Conjugates of Porphyrins and Hyperbranched Polyglycerol for an Application in Photodynamic Therapy

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Abbreviations

aPDT	antimicrobial photodynamic therapy
BSA	bovine serum albumin
CuAAC	copper(I)-catalyzed 1,3-dipolar cycloaddition
DCM	dichloromethane
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DIFO	difluorinated cyclooctyne
DLS	dynamic light scattering
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DT	dark toxicity
EPR	enhanced permeability and retention
FDA	Food and Drug Administration
GSH	glutathione
hPG	hyperbranched polyglycerol
-I	negative inductive effect
IUPAC	International Union of Pure and Applied Chemistry
-M	negative mesomeric effect
MRSA	methicillin-resistant *Staphylococcus aureus*
*m*THPC	5,10,15,20-tetrakis(3-hydroxyphenyl)chlorin
*m*THPP	5,10,15,20-tetrakis(3-hydroxyphenyl)porphyrin
MW	molecular weight

MWCO	molecular weight cut-off
nPG	polyglycerol based nanogels
PBS	phosphate-buffered saline
PDT	photodynamic therapy
PEG	poly(ethylene glycol)
PLGA	Polylactic-co-glycolic acid
PS	photosensitizer
PVA	poly(vinyl alcohol)
rt	room temperature
S_EAr	electrophilic aromatic substitution
siRNA	small interfering ribonucleic acid
S_NAr	nucleophilic aromatic substitution
SPAAC	strain-promoted alkyne-azide cycloaddition
TLC	thin-layer chromatography

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Hyperbranched Polyglycerol and Porphyrins - a Perspective in Photodynamic Therapy

1.1 *Introduction*

1.1.1 *Porphyrins*

Porphyrins are tetrapyrrole-macrocycles with a resonance-stabilized 16-membered ring system. They consist of four pyrrole units connected in a cyclic system via sp^2-hybridized methine-bridges, Different tetrapyrrolic systems are known e.g. porphyrins, corroles, calixpyrroles, and corrins, which depends on the number of the existing bridges and their hybridization (Figure 1).

The 1-24 numbering system is based on the International Union of Pure and Applied Chemistry (IUPAC) rules and shown in Figure 1. The 2, 3, 7, 8, 12, 13, 17 and 18 position are referred to as ”β-positions”. The positions at 1, 4, 6, 9, 11, 14, 16 and 19 are called ”α-positions”, while those at 5,10,15 and 20 are referred to as ”*meso*-positions”. Porphyrins, in particular, the hematoporphyrin are long known and were first described by SCHERER in 1841 although in an impure form.[1] In 1867 THUDICHUM investigated the spectrum of the red substance and its fluorescence,[2] followed 1871 by HOPPE-SEYLER, who first introduced the name “hematoporphyrin”.[3] The first correct structure was proposed by KÜSTER in 1912[4]. Porphyrins have 22 π-electrons and their ring systems are nearly planar with a D_{2h} symmetry with high stability. Pieces of evidence for the stability are the occurrence of porphyrin complexes in larger concentrations in sediments, fossil coal, and petroleum fractions.[5] Only 18 of the 22 π-electrons are necessary to form the aromatic perimeter fulfilling the (4n + 2)-HÜCKEL rule for aromaticity. For porphyrins, the two ”peripheral double bonds” are included in the conjugated π-system but are not necessary for fulfilling the HÜCKEL rule. Hence, these two C-C double bonds in the two pyrrole units exhibit an olefin-type character, which allows the typical reactions e.g. addition, reduction or cycloaddition.[6] Removing one or two the double bonds leads to the corresponding derivatives chlorin and bacteriochlorin, respectively (Figure 1).

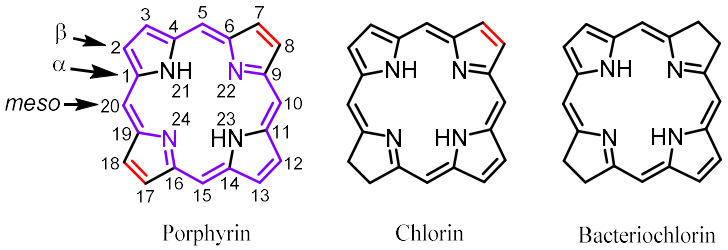
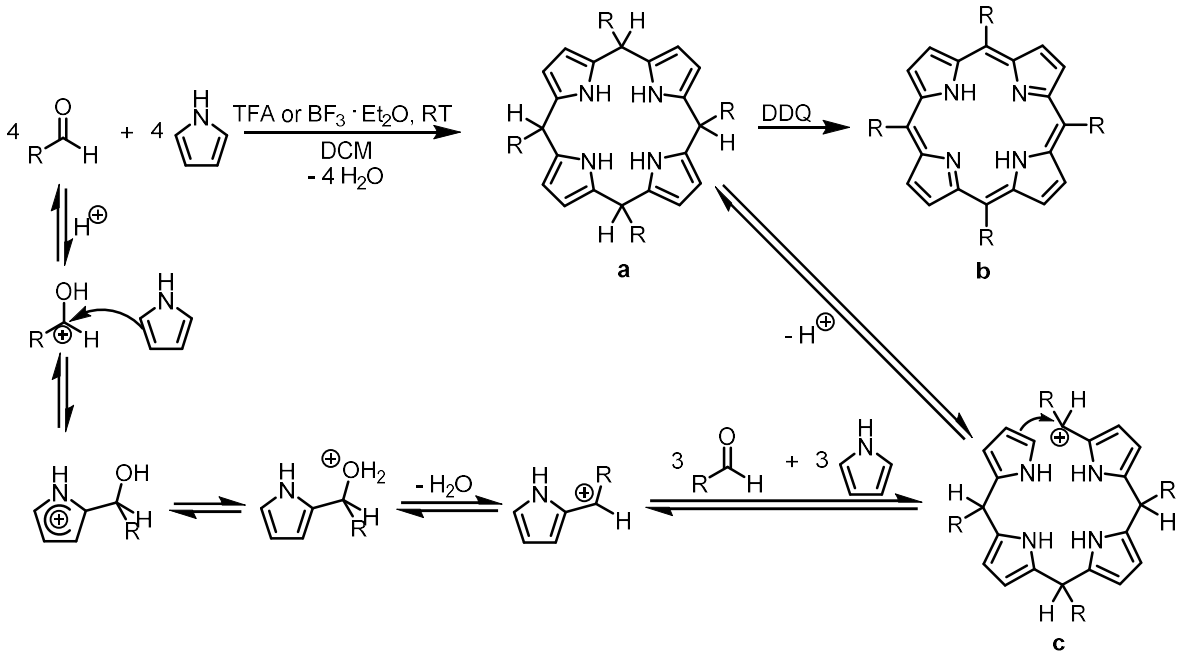


Figure 1 Structural comparison of the tetrapyrroles: porphyrin, chlorin, and bacteriochlorin.

These tetrapyrrolic systems and also the related corrole fulfill the HÜCKEL rule and are aromatic. Because of the conjugated π-electron system porphyrins, other tetrapyrroles, and their metal complexes show an intense absorption of electromagnetic radiation in the visible region and are also called tetrapyrrole dyes.[7] The absorption spectrum of the free-base porphyrin shows a strong SORET-band at around 400 nm and four smaller Q-bands between 450 and 700 nm. To change the absorption behavior, protonation of the central pyrrole units or complexation of the porphyrin with transition metals is possible. For example, porphyrins with a complexed metal exhibit one strong SORET-band and only one or two weaker Q-bands. Another way to influence the absorption behavior is the transformation of the porphyrin into a chlorin or bacteriochlorin. In research porphyrins and their derivatives have found interest in solar cell research,[8] for non-linear optical applications,[9] as catalysts,[8a, 10] as advanced biomimetic models for photosynthesis,[11] and for photodynamic therapy (PDT), which is explained in more detail in Section 1.1.3.

The first synthesis of porphyrins was reported by ROTHEMUND in 1935 from the reactants pyrrole and formaldehyde/acetaldehyde.[12] He had to use very harsh conditions for the synthesis. Therefore only selected aldehydes with a high stability could be employed, and the reaction resulted in very low yields. 32 years later in 1967 ADLER and LONGO reported an improved synthetic method,[13] employing the starting materials in propionic acid yielding the corresponding porphyrins. This new reaction type improved the synthesis of porphyrins by enabling shorter reaction times, higher yields, and slightly milder reaction conditions. However, the reaction conditions still did not allow to use benzaldehydes with sensitive functional groups. Another problem was the tar (polymers) produced during the reaction which complicated purification of the products. Finally, LINDSEY et al. reported 1987 a method with mild reaction conditions at room temperature (rt) for preparing complex porphyrins.[14]

The basic mechanism of the porphyrin formation is explained in Scheme 1.



Scheme 1 Mechanism of the porphyrin synthesis.

The reaction starts with a nucleophilic addition of pyrrole to the carbonyl group, followed by the elimination of water. If this addition-elimination step occurs several times, it possibly leads to unwanted long polymer-like chains instead of the desired formation of the tetrapyrrole ring through intramolecular addition **c**. To avoid the intermolecular chain formation the concentration of the reactants in the solution has to be chosen very low to promote the intramolecular reaction (usually 10^{-2} mol/L). The tetrapyrrole ring product formed in this condensation reaction is called porphyrinogen **a**. Up to this point, all the steps are reversible. The oxidation of the porphyrinogen with 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) irreversibly forms the final porphyrin product **b**.

In nature, many macrocyclic tetrapyrroles occur as metal complexes. One example is the heme protoporphyrin, which is coordinated with a single Fe^{2+}-ion (shown in Figure 2):

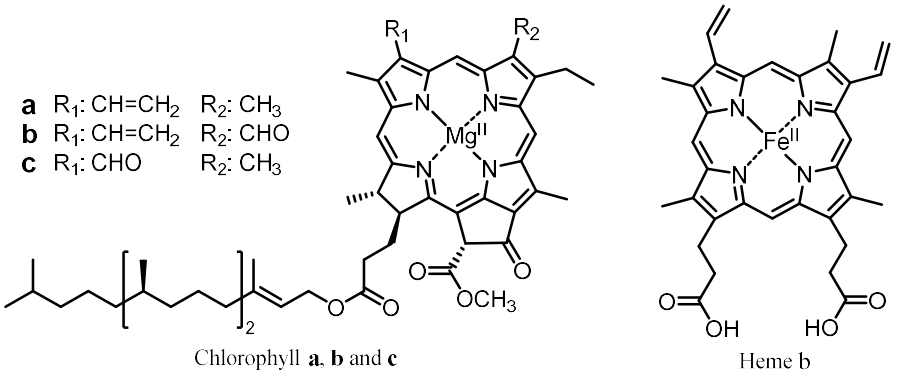


Figure 2 Basic structure of heme b and chlorophyll a, b and c.

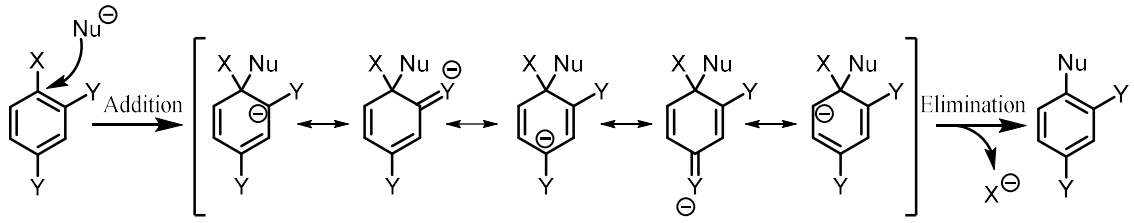
It possesses the ability to bind molecular oxygen reversibly, which is a crucial factor for the transport of oxygen in the human body. The second example for naturally occurring

tetrapyrroles is the chlorophyll shown in Figure 2 with magnesium in its center. This tetrapyrrole is a member of the compound class of the chlorins and gives plants their green color. It is necessary for the photosynthesis in plants, where light energy is absorbed by supramolecular chlorin-containing systems and subsequently is transferred to the photosynthetic reaction center.

