

HT29 cells ($1.5 \cdot 10^4$ cells per ml) were plated into Lab-Tek II chamber Slide (Roskilde, Denmark), incubated in the dark at 37 °C with 1.47 μM of mTHPC alone or complexed with M-β-CD (10 μM) during 1 h, then rinsed with PBS. Fluorescence of mTHPC was observed under an upright epifluorescence microscope (AX-70 Provis, Olympus, France) equipped with a 100 W mercury vapor lamp and a Peltier cooled CCD camera (DP70, Olympus). The filter was set at 400–440 nm band pass excitation associated with a 570 nm dichroic mirror and a 590 nm long pass emission filter for mTHPC fluorescence measurements. Fluorescence images were recorded using an oil immersion ×40 objective.

Fluorescence microscopy technique based on filipin III cholesterol assay kit (ab133116 Abcam, UK) was used to estimate of β-CD effect on plasma membrane cholesterol content. The filter was set at 330–385 nm band pass excitation associated with a 400 nm dichroic mirror and a 420–460 nm long pass emission filter for filipin III fluorescence measurements. A cholesterol inhibitor, U-18666A, was used as a positive control. *In vitro* detection and quantification of cholesterol levels in HT29 cells by filipin III staining were performed as described earlier (Wolfe et al., 2015).

To prevent the photobleaching of dyes, the samples were analyzed immediately after staining. Fluorescence microscopy data were analyzed using ImageJ software.

2.8. Assessment of cell viability

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma] colorimetric assay, which measures the capacity of mitochondrial dehydrogenase in viable cells to reduce MTT to purple formazan crystals. HT29 cells ($5 \cdot 10^4$ cells per ml) were seeded in the 96-well plates, 48 h later the cells were washed twice with PBS and incubated during 3 h or 24 h in RPMI supplemented with 2% FBS containing 1.47 μM of mTHPC only or in presence of 10 μM of M-β-CD or 200 μM of Hp-β-CD. Each series of samples contained non-irradiated plate as the control of the dark cytotoxicity. Irradiation was performed at room temperature with a laser diode (Biolitec AG, Germany) at 652 ± 4 nm at different exposition times varying from 4 s to 500 s, power output was adjusted to 2 mW/cm^2. Following irradiation, cells were washed twice with cold PBS, the fresh medium was added and cells were maintained in the humidified incubator. 24 h later, the medium was removed, the MTT solution was added to each dish and cells were kept in the humidified incubator for 3 h. The formazan crystals were solubilized by adding dimethyl sulfoxide and absorbance was measured at 540 nm with a Multiskan Ascent spectrometer (Labsystems, USA). From these values, cell viability percentages of treated and non-irradiated control samples were calculated. All experiments were carried out in triplicate. The efficacy of mTHPC phototoxicity was based on LD_{50} (Lethal Dose inducing 50% cell death).

2.9. Fluorescence analysis of in vivo distribution of mTHPC

All experiments were performed in accordance with animal care guidelines from European Union and were carried out in an establishment approved by the appropriate authority. The animal project registered under the number 1353–2015080615338957, received a favorable opinion from the Ethics Committee and was definitely approved by the French Higher Education and Research Minister.

All procedures involving animals were performed under general anesthesia with inhaled isoflurane (Forene; Abbott Laboratories, Abbott Park, IL, USA) using a Univentor 400 anesthesia unit (Genestil, Royaucourt, France), with every effort made to minimize suffering. Female NMRI$^{nu/nu}$ mice (Janvier, St Berthevin, France) aged 9–10 weeks were used, with a mean body weight of 29 ± 2 g. Mice were inoculated subcutaneously in the right flank with $8 \cdot 10^6$ exponentially growing HT29 cells. When the tumor volume reached ~100 mm^3, the mice were randomized into three groups (n = 3–4 mice/group) according to the injected formulation: mTHPC alone, mTHPC/M-β-CD and mTHPC/Hp-β-CD. All drugs were administered intravenously by a tail vein injection at a dose of 0.5 mg/kg of mTHPC. Following injection, mice were kept in the dark and experiments were undertaken with minimal ambient light.

PDT fluorometer (JETI Technische Instrumente GmbH, Germany) was used to measure the *in vivo* mTHPC fluorescence after injection. Excitation wavelength was 405 nm, emission wavelength 600–700 nm. At each time interval the mTHPC fluorescence was measured in three points on the surface of tumor through the skin.

To compare tissues and organs distribution of mTHPC alone and complexed to β-CD 24 h after i.v. injection, the excised tissues including liver, spleen, kidney, muscle, lungs, heart and skin as well as the tumor were analyzed by fluorescence imaging. The FluorVivo™ 300 image system (Indec Systems, Santa Clara, CA, USA) equipped with a LED at 510–550 nm and a long wave emission filter (630–690 nm). Imaging data were analyzed using ImageJ software.

2.10. Statistical analysis

Each experiment was repeated at least three times. Data are presented as mean ± SD. The data were evaluated using Student's t-test. The difference was considered significant at $p < 0.05$. Data analysis was carried out with the STATISTICA 10 (StatSoft, Inc., USA).

3. Results

3.1. The spectral characteristics of mTHPC in the serum and β-CDs solutions

Absorption and fluorescence characteristics of monomeric mTHPC in organic solutions are typical for chlorin-type compounds (absorption spectrum with two main peaks at 418 nm and 650 nm and emission spectrum with a peak at 652 nm). Like other hydrophobic tetrapyrroles, mTHPC molecules aggregate in aqueous solutions resulting in significant changes of the spectral properties and almost complete disappearance of PS fluorescence. Introduction of the serum or β-CDs to aqueous solution prevents mTHPC aggregation and restores its spectral properties (Fig. S1–4 in Supplementary material). A comparison of mTHPC spectral features in the serum and β-CDs solution showed only slight differences in the absorption and fluorescence spectra (1–2 nm shifts of the spectra maxima). Fluorescence capacity of mTHPC is increased in 2.0 fold in M-β-CD solution and 1.7 fold in HP-β-CD solutions compared to PS in 2% serum solution. The fluorescence decay time varies slightly from 10.2 ± 0.1 ns in the serum to 10.8 ± 0.1 ns in β-CDs (Fig. S5 and Table S1 in Supplementary material). Significant differences in the presence of polarized fluorescence in the compared solutions are

observed (Fig. S2 and Table S1 in Supplementary material). The ***P*** values of mTHPC complexed with β-CDs molecules are about 4.5% while the fluorescence of PS associated with the serum proteins is characterized by high polarization degree (***P*** = 25%). The gap in ***P*** values at equal decay times indicates the difference in hydrodynamic volumes of small β-CDs molecules and large LDL and HDL being the main carriers for mTHPC in plasma.

3.2. Kinetic and equilibrium distribution of mTHPC between β-CDs and serum

Complexation of mTHPC with β-CDs is a reversible process, *i.e.* each molecule can dissociate from the inclusion complex with the subsequent binding to other molecules (*e.g.* plasma proteins). Therefore, the introduction of mTHPC/β-CD complexes into the serum is accompanied by the processes of PS redistribution to plasma proteins. We have estimated the rates of mTHPC transfer from complexes with β-CDs to the serum proteins and in the opposite direction from the serum to β-CDs. A convenient tool for this purpose is measuring mTHPC fluorescence polarization changes (see 2.3). Fig. 1a shows typical kinetics of ***P*** changes when mTHPC/M-β-CD complexes are mixed with 2% serum solution. A competition between M-β-CD and the serum proteins leads to redistribution of mTHPC molecules from inclusion complexes to proteins, which results in the growth of fluorescence polarization degree. When M-β-CD concentration is low (Fig. 1a, curve 1) the ***P*** value reaches 24–25% in 5–7 min of incubation indicating that almost all mTHPC molecules have changed their location. In the samples containing moderate β-CDs concentration only limited amount of mTHPC transfer from β-CDs to the serum proteins with the achievement of transitional ***P*** values at equilibrium (Fig. 1a, curve 2). Return transfer of mTHPC molecules from serum proteins to β-CDs is controlled in a similar way (Fig. 1a, curves 3, 4). After adding an excess amount of M-β-CD (100 μM) to mTHPC stained 2% serum solution, containing mTHPC, the ***P*** value drops from 25% to 4–5% in several minutes reflecting complete PS redistribution to β-CDs.

Estimation of cyclodextrin effect on mTHPC movement in serum may be obtain from the polarization measurements (Fig. 1b,c). As mentioned above, the binding of mTHPC with LDL and HDL limits PS mobility as manifested in corresponding to the increase in the ***P*** value. The ***P*** value does not change at small mTHPC concentrations (<0.5 μM) in 5% FBS solution (Fig. 1b). When mTHPC concentration overcomes 0.5 μM, a significant decrease of ***P*** is observed and at PS concentrations above 5 μM a completely depolarized fluorescence of mTHPC in serum solution is observed. The mechanism of this phenomenon is well known and is associated with the concentration depolarization. When one lipoprotein molecule binds more than one molecule of mTHPC, there is migration of energy between the dye molecules. A decrease in the distance between dye molecules increases the probability of resonance energy transfer, which leads to significant depolarization (Reshetov et al., 2011a). On the basis of changes in the ***P*** values it is possible to estimate the degree

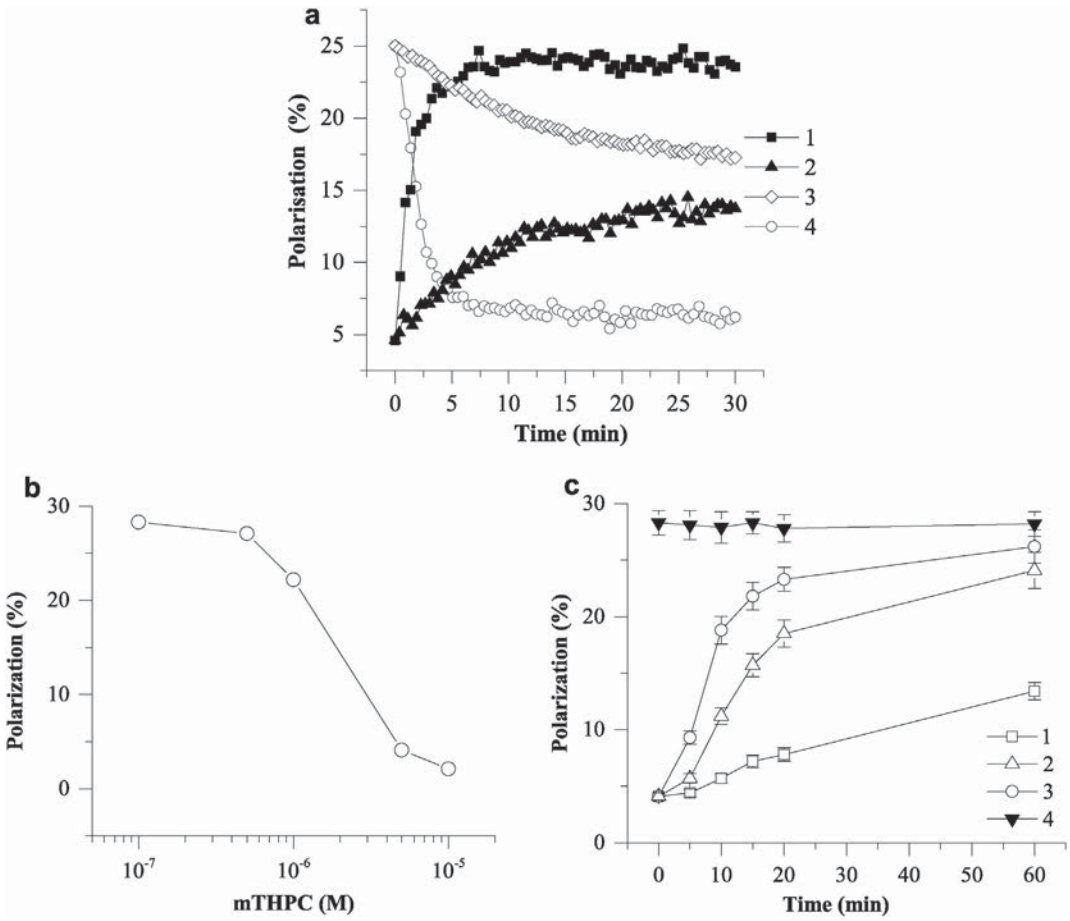

Fig. 1. (a) Kinetics of mTHPC fluorescence polarization degree ***P*** changes after mixing of FBS with mTHPC/M-β-CD solution (curves 1, 2) and after mixing of M-β-CD solution with mTHPC/FBS solution (curves 3, 4). Concentration of mTHPC was 0.2 μM, concentration of FBS was 2% (vol/vol). Concentrations of M-β-CD were 1 μM (curve 1), 10 μM (curves 2, 3) and 100 μM (curve 4). (b) Influence of PS concentration on the ***P*** value of mTHPC fluorescence in 5% FBS solution. (c) Influence of M-β-CD on the rate of mTHPC ***P*** value changes after mixing of mTHPC loaded serum (100 μL) with PS-free FBS solution (1.9 mL) (curves 1-3). Curve 4 corresponds to the changes of mTHPC ***P*** value after introduction of 5 μM M-β-CD to mTHPC-loaded serum solution (0.25 μM mTHPC, 5% FBS). Final concentration of mTHPC was 0.25 μM, concentration of FBS was 5% (vol/vol) (curves 1-3). Concentrations of M-β-CD were 0 (curve 1), 2 μM (curve 2) and 5 μM (curve 3).

of mTHPC redistribution between the serum proteins. Mixing of mTHPC loaded serum with an excess of PS-free serum leads to a gradual slow redistribution of PS, accompanied by the restoration (increase) of ***P*** value (Fig. 1c, curve 1). Introduction of a small amount of β-CDs significantly accelerates the processes of mTHPC redistribution between the serum proteins (Fig. 1c, curves 2,3). It should be noted that the concentration of β-CDs did not affect significantly the equilibrium distribution of mTHPC (Fig. 1c, curve 4).

Fig. 2 shows the dependence of mTHPC fluorescence polarization degree in FBS as the function of cyclodextrins (Fig. 2a) or FBS (Fig. 2b) concentrations. According to the data obtained, the detectable amount of cyclodextrin-bound mTHPC in the 2% serum appears above 2 μM M-β-CD and 50 μM HP-β-CD. The minimal ***P*** values corresponding to complete mTHPC transfer from the serum proteins to β-CDs are observed at 100 μM M-β-CD and 2 mM HP-β-CD. Taking into account the differences in fluorescence capacity of mTHPC we calculated the relative weights of mTHPC fractions bound to β-CDs and serum proteins for intermediate ***P*** values (Fig. 2). This approach allows also analyzing complexation of mTHPC and β-CDs at different serum content. As can be seen from Fig. 2b, at a fixed concentration of β-CDs, the increase in the FBS content leads to the increase of the relative weight of mTHPC fraction bound to serum proteins. It should be noted that M-β-CD binds more molecules of PS even at considerably lower concentrations in comparison with HP-β-CD at the same condition (serum concentration). Evidently, this is a consequence of the greater affinity of M-β-CD to mTHPC molecules. In the case of 100 μM M-β-CD, a greater part of mTHPC associated with cyclodextrin fraction, even at 10% of FBS (Fig. 2b).

To further characterize the influence of β-CDs on the processes of mTHPC distribution in the serum, we used the size exclusive gel chromatography technique. Fig. 3 shows elution profiles for 2% serum samples, containing 1.47 μM mTHPC and different bulk of M-β-CD. In the control samples (mTHPC in FBS) PS passed through the column with the main transport proteins in the range of 20–50 mL (Fig. 3 curve 1). The biochemical analysis of protein eluting through the column shows that mTHPC elution peaks correspond to LDL (elution volume 23 mL) and HDL (elution volume 31 mL) fraction (data not shown). The relative weight of mTHPC bound to the serum albumin (a band with maximum at 40 mL) does not exceed 5%. In serum samples containing higher than 1 μM M-β-CD a decrease in the main elution protein-bound peaks is observed. Simultaneous a new protein free peak of mTHPC bound to M-β-CD appears in the range of 50–70 mL. The relative weight of this new fraction increases with increasing M-β-CD concentrations and it contains all bulk of mTHPC when M-β-CD concentration is above 200 μM (Fig. 3, curve 5). It should be noted that β-CDs do not affect the distribution profile of the drug between the major serum transport proteins (except free inclusion complexes mTHPC/β-CD). The data of mTHPC distribution between the serum proteins and β-CDs obtained by chromatography are in full agreement with those obtained by fluorescence polarization.

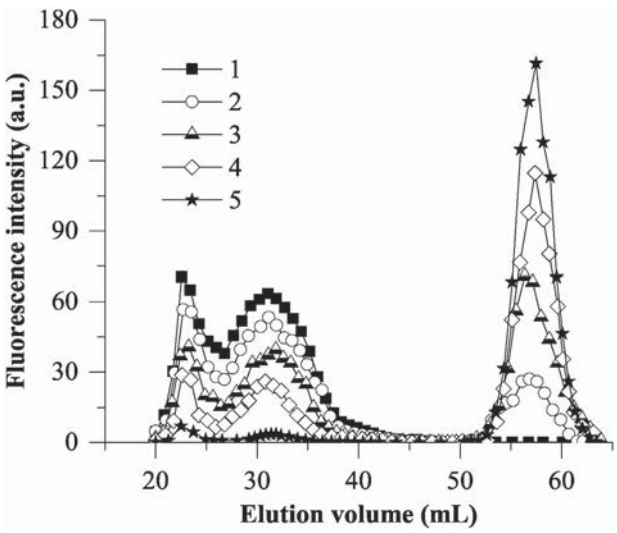

Fig. 3. Elution of mTHPC from column during gel chromatography analysis of 2% FBS solution, containing 1.47 μM of PS and different concentration of *M*-β-CD. Concentration of M-β-CD in the samples was (1) 0, (2) 5 μM, (3) 10 μM, (4) 20 μM or (5) 200 μM. mTHPC was preincubated with FBS at 37 °C during 2 h until complete binding of PS.

3.3. Influence of the β-CDs on the rate of mTHPC cellular uptake

HT29 cells incubated in cyclodextrin-free medium accumulates mTHPC very slow (Fig. 4a). Estimated initial rate of PS accumulation was 0.21 pg cell^{-1} h^{-1} and its value did not changed significantly throughout studied duration of incubation 0-24 h. The presence of β-CDs in incubation medium alters mTHPC uptake by cells, and this effect critically depends on M-β-CD and Hp-β-CD concentrations (Fig. 4b,c). Introduction of β-CDs at low concentrations results in considerable increase of mTHPC cellular uptake. A maximal level of mTHPC accumulation is observed at 10 μM M-β-CD and 200 μM Hp-β-CD independently of incubation time. Further increase in β-CDs concentrations is accompanied by a gradual decrease of mTHPC uptake, so at concentrations

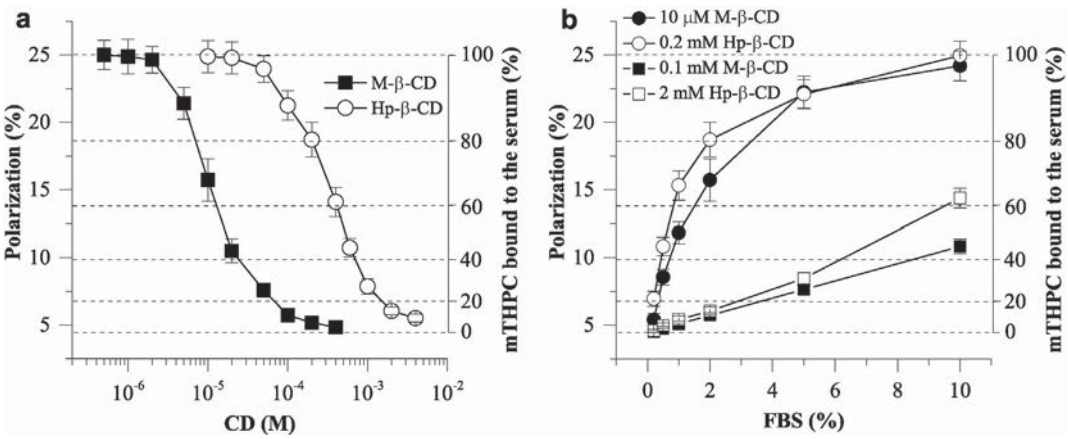

Fig. 2. (a) Influence of β-CD derivatives concentration on the equilibrium distribution of mTHPC in 2% FBS solution. (b) Influence of FBS concentration on the equilibrium distribution of mTHPC in PBS solution containing M-β-CD or Hp-β-CD. mTHPC concentration was 0.2 μM. The left ordinate is equilibrium level for mTHPC fluorescence polarization degree ***P*** in mixed solution of β-CD derivatives and FBS. The right ordinate is mTHPC fraction bound to serum proteins calculated from polarization measurements.

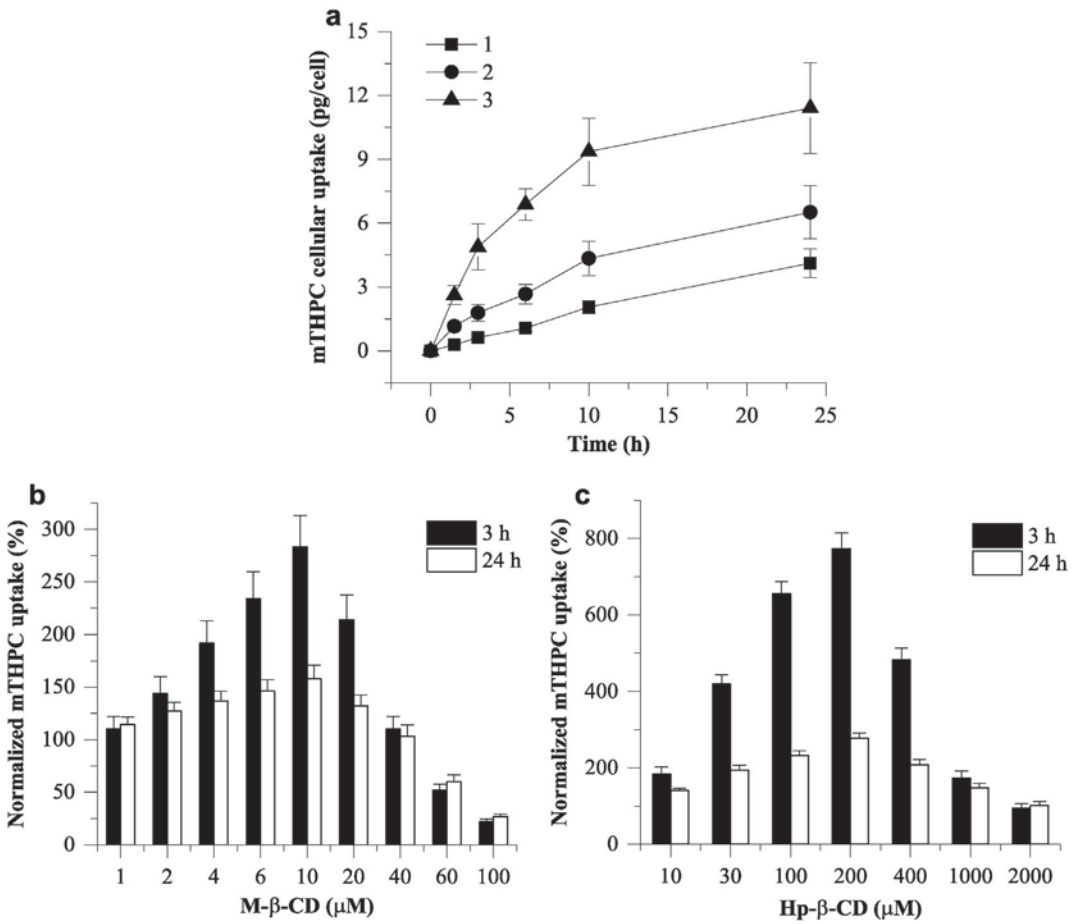

Fig. 4. (a) Influence of 10 μM M-β-CD (2) and 200 μM Hp-β-CD (3) on the rate of mTHPC uptake by HT29 cells. In control samples (1) HT29 cells incubated with mTHPC in β-CDs free medium. (b,c) Effect of (b) M-β-CD and (c) Hp-β-CD on relative level of mTHPC uptake by HT29 cells in culture at 3 h and 24 h of incubation. The concentrations of CDs are shown in the abscissa. mTHPC concentration was 1.47 μM. mTHPC cellular uptake in CDs free medium (control) taken as 100%. mTHPC uptake with CDs was normalized to control sample. The results of three independent experiments are expressed as the mean with the vertical bar showing S.D.

above 60 μM for M-β-CD and 2 mM for Hp-β-CD cells accumulates less quantities of mTHPC as compared to control sample.

It is interesting to estimate the influence of β-CDs at their optimal concentrations (10 μM M-β-CD and 200 μM Hp-β-CD) on the rate of mTHPC uptake by HT29 cells (Fig. 4a). The highest rate of mTHPC accumulation is observed immediately after adding β-CDs: 0.77 and 1.75 pg cell^{-1} h^{-1} for M-β-CD and Hp-β-CD, respectively. Further incubation is accompanied with gradual decrease in the rate of mTHPC uptake until this parameter reaches a value 0.16 pg cell^{-1} h^{-1} for both β-CDs after 10–24 h interval.

It should be noted that all determined cyclodextrin-dependent changes in mTHPC accumulation by cells are not associated with a decrease in cell viability, because its level was not <95% compared to a control sample (according to trypan blue assay). Special studies of cyclodextrins cytotoxicity showed that used in our articles β-CDs concentrations were far from toxic levels (Fig. S6 in Supplementary material).

3.4. Photocytotoxicity of mTHPC/β-CD inclusion complexes

To evaluate photosensitized cytotoxicity of mTHPC alone or its complexes with β-CDs at concentrations providing optimal PS accumulation we have determined the parameter LD_{50} from the corresponding dose-response curves. In this experiments cells, preincubated in dark for 3 h and 24 h with mTHPC, were irradiated by a laser diode ($\lambda = 652$ nm) and in 24 h interval cell viability was determined. LD_{50} values for cells incubated for 3 h and 24 h in the presence of mTHPC alone are equal to 330 mJ cm^{-2} and 39 mJ cm^{-2}, respectively (Fig. 5).

Introduction of β-CDs into the samples enhances mTHPC photosensitizing activity towards HT29 cells. For M-β-CD LD_{50} values are 70 mJ cm^{-2} at 3 h and 14 mJ cm^{-2} at 24 h and for Hp-β-CD these values are 27 mJ cm^{-2} at 3 h and 8 mJ cm^{-2} at 24 h.

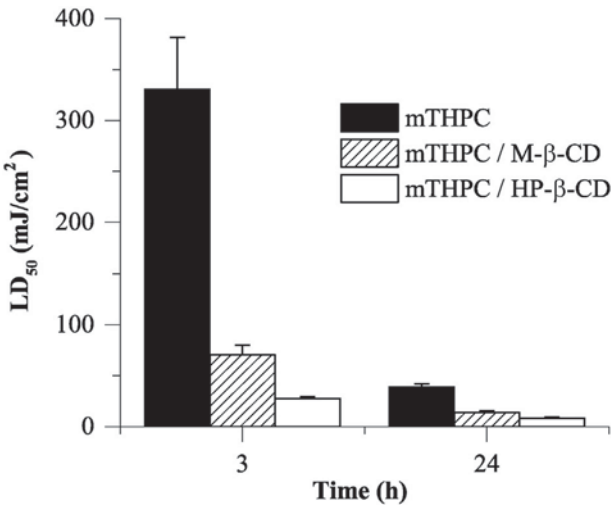

Fig. 5. Influence of 10 μM M-β-CD and 200 μM Hp-β-CD on photosensitivity of HT29 cells incubated in the presence of 1.47 μM mTHPC during 3 h and 24 h. LD_{50} was determined in three independent experiments and expressed as the mean with the vertical bar showing S.D.

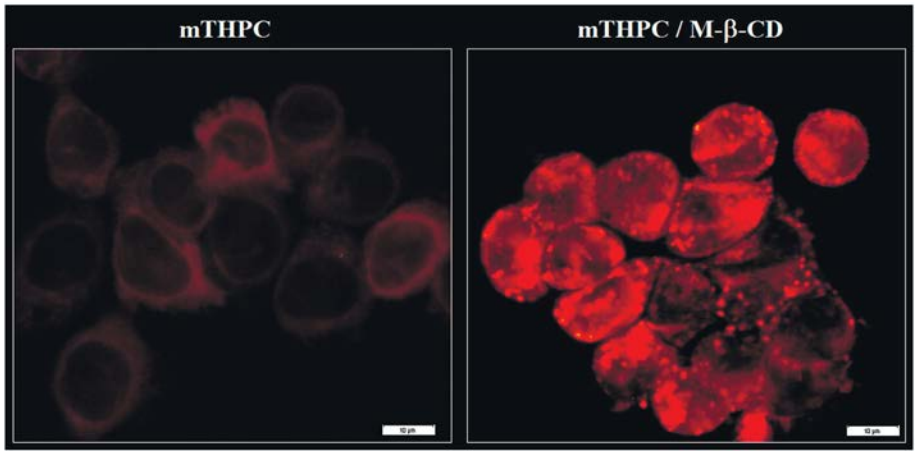

Fig. 6. Epifluorescence images of HT29 cells incubated with 1.47 μM mTHPC alone or with 10 μM M-β-CD. Incubation time was 1 h. Incubation temperature was 37 °C. Scale bar = 10 μm.

3.5. Fluorescent microscopy studies of mTHPC uptake by HT29 cells

The intracellular distribution of mTHPC alone and mTHPC/β-CD complexes was assessed using epifluorescence microscopy (Fig. 6). Fluorescence images of cells treated with mTHPC alone revealed predominant fluorescence in the plasma membrane with a weak diffuse distribution throughout the cytoplasm. After incubation with mTHPC/M-β-CD complexes, the overall level of mTHPC fluorescence in cells increased. In this case, we observed an appearance of bright spots in the periphery of cells fluorescent image indicating about heterogeneous PS localization in the plasma membrane.

Using cholesterol-specific stain filipin III we assumed the involvement of cyclodextrin-mediated changes of plasma membrane cholesterol content into regulation of mTHPC binding to cells. Treatment of cells by 10 μM of M-β-CD or 200 μM of Hp-β-CD causes 15–20% decrease of mean fluorescence intensity of fillipin III in cells indicating reduction of cholesterol content in plasma membrane (data not shown).

3.6. mTHPC biodistribution in tumor-bearing mice

In vivo kinetics of mTHPC fluorescence from the surface of grafted HT29 tumor after i.v. injection of PS alone (curve 1) or its complexes with M-β-CD (curve 2) are presented in Fig. 7. Very low fluorescence level is observed during several hours when mTHPC is injected alone. Then, fluorescence intensity increases gradually for 24 h. In the case of mTHPC/M-β-CD complexes fluorescence signal reaches maximal level very quickly after introduction and keeps it during 24 h.

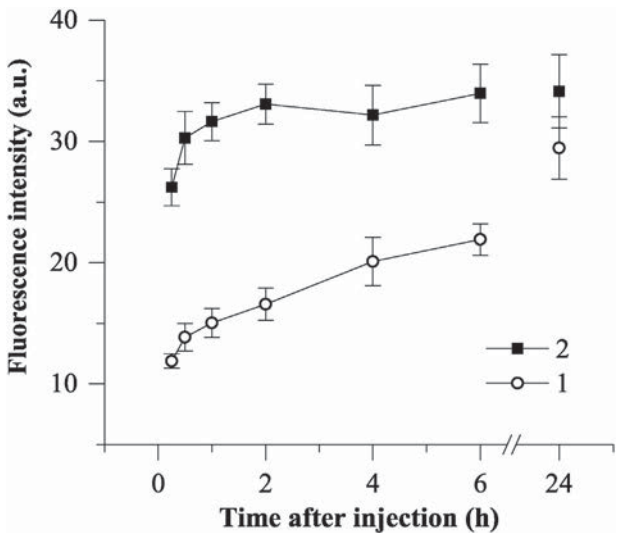

Fig. 7. Kinetics of *in vivo* fluorescence (λ_{ex} = 405 nm, λ_{em} = 600–700 nm) from the surface of HT29 tumor grafted to NMRI$^{nu/nu}$ mice after injection in tale vein of 0.5 mg/kg mTHPC alone (curve 1) or with 2.5 mg/kg M-β-CD (curve 2). Results are expressed as the mean of 3–4 measurements with the vertical bar showing S.D.

Several organs and tissues of the injected mice were harvested for fluorescence imaging to visualize mTHPC distribution (Fig. 8) and to quantify PS accumulation in these samples (Table 1) 24 h after injection. According to the data obtained, studied β-CDs strongly influence mTHPC distribution between different organs and tissues. Indeed, introduction of mTHPC with β-CDs results in 1.5–3 times decreasing of PS accumulation in skin and muscles tissues. Another interesting observation deals with opposite changes in mTHPC accumulation in the kidneys and liver. Introduction of mTHPC/M-β-CD based formulation reduces the level of mTHPC in the liver in 1.5 times and increases mTHPC accumulation by kidneys in 1.8 times compared to introduction of mTHPC alone. Similar changes were also observed in the case of mTHPC/Hp-β–CD based formulation. Changes in the level of PS accumulation associated with the use of both studied β-CDs were expressed weaker in lungs and spleen, and no significant changes were observed in the heart. The PS distribution between the tumor (T) and normal muscle (M) tissue is of a special interest. The highest mTHPC fluorescent signal in the tumor is observed when mTHPC was introduced with M-β-CD. Calculated from the fluorescent data obtained T/M ratio for the formulations mTHPC/M-β-CD, mTHPC/Hp-β-CD and mTHPC alone decreased in the following order 12 > 7 > 5.

4. Discussion

Despite the high efficacy of mTHPC as a photosensitizer for PDT, its low water solubility distorts the biodistribution processes and leads to non-optimal pharmacokinetics and onset of a number of side effects. The use of cyclodextrins as carriers of PSs can be useful to avoid aggregation and, hence, provide favorable characteristics of pharmacokinetics and biodistribution of photosensitizing drugs (Kryjewski et al., 2015). It is well documented that mTHPC similarly to other arylporphyrins is able to bind 1 or 2 molecules of β-CD derivatives and the side phenyl groups penetrate into cyclodextrin cavity (Demore et al., 1999). According to Bautista-Sanchez et al. (2005) binding constants for mTHPC with several β-CDs are very high especially in the case of Hp-β-CD ($4.8 \cdot 10^4\ M^{-1}$) and M-β-CD ($1.2 \cdot 10^5\ M^{-1}$). So, the effective formation of mTHPC/β-CD inclusion complex can be obtained in the whole plasma. Such an effect allows exploiting fluorescence measurements for direct mTHPC pharmacokinetic studies (Desroches et al., 2001).