1.1.2 *Nucleophilic aromatic substitution*

Various transformations are known for aryl systems, depending on their substituents and their reactivity. One typical reaction type is the electrophilic aromatic substitution reaction (S_EAr). Due to the high electron density of the aryl system electrophiles can easily undergo the known two-step addition-elimination mechanism.[15] Increasing the electron density and as a consequence, the reactivity can be achieved by adding electron donating substituents.

In contrast to the S_EAr, the nucleophilic aromatic substitution (S_NAr) is less common and needs highly electron deficient systems. To achieve this deficiency electron withdrawing substituents are required. Two main types of substituents with this behavior are described: functional groups with a negative inductive effect (-I), e.g. halogen atoms or with a negative mesomeric effect (-M), e.g. cyano, nitro or sulfonyl atoms. Most of the substituents show a combination of these two effects, but the mesomeric one is more dominant and has a stronger influence on the system. In the addition step of the S_NAr, substituents with -M-effect in the *ortho*-, *para*-, or both -positions can add one or more additional contributing structures thus stabilizing the occurring negative charge (Scheme 2).



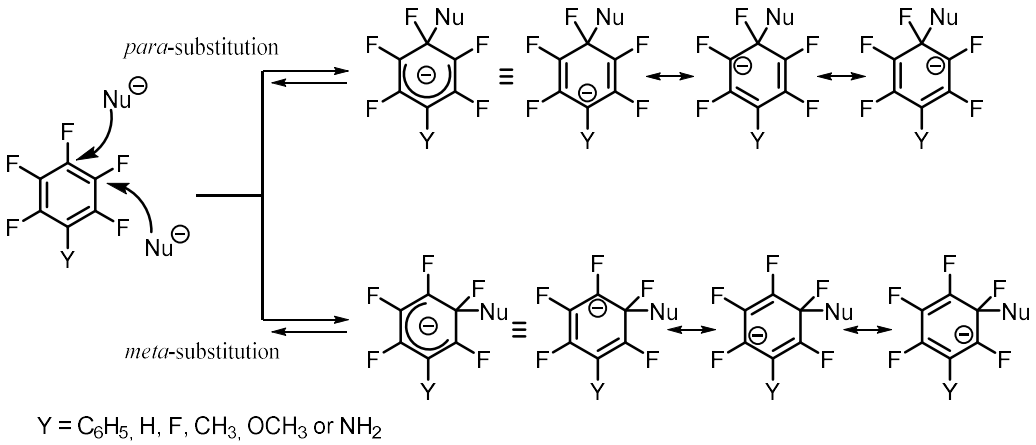
Scheme 2 The general reaction mechanism for a nucleophilic aromatic substitution (S_NAr). X = leaving group, Nu = nucleophile and Y = -M-effect substituent.

Good example nucleophiles for the S_NAr are cyanide, oxygen, nitrogen or sulfur containing molecules, while on the other hand for the elimination suitable leaving groups are necessary, e.g. halogen atoms, mesylate or tosylate.

In the case of fluorine in the S_NAr, fluoride is not the best leaving group, in this reaction. However, the addition step is rate-determining. The addition step is slow because it disturbs the aromaticity, the elimination, on the other hand, is very fast because it restores the aromaticity of the system. Fluoride as a mediocre leaving group slows down the elimination step, but it accelerates the addition step simply by its enormous inductive effect.[15]

A particular case is the pentafluorophenyl group used for the S_NAr in this work. This system is highly electron deficient and does not allow any typical S_EAr reactions, but makes it an ideal candidate for the S_NAr. In literature, it is assumed that the distribution of the electrons in the

aryl system is disturbed by the interaction of the fluorine atom p-orbitals with the π-system. Therefore, this aromatic system has an entirely different behavior compared to the common aryl systems.[16] Due to the difference in electronegativity within the pentafluorophenyl group, the fluorine atoms are partially negatively and the carbon atoms partially positively charged. Concerning regioselectivity, the nucleophilic aromatic substitution is most favored in the *para*-position. Recent studies with discrete FOURIER transform calculations explain the regioselectivity of the reaction by the higher polarizability and the improved stability of the dearomatized transition states of the *para*-position attack. There is no typical MEISENHEIMER-type intermediate (Scheme 3) formed.



Scheme 3 Regioselectivity of the nucleophilic aromatic substitution on monosubstituted pentafluorobenzene.

Instead, a tetrahedral S_N2 mechanism is proposed by PALETA et al.[17] The electron-withdrawing inductive effect of the not-reacting fluorine atoms leads to a charge stabilization, and the substitution is directed to the *ortho*- and *para*-position. On the other hand, electron-donating groups direct the substitution to the *meta*-position. For porphyrins, the *ortho*-position is as well disfavored because of the steric hindrance.

For porphyrins with pentafluorophenyl substituents, various nucleophiles have been described. K. M. KADISH and coworkers described first the substitution with amines in 1990. Followed by this research different nucleophiles for the S_NAr on tetrapyrrolic systems were investigated like amines,[8a, 18] alcohols,[18b, 18c, 18h, 19] carborane,[6b] phosphanes,[18f, 18g] phosphite[20] and thiols.[18b-e, 21] In this thesis the future use in biological systems was an important point in the synthesis, therefore, the reaction with amines which does not require any addition of catalysts or other reagents[18a-j] was the preferred method.

1.1.3 *Photodynamic Therapy*

PDT as a treatment modality for malignant tissues has reached today the level of a routinely applied treatment of certain forms of cancer.[18h, 22] The PDT took its beginning with the phototherapy, which uses light alone for the therapeutic purposes. However, in the most cases, endogenous sensitizers participate in this process. Therefore, the photodynamic processes already occurred in the Phototherapy. With this treatment and the use of bare solar light diseases like vitiligo, psoriasis, rickets, skin cancer and psychosis were treated.[23] The phototherapy dated back 3000 years ago and was already known and used by the Egyptians, Indians, and Chinese. [23] This culminates 1903, where NIELS FINSEN was awarded with the Nobel Prize in the treatment of skin tuberculosis by using light from the carbon arc.[24]

In PDT an active agent, the photosensitizer (PS), and light are combined to induce a toxic effect at the site of action. The history of PDT began in 1900 when OSCAR RAAB investigated the toxic effect of acridine against paramecia. He found out that the paramecia survived longer in the absence of natural light. The results were published wondering if light may play a crucial role in this process – leading to the discovery of the photodynamic action.[25] Typical for this time the results were first published by the supervisor[25a] and later from the student.[25b] The first trials to use the PDT against tumors and other skin diseases were performed between 1903 and 1905 by the group of VON TAPPEINER.[26] They used different dyes in their test like eosin, fluorescein, sodium dichloroanthracene disulfonate and "Grubler's Magdalene red".

Classic methods of cancer treatment like chemotherapy often damage healthy tissue. Compared to these PDT is a mild alternative approach.[18h, 22] Requirements for this approach are a light source with a specific wavelength, the photodynamic active substance (the PS) and oxygen which is already present in all living cells as well in tumor tissue.

For a typical PDT treatment, the patient gets an injection of the PS. After a given time, the PS should accumulate in the tumor tissue where it stays inactive. Irradiation of the tumor tissue with light leads to the formation of reactive oxygen species (most prominently singlet oxygen) through a sequence of photophysical processes. The formed singlet oxygen and other reactive oxygen species damage membrane components in the tumor cells leading to necrosis, apoptosis, or both.[22u, 27] Two different types of photodynamic reactions are known: On the one hand type I photosensitization processes with charge transfers to the nearby oxygen or other substrates. On the contrary the type II with an energy transfer from the PS to triplet oxygen leading to oxygen in the singlet state.[28]

More specifically for type II photosensitization, the PS is excited from the ground state S_0 to the excited singlet state S_1 by a linear light absorption shown in the modified JABLONSKI diagram in Figure 3.

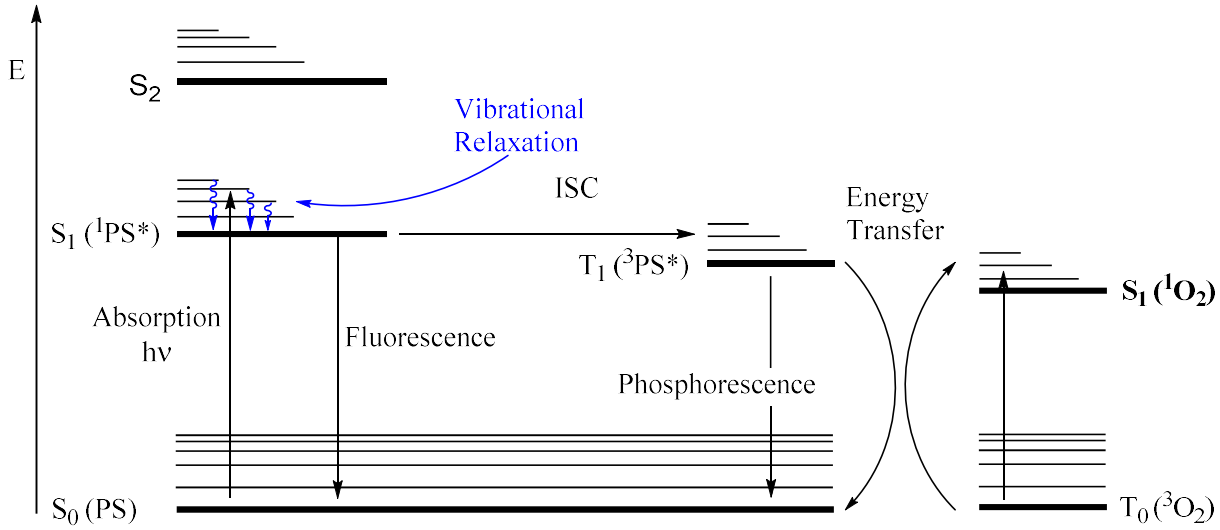


Figure 3 Mechanism of the generation of singlet oxygen demonstrated with a modified JABLONSKI diagram.

In the next step, the PS can return to the ground state S_0 by emitting fluorescence or via intersystem crossing (spontaneous electron spin conversion) the triplet state T_1 of the PS can be formed. The triplet state T_1 can return to the ground state S_0 by emitting phosphorescence or can transfer its energy to a nearby oxygen molecule which is the desired process for PDT (type II mechanism). By this energy transfer from the PS, triplet oxygen is excited to singlet oxygen, which is the most significant cytotoxic species. The lifetime of singlet oxygen in a cellular medium is rather limited: From 100 ns in the lipid region of the membranes up to 250 ns in the cytoplasm.[22u] For singlet oxygen, this leads to a very short range of action of about 45 nm in the cellular medium. Therefore, the cytotoxic effect of singlet oxygen is limited to the immediate environment of its formation. For the type I mechanism the triplet state T_1 can also result in anionic or cationic radical species by charge transfer. The relative distribution of both types depends on the oxygen concentration at the site of formation.

1.1.3.1 Photosensitizers (PS)

The PS is one of the essential components of PDT, if not the most important one. Different requirements for an ideal PS have been named:

1. A high quantum yield of singlet oxygen is needed, as it is the most significant cytotoxic species in PDT.

2. To reach deeper parts of the tissue a strong absorption coefficient in the range of 700 to 800 nm is desirable.[29] Porphyrin-based PSs with the SORET-band have a strong absorption at about 400 nm and weaker Q-bands between 600 and 800 nm. For PDT, only the last Q-band is of particular interest. Different types and positions of the peripheral substituents enable to change the spectral position of these bands.