Inclusion in cyclodextrins exerts a profound effect on physicochemical properties of guest molecules as they are temporarily locked or partially caged within the host cavity giving rise to beneficial modification

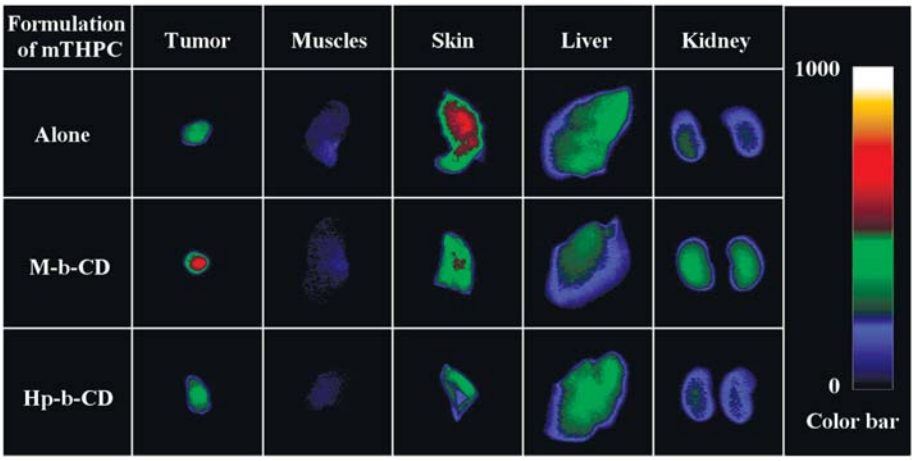

Fig. 8. Fluorescence imaging of different organs and tissues isolated from HT29 tumor bearing mice after 24 h of introduction of 0.5 mg/kg mTHPC alone, with 2.5 mg/kg M-β-CD or with 25 mg/kg Hp-β-CD.

of guest molecule properties. Indeed, in the case of hydrophobic drugs, cyclodextrins increase drug solubility, dissolution, circulation time thus enhancing their bioavailability (Loftsson and Brewster, 2013; Stella and He, 2008). Following this approach, it is reasonable to assume that β-CDs can be used to improve the pharmacokinetic properties of mTHPC.

It is well recognized that the distribution of PS molecules during PDT of solid tumors may be defined by three main stages: i) PS interactions with plasma proteins after injection and transport in bloodstream, ii) PS transfer from the bloodstream to the extravascular tissue and iii) PS interaction with intracellular targets within the tumor environment. Data obtained in this work allow assessing an involvement of β-CDs in mTHPC distribution at all these stages.

Plasma protein binding pattern is an important factor governing PSs biodistribution and photosensitizing efficiency during PDT (Castano et al., 2005b). Previous studies have revealed two main features of mTHPC interaction with plasma proteins. First, about 95% of the sensitizer in plasma is associated with HDL and LDL, and the binding with HDL is twice higher than with LDL (Reshetov et al., 2012; Triesscheijn et al., 2007). Secondly, as compared with other non-polar drugs, mTHPC releases from lipoprotein carriers very slowly and proceeds on a timescale of hours (Sasnouski et al., 2005, 2006). According to our fluorescence polarization measurements, the addition of M-β-CD causes a rapid dissociation of mTHPC molecules from complexes with proteins, resulting in an equilibrium distribution attained in 10–15 min (Fig. 1a curve 4). Since mTHPC dissociation from the inclusion complexes and subsequent binding to the serum proteins is achieved even faster, it can be concluded that β-CDs cause a significant acceleration of PS diffusion movement in plasma. This conclusion is consistent with the evaluation of β-CDs effect on the rate of mTHPC redistribution between serum proteins following from the measurements of fluorescence depolarization after mixing of PS loaded serum with an excess of the PS-free serum (Fig. 1c). Indeed, introduction of a small concentration of β-CDs in medium significantly reduces the time of achieving the equilibrium distribution of mTHPC in mixture.

According to our chromatography data, the equilibrium distribution of mTHPC in serum is dependent on β-CDs concentrations. At low concentrations of M-β-CD (high concentration of FBS) almost all mTHPC molecules are bound to the serum proteins (Fig. 3). That conclusion is consistent with the results of fluorescence polarization measurements (Fig. 2). At M-β-CD concentrations <5 μM and HP-β-CD <50 μM mTHPC fluorescence polarization ***P*** takes the maximum value about 25% and, hence, the relative weight of mTHPC inclusion complexes in the samples is minor. Increasing β-CDs concentration shifts equilibrium of PS distribution towards the formation of inclusion complexes. As a result, when β-CDs concentration is high, most of the sensitizer molecules are not bound to plasma proteins. It is obvious that concentration level of cyclodextrins required to change the mechanism of mTHPC transport in the blood is determined by the constant of inclusion complex formation and may differ significantly in the case of various β-CD derivatives.

The formation of inclusion complexes in the plasma can have a significant impact on mTHPC distribution from the bloodstream and its localization in extravascular tissues including tumor transformed one. Indeed, the interstitial transport of molecules and particles is characteristically described by their effective interstitial diffusion coefficient, which decreases as their size is increased (Pluen et al., 2001). The smaller mTHPC/β-CDs inclusion complexes, which size is 1.5–2.0 nm, must penetrate into extravascular tissues much faster as compare to mTHPC/HDL (8–12 nm) or mTHPC/LDL (20–25 nm) complexes. In addition, lowered cyclodextrins concentration in solid tissue compared to the bloodstream should stimulate dissociation of entering inclusion complexes with subsequent mTHPC binding by tissue structures (cells, interstitial proteins).

Influence of cyclodextrins on the accumulation of arylporphyrins and some other porphyrin-like sensitizers in cellular systems have been studied by several research groups (Dentuto et al., 2007;

Table 1
Fluorescence intensity of mTHPC in various organs and tissues isolated from HT29 tumor bearing mice 24 h after injection obtained from fluorescence imaging data.

Formulations of mTHPC	Fluorescence intensity (a.u.)							
	Tumor	Muscles	Skin	Liver	Kidney	Spleen	Heart	Lung
Alone	196 ± 26	38.7 ± 5.6	323 ± 45	198 ± 41	135 ± 32	60 ± 6	85 ± 12	248 ± 6
M-β-CD	247 ± 30*	20.6 ± 8.9*	203 ± 87*	130 ± 36*	241 ± 39**	54.3 ± 4.2	85 ± 11	150 ± 5**
Hp-β-CD	143 ± 28*	20.3 ± 9.6*	119 ± 23**	157 ± 26	183 ± 72	39 ± 14*	77 ± 5	204 ± 33

The experimental conditions as indicated in the legend to Fig. 8.
Results are presented as mean ± SD (n = 3–4).
Significant difference compared to mTHPC alone: *$p < 0.05$; **$p < 0.01$.

Kitagishi et al., 2015; Kolárová et al., 2003, 2004). It is worth noting that the results on these studies are contradictory. In the study by Kolárová et al. (2004) the one hundred excess of Hp-β-CD in relation to the PSs 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin ($TTPS_4$) and Pd-$TTPS_4$ leads to a 2 fold increase of $TTPS_4$ and Pd-$TTPS_4$ uptake in melanoma G361 cells. At the same time, Kitagishi et al. (2015) observed that the addition of $TTPS_4$ complexed with 2.4 times excess of trimethyl-β-cyclodextrin led to approximately 30% reduction of PS cellular uptake and consequently cell-photokilling ability in HeLa cells. Similarly, Dentuto et al. (2007) showed that the interaction of *chlorophyll a* with trimethyl-β-cyclodextrin at a ratio 1:10, Hp-β-CD (1:100) or Hp-γ-CD (1:100) resulted in a 2–4 fold decrease in phototoxicity observed in Jurkat cell line.

Results presented in this paper confirm that mTHPC accumulation by HT29 cells strongly depends on Hp-β-CD and M-β-CD concentrations, the β-CDs can cause both acceleration and inhibition of mTHPC cellular uptake (Fig. 4). Most of water soluble cyclodextrins are not able to penetrate into cells. So, their effect on accumulation of hydrophobic drugs should be attributed to an increase of the permeability by enhancing drug solubility or dissolution and thus making the drug available at the surface of plasma membrane of cells, from where it partitions into intracellular compartments (Loftsson and Brewster, 2013). In such cases it is important to use proper cyclodextrins concentration since excess may decrease the drug availability. Actually, at high β-CDs concentrations, the rate of mTHPC accumulation in the cells is reduced due to the high probability of formation of inclusion complexes in the extracellular environment.

Another mechanism affecting mTHPC absorption by cells may be mediated by cyclodextrin-dependent perturbations in cellular membrane. Cyclodextrins are able to bind several components of plasma membrane of cell that causes changes in membrane composition and structure, modifying its binding and barrier functions (López et al., 2011; Zidovetzki and Levitan, 2007). In this connection, it is interesting to consider the involvement of cellular cholesterol, the content of which significantly affects the relative affinity of porphyrins to cellular and model biological membranes (Cohen and Margalit, 1985; Zorin et al., 1997). Much of evidence reported suggests that β-CD derivatives very effectively (up to 90%) eliminate cholesterol from plasma membrane of various cells (Zidovetzki and Levitan, 2007). According to our results on the fluorescence of cholesterol-specific stain filipin III the treatment of HT29 cells with 10 μM of M-β-CD or 200 μM of Hp-β-CD led to depletion of 15–20% cholesterol from the plasma membrane. Such a moderate change in the level of cholesterol apparently cannot be the main reason for multifold acceleration of sensitizer accumulation by treated cells.

It is essential to take into account that exposing cells to β-CDs also alter the relative distribution of cholesterol between different membrane compartments with corresponding physiological effects (Zidovetzki and Levitan, 2007). According to Decker et al. (2013) mTHPC prefers cholesterol poor membrane compartments. In this connection an appearance of bright spots at the fluorescent images of the HT29 cells treated with β-CDs apparently could be attributed to an increased level of mTHPC accumulation in cholesterol depleted areas of plasma membrane.

Photosensitization studies have shown that the β-CDs enhance mTHPC photocytotoxicity in respects of HT29 cells in culture (Fig. 5). It is unlikely that this effect is connected with the direct influence of β-CDs on photodynamic activity in inclusion complexes. According to IR luminescence measurements of singlet oxygen described in (Bautista-Sanchez et al., 2005) mTHPC association with β-CDs is accompanied with decreasing in the value of singlet oxygen quantum yield in 1.5 times. Beside that as it was mentioned above, the penetration of mTHPC into the cell follows processes of its dissociation from the inclusion complexes with β-CDs. It is well known that the photocytotoxicity of PS depends on the level of its accumulation in cells (Castano et al., 2005a). According to the data obtained in this work cyclodextrin-depending acceleration of mTHPC uptake by HT29 cells provides increasing in cells sensitivity to the phototreatment. There are strong correlations between increasing of PS cellular uptake and decreasing of LD_{50} value at different protocols of cells treatment (type of β-CD, incubation time).

Changes of physico-chemical properties of sensitizer *via* inclusion complexation with β-CDs are ought also to modify its biodistribution in the whole body. Our results of *in vivo* fluorescence kinetics (Fig. 7) and fluorescent imaging of mTHPC distribution in various tissues (Fig. 8) confirm this assumption. Indeed, intravenous administration of mTHPC alone leads to formation of large non-fluorescent aggregates distorting the pharmacokinetics and reducing drug bioavailability (Sasnouski et al., 2005; Triesscheijn et al., 2007). The gradual destruction of these aggregates is accompanied by slow increase of monomeric mTHPC concentration in bloodstream and consequently its fluorescence intensity (Fig. 7, curve 1). Co-administration with β-CDs abolishes mTHPC aggregation in blood, thus the highest levels of mTHPC fluorescence intensity are achieved in 15–30 min (Fig. 7, curve 2). Change of mTHPC kinetic pattern should enhance of PS bioavailability immediately after injection.

In our work fluorescence imaging technique was used to evaluate the β-CDs effect on mTHPC accumulation by different tissue and organs. It should be noted that the correlation between PS accumulation and its fluorescent signal obtained by this non-invasive technique is a function of tissue optical properties (scattering, reflection, endogenous chromospheres *etc.*). For this reason, our fluorescent imaging data are suitable only for qualitative comparison of PS accumulation in different tissues. For the same tissue measured changes in the level of fluorescence signal should clearly reflect variations in the accumulation of PS and fluorescent imaging technique allows quantifying β-CDs effect on mTHPC accumulation in different tissues.

Data obtained in this article suggest that co-administration of mTHPC with M-β-CD and Hp-β-CD modifies mTHPC distribution in tumor bearing mice. The tumor and muscles uptake indicates that complexation of mTHPC with β-CDs especially with M-β-CD results in higher tumor-to-muscles ratio than mTHPC alone that may induce a better selectivity of PDT treatment (Roby et al., 2007). Moreover, formulation of mTHPC with M-β-CD and Hp-β-CD showed lower drug levels in the skin compared to mTHPC alone (Fig. 8), thus indicating that the side effects with both β-CDs may prove less severe. It should be noted, that cyclodextrins are rapidly eliminated in the urine and can increase renal clearance of lipophilic water-insoluble drugs (Stella et al., 1999). This is in accordance with a significant increase in the fluorescence level of mTHPC in the kidney as well as a reduction in the PS fluorescence level in the liver when applying a formulation with M-β-CD compared to mTHPC alone. This indirectly confirms that even after administration, a certain part of mTHPC remains associated with M-β-CD.

5. Conclusion

Application of β-CD derivatives can open up new possibilities to modify mTHPC biodistribution in the course of PDT. mTHPC formulations with Hp-β-CD and M-β-CD showed sufficiently different behavior in several studied biological systems: serum solutions, cellular cultures and HT29 tumor bearing mice. Association of mTHPC with the β-CDs completely abolishes its aggregation after introduction into blood that should improve its pharmacokinetics and bioavailability. It was demonstrated that the β-CDs have a concentration-dependent effect on the process of mTHPC distribution in blood serum. At high concentrations the β-CDs can form inclusion complexes with mTHPC in the blood that can have a significant impact on PS distribution out of the vascular system in solid tissues. Besides, the β-CDs increase diffusion movement of mTHPC molecules that can significantly accelerate the delivery of PS to the targets cells and tissues. *In vivo* study confirms the fact that the use of β-CDs allows to modify mTHPC distribution processes in tumor

bearing animals that is reflected in the decreased level of PS accumulation in skin and muscles, as well as in the increased PS accumulation in tumor. Further studies are underway to verify the optimal protocols of mTHPC/β-CD formulation for PDT.

Abbreviations

PDT	Photodynamic therapy
PS	Photosensitizer
β-CD	β-Cyclodextrin
M-β-CD	Methyl-β-cyclodextrin
Hp-β-CD	2-Hydroxypropyl-β-cyclodextrin
mTHPC	Meta-tetra(hydroxyphenyl)chlorin
PBS	Phosphate buffered saline
FBS	Fetal bovine serum
P	The degree of fluorescence polarization
LD_{50}	Lethal dose, inducing 50% cell death
LDL	Low density lipoproteins
HDL	High density lipoproteins

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejps.2016.06.012.

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2. Développement de méthodes de fluorescences pour le monitoring du relargage de la mTHPC du complexe d'inclusion.

La seconde partie des résultats est présentée dans l'article « Soret band shape indicates mTHPC distribution in biological systems » accepté dans le journal « *Dyes and Pigments* » et relate les méthodes de mesure de relargage et de distribution de la mTHPC.

Les caractéristiques de fluorescence et d'absorbance de la mTHPC solubilisée dans différents solvants organiques et complexée avec différentes structures biologiques ont été analysées. Une nouvelle technique de mesure des spectres de fluorescence pour le contrôle non-invasif de la distribution de la mTHPC entre les nanotransporteurs β-CD et différentes structures biologiques a été décrite. Une analyse des données obtenues permet de conclure que les caractéristiques spectrales de la bande de Soret sont les plus sensible à la liaison de la mTHPC aux structures biologiques. La forme de la bande de Soret du spectre d'absorbance de la mTHPCs et la bande correspondante du spectre d'excitation de fluorescente sont fortement dépendantes du type de structures biologiques liées aux molécule pigmentaires.

Le ratio de deux pics de bande de Soret est fortement dépendant du type de structures biologiques liée aux molécule pigmentaires. Une comparaison des résultats numériques obtenu à la suite de la déconvolution des spectres d'absorption et d'excitation démontre que le ratio B_x/B_y varie d'une valeur inférieure à 0,7 (mTHPC conjuguée aux vésicules lipidiques et aux protéines du sérum) à des valeurs de 1,03 – 1,42 pour le complexe mTHPC/β-CD. En prenant en compte les mécanismes possibles d'incorporation des molécules de mTHPC dans les cavités des β-CDs ou dans les membranes lipidiques, on peut suggérer que la variabilité de la forme de la bande de Soret est associée aux changements de conformation des molécules de pigment. En se basant sur les variations de formes de la bande de Soret, la technique spectrale de monitoring en continu du relargage de la mTHPC des transporteurs β-CD dans un système biologique (e.g., distribution dans le sérum) a été développée. Cette approche peut être aussi utilisée dans l'étude de l'équilibre de distribution de la mTHPC dans un system complexe. Ces découvertes sont intéressantes pour le développement des techniques spectrales de monitoring non-invasif de la distribution de la mTHPC dans un system biologique.

Cette partie du travail a été accepté dans le journal « *Dyes and Pigments* » et présenté si après sous sa version publiée.

2. Development of fluorescence methods suitable for monitoring of mTHPC release from inclusion complexes

The second part of the results is presented in the article "Soret band shape indicates mTHPC distribution between β-cyclodextrins and serum proteins" accepted to *Dyes and Pigments* and is related to the methods of mTHPC release and distribution measurements.

Fluorescence and absorbance characteristics of mTHPC in several organic solutions and in complexes with various biological structures have been analyzed. A new spectroscopic technique for noninvasive control of mTHPC distribution between β-CD nanocarriers and various biological structures was described. An analysis of received data allows concluding that Soret band is the most variable spectral characteristic sensitive to the binding of mTHPC to biological structures. The shapes of the Soret band of mTHPC absorbance spectrum and corresponding band of fluorescence excitation spectrum are strongly depended on the kind of biological structure bound with the pigment molecule.

The ratio of two Soret band peaks are strongly depended on the kind of biological structure bound with the pigment molecule. A comparison of the numerical results for mTHPC absorption and excitation spectra deconvolution shows that the ratio B_x/B_y varies from less than 0.7 (mTHPC bound to lipid vesicles and serum proteins) to 1.03-1.42 for mTHPC/β-CD complexes. Taking in account the possible mechanism of incorporation of mTHPC molecule into the β-CD cavities or lipid membranes, one can suggest that the variability of Soret band shape is associated with the changes of pigment molecule conformation. On the basis of Soret band shape variations, the spectral technique for continuous monitoring of the release of mTHPC from β-CD carriers in biological systems (e.g., distribution in blood serum) was developed. This approach can be also applied in a study of equilibrium distribution of mTHPC in complex system. This finding is useful for the development of spectral technique for noninvasive continuous control of mTHPC distribution in biological systems.