3. In the absence of light, the PS should not exhibit any toxicity (no dark toxicity (DT)).

4. Most desirable for PDT treatment is a fast and selective accumulation of the PS in the tumor tissue.

5. The PS should show a high stability and solubility in the solvent (preferably water solubility) for injection.

Several PS based on the tetrapyrrolic structures are described in literature: e.g. chlorins,[22h-j, 22m, 22p] porphyrins,[18h, 18j, 22d, 22k, 22n, 22o] phthalocyanines[22c, 22g, 22l, 22q] and corroles[22a, 22b, 22f]. By choosing porphyrins as the PS, these tetrapyrrolic systems may also be transformed into their corresponding chlorin analogs, which show even more potent properties as PS.[6c, 6f, 22m, 30] One example for an important clinically applied PS is the chlorin Temoporfin shown in Figure 4, used in the treatment of head and neck squamous cell carcinomas. This PS is based on a tetrapyrrolic chlorin system and is the active substance in the medicinal product Foscan®.

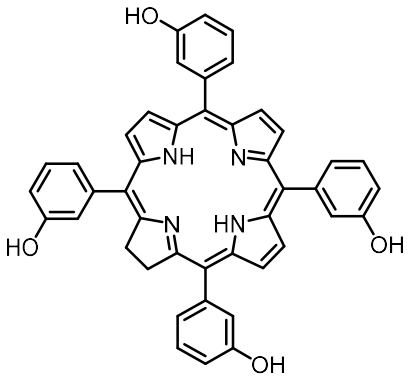


Figure 4 Structure of Temoporfin.

It can be synthesized in only a few steps. Temoporfin with its amphiphilic behavior accumulates in membrane structures in the cells. It is not soluble in water and is currently administered in an ethanol/propylene glycol solution. Alternative formulations based on liposomes are under development.[31] To increase the bioavailability and to shorten the time for accumulation in the tumor tissue it may be feasible to use carrier systems which can transport such lipophilic substances and make the combination with targeting molecules easier. One of these transport systems could be water-soluble polymers, such as hyperbranched polyglycerols (hPG).

In general, the uptake of drugs can broadly be classified as active and passive transport. For the active mechanism targeting groups are required, which are often expressed on the desired target substrate. On the other hand, the passive mechanism arises from non-specific interactions. The lack of selective targeting is an issue which all accredited PS have in common. The activity of these PS stems from the non-specific uptake promoted by the increased metabolic uptake of the cancer cells. Photosensitivity is one side-effect which occurs if the PS is distributed all over the body and accumulates in healthy tissue (e.g. the skin) as well as in the tumor tissue. A cadre of glycosylated porphyrins is of major concern for the use in PDT and other fields, as they add the advantage of cellular recognition and could make PDT more specific and efficient.[32] To fulfill their energy requirement cancer cells have an increased uptake of glucose compared to normal cells, which provides metabolic energy and maintains their proliferation.[33] Glucose transporter proteins are over-expressed in different cancer cells.[33b, 34] Another point for adding glycosides to tetrapyrrolic systems, is the increased hydrophilicity, which may be favorable also for other biological applications.

1.1.3.2 Antimicrobial photodynamic therapy

Up to the middle of the last century, the antimicrobial photodynamic therapy (aPDT) was overlooked due to the development of antibiotics. Penicillin was the first of them, found by ALEXANDER FLEMING in 1928.[35] The discovery of penicillin was the start of the Golden Age of antibiotics. Over ten years of work were required by H. FLOREY, and E. CHAIN[36] before mass-delivered penicillin made its clinical presentation in the 1940s;[37] at this time all strains of *Staphylococcus aureus* were vulnerable. With this success at beginning different antibiotics, like chloramphenicol,[38] streptomycin,[39] and tetracycline[40] were discovered soon after, and by the 1950s, these and other antibiotic agents were clinically utilized. Unfortunately, in 1942 only two years after the discovery of penicillin the first resistance against this antibiotic occurred.[41] In the 1950s, already half of all *S. aureus* strains were immune against penicillin. Therefore, different antibiotics followed as a replacement, e.g. methicillin (BEECHAM 1959). This development eventually led to strains of *S. aureus*, *Staphylococcus epidermis*, and *Staphylococcus hemolyticus* becoming resistant against all antibiotics based on the beta-lactam structure.[42] With these resistance problems, only vancomycin[43] based on a different glycopeptide structure stayed as the effective antibiotic agent of the last resort against gram-positive bacteria. However, again in 1996, the first methicillin-resistant *S. aureus* (MRSA) strain with a reduced susceptibility to vancomycin was isolated.[44] This cat-and-mouse game of MRSA makes other options to the standard antimicrobial treatment relevant.

A newer approach is the use of aPDT as a possible alternative treatment for (localized) microbial infections which has shown promising results in killing pathogenic microorganisms.[45] The mechanism of the inactivation of microorganisms is similar to the PDT of tumor cells in that the PS is activated with light which leads to the generation of singlet oxygen in the near environment, which in turn destroys the bacterial membranes. Studies have demonstrated that there is a difference in the aPDT effectiveness against gram-positive or gram-negative bacteria.[45c, 46] Anionic or neutral PS are more effective against gram-positive bacteria, because of their better binding properties. On the other hand, gram-negative bacteria are typically highly resistant against aPDT.[46b, 46d, 47] This may be explained by the different outer membrane structure of these two types (Figure 5).

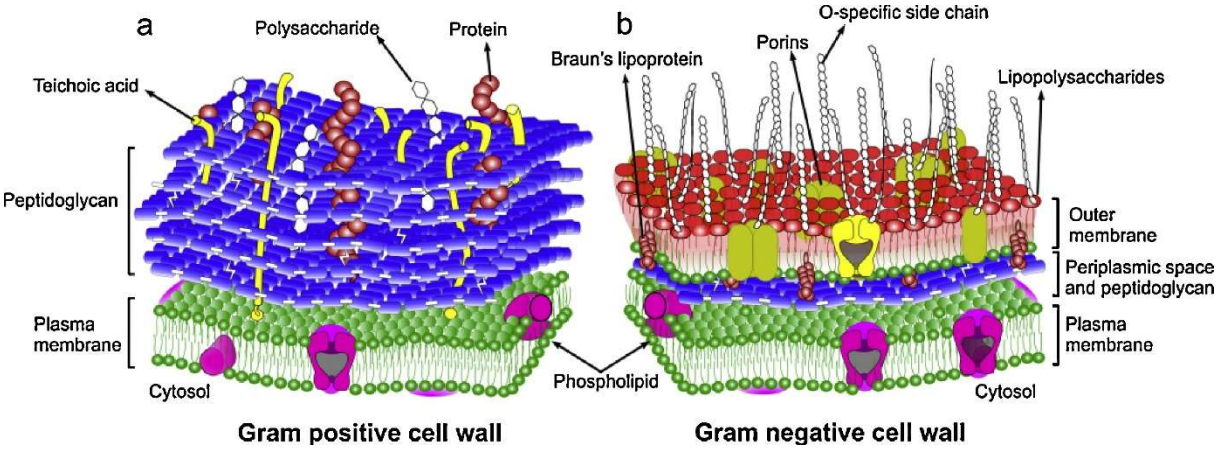


Figure 5 Schematic representations[48] of the structure of the cell membrane of gram-positive (a) and gram-negative (b) bacteria. The cell wall of gram-positive bacteria is a 15–80 nm thick peptidoglycan layer which consists of up to 100 interconnecting layers. Teichoic acids are merged into these layers, and part of them, called lipoteichoic acid have a lipid attached. The cell wall of Gram-negative bacteria comprises an outer lipid bilayer (7 nm), and a thin, middle wall constituted of two to three layers of peptidoglycan (2–3 nm thick). The outer membrane consists of phospholipids, lipid proteins, lipopolysaccharides and proteins (porins).[42]

For the gram-positive bacteria, the membrane consists of a more or less porous peptidoglycan wall with a thickness of 40 to 80 nm.[42] Compared to gram-negative bacteria the thickness of the layer is lower but does not have a significant influence as a permeability barrier because of its porosity. The penetration of antimicrobial peptides, glycopeptides, and polysaccharides with a molecular weight between 30 and 57 kDa has been shown.[49] Resistance against Antibiotics only occurs with a mechanism like active discharge, changes in the target membrane or inactivation.[50]

Gram-negative bacteria have an additional layer outside the peptidoglycan wall composed of highly negative charged lipopolysaccharides, lipoproteins, and proteins for the porin function. Only small hydrophilic molecules under 600 Da can diffuse through this layer via the porins,

known as aqueous channel-forming porins,[51] which makes gram-negative bacteria resistant to most of the common PS tested for aPDT.

During the last 20 years considerable improvement has been achieved in the field of the aPDT: An advantage is the short overall application time of aPDT. The PS is taken up fast by the bacteria within a few minutes followed by a mild irradiation treatment (e.g. 40 to 100 mW cm^{-2}). This treatment diminishes the bacteria population effectively.[42]

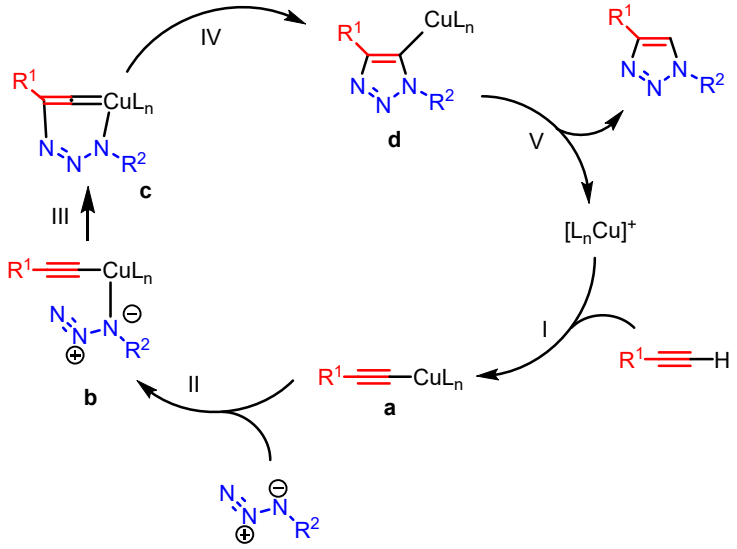
A broad range of PSs has been reported for use in aPDT concerning the photodynamic inactivation of antibiotic resistant bacteria.[42, 45b, 45d, 45h] Examples for the medical application of aPDT include e.g. dental usage,[45b, 45c, 45f, 45g, 45i] burn wounds,[45d, 45h] and acne.[42, 45e] An interesting approach is the recently reported combination of aPDT with nanoparticles to increase the effectiveness of this treatment. So far, four different known combinations of nanoparticles and PS have been reported:

- PS encapsulated into polymeric nanoparticles (e.g. liposomes,[52] polylactic-co-glycolic acid (PLGA),[53] or cyclodextrins[54]) or photosensitizer can be loaded on to nanomaterials (graphene[55])
- PS covalently bound to the surface of nanoparticles (e.g. rose bengal bound to glass[56] or polystyrene,[57] toluidine blue linked to gold nanoparticles,[58] or porphyrin linked to carbon nanotubes[46d])
- PS-accompanying nanoparticles, where the particles are too big and cannot penetrate the bacteria cell wall. Only physical/chemical interactions with the microbial surrounding occur. The nanoparticle, mostly gold nanoparticles have been used in combination with PS (e.g. toluidine blue or methylene blue in solution or encapsulated in polymers[59]).
- nanoparticles as PS themselves (e.g. TiO_2[60], fullerenes[61], black phosphorous nanosheets[62], MoS_2[63])

Active nanoparticles are nanoparticles which act themselves as PS (as in the last case) whereas the other three cases relate to passive nanoparticles.[64]

Their activity is independent of the resistance of bacteria, which makes aPDT a promising option for the treatment of multi-resistant bacteria (e.g. MRSA). Besides, the mode of action allows aPDT to treat bacteria without inducing new resistances against the PS.

1.1.4 *Cu-catalyzed azide-alkyne cycloaddition*

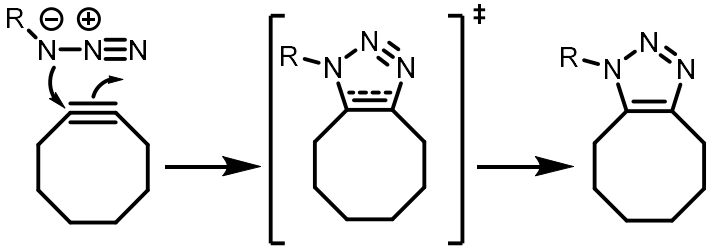


Scheme 4 Proposed schematic representation of the mechanism for the formation of the triazole by SHARPLESS et al.[65]

HUISGEN first reported the 1,3-dipolar cycloaddition between an azide and an alkyne forming the triazole bond, known as the HUISGEN 1,3-dipolar cycloaddition which is today one of the most investigated organic reactions.[66] A drawback of this reaction is the slow reaction kinetics. Therefore, a versatile, easy and fast variant of this reaction is the copper(I)-catalyzed 1,3-dipolar cycloaddition (CuAAC) first reported by groups of MELDAL and SHARPLESS independently.[65, 67] SHARPLESS et al. defined the CuAAC as one of the most perfect reactions in organic chemistry and classified it as the today well-known click chemistry. The requirements of click reactions are high yields, no or only harmless by-products, proceeding at rt, readily available starting materials, and non-toxic solvent preferably water.[68] The CuAAC reaction is widely used in organic synthesis,[69] polymer chemistry,[70] materials chemistry,[71] and medicinal chemistry.[72] The suggested mechanism is shown in Scheme 4.[65] In the first step **I**, the formation of copper(I) acetylide **a** is depicted. Afterward, the azide adds to the copper(I) **II** and builds the intermediate **b**. In step **III** a rigid six-membered ring **c** is formed. This is followed by step **IV** which generates the triazole with the copper(I) ligand **d**. At the end in step **V**, copper(I) is regenerated as the catalyst and the final triazole product is formed.

1.1.5 *Strain-promoted azide-alkyne cycloaddition*

The strain-promoted azide-alkyne cycloaddition (SPAAC) is a reaction which improves the existing conditions of the CuAAC and does not need any additional catalyst or transition metals.[73] KREBS and co-workers described 1961 the cycloaddition of phenyl azide and cyclooctyne as a highly exothermic and fast reaction which proceeds at rt.[74] The alkyne functionality in the eight-membered ring leads to an increased ring strain and a reaction rate enhancement compared to linear alkynes. With these properties, the activation energy of the cycloaddition is considerably reduced, and the reaction proceeds accelerated without any addition of a catalyst. In 2006 the idea of the SPAAC (Scheme 5) was newly adopted from BERTOZZI and co-workers which realized its great potential and investigated cyclooctynes and their reaction rates.[75] They demonstrated that a modified cyclooctyne with two fluorine atoms next to the alkyne functionality (DIFO) showed an about 15 times higher reaction compared to the STAUDINGER reaction. With this method, the research group was even able to label cell membranes in vitro[76] and also in vivo.[77] Nonetheless, the tedious synthetic effort for the preparation of cyclooctynes limited the broad biomedical application of the SPAAC. DIFO as an example for the second generation cyclooctynes needs eight synthetic steps.[78] The number of steps was reduced to four by VAN DELFT and co-workers, and they also increased the reactivity of the cyclooctyne toward the azide with an additional cyclopropane ring connected to the cyclooctyne. This leads to an increased ring strain and therefore a higher reaction rate.[79] For the present work, the cyclooctyne functionality is a decisive improvement compared to the CuAAC because it avoids the additional use of any (toxic) catalysts/substances. Therefore, there is no need for complexation of metals into the porphyrin before the cycloaddition step.



Scheme 5 Schematic representation of the mechanism for the SPAAC.[75]

1.1.6 *Polyglycerol in biomedical applications*

Conjugates of polymers and drug molecules for tumor-targeted delivery were introduced in literature around 60 years ago, using passive tumor targeting like the EPR-effect (see below) or active targeting by adding specific ligands.[80] Several examples of polymer-chemotherapeutics (e.g. doxorubicin, paclitaxel and, camptothecins) conjugates have been reported in clinical trials in literature.[81]

There are different requirements for the use of polymers in biomedical applications, such as non-toxicity, non-immunogenicity, biodegradability, and water solubility.[82] The research group of BROOKS has shown in studies that polyglycerols are non-toxic[83]and non-immunogenic. Due to their abundant hydroxyl groups, hPGs are highly water soluble and can be functionalized by various methods.[84] In the 1980s, VANDENBERG reported the synthesis of relatively monodisperse and highly branched PG based on an anionic ring opening polymerization of glycidol. With this method hPGs, with a molecular weight of a few thousand daltons could be obtained.[85] Later the research group of BROOKS increased the molecular weight of hPG up to 1 MDa with an average diameter of 10 nm by using an emulsion type polymerization with bulk heterophases and an emulsifying non-solvent.[86] Another method is the polymerization in a mini-emulsion.[87] Several in vitro and in vivo biocompatibility tests of hPGs with molecular weights around 6 kDa have shown better properties compared to poly(ethylene glycol) (PEG) and other clinical applied polymers.[83d, 88] Similarly excellent properties were found for hPG with higher molecular weights up to 800 kDa.[83a, 83e]

1.1.6.1 Hydrogels and Nanogels

Hydrogels consist of polymer network chains and are highly water absorbent with a swelling behavior. The water molecules are absorbed and integrated into the hydrophilic parts of the polymer chain with functionalities such as -OH, -CONH, -$CONH_2$ and SO_3H, but due to the critical crosslinks they cannot dissolve in water.[89] Hydrogels show a low surface tension and high water content which exhibits similar characteristics to body tissues, leading to a high biocompatibility.[90] These properties make hydrogels a highly relevant research field for biomedical and pharmaceutical applications.[91] One example is the field of drug delivery, where the drug can be encapsulated into the hydrogel and afterward be released by degradation or increased swelling.

Hydrogels can consist out of natural or synthetic polymers. Examples of naturally occurring polymers are alginate,[92] chitosan,[93] collagen,[94] hyaluronic acid,[95] and fibrin.[96] These types

of polymer, however, are only available as mixtures which can interact in an uncontrolled way with biomolecules and cells. Besides, the functionalization of natural polymers is hard to control, and also the degradation rate cannot be controlled.[97] Therefore, synthetic hydrogels have been developed, e.g. PEG,[98] poly(vinyl alcohol) (PVA),[99] hPG,[100] and polymerization of different methacrylates.[101]

Hydrogels with an average diameter in the sub-micrometer range are called nanogels. With this size, they are ideal candidates for pharmaceutical applications due to their ability to circulate in the blood stream and to transport the active substance to the desired target.

Several active agents (e.g. anticancer drugs, imaging agents, and oligonucleotides) show a low solubility in blood, a low stability, higher toxicity, and lower selectivity and can, therefore, not easily be applied and administered.[102] With nanogels, it is possible to circumvent these drawbacks and link the active substance covalently to the polymer or to encapsulate it into the polymeric matrix. Ideally, for a secure biomedical application, the nanogel is made of polymers which are non-toxic, non-immunogenic, and degrade under defined conditions.[82]

One example which benefits from the properties of the nanogel is described in the following passage. The passive accumulation of macromolecules and nanoparticles in solid tumors was investigated by MAEDA et al.[103] and JAIN et al.[104] Small drug molecules can diffuse in tumor cell through endothelial cell layers, but as well into the healthy ones. There is no selectivity between healthy and tumor cells which leads to undesired side-effects and a lower concentration of the drug has to be chosen. Macromolecules, on the other hand, are only capable of diffusing through the porous defective vascular structure of the tumor tissue. Another difference and a possible advantage is the deficient lymphatic system which aggravates the removal of the active substance. These factors lead to an easier diffusion (permeation) of macromolecules, liposomes, and nanoparticles into the tumor tissue compared to the healthy tissue. Moreover, the removal of the diffused particles is hindered by the insufficient lymphatic system (retention). Therefore, this passive accumulation in tumor tissue is called the enhanced permeability and retention (EPR) effect (shown in Figure 6).[81e, 103, 105]

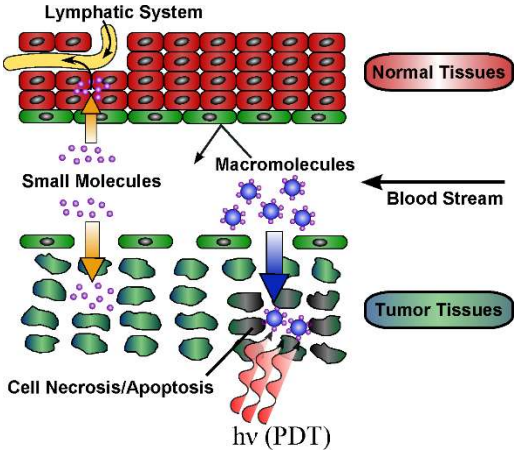


Figure 6 Schematic representation of the EPR-effect in combination with PDT.

1.1.7 *Cleavable Linkers*

Tumor tissues have several specific characteristics, such as angiogenesis,[106] overexpression of enzymes and the EPR effect (Figure 6),[81e, 103, 105] which are different from the healthy tissue. This particular environment in the tumor opens different possibilities for drug delivery systems improving therapeutic effectiveness. So far, various drug delivery systems have been described and partially also applied for clinical use.[81a, 81c, 81d, 81f]

In the application of antitumor drugs, various drawbacks must be dealt with, e.g. a low solubility of the drug in water, improvable selectivity, and low uptake and internalization into the cell. Given these challenges tumor-specific delivery systems (such as dendrimers, inorganic nanoparticles, liposomes, micelles, nanogels, or polymer nanoparticles) which can release the drug under certain stimuli (e.g. enzymes, pH changes, reductive environment) have been described in the literature.[81c, 81d, 81f, 84d, 107] Nevertheless, the pure physical loading of a drug onto a delivery system leads to certain problems, e.g. instability during storage and application, cytotoxicity of the functional material, low embedding strength with fast drug release, and low drug encapsulation efficacy.[108] For an efficient and controlled delivery covalently bound drug molecules have been used, which are connected to the carrier, e.g. the polymer, to form polymer-drug-conjugates avoiding the problem of burst release. Covalent conjugation to a water-soluble polymer combines the advantage of high loading capacity and a longer circulation time with a reduced unwanted premature release compared to pure physical embedment.[108] Furthermore, these systems can make use of the EPR-effect delivering the drug selectively to the tumor site.[81e, 103b, 105]

Cleavable linkers connect different molecules or substrates and are capable of releasing the load under certain conditions. Linkers used in nanocarriers can serve various functions, e.g. act as linkers that fulfill molecular co-delivery preconditions or specific cleavage under environmental stimuli. Several effective linker types (e.g. acetal,[73f, 73g, 84d, 107c, 107h] disulfide,[107i, 107l, 109] hydrazone,[107d-f, 107k, 107o, 110] peptide,[107a, 107f, 107g, 107n, 107p, 107r, 111] and azo[81c, 81d, 107r, 107u, 108]) are known for targeted release of the active substance from the drug delivery system. These linkers should preferably remain stable under normal physiological conditions and should only be cleaved under particular internal (e.g. reductive environment, pH changes, and specific enzymes) or external stimuli (e.g. light, magnetic field, temperature, and ultrasound). The combination of bioactive substance and delivery system via a cleavable linker allows for the safe transportation inside cells and even to different intracellular compartments where it can be released under specific conditions.