This part of the work was accepted in *Dyes and Pigments* and is presented thereafter in its published form.

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Soret band shape indicates mTHPC distribution between β-cyclodextrins and serum proteins

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ABSTRACT

Fluorescence and absorbance characteristics of 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (mTHPC) in several organic solutions and in complexes with various biological structures have been analyzed. A comparison of data showed that the most variable mTHPC spectral characteristic is the Soret band shape. The ratio of two Soret band peaks are strongly dependent on the kind of biological structure bound with the pigment molecule. It has been suggested, that the observed variations of Soret band shape are associated with conformational changes of mTHPC molecule, caused by the forced rotation of the aryl-substitutes. On the basis of Soret band shape variations, the spectral technique for noninvasive monitoring of mTHPC release from inclusion complex with β-cyclodextrin carriers in blood serum was developed.

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1. Introduction

The expanding application of tetrapyrroles in photomedicine techniques (photoimaging, photodynamic diagnosis and therapy (PDT)) increases interest in the study of the porphyrin spectral characteristics in biological systems [1–4]. The spectral properties of porphyrins are often highly informative in analysis of the porphyrin interaction with biological structures, for instance in study of photosensitizer (PS) distribution in cellular and tissue systems.

5,10,15,20-Tetra(*m*-hydroxyphenyl)chlorin (mTHPC) (Scheme 1), the potent second-generation PS, which is currently under clinical trial for the palliative treatment of head and neck cancer, appears as a promising PS exhibiting a high therapeutic ratio [5–7]. mTHPC possesses optimal photophysical characteristics for PDT [8], selectively accumulates in the tumor tissue and sensitizes its photodamage [7]. However, this drug is hydrophobic and is prone to aggregation in biological media, resulting in a decreased photodynamic efficacy, moderate selectivity and prolonged skin photosensitivity [6,9]. To abolish these problems, special pharmacological forms such as liposomes [10,11], polymeric nanoparticles [12] and bioconjugates [13] are proposed to use for mTHPC administration.

β-Cyclodextrin (β-CD) derivatives can be successfully used for nonpolar porphyrin administration [14–16]. Introduction of β-CDs into aqueous solutions of porphyrins causes PS disaggregation by forming the inclusion complexes [17]. It is also reported that porphyrins in combination with β-CDs efficiently accumulate in tumor cells *in vitro* [18–20]. In our recent study we have proposed to use β-CDs as a delivery system in mTHPC-PDT [20]. It was demonstrated that the β-CDs have a concentration-dependent effect on the process of mTHPC distribution in blood serum and significantly alter the selectivity of mTHPC accumulation in the tumor tissue. It was suggested, that β-CDs concentration dependent effect strongly depends on mTHPC distribution between inclusion complexes and

Abbreviations: PDT, photodynamic therapy; PS, photosensitizer; mTHPC, 5,10,15,20-Tetra(*m*-hydroxyphenyl)chlorin; β-CD, β-cyclodextrin; DPPC, dipalmitoyl phosphatidyl choline; DPPG, dipalmitoylphosphatidylglycerol; Me-β-CD, methyl-β-cyclodextrin; TM-β-CD, trimethyl-β-cyclodextrin; DPBS, dulbecco's phosphate-buffered saline; TGF, tetrahydrofuran; φ_f, fluorescence quantum yields; *P*, fluorescence polarization degree; r^2, adjusted coefficient of determination; N, the fraction of mTHPC molecules bound to Me-β-CD.

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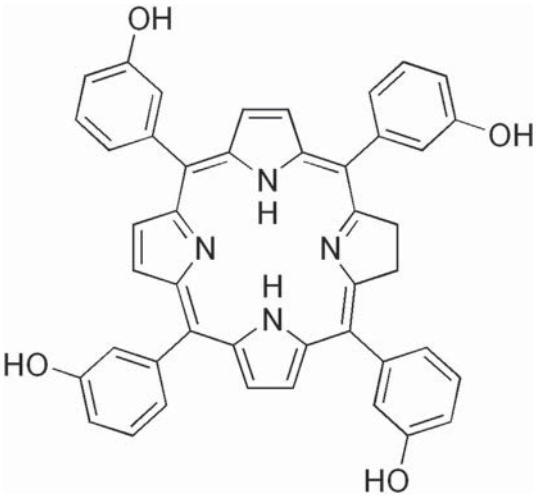

Scheme 1. Representation of the molecular structure of the 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (mTHPC).

biological structures in the blood stream or interstitial media (serum proteins and cellular membranes). From this point of view, the rate and the extent of mTHPC transfer from the inclusion complex is an important determinant of this PS pharmacokinetics. In the present work we describe a new fluorescent spectral technique for noninvasive control of mTHPC distribution between β-CD nanocarriers and various biological structures.

2. Materials and methods

2.1. Chemicals and reagent preparation

The photosensitizer mTHPC was kindly provided by Biolitec Research GmbH (Jena, Germany) with the purity > 99%. Neutral detergent Triton® X-100 was purchased from Sigma Aldrich (France). Embryonal calf serum, dipalmitoylphosphatidyl choline (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were purchased from Sigma Chemical Co. (USA). The cyclodextrins methyl-β-cyclodextrin (Me-β-CD) and trimethyl-β-cyclodextrin (TM-β-CD) were purchased from AraChem (Tilburg, NL).

mTHPC stock solution (1 mM) was prepared in absolute ethanol (99.6%) and was kept at 4 °C in the dark. The concentration of mTHPC in the solution was estimated by a spectrophotometric method using molar extinction coefficient of 30 000 M^{-1} cm^{-1} at 650 nm in ethanol.

All aqueous solutions were prepared in Dulbecco's phosphate-buffered saline (DPBS) (pH 7.4) at 25 °C. To prepare mTHPC solution in organic solvents, mTHPC stock solution was dissolved 100 times. To prepare the solution with serum proteins, mTHPC was incubated for 3 h with 2% serum at 37 °C under light protection.

Me-β-CD and TM-β-CD complexes with mTHPC were formed using the co-precipitation method [21]. Briefly, β-CDs were dissolved in DPBS at the required concentrations with subsequent addition of the stock solution of mTHPC. The final content of ethanol in the mTHPC/β-CD solutions did not exceed 0.5%. The solution was thoroughly mixed for 15 min under magnetic stirring.

All other chemicals and solvents (ethanol, methanol, tetrahydrofuran (TGF), acetone) used in our experiments were commercial products of the highest grade of purity and its manufacturers will be specified below.

2.2. Preparation of liposomes

Unilamellar liposomes containing mTHPC were made by filter extrusion technique as described in Ref. [22]. Briefly, 18 mg mL^{-1} of DPPC (Avanti, USA) and 2 mg mL^{-1} of DPPG (Avanti) were dissolved in 1 mL of 99.6% ethanol. A thin film was obtained by removal of the solvent by rotary evaporation at 60 °C. The film was hydrated in 1 mL of DPBS and underwent three freeze-thaw cycles. The suspension was extruded 21 times through 100 nm polycarbonate Nuclepore® membranes using Avanti Mini-Extruder (Avanti) at 50 °C. After extrusion liposomes were stored at 4 °C. mTHPC was added to the lipid vesicles on the stage of receiving of the lipid film to obtain dye: lipid ratio of 1: 100.

2.3. Spectroscopic measurements

The absorption spectra were measured on Solar PV1251 (Solar, Belarus) spectrophotometer using 1 cm optical path quartz cuvettes. Fluorescent measurements were performed on Solar CM 2303 (Solar, Belarus) fluorescence spectrometer equipped with thermostat cuvette compartment and magnetic stirring. The error in the wavelength measurements was estimated as ± 1 nm.

The concentration of mTHPC in all samples was 0.5 μM. The spectroscopic measurements were carried out at room temperature 22–24 °C. Optical density at all measurements did not exceed 0.15 a.u. The concentrations of serum and Triton® X-100 were 5% (vol/vol) and 0.2% (vol/vol) respectively. The concentration of β-CDs was 0.1 mM.

2.4. Quantum yield measurements

The fluorescence quantum yields (φ_f) were determined using mTHPC in methanol ($\varphi_f = 0.089$) [8] as the standard, and an excitation wavelength of 416 nm. The absolute error in quantum yields values was estimated as ± 0.01.

2.5. Fluorescence polarization measurements

Measurements of mTHPC fluorescence polarization degree (*P*) were performed on Solar CM 2303 equipped with polarizers. In this case, samples were excited at 430 nm and fluorescence was registered at 652 nm as published before [20]. The polarization degree was calculated using a mean values of fluorescence intensities with the polarizers in parallel and perpendicular positions. The absolute error value for all measurements was less than 0.02.

2.6. Deconvolution of curve into component Gaussians

The experimental spectra were obtained and then were deconvoluted using a MATLAB software (The Math Works, USA). Using this program for an experimental curve, a multiple Gaussian fit can be done. With the approximate input parameters as above for each of the Gaussian curves an iterative nonlinear least square fit method was used. For optimization of the fit conditions, iterations ware carried out until the difference in the value of chi-square for successive iterations was less than 5% of the previous value. The adjusted coefficient of determination (r^2) was more than 0.995 for all calculations.

2.7. Statistical analysis

Each experiment was repeated at least three times. Data are presented as mean ± SD. The data were evaluated using Student's t-test. The difference was considered significant at $p < 0.05$. Data analysis was carried out with the STATISTICA 10 (StatSoft, Inc., USA).

3. Results and discussion

3.1. Absorption and fluorescence characteristics of mTHPC in solvents and solutions with different biological structures

The results obtained from spectral studies of mTHPC in neat solvents and different biological solutions are summarized in Table 1. The spectral characteristics of mTHPC are generally defined by PS belonging to chlorin-type compounds. Absorption spectra of mTHPC reveal most prominent peaks in the region of 420 nm (B-band or Soret band) and 651 nm (Q-band). In organic solvents mTHPC intensively fluoresces at 652 nm with a quantum yield (φ_f) around 0.09 [8] and the fluorescence lifetime of 10 ns [23]. In different organic solvents we detected only minor spectral changes: shifts of 1–2 nm in the peaks and the relative fluorescence quantum yield varying within 10% compared to ethanol solution.

Like many hydrophobic tetrapyrroles, mTHPC molecules in aqueous solutions tend to aggregate. The formation of large J-aggregates leads to a significant change in the absorption and mTHPC fluorescence properties [24,25]. The aggregated species are characterized by the decreased extinction coefficients for all bands and exhibit bathochromic shifts of the peaks (Table 1). According to the data obtained, the fluorescence quantum yield of mTHPC in DPBS decreases hundreds times.

The aggregation of mTHPC in aqueous media is completely prevented *via* interaction and binding with different biological structures *i.e.* liposomes, proteins, cyclodextrins [17,26–28]. Depending on the type of biological structure several features of mTHPC spectral properties are observed (Table 1):

i) the positions of all spectral bands (except Soret band) remain practically unchanged;
ii) an enhanced quantum yield of mTHPC fluorescence in solutions with β-CDs and liposomes, while in Triton® X-100 and serum solution this parameter is decreased;
iii) the presence of mTHPC polarized fluorescence (in liposomes, neutral detergent micelles and serum proteins *P* up to 0.30, in inclusion complexes with β-CDs *P* up to 0.05 only), while in organic solutions fluorescence is totally depolarized (*P* less than 0.02). The fluorescence decay time varies slightly in studied samples [20], that allow us to use the detection of polarized fluorescence to confirm mTHPC binding to studied biological structures.

An analysis of the collected data allows the conclusion that the Soret band is the most variable characteristic, when we compared spectra of mTHPC bound to the studied biological structures (Fig. 1). This statement concerns both the position and the shape of the band.

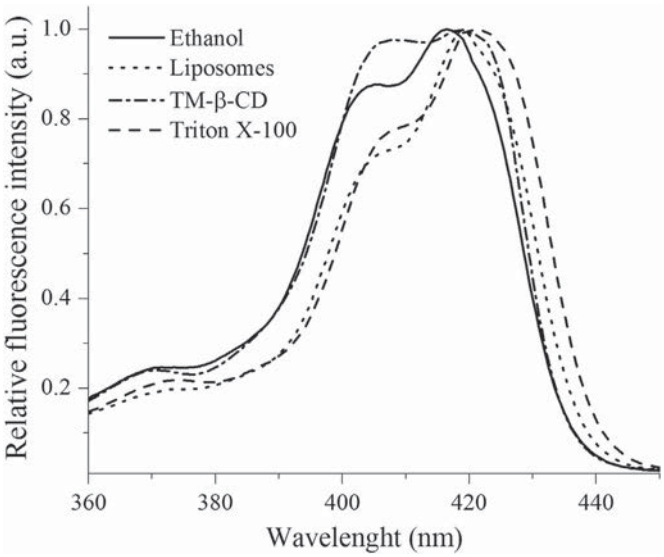

Fig. 1. Normalized fluorescence excitation spectra of mTHPC in various biological media. The solid black line is the mTHPC Soret band spectrum in ethanol, the dots are in liposomes, the dash dots are spectrum of mTHPC in complexes with TM-β-CD and the dash line is Soret band spectral region of mTHPC in micelles of Trinton® X-100. The concentration of mTHPC was 0.5 μM, the concentrations of serum and Triton® X-100 were 5% (vol/vol) and 0.2% (vol/vol) respectively. The concentration of TM-β-CD was 0.1 mM.

Soret band has a semi-resolved structure with two distinct peaks. In organic solvents the maxima of these peaks are located at 410 nm and 417 nm respectively with the intensity ratio 0.85. The binding of mTHPC to liposomes, neutral detergent micelles and serum proteins leads to 3–4 nm bathochromic shift of Soret band and the peaks intensity ratio decreasing by 15–20% compared to ethanol solution. In β-CDs solutions similar spectral shift (2–3 nm) is accompanied by the opposite changes of its shape (intensity of shortwave shoulder almost reaches the value of main peak).

3.2. Formalization of mTHPC Soret band

To formalize the description of mTHPC Soret band shape variations in different solutions we used the deconvolution technique. According to Palummo et al. [29], the absorption spectrum of free-base porphyrins and their derivatives over the range 350–450 nm may be approximated using five Gaussian components: B_x, B_y and N_x, N_y, L_x (Fig. 2). The components of this system are labeled according to the directions of polarization in the (x,y) planes of a macrocyclic system. B-band also known as the Soret band is located within the range 370–450 nm and is associated with the high-

Table 1
Spectral-fluorescent characteristics of mTHPC.

Surrounding	Absorbance		Fluorescence		
	λ_{max}, nm (the Soret band)	λ_{max}, nm (Q(0,0)-band)	λ_{max}, nm	φ_f	*P*, a.u
Ethanol	417	651	652	0.092	0.02 ± 0.01
Methanol	416	650	652	0.089	0.01 ± 0.01
Acetone	417	651	653	0.085	0.01 ± 0.01
TGF	418	653	654	0.083	0.01 ± 0.01
DPBS	430–438	654	654	<0.001	–
Triton X-100®	421	653	654	0.074	0.23 ± 0.02
Liposomes	420	651	652	0.132	0.30 ± 0.01
Serum proteins	421	653	654	0.063	0.26 ± 0.02
Me-β-CD	420	650	652	0.125	0.04 ± 0.01
TM-β-CD	419	650	652	0.142	0.05 ± 0.01

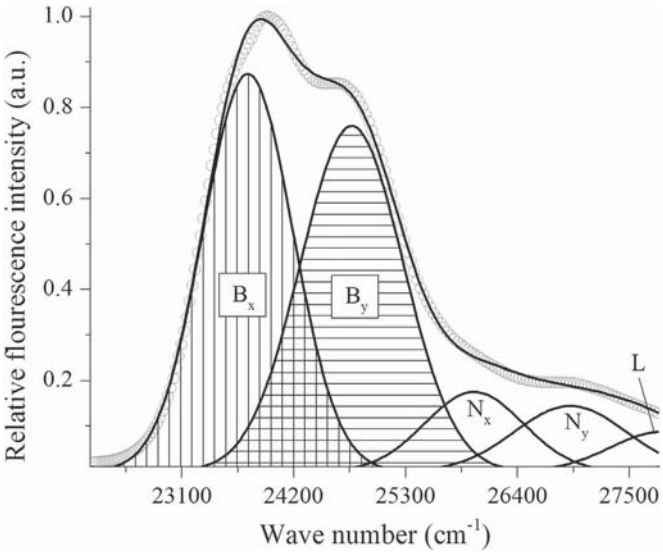

Fig. 2. Gaussian deconvolution for mTHPC in ethanol for the Soret band region. The open circles are the experimental data, the solid black lines are the theoretical curves obtained from Gaussian deconvolution.

energy π-π* transition to the 2nd degenerate excited state, ($S_0 \rightarrow S_2$) [29–31]. The B-band degenerates for the D_{4h} high symmetry porphyrins and splits in the case of lower symmetry derivatives such as mTHPC [31,32]. In the near UV region from 360 to 400 nm the higher-energy transitions known as the N-, L-bands are also found. These bands are associated with the allowed π-π* (N-band) and n-π* (L-band) electronic transitions to the high-energy states ($S_0 \rightarrow S_n$) [33,34].

The applied model may be used for the description and quantitative analysis of the observed spectral variations by Gaussian function expansion of the electronic spectra using the wavenumbers as coordinates [35,36]. The applicability of model proposed was checked by adjusted coefficient of determination (r^2) for each sample.

To compare the contribution of each component to the changes in the shape of the Soret band the relative weights of sub-bands were calculated (Table 2). A comparison of the numerical results for mTHPC absorption and excitation spectra deconvolution shows that the main factor determining observed mTHPC spectral features in the 360–450 nm region is a variation of ratio B_x/B_y. For monomeric unbound mTHPC molecules in organic solvents, the ratio B_y/B_x is within the range 0.73–0.93, whereas for mTHPC/β-CD complexes its values are 1.03–1.42. For mTHPC bound to lipid vesicles and serum proteins, the value of B_y/B_x is less than 0.7.