For the reductive environment glutathione (GSH), a tripeptide consisting of glutamate, cysteine, and glycine, is an available thiol containing molecule in the cytoplasm.[112] The sulfhydryl functionality of the cysteine part is a strong reducing agent and a strong nucleophile which can react with several toxic substances. The GSH concentration depends on the type of tissue, for breast, head and neck, lung, and ovarian tumors the level is elevated however it is decreased in brain and liver tumors compared to the healthy tissues.[112d] Furthermore, the concentration of GSH is considerably higher within the cell (~100–1000 fold[113]) compared to the extracellular compartment and its counterpart GSSG.[112-114] Due to these properties, the reductively mediated release mechanism has been investigated for tumor targeting with different bioactive molecules. The disulfide linker,[107i, 107l, 109] as one of the most important and applied redox-sensitive examples, is easily cleaved reductively by high concentrations of GSH. The redox potential between the intracellular compartment (reductive) and the blood stream (mildly oxidative) allows the use of the disulfide bridge.[107s, 115] As a consequence of the higher GSH concentration in tumor cells, loaded substance should be mainly released at the tumor site than in the blood stream or healthy cells. Furthermore, a reductive environment has been evidenced in intracellular endosomes and lysosomes.[116] Several disulfide-containing nanocarriers for drug delivery, such as capsules, micelles, nanogels, nanoparticles, and polymersomes have been described.[117] In conjugates, the disulfide moiety can be located in the main chain,[118] side chain,[119] or inside a dendritic structure[120] allowing the delivery of various active biomolecules [e.g. antisense oligonucleotides,[109i, 109p] anticancer drugs,[107i, 109a, 109e, 109l, 109m] plasmid deoxyribonucleic acid (DNA),[109b, 109f, 109h, 109n, 109o] imaging molecules, peptide nucleic acids,[109c] proteins,[121] toxins,[109j, 109k] and small interfering ribonucleic acid (siRNA)[109g]]. Possible drug delivery systems are e.g. organic,[107l, 107u, 122] and inorganic nanoparticles,[123] nanotubes,[124] and polymeric micelles.[107s, 125]

Glycolysis is increased in extracellular environments after the upregulation by hypoxia. This leads to a lower pH in tumor tissue (pH 6.5 to 7.2), endosomes (pH 5.0 to 6.5) and lysosomes (pH 4.5 to 5.0) compared to healthy tissue and the blood stream (pH 7.4).[107s, 126] This pH gradient is the basis for the development of drug release systems which rely on the pH difference at the active site. There, the drug should be uptaken into endosomes and lysosomes via an endocytic way and leads to the release of the active agent. Therefore, hydrolysis under acidic pH is very promising for controlled drug delivery systems. Different acid-sensitive cleavable linkers have been investigated including acetal linkers,[73f, 73g, 84d, 107c, 107h] *cis*-aconityl

linkers,[107b, 107d, 127] hydrazone linkage,[107d-f, 107k, 107o, 110, 128] imine,[107s, 107u, 128-129] orthoester,[128] and polyketal (Figure 7).[128] Acetals are employed as delivery systems for proteins,[107c, 107h] cells[73f, 73g] and enzymes.[84d]

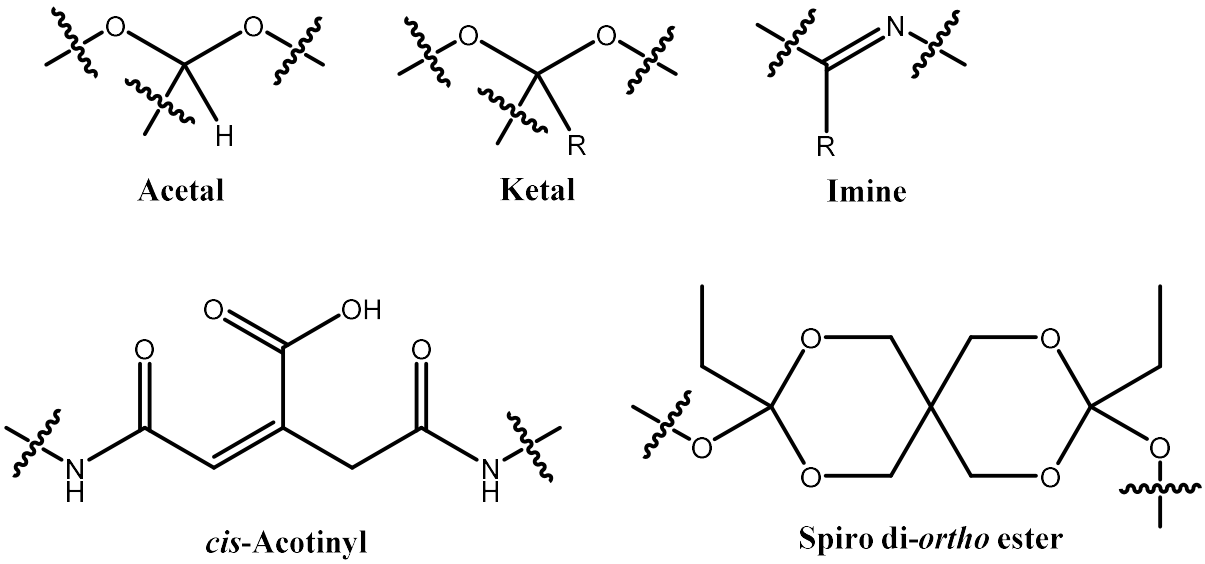


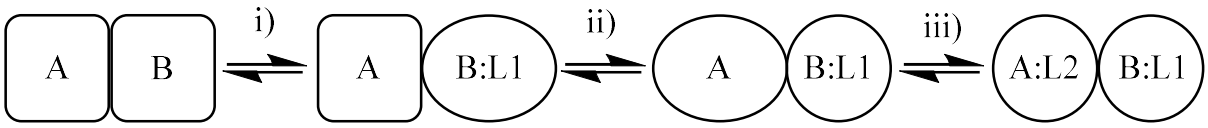
Figure 7 Examples of acid sensitive bonds.

1.1.8 *Multivalency*

Valence is defined as the maximum number of the same interaction between two sites. Multivalency describes the interaction between two or more complementary binding sites in a host-guest system. In nature, multivalency is a fundamental principle, which uses strong but simultaneously reversible interactions. One vivid example is the burr in nature on the one hand and the Velcro® as artificially made by men on the other hand (Figure 8).

Figure 8 Images of the burr in nature (left image) and the artificial human-made hook and loop fastener Velcro® (right image). This principle can also be assigned to the molecular level. (Right image: by Natural Philo (Own work) [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)], via Wikimedia Commons)

Multiple hooks on one side and loops on the other one become entangled. The strength of one of these connections itself is considerably lower compared to several interactions. The bigger the surface area of these two parts is, the stronger is the binding. One striking example is the hook and loop fastener Metaklett, which can hold a load up to 35 t/m^2. Another important property of multivalent interactions compared to covalent interactions is the reversibility. Velcro® can easily be opened again if the hooks and loops are detached sequentially.



Scheme 6 Schematic representation of cooperative ligand binding. i) Conformational change in B caused by the binding of a ligand (L1). ii) Alteration of the binding subunit A by the conformational change in B. iii) The second ligand (L2) binds more or less readily to the subunit A.

In literature cooperativity and chelate effect are sometimes used in the same context for multivalency in an inconsistent manner due to outmoded terminology.[130] Cooperativity (shown in Scheme 6) does not require multivalency, and it can, for example, be described by the binding of a guest at the host's binding site B, which influences the second binding step at

the location A of the same host. Positive cooperativity ($-\Delta G_{avg}^{poly} > -\Delta G^{mono}$) makes the binding easier for the second molecule, which means the GIBBS binding energy of the second molecule is more negative compared to the first one. Whereas negative cooperativity ($-\Delta G_{avg}^{poly} < -\Delta G^{mono}$) makes the second binding more difficult, which means the Gibbs binding energy of the second molecule is more positive compared to the first one.[131] A prominent example of a positive, cooperative system is the binding of oxygen to hemoglobin.[132] In this positive cooperative system, the binding of the first oxygen molecule facilitates the binding of a second molecule.

The chelate effect on the other hand also refers to the increased binding of ligands to multivalent hosts. This effect is long known and was introduced by SCHWARZENBACH in 1952.[133] However, in this case, it is mainly used for small molecules (primarily metals and ions) binding to multivalent hosts (often cyclic). The chelate effect should be classified as a subclass under the multivalency effect. One classic example is e.g. ethylene diamine or 2,2'-bipyridine as the bidentate ligand which forms more stable complexes with metals, compared to the corresponding monodentate ligands e.g. ammonia or pyridine.

The multivalency interaction can also be observed at the molecular level, where specific binding molecular units (ligands) depict the hooks and the binding pockets on the other side (receptor) depict the loops. These units can be small molecules, oligosaccharides, proteins, nucleic acids, lipids or aggregates of these molecules; membranes or organelles like viruses, bacteria or cells.[84c, 131, 134] In biological systems the multivalent interactions between cells and other classes like viruses, bacteria or molecules are for major interest for medical research. Multivalency occurs in many biological fields, and the mechanism can be both, agonistic and antagonistic.[135]

One example are lectins which are well studied and therefore a good model for the understanding in multivalent interactions.[121, 136] The interaction on a biological surface with its multivalent interactions of lectins with glycosides or glycans is of fundamental importance. Lectins are proteins with a defined recognition structure for glycosides/glycans on the surface of viruses, bacteria, plant cells, and animal cells. An important field of research is the invention of inhibitors with an affinity to lectins. They can help to understand the function of defined carbohydrate structures (as competitive binding partners), leading to new options for a possible pharmacological use (as a viral or bacterial inhibitor). Another reason for preparing such multivalent systems is to achieve very high binding to the target, thus making drug and diagnostics delivery more efficient. The phenomenon of the multivalent glycan-lectin-

interaction well-known in glycoside research is also described as glycoside cluster effect.[137] Although the presentation of ligands on linear polymers (e.g. sialic acid functionalized polyacrylamides as virus inhibitors) or spherical (e.g. sialic acid functionalized hPG) and planar surfaces (e.g. metal surface functionalized with organic molecules) is statistically distributed and not rationally designed for the corresponding receptor position, a significant increase of the binding strength has been observed.[134f, 134g]

Multivalency can be defined as multiple, supramolecular binding modules in biological- as well as in synthetic-systems. Research in supramolecular chemistry can significantly contribute to fundamental understanding of the underlying principles. The number of ligands and receptors is known and can, therefore, be controlled in synthetic systems, which makes a quantitative thermochemical analysis possible. The synthetic systems can be systematically varied with appropriate effort. With this method, host-guest systems can be investigated in detail, where not only the monovalent and multivalent case is relevant, but also the in between intermediate interactions are of interest. Another part is the spacer chemistry which connects the two binding sites: The influence of different varied spacers and their positive and negative interactions with the receptor itself.[134f, 134g]

Multivalency is therefore not only of interest in medical research[131, 134a] and biochemistry,[134b, 134c] but as well for the synthesis of defined functional molecules in supramolecular chemistry[130a, 130b, 138] and material science.[139]

1.2 *Scientific goals*

As illustrated in Figure 9 the main scientific goals for the synthesis of porphyrin-hPG-conjugates are the A) synthesis of A_3B alkyne zinc-porphyrins and the connection to hPG and expanding the preparation of the A_3B porphyrin and their connection to hPG to connections with transition metal-free chemistry, B) adding mannose as a targeting group for aPDT and investigating the multivalency effect of the conjugates in vitro by using systematically varied mannose loadings with a fixed hPG-core size or C) investigate the multivalency effect with systematically varied hPG-core sizes (up to nanogel size), and D) controlled release of the porphyrin by linker cleavage and release. In this thesis, the author focused on these points to improve the properties of free porphyrin dyes and investigate the multivalency effect by using porphyrin-hPG-conjugates with mannose targeting groups and conjugates for the controlled release of the porphyrin.

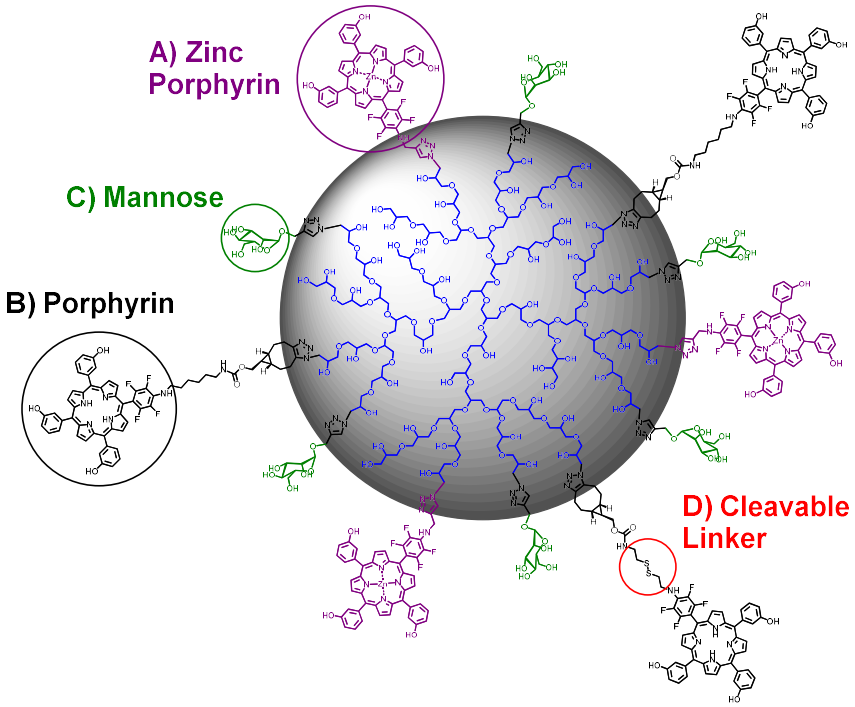


Figure 9 Schematic representation of the four main scientific goals for the synthesis of porphyrin-hPG-conjugates (A) Preparation of A_3B-type zinc-porphyrins with alkyne linkers for the connection to other molecules and substrates, in particular with hPG. (B) Expanding the synthesis of metal-free A_3B-type porphyrins with different linkers, allowing the transition metal-free connection to hPG. (C) Adding targeting groups to the porphyrin-hPG-conjugates and investigating their multivalency effect in aPDT. (D) After the conjugate has reached its biomedical target a controlled release is desired. Cleavage of the linker between the porphyrin and hPG, followed by the release of the covalently bound porphyrin is a promising strategy.

A) 5,10,15-Tris(3-hydroxyphenyl)-20-(pentafluorophenyl)porphyrin[19a, 140] (and its zinc complex) was selected as the basic structure for the synthesis of the porphyrin-hPGs. The structure of this porphyrin with its 3-hydroxyphenyl groups is inspired by the clinical approved PS Temoporfin (tetrakis(3-hydroxyphenyl)chlorin, *m*THPC).[141] Propargylamine

was chosen for the functionalization of the pentafluorophenyl moiety which results in the alkyne-substituted porphyrin and allows the following CuAAC as a versatile, fast and easy reaction. hPG is an ideal drug carrier for medical applications.[83b-e, 84b-d, 111a, 142] This allows the synthesis of zinc-porphyrin-hPG-conjugates as first examples of conjugates combining porphyrins and the hPG carrier system.

To expand the connection of the porphyrin to hPG, a toolset with diverse reactive groups (alkenyl-, amino-, cyclooctyne-, hydroxyl-, and maleimido-groups) by reactions of the pentafluorophenyl-substituted A_3B-porphyrins with amines will be developed. In particular, these groups allow the further functionalization and conjugation to different substrates or materials under transition metal-free conditions. This prevents the complexation of metals into the porphyrins and permits the use of metal-free porphyrins in the conjugation step. The conjugation of the porphyrin (with cyclooctyne functionality) with hPG will be shown without any addition of a catalyst.

B) Several applications of nanoparticles in addition with PDT against tumors have been investigated in the literature,[143] though only little research has been done in the area of aPDT in the combination of nanoparticles.[18j, 46a, 46d, 52a, 52c, 52d, 54, 144] Such research could have a significant medical impact as the development of antibiotic multiresistant bacteria is a major health problem, which may be solved with the help of aPDT and appropriate drug carrier systems.[42, 45b, 45d, 45h] The use of porphyrins in combination with hPG as carrier system is, therefore, an important and interesting field for medical development.

   Another part is the investigation of a combination of three approaches – aPDT, nanoparticles in the PDT, and the multivalency effect. The functionalization of hPG with zinc porphyrins as PS molecules and mannose groups is used for the investigation of the multivalent interaction. These mannose units are capable of interacting with mannose receptors on the bacterial membrane.[145] The multivalency effect will be assessed by measuring the antibacterial phototoxicity of such conjugates against *S. aureus*, employing conjugates with fixed polymer hPG size and a fixed porphyrin loading with a systematically varied mannose loading or

C) Investigation of the multivalency effect by functionalization of hPG with zinc porphyrins (see B)) by using different hPG-core sizes.

D) One of the biggest problems concerning drug carrier systems is the specific release of the substance from the carrier at the active site. The focus in this part of the thesis is the synthesis of substituted porphyrins with cleavable linkers (namely disulfide and acetal) and the conjugation to hPG allowing the release of active substance. To the best of our

knowledge, only little work has been done in the preparation of porphyrins with cleavable linkers that permit the release of a carrier system. The release of a drug carrier system is of interest in many biomedical applications.[107s, 146] To achieve the release of porphyrins it is necessary to introduce cleavable linker bonds.

1.3 *Publications*

1.3.1 *A toolset of functionalized porphyrins with different linker strategies for application in bioconjugation*

M. H. Staegemann, S. Gräfe, R. Haag and A. Wiehe
Org. Biomol. Chem. **2016**, *14*, 9114–9123.
DOI: https://doi.org/10.1039/C6OB01551D
http://pubs.rsc.org/en/content/articlelanding/2016/ob/c6ob01551d#!divAbstract

Author contributions

M. H. Staegemann: Porphyrin and porphyrin-hPG-conjugate synthesis, hPG functionalization, characterization, preparation of the manuscript.
S. Gräfe: Cellular assays
R. Haag: Supervision, correction of the manuscript, scientific discussion of the data.
A. Wiehe: Supervision, correction of the manuscript, scientific discussion of the data.

Abstract

The reaction of amines with pentafluorophenyl-substituted A_3B-porphyrins has been used to obtain different useful reactive groups for further functionalization and/or conjugation of these porphyrins to other substrates or materials. Porphyrins with alkenyl, alkynyl, amino, azido, epoxide, hydroxyl, and maleimido group moieties have thus been synthesized. For the first time such functionalized porphyrins have been conjugated to hPG as a biocompatible carrier system for PDT using the CuAAC. The photocytotoxicity of selected porphyrins as well as of the porphyrin-hPG-conjugates has been assessed in cellular assays with human epidermoid carcinoma A-253 and squamous carcinoma CAL-27 cells. For several biomedical applications a release of the active drug and/or fluorescent dye is desired. Therefore, additionally, the synthesis of A_3B-porphyrins with cleavable linker moieties is presented, namely disulfide, cleavable in a reductive environment, and acetal linkers whose cleavage is pH triggered.

1.3.2 *Mannose-Functionalized Hyperbranched Polyglycerol Loaded with Zinc-Porphyrin: Investigation of the Multivalency Effect in Antibacterial Photodynamic Therapy*

M. H. Staegemann, B. Gitter, J. Dernedde, C. Kuehne, R. Haag and A. Wiehe
Chem. Eur. J. **2017**, *23*, 3918–3930.
DOI: https://doi.org/10.1002/chem.201605236
http://onlinelibrary.wiley.com/doi/10.1002/chem.201605236/full

Author contributions

M. H. Staegemann: Porphyrin and porphyrin-hPG-conjugate synthesis, hPG functionalization, characterization, fluorescence experiments, preparation of the manuscript.
B. Gitter: Antibacterial assays
J. Dernedde, C. Kuehne: Surface plasmon resonance (SPR) investigations
R. Haag: Supervision, correction of the manuscript, scientific discussion of the data.
A. Wiehe: Supervision, correction of the manuscript, scientific discussion of the data.

Abstract

The antibacterial photodynamic activity of hPG loaded with zinc porphyrin PS and mannose units is investigated. hPG with a MW of 19.5 kDa has been functionalized with ~15 molecules of the PS {5,10,15-tris(3-hydroxyphenyl)-20-[4-(prop-2-yn-1-ylamino)¬tetra¬fluoro¬phenyl]¬por¬phyri¬nato-zinc(II) using the CuAAC. These nanoparticle conjugates have been functionalized systematically with increasing loadings of mannose in the range of approx. 20 to 110 groups. With higher mannose loadings (approx. 58-110 groups) the water-insoluble zinc-porphyrin PS can thus be transferred into a water-soluble form. Targeting of the conjugates was proven in binding studies to the mannose-specific lectin Concanavalin A (Con A) using surface plasmon resonance (SPR). The antibacterial phototoxicity of the conjugates on Staphylococcus aureus (as a typical Gram-positive germ) is investigated in PBS. It is shown that conjugates with approx. 70-110 mannose units exhibit a significant antibacterial activity, whereas conjugates with approx. 20-60 units did not induce bacterial killing at all. These results give an insight into the multivalency effect in combination with PDT. On addition of serum to the bacterial cultures a quenching of this antibacterial phototoxicity is observed. In fluorescence studies with the conjugates in the presence of

increasing bovine serum albumin (BSA) concentrations protein-conjugate associations could be identified as a plausible cause for this quenching.

1.3.3 *Hyperbranched Polyglycerol Loaded with (Zinc-)Porphyrins: Photosensitizer Release Under Reductive and Acidic Conditions for Improved Photodynamic Therapy*

M. H. Staegemann, S. Gräfe, B. Gitter, K. Achazi, E. Quaas, R. Haag, A. Wiehe
Biomacromolecules, **Article ASAP**.
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Author contributions

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S. Gräfe: Cellular photocytotoxicity assays
B. Gitter: Antibacterial assays
K. Achazi: Cellular Uptake Experiments
E. Quaas: Cellular Uptake Experiments
R. Haag: Supervision, correction of the manuscript, scientific discussion of the data.
A. Wiehe: Supervision, correction of the manuscript, scientific discussion of the data.

Abstract

An adaptable approach toward cleavable nanoparticle carrier systems for photodynamic therapy (PDT) is presented, comprising a biocompatible carrier loaded with multiple photosensitizer (PS) molecules related to the clinically employed PS Temoporfin, two linkers cleavable under different triggers and glyco-targeting with mannose. A synthetic pathway to stimuli responsive hyperbranched polyglycerol (hPG) porphyrin conjugates via the copper(I)-catalyzed 1,3-dipolar cycloaddition (CuAAC) or the strain-promoted alkyne−azide cycloaddition (SPAAC) has been developed. The PS 10,15,20-tris(3-hydroxyphenyl)-5-(2,3,4,5,6-pentafluorophenyl)porphyrin was functionalized with disulfide containing cystamine and acid-labile benzacetal linkers. Conjugates with reductively and pH labile linkers were thus obtained. Cleavage of the active PS agents from the polymer carrier is shown in several different release studies. The uptake of the conjugates into the cells is demonstrated via confocal laser scanning microscopy (CLSM) and flow cytometry. Finally, the antitumor and antibacterial phototoxicity of selected conjugates has been assessed in four different tumor cell lines and in cultures of the bacterium *Staphylococcus aureus*. The conjugates exhibited phototoxicity in several tumor cell

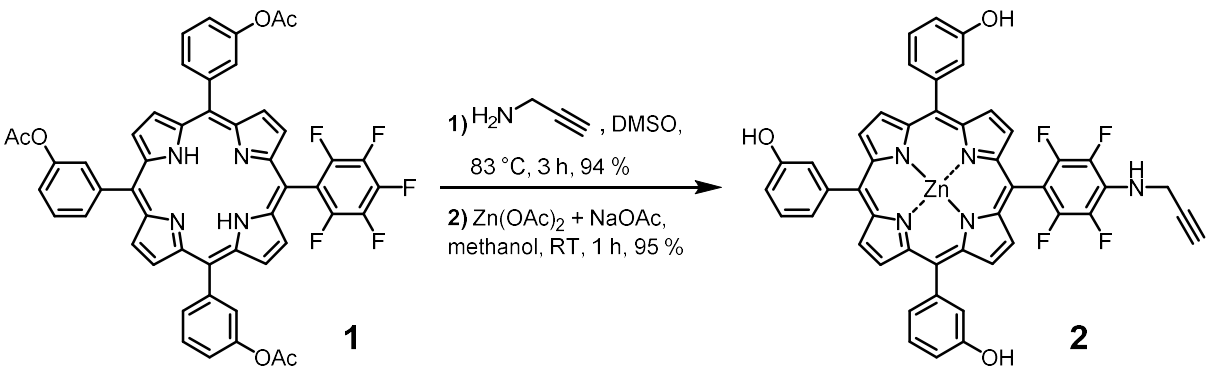
lines in which conjugates with reductively cleavable linkers were more efficient compared to conjugates with acid-cleavable linkers. For *S. aureus*, strong phototoxicity was observed for a combination of the reductively cleavable and the pH labile linker and likewise for the cleavable conjugate with mannose targeting groups. The results thus suggest that the conjugates have potential for antitumor as well as antibacterial PDT.