Table 2
Results for deconvolution of the mTHPC Soret band in various solvents and solutions with different biological structures.

Medium	B_x	B_y	N_x, N_y, L_x	B_y/B_x	r^2
TM-β-CD	0.31	0.44	0.25	1.42	0.995
Me-β-CD	0.38	0.39	0.23	1.03	0.997
Ethanol	0.44	0.33	0.23	0.74	0.999
Methanol	0.45	0.33	0.22	0.73	0.998
Aceton	0.42	0.36	0.22	0.86	0.998
TGF	0.40	0.37	0.23	0.93	0.997
Triton X-100	0.45	0.35	0.20	0.78	0.998
Serum proteins	0.48	0.32	0.20	0.67	0.999
Liposomes	0.49	0.31	0.20	0.63	0.998

3.3. The analysis of Soret band shape of mTHPC in solvents and solutions with different biological structures

It is recognized, that the solvent impacts the spectral characteristics of free base porphyrins and chlorins [3]. Quantum calculations suggest that the main mechanism of this effect deals with the slight change of natural porphyrin ring conformation due to the interactions of solvent molecules with the lateral substituents. Based on mTHPC crystallographic data [37], we can suggest that in solvents the planar chlorin ring of free mTHPC molecule is surrounded by pairs of face-to-face positioned phenyl groups, with two of them related to the macrocycle meanplane dihedral angles (Fig. 3a). Organic solvents can have a minor impact on the rotation of phenyl groups of mTHPC and only slightly change the mTHPC Soret band shape. More significant changes of mTHPC conformation related to the phenyl groups are supposed to occur in organized biological structures as liposomes, serum proteins and inclusion complexes.

An incorporation in lipid membrane suggests strong interaction between mTHPC and lipid molecules [38]. Data concerning the mTHPC fluorescence quenching by membrane impermeable quenchers, mTHPC molecules penetrate into the lipid bilayer [39]. The inclusion of the mTHPC molecule into biological membranes in intact conformation should lead to a significant disordering of lipid bilayer and as the consequence to the modification of structural characteristics and stability of lipid vesicles. In fact, mTHPC loading into liposomes has practically no effect on the stability of the vesicles. Even at high dye: lipid ratio of 1: 12 the size of DPPC liposomes changes by only a few nanometers while the liposomal membrane retains the ability to change phase state when heated [40].

Absorption and fluorescence wavelengths provide valuable information of the microenvironment experienced by mTHPC in lipid membranes. It is proposed that the influence of the lipid bilayer on mTHPC spectral characteristics can occur through the effect of apolar environment or as a result of steric interactions in the bulk of the lipid bilayer. In accordance with our results an alteration of solvent polarity keeps the parameters of the Soret band unchanged (Table 1). At the same time for mTHPC molecules incorporated into lipid vesicles the membrane lateral pressure should change the orientation of two or more phenyl meso-substituents and arrange them in parallel relatively tetrapyrrolic cycle (Fig. 3b). This conformation of mTHPC molecule provides a minimal effect on the lipid bilayer structure. Discussed structural modification of mTHPC molecule in lipid membrane is associated with a significant increase of overlapping of the phenyl rings and chlorin core π-systems, and consequently with changes in the energy states and electronic spectra.

In accordance with our assumption, modifications of the mTHPC molecule conformation should depend on the rigidity of lipid bilayer. In this connection it is interesting to compare the temperature-depended variations of the ratio B_x/B_y and polarization degree of fluorescence of mTHPC incorporated into DPPC lipid vesicles (Fig. 4).

Highly polarized fluorescence ($P = 33\%$) of mTHPC in liposomes in a quasi-crystal state (at the temperature less than 35 °C) indicates a significant restriction of fluorophore rotation in the lipid matrix [42]. The heating above 40 °C leads to the DPPC lipid bilayer phase transition, accompanied with the changing of lipid packing type [39]. The noted structural changes result in the increased mobility of mTHPC molecules as evidenced by the lowering of the fluorescence polarization degree by up to 24%. The decreased packing density of lipid molecules and lateral pressure in a bilayer [43], high conformational mobility of fatty-acid chains at temperatures above the phase-transition temperature should provide

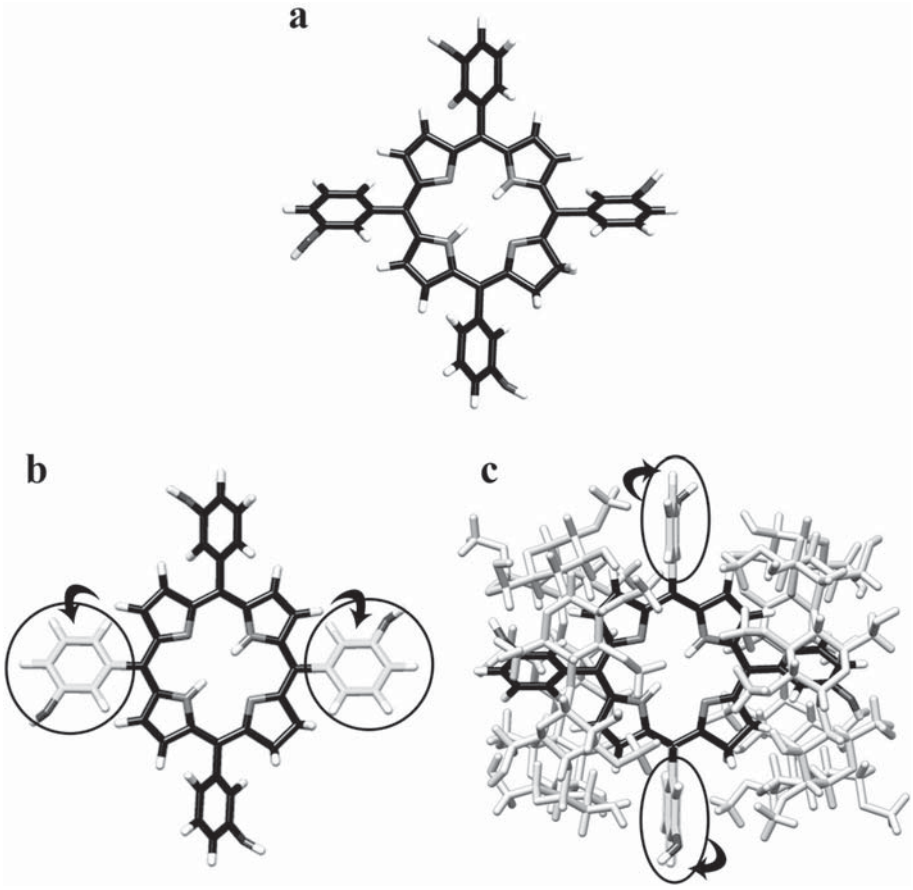

Fig. 3. Possible steric structures of mTHPC molecules: a – in organic solvent (natural conformation: phenyl groups are related to the macrocycle meanplane with dihedral angles of 61° and 76°), b – in lipid bilayer or serum lipoproteins (phenyl groups (grey coloured) tends to turn parallel to the macrocycle), c – in inclusion complex with β-CDs tends to turn perpendicular to the macrocycle). The structures were modeled in UCSF Chimera [41].

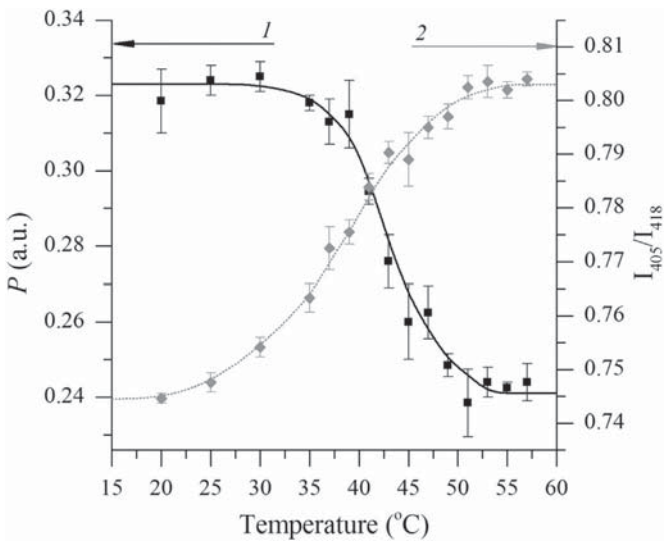

Fig. 4. The dependence of polarization degree of mTHPC fluorescence (1) and I_{405}/I_{418} value (2) from temperature for mTHPC in lipid bilayer. mTHPC concentration was 0.5 μM. The liposome ratio dye: lipid = 1: 100. The results of three independent experiments are expressed as the mean with the vertical bar showing S.D.

enhanced freedom of mTHPC aryl substituents rotation. As a result, for mTHPC in liposomes at high temperatures the B_y/B_x ratio is growing.

It is recognized, that serum lipoproteins are the main binding sites for mTHPC (95% of mTHPC in serum plasma are bound to high and low density lipoproteins) [26]. This type of protein consists of a core of hydrophobic lipids surrounded by a shell of proteins and phosphatidyl glycerols and has quite similar structure as lipid micelles. As in the liposomes, a high value of the fluorescence polarization degree of mTHPC in serum indicates an extremely strong fixation of PS molecules. Thus, based on studies of mTHPC spectral characteristics in solutions of serum proteins, we can conclude that the interaction of the PS molecule with lipoproteins leads to the same conformational modifications as were observed in a lipid bilayer.

mTHPC effectively forms inclusion complexes with β-CDs [15,17]. β-CD/mTHPC complexes are reported to have a 1: 2 stoichiometry [17] and are characterized by a high values of binding constants (1.2 10^5 M^{-1} for Me-β-CD) [15]. Molecular modeling and NMR spectroscopy of β-CD/aryl-porphyrins complexes structure indicate that two opposite porphyrin aryl-groups would be totally located in the hydrophobic β-CD cavity from the secondary hydroxyl-groups side (Fig. 3c) [17,44,45]. The proximity of β-CD's face groups has to limit the rotation of two hydroxyphenyl-groups, which are not involved into the formation of host-guest complex. On the contrary to mTHPC in lipid bilayer, mTHPC binding to β-CD induces steric restrictions leading to the increase of the average value of dihedral angles between macrocycle and phenyl

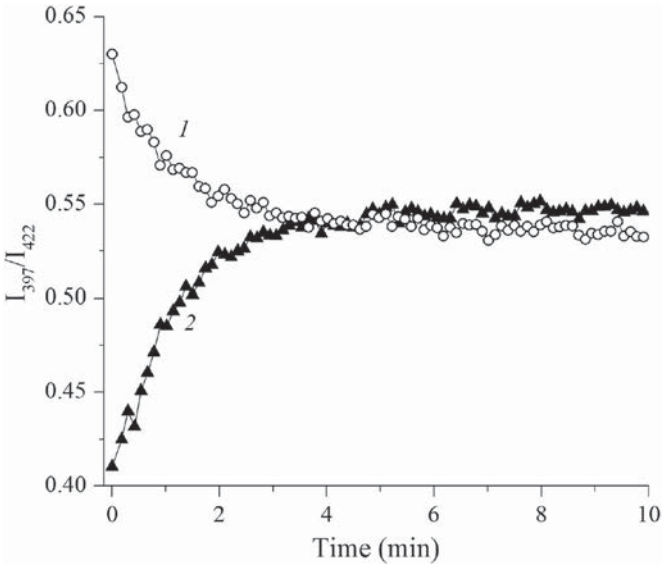

Fig. 5. Kinetics of I_{397}/I_{422} value changes after mixing of serum with mTHPC/Me-β-CD solution (curve 1) and after mixing of Me-β-CD solution with mTHPC/serum solution (curve 2). The concentration of mTHPC was 0.5 μM, concentration of serum was 5% (vol/vol). The concentration of Me-β-CD was 60 μM.

substituents (Fig. 3c). A similar conclusion on conformational changes of aryl-substituted porphyrins in complexes with β-CD has been obtained by computational studies [17,44]. The conformation changes of mTHPC molecule result in decreasing conjugation degree between hydroxyphenyl-group and chlorin-ring systems. Alteration of mTHPC electronic system is manifest as the decrease of B_y and B_x relative weight disbalancing, which corresponds to increasing of mTHPC B_y/B_x value in the complexes with β-CD.

It is obvious, that the role of discussed conformation-linked mechanism of mTHPC spectral alterations depends on characteristics of biological structures. Values of dihedral angles determine the degree of orbital-overlapping π-conjugated electron systems of aryl-groups and chlorin ring that affects the energy spectrum of mTHPC in complexes with biological structures [44,45]. It has been shown that increasing the degree of etherification of the hydroxyl groups of the β-CD leads to the enhancement of affinity between β-CD and mTHPC [17]. According to the obtained data, the etherification of hydroxyl-groups results in increasing of binding constants as well as increasing of the penetration depth of mTHPC's aryl-groups in the β-CD's molecule.

3.4. Application of mTHPC spectral features for the control its redistribution in serum

Our results show that Soret band shape is dependent on mTHPC binding to the different biological structures (β-CDs and serum proteins/lipid membranes). We suggest that this feature allow one to control whether mTHPC molecules are in inclusion complex with β-CDs or they are bound to the serum protein/membrane.

Fig. 5 shows the kinetics of $I_{\lambda 1}/I_{\lambda 2}$ value changes when mTHPC/Me-β-CD complexes are mixed to the 5% serum solution (curve 1) and when Me-β-CD is added to the 5% serum solution preincubated with mTHPC (curve 2). We analyzed mTHPC excitation spectrum at 652 nm using $\lambda_1 = 397$ nm and $\lambda_2 = 422$ nm excitation wavelengths, providing the greatest difference in mTHPC emission intensities ratio. The I_{397}/I_{422} value of mTHPC complexed with Me-β-CD molecules is equal to 0.63, while the fluorescence spectrum of PS associated with serum proteins is characterized by $I_{397}/I_{422} = 0.41$. A competition between Me-β-CD and the serum proteins leads to redistribution of mTHPC molecules from inclusion complexes to serum proteins, which results in the drop of I_{397}/I_{422} value. The equilibrium value corresponding to used Me-β-CD and serum concentrations is equal to 0.55. Return transfer of mTHPC molecules from serum proteins to Me-β-CDs is controlled in a similar way (Fig. 5, curve 2). The application of this technique allows the estimation of the fraction of mTHPC molecules bound to serum proteins/Me-β-CD at each time point and the kinetic characteristics of mTHPC transfer.

Fig. 6a illustrates the kinetics of mTHPC redistribution from serum proteins to Me-β-CD depending on β-CD concentration. When Me-β-CD concentration is low (less that 20 μM) the Soret band shape changes not significantly indicating that almost all mTHPC molecules remained bound to serum proteins (Fig. 6a, curves 1, 2, 3). In the samples containing moderate concentration of Me-β-CD, the I_{397}/I_{422} ratio value reaches 0.52 in 10 min of incubation meaning the transport of only limited amount of mTHPC molecules from the serum proteins to Me-β-CD (Fig. 6a, curve 4). Finally, after adding an excess amount of Me-β-CD (200 μM) to the

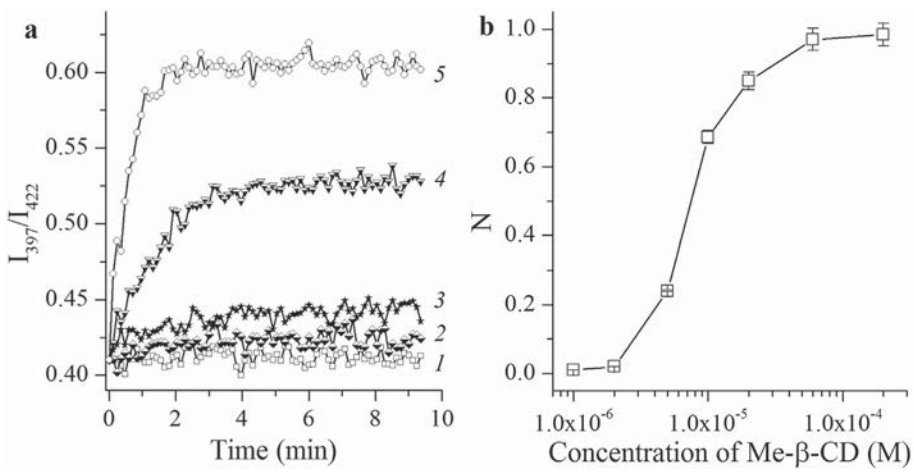

Fig. 6. A – The kinetics of I_{397}/I_{422} value changes after mixing of Me-β-CD solutions with mTHPC/serum solution. The concentration of Me-β-CD in the samples was (1) 5 μM, (2) 10 μM, (3) 20 μM, (4) 50 μM or (5) 200 μM. B – Influence of Me-β-CD concentration on the equilibrium distribution of mTHPC in serum solution after 24 h of incubation at 37 °C. N – the fraction of mTHPC molecules bound to Me-β-CD. The concentration of mTHPC was 0.5 μM, the concentration of serum was 5% (vol/vol). mTHPC was preincubated with serum at 37 °C during 2 h until complete binding of photosensitizer.

serum solution, containing mTHPC, the I_{397}/I_{422} value grows from 0.41 to 0.62 in several minutes confirming complete PS redistribution to Me-β-CD (Fig. 6a, curve 5).

Taking into account spectral shifts and variations of quantum yield of mTHPC fluorescence, it is possible to convert the value of $I_{\lambda 1}/I_{\lambda 2}$ ratio to the relative weights of fractions of mTHPC molecules bound to serum proteins or β-CD. The dependence of the fraction of mTHPC molecules bound to Me-β-CD (N) on the concentration of Me-β-CD is shown on Fig. 6b. The data obtained allow to estimate the interval of Me-β-CD concentrations in which mTHPC distribution pattern in serum has undergone changes.