1.4 *Unpublished results and Discussion*

1.4.1 *Synthesis of the Porphyrin-Mannose-Conjugates*

The following chapter gives an overview of those synthetic and experimental results of the Ph.D. thesis which have not been published. The previously published results show the synthesis and antibacterial effect of porphyrin-mannose-hPG conjugates with a fixed polymer core size and systematically varied mannose loadings. Another approach is the synthesis of different conjugate sizes and the effect of antibacterial phototoxicity. These comprise the synthesis of additional conjugates of hPG, PS, and mannose which were not included in the publications and specifically a new method for the synthesis of the nanogels. With multiple hydroxyl groups present there are different methods to functionalize hPG further.[84] One approach which has been described before is the mesylation of the hydroxyl groups followed by a nucleophilic substitution with sodium azide leading to the azido-substituted hPG.[84a] The degree of functionalization of the hPG can easily be controlled by the stoichiometry of the reactants.[84a] With the CuAAC, known as click-reaction,[69-70, 71-72, 72c] the azido functionalized hPG conjugates can be further functionalized. Like in our case with corresponding alkyne-substituted porphyrin and the alkyne-mannose as active agent and targeting group, respectively. Different tetrapyrrolic PS have been described in literature, including porphyrins, [18h, 18j, 22d, 22k, 22n, 22o] chlorins, [22h-j, 22m, 22p] corroles[22a, 22b, 22f] and phthalocyanines.[22c, 22g, 22l, 22q] We choose porphyrins for systems since they are well described as PS, and the transformation to chlorins can easily be achieved.[6c-e, 147] Chlorins show even more supportive properties as PS.[6f, 22m] In this thesis we choose the zinc-porphyrin **2** as PS where the alkyne functionality has been introduced via nucleophilic aromatic substitution on the pentafluorophenyl group.[148] This porphyrin has similar structure elements to the known active PS tetrakis(3-hydroxyphenyl)porphyrin (*m*THPP) and its corresponding chlorin *m*THPC.[22m]

We started from 5,10,15-tris(3-acetoxyphenyl)-20-(pentafluorophenyl)porphyrin **1**[19a, 140] – possessing three acetoxy-protected hydroxyl groups and one pentafluorophenyl group which is capable for the further functionalization.

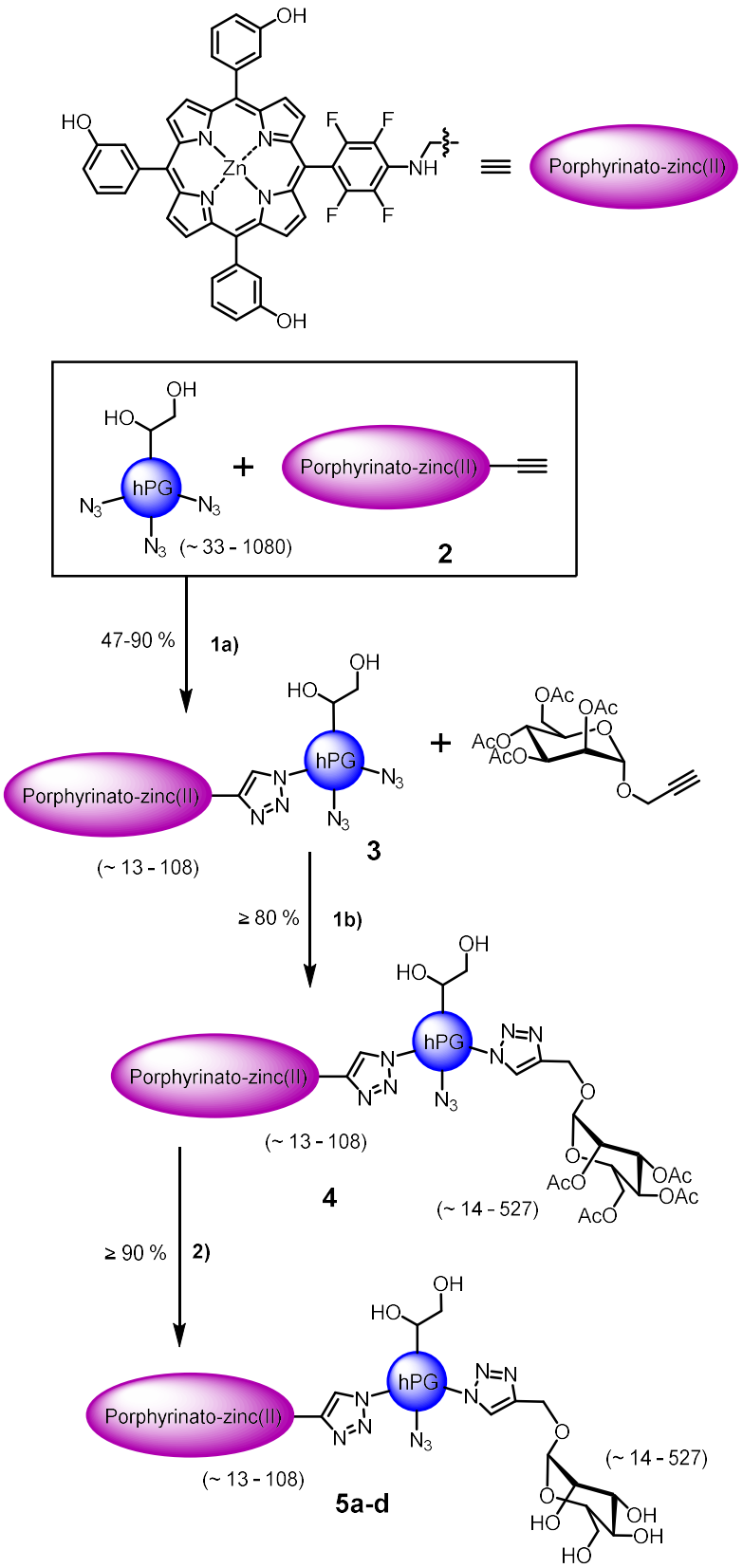


Scheme 7. Synthesis of zinc-porphyrin PS **2**.[148]

To include the alkyne functionality into our porphyrin system, propargylamine was reacted with 5,10,15-tris(3-acetoxyphenyl)-20-pentafluorophenylporphyrin **1**[19a, 140] at 83 °C in dimethyl sulfoxide (DMSO) in a two-step one-pot reaction (Scheme 7, step 1) followed by the zinc complexation into the porphyrin core by using Zn(OAc) yielding **2** (Scheme 7, step 2).[148] The nucleophilic aromatic substitution with amines does not need catalysts or other reagents (e.g. addition of a base),[18a-j] which is favorable due to different reactive sites of porphyrins (Scheme 7, step 1). Copper can be complexed from the metal-free porphyrin in the following CuAAC. Therefore, zinc was incorporated in the porphyrin core (Scheme 7, step 2). For the aPDT insertion of zinc into the porphyrin can even be favorable.[46b] The hPG-azides with different molecular weights (MW) (azide loading between approx. 65 and 80 %) were synthesized according to a known procedure.[84a] The core sizes used for the hPG were 3.7, 9.8, 19.5 and 100 kDa.

The porphyrin-mannose-hPG-conjugate with 19.5 kDa core size has been synthesized previously in our group, and the synthesis of the different sizes was based on this procedure.[149]

The porphyrin-mannose-hPG-conjugates were then obtained by CuAAC reactions (Scheme 8).[149] hPG-azides with approx. 65–80 % loading were reacted with the propargylated porphyrinato-zinc(II) complex **2** in DMSO/H_2O mixtures with $CuSO_4$ x $5H_2O$ and ascorbic acid sodium salt (Scheme 8, step 1a).



Scheme 8. Schematic representation of the synthesis of the porphyrin-mannose-hPG-conjugates **5a-d**. The structure of hPG is only representative. hPGs with an average molecular weight of 3.7, 9.8, 19.5 and 100 kDa were used. 1a-b) $CuSO_4$ x $5H_2O$, ascorbic acid sodium salt, DMSO/H_2O mixtures, 75 h, rt–60 °C (click chemistry). 2) NaOMe, dimethylformamide (DMF)/MeOH mixtures, 16 h, rt.

The solutions were stirred for 3 d at rt, and 3 h at 60 °C. Thin-layer chromatography (TLC) of the individual reaction mixtures showed that all or almost all of the fluorescent visible starting material (porphyrin **2**) had been transformed into a new red-colored substance, which stayed at the baseline. The crude products in the resulting reaction mixtures were purified by dialysis in acetone/H_2O (ratios are given in the individual procedures in the experimental section) to remove unreacted porphyrin, DMSO, $CuSO_4$ x $5H_2O$ and ascorbic acid sodium. The loading of the porphyrin covalently bound to hPG was determined via NMR spectroscopy.[150] Conjugates **3** with approx. 5–10 % porphyrin (approx. 13–108 groups) loading could be obtained. To introduce the targeting group acetylated propargyl α-$_D$-mannopyranoside [151] was reacted with these conjugates **3** in DMSO/H_2O mixtures. $CuSO_4$ x $5H_2O$ and ascorbic acid sodium salt were used as catalyst (Scheme 8, step 1b), followed by dialysis in acetone/H_2O (ratios are given in the individual procedures in the experimental section) yielding the conjugates with protected mannose units **4**. To obtain the unprotected glyco-functionality, the porphyrin-mannose-hPG-conjugates **4** were treated with NaOMe (Scheme 8, step 1c) followed by dialysis in H_2O yielding the conjugates **5a-d**. In short, conjugates with a mannose loading in the range of ~14 and ~527 groups could be obtained via this synthetic route (Table 1), using different hPG sizes, including nanogels (see below). With this method, the complete water insoluble porphyrin **2** could be transformed into a water-soluble formulation for all conjugates.