Similar results of mTHPC distribution between β-CD and serum proteins were obtained recently by means of fluorescence polarization measurements [20]. In this connection it is worth noting, that the spectral technique described in this paper is less sensitive to the influence of various physical factors, such as light scattering, fluorescence quenching. And for this reason the technique based on spectral features of the Soret band shape is more reliable for the non-invasive studies of mTHPC distribution in blood serum.

4. Conclusion

Spectral characteristics of Soret band is considered to be uninformative for the analysis of peculiarities of aryl-porphyrin microenvironment. In the present study it was shown that the shapes of the Soret band of mTHPC absorbance spectrum and corresponding band of fluorescence excitation spectrum are strongly dependent on the kind of biological structure bound with the pigment molecule. The well-detected difference in ratio of two Soret band peaks is observed for mTHPC molecules bound to β-CD on the one hand and to serum proteins/lipid membranes on the other. Taking in account the possible mechanism of incorporation of mTHPC molecule into the β-CD cavities or lipid membranes, one can suggest that the variability of Soret band shape is associated with the changes of pigment molecule conformation. This finding is useful for the development of spectral technique for noninvasive continuous control of mTHPC distribution from β-CDs to biological structures.

Acknowledgments

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GENERAL DISCUSSION

The most effective clinically approved PSs have poor solubility in water resulting in unfavorable physico-chemical and biopharmaceutical properties. An important approach in this regard is the use of appropriate DDS. Since both CDs and tetrapyrrole PSs have been widely examined for applications in supramolecular chemistry (Kryjewski et al., 2015), it is not surprising that the CDs have been proposed as a DDS in PDT (Mazzaglia, 2011). Numerous studies have shown that CDs have a high affinity to porphyrinoid PSs especially aryl substituted (Lang et al., 2004; Wu et al., 2005). Inclusion in CDs exerts a profound effect on physicochemical properties of guest molecules as they are temporarily locked or partially caged within the host cavity giving rise to beneficial modifications of guest molecule properties. These modifications may enhance the therapeutic potential of PSs by increasing their aqueous solubility and dissolution rate, diminishing their decomposition before drugs enter tissues and by altering how they enter tissues (Gidwani & Vyas, 2015). In this perspective, it is reasonable to assume that β-CDs can be used to improve the pharmacokinetic and biodistribution properties of mTHPC affecting its PDT efficiency.

It is known that in water solutions mTHPC forms large J-aggregates leading to a significant change in the absorption and fluorescence properties (Ball et al., 1998; Sasnouski et al., 2005). Introduction of β-CD derivatives to the aqueous mTHPC solution results in monomerisation of mTHPC as shown by its spectral and fluorescence parameters: the value of relative quantum yield of mTHPC fluorescence in PBS shows more than 100 times increase in the presence of M-β-CD. Similar effects are observed when mTHPC aggregates interact with different structures, such as serum, lipid vesicles or detergent molecules, and the mechanism of this dissociation have been studied in details experimentally (Ball et al., 1998; Kachatkou et al., 2009; Rezzoug et al., 1998; Sasnouski et al., 2005). On the other hand, simultaneous administration of mTHPC with β-CD derivatives prevents its aggregation. Demore et al. (1999) showed that in water solution mTHPC forms readily inclusion complexes with β-CD derivatives confirmed by the presence of strong induced circular dichroism at the main band of mTHPC absorption spectrum (Desroches et al., 2001). mTHPC similarly to other aryl-porphyrins is able to bind 1 or 2 molecules of β-CD derivatives and the side phenyl groups penetrate into CD cavity (Demore et al., 1999).

An interesting peculiarity of mTHPC interaction with the microenvironment is a strong dependence of several photophysical characteristics on the type of biological

compound bound to mTHPC molecule. An analysis of received data allows concluding that both the degree of fluorescence polarization and the Soret band (the position and the shape) are the most informative and variable spectroscopic characteristics in respect to mTHPC microenvironment. It allows us to use the registration of these characteristics to confirm mTHPC binding to studied biological compounds. In case of mTHPC/CD inclusion complexes the value of fluorescence polarization degree is relatively low P=0.05 compared the PS bound to liposomes and serum proteins ($P \leq 0.30$). Fluorescence of PS bound to proteins, liposomes, micelles and CDs is polarized due to mobility restrictions. Since the fluorescence decay time varies slightly in compared samples, the difference in the values of fluorescence polarization degree is determined by hydrodynamic volumes of each structure (proteins, liposomes, micelles, CDs) bound to mTHPC. It is worth noting that a measurement of fluorescence polarization degree is sensitive to the various factors, such as sample turbidity and PS concentration. Indeed, fluorescence polarization of mTHPC in lipid vesicles and serum proteins is strongly dependent on the local concentration of PS and increase of PS concentration leading to progressive depolarization of fluorescence (Kachatkou et al., 2009; Reshetov et al., 2011). On the other hand, excitation fluorescence-based measurements of mTHPC Soret band are more informative and less sensitive to the above mentioned factors.

Unlike porphyrins with their symmetrical structure, the chlorin-type molecules are tetrapyrroles with the double bond in one pyrrole ring reduced, and, as a result, Soret band has a semi-resolved structure with two distinct peaks that can be represented by two Gaussian components (B_x, B_y) (Shkirman et al., 1999; Steiner & Fowler, 2006). Although in the near UV region from 360 to 400 nm the higher-energy transitions known as the N-, L-bands are also found (Palummo et al., 2009), according to our analysis they are not practically affected by different mTHPC surroundings and their contribution to the Soret band can be fixed. A comparison of the numerical results for mTHPC absorption and excitation spectra deconvolution shows that the ratio B_x/B_y varies from less than 0.7 (mTHPC bound to lipid vesicles and serum proteins) to 1.03-1.42 for mTHPC/β-CD complexes. Most probable that the main mechanism of this effect deals with the slightly change of tetrapyrrole ring conformation due to the interactions of the lateral substitutes with surrounding microenvironment (Noss et al., 1997; Rosa et al., 2006). So, an incorporation of mTHPC in lipid membrane or CD cavity should cause the rotation of phenyl groups around PS macrocycle; however, due to the different insertion mechanisms, rotation of phenyl substituents has a different direction in these cases.

After injection into the bloodstream the PS interacts primarily with plasma proteins. Drug release from vehicles and subsequent pattern of plasma protein binding are important factors governing the *in vivo* pharmacokinetics and biodistribution of PSs during PDT (Castano et al., 2005b). In the case of mTHPC about 95% of PS in plasma is associated with HDL and LDL (Reshetov et al. 2012, Triesscheijn et al. 2007) and mTHPC releases from lipoprotein carriers very slowly proceeding on a timescale of hours (Sasnouski et al. 2006, 2005). According to Bautista-Sanchez et al. (2005) association constants for mTHPC with several β-CDs are very high (~10^5 M^{-1}) and evidently the effective formation of mTHPC/β-CD inclusion complex can be obtained in the whole plasma. Desroches et al. (2001) suggested using β-CDs for pharmacokinetic studies in order to improve the sensitivity of the detection of mTHPC in human plasma.

We have proposed the use of techniques described above (fluorescence polarization, Soret band) for controlling the distribution of mTHPC between inclusion complexes with β-CDs and serum proteins. Analysis of the data showed that these methods can be used for both kinetic and equilibrium distribution of mTHPC between β-CDs and serum. The data of mTHPC distribution between the serum proteins and β-CDs obtained by Soret band correlate well with those received by fluorescence polarization that is supported by the gel chromatography data. Complexation of mTHPC with β-CDs is a reversible process, i.e. each molecule can dissociate from the inclusion complex with the subsequent binding to other molecules like plasma proteins. Indeed, the introduction of mTHPC/β-CD complexes into the serum is followed by a competition between CD and the serum proteins leading to redistribution of mTHPC molecules from inclusion complexes to serum proteins. It is obviously that return transfer of mTHPC molecules from serum proteins to CDs is controlled in a similar way.

The rate and the extent of mTHPC transfer from inclusion complex is an important determinant of PS pharmacokinetics. According to our studies, β-CDs cause a significant acceleration of mTHPC diffusion movement in plasma. The addition of M-β-CD causes a rapid dissociation of mTHPC molecules from complexes with proteins, resulting in an equilibrium distribution attained in 10-15 min. Further, introduction of a small content of β-CDs in serum significantly accelerates the rate of mTHPC redistribution between serum proteins. Besides studying the release rate of mTHPC from CD carriers to serum proteins, it is equally important to determine the exact protein fractions that bind mTHPC, since this has a significant effect on the drug tumor binding. According to gel chromatography data, β-CDs do not affect the distribution profile of the drug between the major serum transport proteins

except an appearance of free inclusion complexes of mTHPC/β-CD. It should be noted that the β-CDs effect on the process of mTHPC distribution in blood serum is a concentration-dependent. At low concentrations of CD (high concentration of serum) almost all mTHPC molecules are bound to the serum proteins. Increasing β-CDs concentration shifts equilibrium of PS distribution towards the formation of inclusion complexes. The final pattern of mTHPC equilibrium distribution in the blood serum is determined by relative content of CDs and affinity of CDs to the PS. From this we can conclude that mTHPC transport in the blood is defined by the constant of inclusion complex formation and may differ significantly in the case of various β-CD derivatives.

Despite a considerable amount of papers relate to study the influence of CDs on PS interaction with cells *in vitro*, there are no clear criteria for determining obtained effect of CDs (an increase or inhibition of PS accumulation). Moreover, an increase in the accumulation due to the increase of water solubility of the PS is not always the case (Dentuto et al., 2007; Kitagishi et al., 2015). The obtained results showed that mTHPC accumulation by cells strongly depends on type of CDs and its concentration: the β-CDs can cause both acceleration and inhibition of mTHPC cellular uptake. The effect of CDs on accumulation of hydrophobic drugs should be attributed to an increase of the permeability by enhancing drug solubility and thus making the drug available at the surface of plasma membrane of cells, from where it partitions into intracellular compartments (Loftsson & Brewster, 2013). Since most of water soluble CDs are not able to penetrate into cells, it is important to use appropriate CD concentration because excess may decrease the drug availability (Másson et al., 1999). Actually, at high β-CDs concentrations, the rate of mTHPC accumulation in the cells is reduced due to the high probability of formation of inclusion complexes in the extracellular environment. Furthermore, accumulation of mTHPC/CD inclusion complexes may be determined by specific mechanisms of CD interaction with cells. CDs are able to bind several components of cellular plasma membrane especially cholesterol that causes changes in membrane composition and structure, modifying its binding and barrier functions (López et al., 2011; Zidovetzki & Levitan, 2007). On the other hand, the level of membrane cholesterol significantly affects the relative affinity of porphyrins to cellular and model biological membranes (Cohen & Margalit, 1985; Zorin et al., 1997). According to our results on the fluorescence of cholesterol-specific stain filipin III the treatment of HT29 cells with 10 μM of M-β-CD or 200 μM of Hp-β-CD led to depletion of 15-20% cholesterol from the plasma membrane. Such a moderate change in the level of cholesterol apparently can not be the main

reason for multifold acceleration of mTHPC accumulation by treated cells, but it may be the cause of bright spots in the periphery of cells loaded with mTHPC.

It is well known that the photocytotoxicity of PS depends on the level of its accumulation in cells (Castano et al., 2005a). According to the data obtained CD-depending acceleration of mTHPC uptake by cells provides better cells sensitivity to phototreatment. It is unlikely that this effect is connected with the improved photophysical properties of mTHPC in the presence of β-CDs on mTHPC. According to Bautista-Sanchez et al. (2005), depending on used techniques of singlet oxygen measurements, the values $\Phi_{\Delta}(^{1}O_{2})$ were either not affected or were even 1.5 lower by the mTHPC/CDs associations. Besides, the penetration of mTHPC into the cell follows the processes of its dissociation from the inclusion complexes with β-CDs.

Finally, the processes of mTHPC biodistribution in HT29 tumor bearing mice after intravenous injection of PS alone or with the β-CDs were compared. Co-administration with β-CDs abolishes mTHPC aggregation in blood which was observed in the case of standard formulation, thus probably enhancing PS bioavailability immediately after injection. The formation of inclusion complexes in the plasma can have a significant impact on mTHPC distribution from the bloodstream and its localization in extravascular tissues including tumors. Indeed, the interstitial transport of molecules and particles is characteristically described by their effective interstitial diffusion coefficient, which decreases as their size is increased (Pluen et al. 2001). In addition, Chauhan et al. (2012) showed that smaller particles penetrate tumors better than larger particles. Thus, the smaller mTHPC/β-CDs inclusion complexes, with the size about 1.5-2.0 nm, must penetrate into extravascular tissues much faster as compare to mTHPC/HDL (8-12 nm) or mTHPC/LDL (20-25 nm) complexes.

In vivo study confirms the fact that the use of β-CDs allows modifying mTHPC distribution processes in tumor bearing animals that is reflected in the decreased level of PS accumulation in skin and muscles, as well as in the increased PS accumulation in tumor. Enhancement tumor-to-muscles ratio may induce a better selectivity of PDT treatment (Roby et al., 2007). Moreover, formulation of mTHPC with M-β-CD and Hp-β-CD showed lower drug levels in skin compared to mTHPC alone, thus indicating that the side effects with both β-CDs may prove less severe. We can assume that even after administration, a certain part of mTHPC remains associated with M-β-CD. According to the literature the highest level of mTHPC was found in the liver (Senge & Brandt, 2011) and the PS is excreted unchanged via biliary excretion in the faeces (Cai et al., 1999a, 1999b). On the other hand, CDs are rapidly eliminated in the urine and can increase renal clearance of lipophilic water-insoluble drugs

(Stella et al., 1999). This is in accordance with a significant increase in the fluorescence level of mTHPC in the kidney as well as a reduction in the PS fluorescence level in the liver when applying a formulation with M-β-CD compared to mTHPC alone.

Taken as a whole, complexation of mTHPC with β-CDs leads to increased water solubility, accelerated delivery of PS to the targets cells and tissues, improved the tumor-to-muscles ratio and expected low skin photosensitivity. Thus, β-CDs are very attractive delivery systems of aryl-substituted PSs and these findings might have potential relevance in improvement of PDT treatment with mTHPC.

CONCLUSIONS AND OUTLOOK

The present thesis covers the study of inclusion complexes of CDs and second generation photosensitizer mTHPC. The main research results are summarized as follows:

1. According to the spectral data, mTHPC efficiently forms the inclusion complexes with β-CD molecules in solutions and in biological systems. The shape of mTHPC Soret band and fluorescence polarization degree are the most informative spectral features for the purposes of controlling mTHPC distribution between inclusion complex and biological compounds (e.g., serum proteins and biological membranes).

2. β-CDs have a concentration-dependent effect on the process of mTHPC distribution in blood serum. At low concentrations (10^{-6}-10^{-5} M) the CDs do not affect the equilibrium distribution of mTHPC in blood serum, but significantly increase its diffusion mobility accelerating sensitizer binding to serum proteins. At high concentrations (>10^{-4} M) β-CDs are strongly bound to mTHPC molecules.

3. mTHPC accumulation by HT29 cells is strongly depended on β-CD concentrations. The β-CDs can cause both acceleration and inhibition of mTHPC cellular uptake. The main mechanism of mTHPC cellular uptake increase is the acceleration of mTHPC transfer from serum proteins to cellular membranes. On the other hand, most of water soluble CDs are not able to penetrate into cells. Thus, at high β-CDs concentrations, the rate of mTHPC accumulation in the cells is reduced due to the high probability of inclusion complexes formation in the extracellular environment.

4. Variation of β-CD type leads to the shifts of optimal concentrations providing maximal increase of the PS accumulation due to the changes in binding constants with mTHPC. β-CDs increase mTHPC photocytotoxicity towards HT29 cells due to the increase of mTHPC accumulation level.

5. Co-administration with β-CDs completely abolishes mTHPC aggregation in blood and enhances mTHPC bioavailability immediately after injection. Besides, use of β-CDs modifies mTHPC biodistribution pattern in tumor bearing mice *in vivo*.

6. The tumor and muscles uptake indicates that complexation of mTHPC with β-CD, especially with M-β-CD, results in higher tumor-to-muscles ratio than mTHPC alone that may probably induce a better selectivity of PDT treatment and overcome some side effects.

On the basis of data obtained, new pharmacological forms of the sensitizer based on CDs in the PDT may be designed.

Outlook

Further continuation of research is related to the definition of optimal parameters of PDT treatment with mTHPC/CD inclusion complexes. Optimization of PDT regimens necessarily requires knowledge of mTHPC/CD complexes pharmacokinetics and biodistribution, including the intratumoral spatial distribution of the PS.

Firstly, the *in vivo* study shall be conducted, comparing the pharmacokinetics (including drug release) and biodistribution of mTHPC inclusion complexes with different CDs. Based on these findings the type of CD providing the most favorable biodistribution of mTHPC in tumor-bearing animals (i.e., higher tumor/muscles ration and low skin photosensitivity) will be defined. Further, the optimal concentrations of the CDs and PSs will be determined.

Secondly, the localization of the mTHPC/CD complexes will be studied in the parenchyma and the vascular system of the tumor. The results obtained above suggest that CDs allow accelerating the rate of mTHPC uptake in target tissues, thus optimizing DLI of PDT. Based on the intratumoral mTHPC localization the optimal DLI for PDT treatment will be selected.

Thirdly, another direction of future research would be development of spectral technique based on Sored band variation for continuous control of mTHPC distribution *in vitro* and *in vivo*.

Fourthly, CDs can be used for controlled acceleration of mTHPC release from the various types of nanocarriers (liposomes, micelles, etc.). Indeed, CDs have high affinity to mTHPC molecules and, therefore, additional introduction of CDs should cause mTHPC release from nanocarriers in biological systems (*in vitro* and *in vivo*).

Finally, the results of this work can be useful for the development of CD-based nanocarriers for PDT, including CD nanoassemblies, CD-nanoconjugates with different targeting molecule and different nanoparticles functionalized with CD molecules.

The overall results of such work would be beneficial for understanding the behavior of CD-PDT drug.

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SYNTHESE DES TRAVAUX DE THESE

INTRODUCTION

Généralités

La thérapie photodynamique (PDT) est un traitement peu invasif, alternatif à la chirurgie, employé en oncologie et dans diverses pathologies. Cette technique est basée sur l'activation par la lumière d'une molécule, appelée photosensibilisateur (PS) qui génère sous l'effet d'une irradiation lumineuse et en présence d'oxygène des espèces réactives de l'oxygène (ROS) cytotoxiques responsables de dommages irréversibles dans le tissu cible. Les composés tetrapyrroliques tels que la méta-tétra(hydroxyphenyl)-chlorine (mTHPC) constituent la classe majeure des PS utilisée en PDT. Cependant, l'hydrophobicité élevée de ces composés, qui entraine leur faible solubilité en milieu biologique et leur agrégation dans le sang après injection intraveineuse, réduit leur application clinique. En effet, l'agrégation des PS génère une biodistribution et une sélectivité vis-à-vis du tissu cible médiocre se traduisant par une diminution de l'efficacité photodynamique combinée à une photosensibilité cutanée prolongée.

Pour pallier ces problèmes, des formulations pharmaceutiques telles que liposomes, nanoparticules polymériques et bioconjugués ont été proposées pour une meilleure administration du PS. Parmi les composés biodégradables et non toxiques qui peuvent être utilisés pour l'administration de substances actives, les cyclodextrines (CD) sont attractives. Les CD constituent une famille d'oligosaccharides cycliques possédant une cavité interne hydrophobe et une surface externe hydrophile. Elles sont solubles en milieu aqueux, biocompatibles et peuvent former des complexes d'inclusion stables avec un large éventail de composés qu'ils soient liquides ou gazeux. L'inclusion dans la cavité interne de la CD d'une substance hydrophobe permet d'en améliorer la solubilité et d'en facilité la dissolution. Sa stabilité chimique et physique ainsi que sa durée de circulation dans le sang est également augmentée facilitant ainsi sa biodisponibilité. En outre, les CD sont utilisés dans l'élaboration de complexes dirigés vers le tissu cible, y compris de façon active (incorporation d'un ligand spécifique de certains récepteurs cellulaires), permettant la libération immédiate ou retardée des substances médicamenteuses.

La mTHPC, actuellement évaluée en essai clinique pour le traitement palliatif des cancers de la tête et du cou est un PS de seconde génération très puissant et attractif en raison

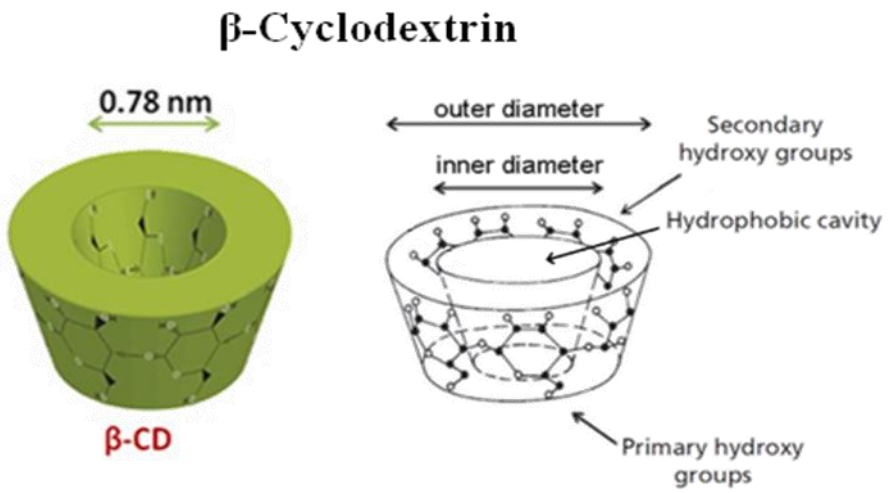
de son ratio thérapeutique élevé. Des études précédentes ont montré que la mTHPC forme des complexes d'inclusion avec les dérivés de la ß-CD ce qui a pour conséquence d'améliorer sa solubilité et ses propriétés photophysiques en solution aqueuse. A ce jour, l'application de complexes mTHPC-CDs dans des modèles précliniques *in vitro* et/ou *in vivo* n'a pas été reportée.

Nanovectorisation de substances actives

Nombreuses sont les molécules médicamenteuses possédant des propriétés pharmacologiques non-optimales : faible solubilité, nature irritante, absence de stabilité, métabolisme rapide et distribution non sélective. La nanovectorisation, développée pour pallier ces inconvénients et améliorer l'administration médicamenteuse, est un domaine en plein développement de la nanomédecine en particulier pour la thérapie oncologique y compris la PDT (Calixto et al., 2016 ; Marchal et al., 2015). Ainsi, des nanoparticules (NP) jusqu'à 200 nm ont été développées (Khodabandehloo et al., 2016). La libération spatiotemporelle de la substance active à partir du nanovecteur pouvant être contrôlée, une augmentation de l'efficacité thérapeutique tout en réduisant les effets secondaires systémiques est attendue. L'observance thérapeutique par le patient est également améliorée car la dose et la fréquence d'administration sont diminuées (Lee & Yeo, 2015).

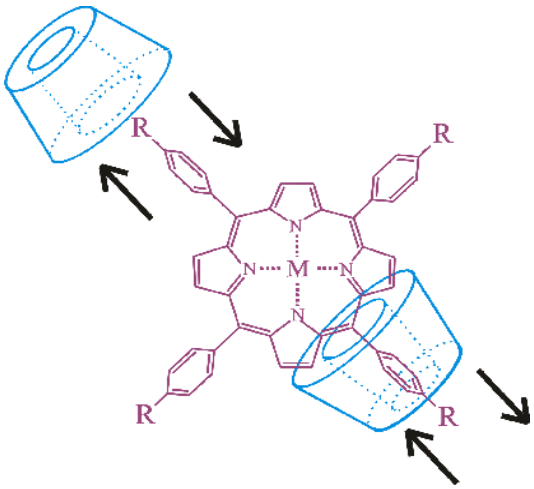
Cyclodextrines

Oligosaccharides cycliques, les CD possèdent une cavité interne hydrophobe et une surface externe hydrophile constituée par des unités D-glucopyranose à six α-CD, sept ß-CD ou huit γ-CD. Les complexes issus de CD incluant une large variété de composés organiques ou non constituent la classe la plus simple des systèmes supramoléculaires étudiés dans les dernières années d'un point de vue théorique et pratique. C'est pourquoi, les CD ont été utilisés dans l'alimentation, les cosmétiques et l'industrie pharmaceutique où leur application est en constante évolution. Actuellement, il y a 40 formulations contenant des CD, et plus particulièrement des ß-CD et dérivés (Gidwani & Vyas, 2015).



Utilisation des cyclodextrines en thérapie photodynamique

A ce jour, plus de 150 communications (articles et conférences) ont été publiées sur l'application des CD en PDT. La plupart de ces études concernent les PS tetrapyrroliques mais d'autres PS tels que l'hypericine (Sattler et al., 1997 ; Zhang et al., 2013), l'acide aminolévulinique (Aggelidiou et al., 2014), le fullerène (Ikeda et al., 2013 ; Iohara et al., 2012 ; Zhao et al., 2009), le polyméthine squaraine (Arun et al., 2011), la curcumine (Hauvik et al., 2009 ; Wikene et al., 2014) et la phéno-safranine (Kundu et al., 2016). Par l'utilisation de CD, ces études ont abordé (i) l'augmentation de la solubilité du PS en milieu aqueux ; (ii) les changements des caractéristiques photophysiques ; (iii) l'amélioration de la distribution du PS dans les cellules ou tissus cibles. Les solutions à ces problématiques ont été apportées par différentes approches telles que la complexation par inclusion non-convalente, la conjugaison covalente ou la formation d'assemblage nanométriques entre le PS et les CD.



Complexe d'inclusion avec une molécule tétrapyrrolique

OBJECTIF

L'objectif de ce travail a été de déterminer l'effet de certains dérivés des ß-CD sur les propriétés *in vitro* et *in vivo* de la mTHPC et sa libération à partir de son vecteur CD. Dans ce but, les études suivantes ont été menées :

- Déterminer la cinétique de distribution et l'état d'équilibre de la mTHPC entre les ß-CD et les protéines sériques ;
- Evaluer l'influence des dérivés de ß-CD sur le taux de mTHPC incorporée dans les cellules ;
- Estimer l'influence des dérivés de ß-CD sur la l'accumulation intracellulaire de la mTHPC et l'efficacité photodynamique *in vitro* ;
- Etudier la biodistribution des complexes mTHPC/CD chez la souris porteuse de tumeur ;
- Déterminer les caractéristiques d'absorption et de fluorescence de la mTHPC en solutions et en milieux biologiques. Les différences entre les caractéristiques spectrales de fluorescence du PS lié aux CD et à d'autres structures biologiques (protéines du sérum, membranes lipidiques par exemple) ont été identifiées ;
- Développer la méthode basée sur la variation de la bande de Soret de la mTHPC pour estimer la redistribution du PS à partir des CD dans les milieux biologiques. Les champs d'application potentielle de cette technique ont été examinés ;
- Comparer les méthodes basées sur les caractéristiques spectrales et de fluorescence de la mTHPC pour estimer la relargage de la mTHPC des ß-CD en milieu biologique.

RESULTATS ET DISCUSSION

Il est connu que la mTHPC en solution aqueuse forme de larges agrégats de type J conduisant à un changement significatif des propriétés d'absorption et de fluorescence (Ball et al., 1998 ; Sasnouski et al., 2005). L'introduction de dérivés ß-CD à la solution aqueuse de mTHPC provoque une monomérisation de la mTHPC se traduisant par une modification des paramètres spectraux et la fluorescence. Des effets similaires sont observés quand les agrégats de mTHPC interagissent avec différentes structures telles que le sérum, les vésicules lipidiques ou les molécules de détergent et le mécanisme de cette dissociation a été étudié

expérimentalement en détail (Ball et al., 1998 ; Rezzoug et al., 1998 ; Sasnouski et al., 2005 ; Kachatkou et al., 2009). L'administration simultanée des dérivés ß-CD avec la mTHPC empêche l'agrégation de celle-ci. Demore et al. ont montré qu'en solution aqueuse la mTHPC forme rapidement des complexes d'inclusion avec les dérivés ß-CD (Demore et al., 1999).

Les caractéristiques photophysiques de la mTHPC sont fortement dépendantes du composé biologique à laquelle elle est liée. L'analyse des données permet de conclure qu'à la fois le degré de polarisation de fluorescence et la bande de Soret (position et forme) sont les plus informatives sur le microenvironnement de la mTHPC. La modification de ces caractéristiques photochimiques permet de confirmer la liaison de la mTHPC au composé biologique étudié.

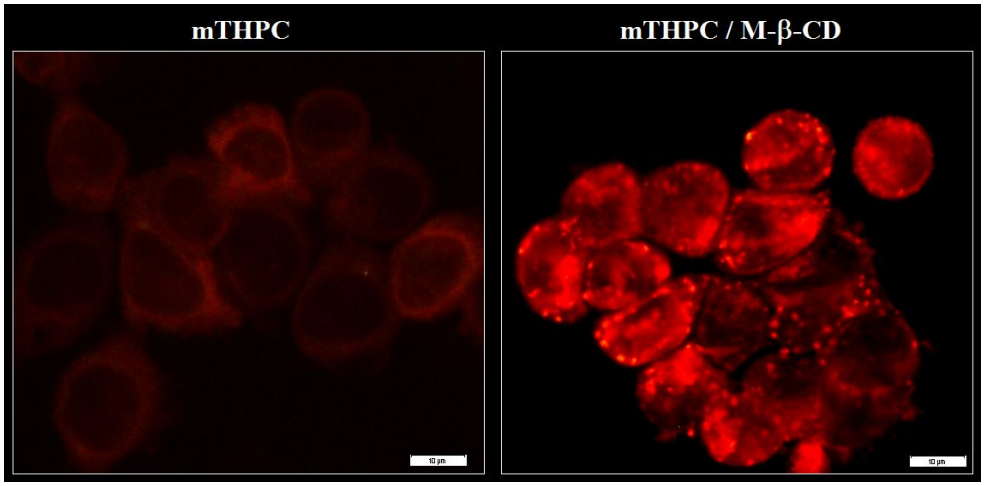
Dans le cas des complexes d'inclusion mTHPC/CD la valeur du degré de polarisation de fluorescence est relativement bas (P = 0,05) comparé à celle du PS lié aux liposomes ou aux protéines du sérum (P = 0,3). La fluorescence du PS lié aux protéines, aux liposomes ou aux CD est polarisée du fait des restrictions de mobilité. Puisque la durée de la décroissance de la fluorescence ne varie que légèrement dans ces situations, la différence de la valeur du degré de polarisation est due au volume hydrodynamique de chaque structure (protéines, liposomes, CD…) liée à la mTHPC.

Les molécules de type chlorine sont tetrapyrroliques avec une double liaison dans un des cycles pyrroles. Par conséquent, la bande de Soret est un spectre non résolu comportant deux pics représentés par deux paramètres gaussiens (B_x, B_y) (Shkirman et al., 1999 ; Steiner & Fowler, 2006). Une comparaison des résultats numériques issus de la déconvolution des spectres d'absorption et d'excitation montre que le ratio Bx/By varie d'environ 0,7 (mTHPC liée aux vésicules lipidiques ou aux protéines sériques) à 1,03-1,42 pour les complexes mTHPC/CD. L'explication la plus probable est qu'une légère modification de conformation des cycles tetrapyrroliques se produit lors des interactions des substituts latéraux avec le microenvironnement (Noss et al., 1997 ; Rosa et al., 2006). En se basant sur les variations de formes de la bande de Soret, la technique spectrale de monitoring en continu du relargage de la mTHPC des transporteurs β-CD dans un système biologique (e.g., distribution dans le sérum) a été développée. Cette approche peut être aussi utilisée dans l'étude de l'équilibre de distribution de la mTHPC dans un system complexe. Ces découvertes sont intéressantes pour le développement des techniques spectrales de monitoring non-invasif de la distribution de la mTHPC dans un system biologique.

Après injection dans la circulation sanguine, le PS interagit immédiatement avec les protéines du plasma. Nous avons appliqué les techniques décrites ci-dessus (polarisation de fluorescence, bande de Soret) pour contrôler la distribution de la mTHPC entre les ß-CD et les protéines sériques. L'analyse des data montre que ces méthodes donnent de bons résultats pour déterminer la cinétique de distribution et l'état d'équilibre de la mTHPC entre les ß-CD et les protéines du sérum. L'introduction des complexes mTHPC/ß-CD dans le sérum est suivie par une compétition entre les CD et les protéines sériques se traduisant par une redistribution des molécules de mTHPC des complexes d'inclusion vers les protéines du sérum. Le taux et la cinétique du transfert vers ces protéines sont déterminants pour la pharmacocinétique du PS. Selon nos études, les ß-CD causent une accélération de la diffusion de la mTHPC dans le plasma. L'ajout de M-ß-CD produit une rapide dissociation des molécules de mTHPC des complexes vers les protéines. De plus, l'effet des ß-CD sur le processus de redistribution de la mTHPC dans le sérum est concentration dépendant. A faible concentration de ß-CD presque toutes les molécules de mTHPC sont liées aux protéines sériques. L'augmentation de la concentration en ß-CD produit un shift de l'équilibre de la distribution vers la formation des complexes d'inclusion.

Les résultats que nous avons obtenus sur l'accumulation de la mTHPC complexée aux CD montrent qu'elle dépend du type de CD et de leur concentration. Les ß-CD produisent à la fois une accélération et une inhibition de l'accumulation intracellulaire. L'effet des CD sur l'accumulation intracellulaire des molécules hydrophobes pourrait être attribué à une augmentation de la perméabilité membranaire due à la meilleure solubilisation et dissolution de la molécule en présence de CD (Loftsson & Brewster, 2013). Il semble cependant que le mécanisme principal soit une accélération du transfert de la mTHPC des protéines du serum aux membranes cellulaires. La plupart des ß-CD solubles dans l'eau n'étant pas capables de pénétrer dans les cellules, le taux d'accumulation de la mTHPC est réduit si les ß-CD sont hautement concentrées, ceci étant dû à la probable formation de complexes d'inclusion de mTHPC dans l'environnement extracellulaire.

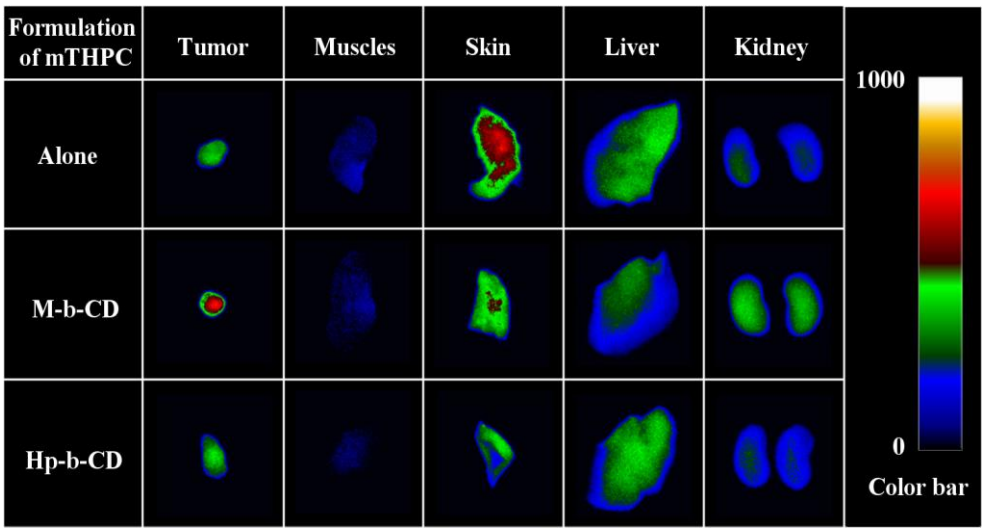
En ce qui concerne la photocytotoxicité des PS, il est évident qu'elle dépend essentiellement du niveau d'accumulation intracellulaire (Castano et al., 2005a). D'après nos résultats, l'accélération de l'accumulation intracellulaire en présence de ß-CD résulte en une phtocycotoxicité accrue. Il est peu probable que cet effet soit dû aux propriétés photophysiques améliorées de la mTHPC en présence de ß-CD. En effet, la pénétration de la



Images en épifluorescence des cellules HT29 incubées à 37°c pendant 1 heure avec 1.47 µM de mTHPC seule ou avec 10 µM de M-ß-CD

mTHPC dans les cellules est consécutive à sa dissociation des complexes d'inclusion avec la ß-CD. La variation du type de ß-CD conduit au shift de la concentration optimale générant une accumulation intracellulaire de PS maximale, les constantes d'affinité étant alors modifiées.

La dernière partie du travail concerne la biodistribution de la mTHPC vectorisée par les ß-CD, comparée à la mTHPC seule, chez la souris porteuse d'une tumeur d'adénocarcinome de colon humain (HT29). La co-administration de la mTHPC avec les ß-CD abolit l'agrégation de la mTHPC dans le sang, observée dans le cas de l'injection de la formulation standard, ce qui probablement augmente sa biodisponibilité immédiatement après injection. La formation des complexes d'inclusion dans le plasma ont probablement un impact significatif sur la distribution de la mTHPC à partir du flux sanguin et la localisation de cette dernière au niveau des tissus extravasculaires y compris tumoraux. L'étude *in vivo* confirme que les ß-CD modifient les processus de biodistribution car l'accumulation de PS dans la peau et les muscles semble diminuée alors qu'elle est augmentée dans la tumeur. L'augmentation du ratio tumeur/muscle indique une meilleure sélectivité pour la PDT (Roby et al., 2007). De plus, la quantité de mTHPC dans la peau, après injection des formulations mTHPC complexée aux M-ß-CD ou Hp-ß-CD, est moindre par rapport à la mTHPC seule ce qui suggère que les effets secondaires peuvent être moins sévères.



Imagerie de fluorescence de differents organes et tissus prélevés d'une souris porteuse d'une tumeur HT29 24 h après l'injection de 0, 5 mg/kg de mTHPC seule ou avec 2,5 mg par kg de M-ß-CD ou 25 mg/kg de Hp-ß-CD

CONCLUSIONS ET PERSPECTIVES

Au cours de cette thèse, nous nous sommes intéressés aux complexes d'inclusion des CD avec le PS de deuxième génération mTHPC. Les principaux résultats peuvent être résumés ainsi :

1. Selon les données spectrales, la mTHPC forme effectivement des complexes d'inclusion avec les molécules de ß-CD que ce soit en solution ou en milieu biologique. La forme de la bande de Soret de la mTHPC ainsi que le degré de polarisation de fluorescence sont les deux paramètres les plus informatifs permettant de contrôler la distribution de la mTHPC entre le complexe d'inclusion et les composants biologiques tels que les protéines sériques et les membranes biologiques.

2. Les ß-CD ont un effet dépendant de leur concentration sur processus de distribution de la mTHPC dans le sérum. A faible concentrations (10^{-6}-10^{-5}M) les CD n'affectent pas l'état d'équilibre de la mTHPC mais augmente significativement sa diffusion en accélérant la liaison aux protéines. A concentrations élevées (>10^{-4} M) ß-CD et mTHPC sont solidement complexés.

3. L'accumulation de la mTHPC dans les cellules HT29 est fortement dépendante des concentrations de ß-CD. Celles-ci causent à la fois l'accélération et l'inhibition de l'entrée de la mTHPC dans les cellules. L'accélération du transfert de la mTHPC des protéines du

serum aux membranes cellulaires en est le principal mécanisme. A forte concentration de ß-CD, celles-ci solubles dans l'eau n'étant pas capables de pénétrer dans les cellules, le taux d'accumulation de la mTHPC est réduit de fait de la formation probable de complexes d'inclusion de mTHPC dans l'environnement extracellulaire.

4. La variation du type de ß-CD modifiant les constantes d'affinité avec la mTHPC, il en résulte un shift de la concentration optimale générant une accumulation intracellulaire de PS maximale. Les ß-CD augmentent la photocytotoxicité par l'augmentation de l'accumulation intracellulaire de la mTHPC.

5. La co-administration de la mTHPC avec les ß-CD abolit l'agrégation du PS dans le sang et augmente sa biodisponibilité. Des modifications de biodistribution *in vivo* de la mTHPC issue des complexes d'inclusion en résultent.

6. Particulièrement avec la M-ß-CD, l'accumulation de la mTHPC dans la tumeur et dans le muscle produit un ratio de mTHPC tumeur/muscle augmenté par rapport à celui de la mTHPC seule. Ceci indique une meilleure sélectivité tumorale avec des effets secondaires réduits.

Sur la base des résultats obtenus, des formulations de mTHPC ou d'autres PS impliquant des complexes d'inclusion ß-CD peuvent être développées pour la PDT.

Perspectives

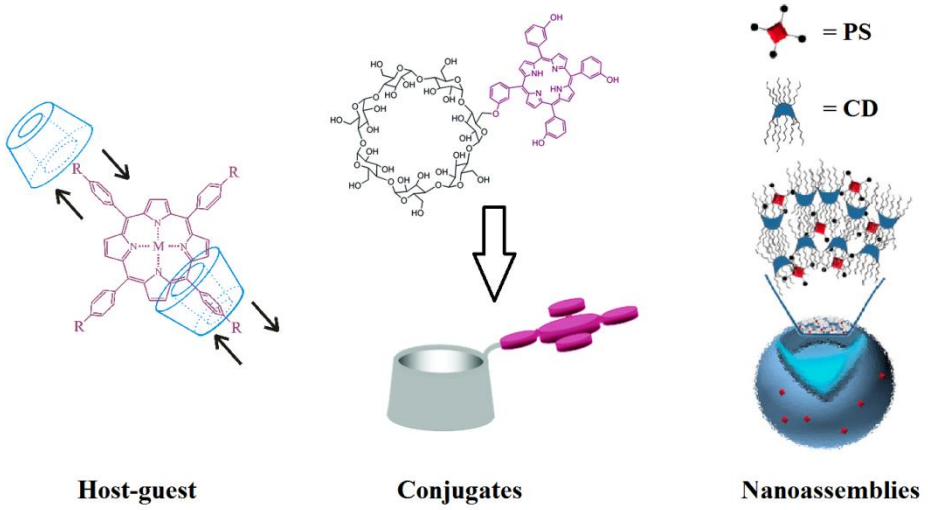
La continuation de ces travaux de recherche est liée à l'optimisation des paramètres de la PDT utilisant les complexes d'inclusion mTHPC/CD. Cela demande nécessairement une meilleure connaissance de la pharmacocinétique et de la biodistribution en particulier par une mise en évidence de la distribution spatiotemporelle du PS issu de ces complexes dans la tumeur.

En premier lieu une étude *in vivo* comparant la pharmacocinétique et la biodistribution de la mTHPC complexée à différentes CD peut être envisagée. Ainsi, le type de CD donnant la biodistribution la plus favorable chez la souris porteuse de tumeur pourra être connue. La concentration optimale de CD et de PS devra être déterminée.

Ensuite, la localisation des complexes mTHPC/CD pourrait être étudiée dans le parenchyme et le compartiment vasculaire de la tumeur. En fonction de la localisation intratumorale de la mTHPC, l'intervalle de temps optimal entre l'injection du PS et l'irradiation (IDL ; intervalle drogue-lumière) pourra être sélectionné.

Un autre aspect pour les recherches ultérieures pourrait être le développement de la méthode spectrale basée sur la variation de la bande de Soret pour un contrôle continu de la distribution de la mTHPC *in vitro* et *in vivo*.

Pour finir, les CD peuvent être utilisées pour assurer une accélération contrôlée du relargage de la mTHPC à partir de différent types de nanovecteurs tels que les liposomes ou les micelles. De plus ces résultats sont encourageants pour développer d'autres nanovecteurs basés sur les CD pour une utilisation en PDT. Ce pourraient être des assemblages ou des nanométriques de CD, des conjugués à base de CD pour cibler les tumeurs et différentes nanoparticules fonctionnalisées comportant des molécules de CD.



Types de nanovectorisations impliquant CD et PS de type tetrapyrrolique

De tels travaux ne pourraient qu'être bénéfiques pour comprendre le comportement des PSs associés aux CDs dans le cadre de la PDT.

SCIENTIFIC OUTPUT

1. ARTICLES IN PEER-REVIEWED JOURNALS

1) **Yankovsky I.**, Bastien E., Yakavets I., Khludeyev I., Lassalle H-P., Gräfe S., Bezdetnaya L., Zorin V. Inclusion complexation with β-cyclodextrin derivatives alters photodynamic activity and biodistribution of meta-tetra(hydroxyphenyl)chlorin / *Eur J Pharm Sci. 2016 Aug 25;91 P.:172-82.*
2) Ilya Yakavets[1], **Igor Yankovsky[1]**, Bezdetnaya L., Zorin V. Soret band shape indicates mTHPC distribution between β-cyclodextrins and serum proteins // *Dyes and Pigments 137 (2017) P.: 299-306.* [1]*These authors have contributed equally to this work.*
3) Zorina T.E., **Yankovsky I.V.**, Kravchenko I.E., Shman T.V., Zorin V.P. The Liposomal formulations of chlorin e6 esters and the features of their accumulation by cells // *J. BSU Herald, Series 2 Biology, 2013. № 3, - P. 30-35.*
4) Ilya Yakavets, **Igor Yankovsky**, Lina Bezdetnaya, Vladimir Zorin. "Spectral characteristics of meta-tetra(hydroxyl-phenyl)chlorin in biological systems". *J. BSU Herald, Series 1 Physics,2015, Vol. 2, P. 39-45.*
5) T.E. Zorina , **I.V. Yankovsky**, I.E. Kravchenko, T.V. Shman, M.V. Belevtsev, V.P. Zorin. "Evaluation of Phototoxicity and cytotoxicity for chlorin e6 ester derivatives and their liposomal forms" *Biofizika September 2015, Volume 60, Issue 5, P.:922-930.*

2. MAJOR ORAL COMMUNUCATIONS

1) **Yankovsky I.,** V. Zorin, L. Bezdetnaya Use of cyclodextrins to improve antitumor activity of mTHPC in human colon carcinoma HT-29 cells *// 16th European Society for Photobiology Congress – Aveiro (Portugal), 31 August - 4 September 2015: P.59.*

2) **Yankovsky I.,** Yakovets I., Khludeyev I., Bolotine L., Zorin V. Influence of methyl-β-cyclodextrin on mTHPC interaction with blood components *// «Open Readings 2015»: 58-th Scientific Conference for Students of Physics and Natural Sciences, 24-27 March, 2015 , Vilnius, Lithuania. P. 80.*

3) **Yankovsky I.V.,** Yakavets I.V., Zorin V.P., Bolotine L.N. Development of model for an intratumoral distribution of mTHPC/cyclodextrin complexes / *XII International conference "Molecular, membrane and cell fundamentals of biosystems' functioning", June 28-30, 2016: Collected articles. – Belarus. Minsk. – 2014. – P. 225-228.*

4) **Yankovsky I.V.,** Yakovets I.V., Bolotine L.N., Zorin V.P. Investigation of supramolecular complexes between mthpc and methyl-β-cyclodextrin *// "Low Temperature Physics" (ICYS-LTP-2014): V Int. Conf. Young Scient., 2-6 June, 2014 , Kharkiv, Ukraine. P. 117.*

5) **Yankovsky I.V.,** Yakovets I.V., Bolotine L.N., Zorin V.P. meta-tetra(hydroxyphenyl)chlorin accumulation in blood components: influence of methyl-β-cyclodextrin *// XI International conference "Molecular, membrane and cell fundamentals of biosystems' functioning", June 17-20, 2014: Collected articles. – Belarus. Minsk. – 2014. – P. 180-183.*

6) **Yankovsky I.,** Yakovets I., Bolotine L., Zorin V.P. Alteration of meta-tetra(hydroxyphenyl)chlorin biodistribution by β-cyclodextrin derivatives *// IV International conference " Topical issues of photodynamic therapy and photodiagnostics", Saint Petersburg, Russia, September 25-26, 2015: P. 14.*

3. POSTERS

1) **Yankovsky I.V.**, Yakavets I.V., Tihonov K.D., Bolotine L.N., Zorin V.P. Determination of aryl-porphyrins binding constants to biological structures by indirect spectral approach / *XII International conference "Molecular, membrane and cell fundamentals of biosystems' functioning", June 28-30, 2016: Collected articles. – Belarus. Minsk. – 2014. – P. 37-39.*

2) **Yankovsky I.**, I. Yakovets, L. Bezdetnaya, V. Zorin New spectral approach for monitoring of mTHPC redistribution in biological systems // *16th European Society for Photobiology Congress – Aveiro (Portugal), 31 August - 4 September 2015: P.45.*

4. COMMUNICATIONS PRESENTED BY COLLABORATORS

1) Yakovets I., **I. Yankovsky**, V. Zorin, L. Bezdetnaya The alteration of mthpc intratumoral distribution in ht-29 spheroids by cyclodextrins // *59th Scientific Conference for Students of Physics and Natural Sciences "Open Readings 2016", Vilnius, Lithuania 15-18th of March. – 2016. - P. 18.*

2) Yakovets I., **I. Yankovsky**, L. Bezdetnaya, V. Zorin The fluorescent spectral technique for determination the affinity of aryl-porphyrins to biological structures // *59th Scientific Conference for Students of Physics and Natural Sciences "Open Readings 2016", Vilnius, Lithuania 15-18th of March. – 2016. - P. 262.*

3) Yakovets I., **I. Yankovsky**, V. Zorin, L. Bezdetnaya Spectral method for controlling of mTHPC redistribution between cyclodextrins and biological structures // *58th*

Scien. Conf. for Students of Physics and Natural Sciences "Open Readings 2015", Vilnius, Lithuania. – 2015. - P. 36.

4) Zorina T.E., **Yankovsky I.V**., Kravchenko I.E., Ermilova T.I., Shman T.V., Zorin V.P. Nanosized photosensitizers accumulation and elimination from cells with different structural and morphological properties // *XII International conference "Medico-social ecology of the person: state and perspectives", April 11-12 2014., – Collected articles. – Belarus. Minsk. – 2014. – P. 149-151.*
5) Zorin V.P., **Yankovsky I.**, Yakovets I., Bolotine L. Influence of β-cyclodextrin derivatives on photosensitizer meta-tetra(hydroxyphenyl)chlorin distribution in biologica systems // *V congress of Russian biophysicists, 4-10 October 2015, Rostov-on-Don, Russia. T. 1. – P. 340.*

ABSTRACT

Photodynamic therapy (PDT) is a minimally invasive photochemical treatment with a promising clinical track record for oncological and some other diseases. Most PDT-drugs (photosensitizers) including a second-generation PS meta-tetra(hydroxyphenyl)chorin (mTHPC) are highly hydrophobic and require delivery systems. To improve mTHPC solubility and pharmacokinetic properties, β-cyclodextrins (β-CDs) derivatives were proposed.

The present study investigates the effect of β-CDs on mTHPC behavior at various stages of its distribution *in vitro* and *in vivo.* Interaction of mTHPC with β-CDs leads to the formation of inclusion complexes that completely abolishes its aggregation after introduction into serum. It was demonstrated that the β-CDs have a concentration-dependent effect on the process of mTHPC distribution in blood serum and cellular cultures *in vitro*. *In vivo* study confirms the fact that the use of β-CDs allows modifying mTHPC distribution processes in tumor bearing animals that is reflected in the decreased level of PS accumulation in skin and muscles, as well as in the increased PS accumulation in tumor. In conclusion, application of β-CD derivatives can open up new possibilities to modify and control biodistribution and pharmacokinetics of mTHPC in the course of PDT.

Key-Words : Photodynamic therapy, mTHPC, cyclodextrins, biodistribution, protein binding.

RESUME

La thérapie photodynamique (PDT) est un traitement minimalement invasif basé sur une approche photochimique. Ce traitement est prometteur en oncologie et pour d'autres pathologies. La plupart des drogues utilisées en PDT (photosensibilisateurs) y compris le PS de seconde génération, la méta-tétra (hydroxyphényl)chlorine (mTHPC), sont hautement hydrophobiques et nécessitent un système de transport. Pour améliorer la solubilité de la mTHPC et ses propriétés pharmacocinétiques, les dérivés des β-cyclodextrines (β-CDs) ont été proposés.

L'étude présente a analysé l'effet des β-CDs sur le comportement de la mTHPC à différentes étapes de sa distribution in vitro et in vivo. L'interaction de la mTHPC avec les β-CDs conduit à la formation de complexes d'inclusion qui abolissent complètement son agrégation après introduction dans le sérum. Il a été démontré que les β-CDs ont un effet lié à la concentration sur le processus de distribution de la mTHPC dans le sérum sanguin et les cultures cellulaires in vitro. L'étude in vivo confirme le fait que l'utilisation de β-CDs permet de modifier les processus de distribution de la mTHPC chez les animaux porteurs de tumeurs, ce qui se traduit par un taux moins élevé d'accumulation du PS dans la peau et les muscles, ainsi qu'une accumulation plus élevée du PS dans la tumeur. En conclusion, l'application des dérivés β-CDs peut ouvrir de nouvelles possibilités pour modifier et contrôler la biodistribution et les pharmacocinétiques de la mTHPC au cours de la PDT.

Mots-clés : Thérapie photodynamique, mTHPC, cyclodextrines, biodistribution, liaison aux protéines.