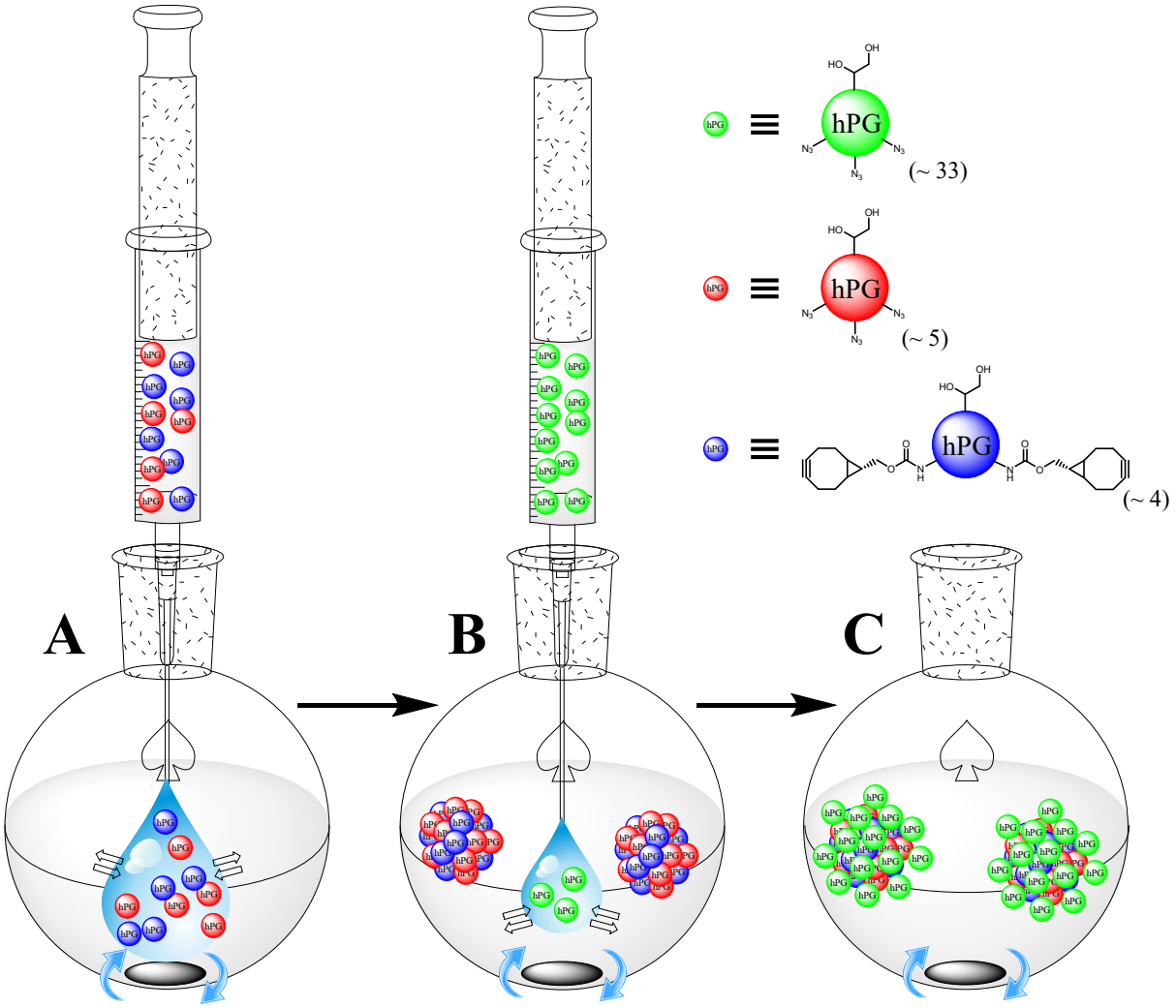
Table 1. Approximate porphyrin and mannose loading and hPG-core size for the porphyrin-mannose-PG-conjugates **5a-d** and **8**.

Entry	Approx. porphyrin loading [groups] ([loading])	Approx. mannose loading [groups] ([loading])	hPG-core size [kDa]	Product
1	4 (7 %)	14 (28 %)	3.7	**5a**
2	13 (10 %)	38 (29 %)	9.8	**5b**
3	13 (5 %)	108 (41 %)	19.5	**5c**
4	108 (8 %)	527 (39 %)	100	**5d**
5	- (6 %)[a]	- (10 %)[a]	nanogel	**8**

[a]: The nanogel was synthesized via inversed nanoprecipitation and subsequent functionalization. For this case no absolute number of substituents on the surface could be determined. The relative loading was determined via NMR.

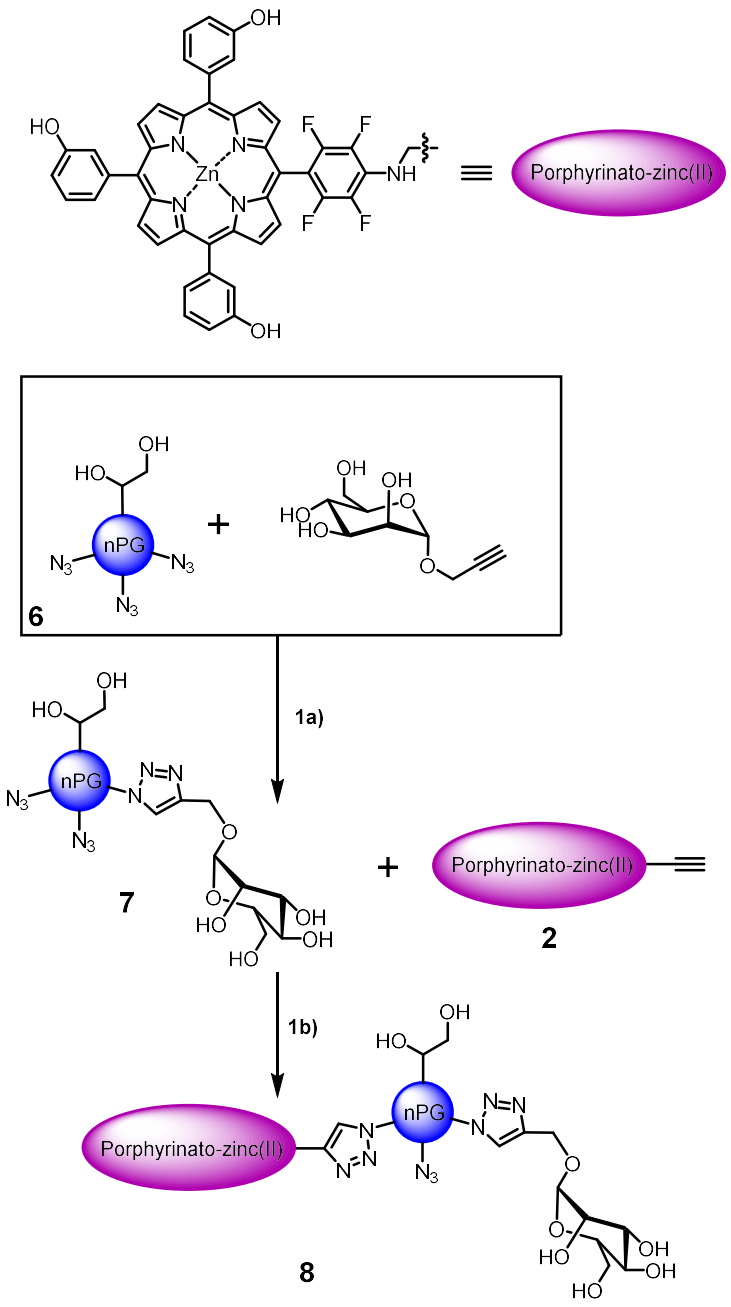
Nanogels are typically synthesized by using reactive monomers and template them on the nanometer scale. In the next, step the monomers are crosslinked in the template and form the hydrogel nanoparticles. Commonly used methods for the synthesis are mini[87, 100a, 152]- and microemulsions.[153] Drawbacks of this technics are high energy input by ultrasonication (for the mini-emulsion) and the use of surfactants. This leads to purification problems and does not allow the encapsulation of labile biological compounds.

Nanoprecipitation is a known and applied technology. This method does not require any addition of surfactants or ultrasonication as high energy input. A highly diluted polymer solution is added to a polymer non-solvent. This new inverse nanoprecipitation method for the formation of hydrophilic nanogel particles has previously been reported by Haag et al.[84d] In the present work this new method has been expanded by using a combination hydrophilic and lipophilic hPG macromonomers, leading to an azide functionalized nPG which can be combined with cyclooctyne-modified polymer units via SPAAC. SPAAC shows high conversions, bioorthogonality, and fast reaction kinetics and was therefore selected as crosslinking reaction.[73a-e] hPG-cyclooctyne was obtained by reacting hPG-amine with (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate *endo* in DMF. After 16 h the reaction mixture with the crude product was purified by dialysis in methanol. hPG-azide with 7 azide groups was synthesized according to a modified procedure from our group.[84a] Of both polymers, the hPG-cyclooctyne and the corresponding azide functionalized hPG, highly diluted solutions in methanol were prepared. These solutions were cooled down to 4 °C. Template formation of the nanoparticles was induced by the nanoprecipitation in DCM. Methanol will diffuse into the DCM phase and starts the gelation reaction process and hPG network formation by increasing the macromonomer concentration. The remaining active surface cyclooctyne groups were quenched with a high loaded hPG-azide followed by azidopropanol, after gelation. With this method nanoparticles with low polydispersity and a size of 150 nm could be obtained. The nanogel dispersion was purified by dialysis in acetone. Nanogels tend to aggregate when the solvent is evaporated completely. Therefore a small amount DMSO was added to the dialyzed nanogels dissolved in acetone. Afterward, the acetone was evaporated in vacuo yielding the nanogels **6** in DMSO for further reactions.



Scheme 9. Schematic representation of the nanoprecipitation process. A) Injection of cyclooctyne functionalized (blue spheres) and low azid functionalized (red spheres) hPG macromonomers dissolved in methanol into the dichloromethane (DCM) phase. Particle templation by diffusion from the methanol phase (blue droplet) into DCM (gray phase in the flask). B) Particle formation by SPAAC. Afterward, injection of high azid functionalized (green spheres) hPG macromonomers. C) Final nanogel formation by SPAAC.

The porphyrin-mannose-nPG-conjugate **8** was then obtained by CuAAC reaction (Scheme 10). In this reaction, the unprotected propargyl α-D-mannopyranoside was used to avoid the necessity of subsequent deprotection with a strong base to avoid damaging the nanogel in this reaction. nPG-azide 6 was reacted with propargyl α-D-mannopyranoside in DMSO/H_2O mixture with $CuSO_4$ x $5H_2O$ and ascorbic acid sodium salt (Scheme 10, step 1a). The solution was stirred for 12 h at 60 °C and 6 h at rt. The crude product in the resulting reaction mixture was purified by dialysis in H_2O to remove unreacted propargyl α-D-mannopyranoside, DMSO, $CuSO_4$ x $5H_2O$ and ascorbic acid sodium salt. A small amount of DMSO-d_6 was added to the dialyzed solution, and the H_2O was evaporated. The loading of the mannose covalently bound to nPG was determined via NMR spectroscopy. Conjugate **7** with approx. 10 % mannose loading could be obtained.



Scheme 10. Schematic representation of the synthesis of the porphyrin-mannose-nPG-conjugate **8**. The structure of nPG is only representative. 1a,b) $CuSO_4$ x $5H_2O$, ascorbic acid sodium salt, DMSO/H_2O mixtures, 18 h, rt–60 °C (click chemistry). Detailed conditions and yields are given in the experimental section.

nPG-mannose **7** with approx. 10 % loading was then reacted with the propargylated porphyrinato-zinc(II) complex **2** in DMSO/H_2O mixture with $CuSO_4$ x $5H_2O$ and ascorbic acid sodium salt (Scheme 10, step 1b). The solution was stirred for 12 h at 60 °C and 6 h at rt. TLCs of the reaction mixture showed almost all of the fluorescent visible starting material (porphyrin **2**) had been transformed into a new red-colored substance, which stayed at the baseline. The crude product in the resulting reaction mixture was purified by dialysis in DMSO to remove unreacted porphyrin followed by dialysis in H_2O to obtain the nanogel in a more suitable

solvent. The loading of the porphyrin covalently bound to nPG was determined via NMR spectroscopy. Conjugate **8** with approx. 6 % porphyrin and 10 % mannose loading could be obtained.

In summary a nanogel with a mannose loading with approx. 10 and porphyrin with approx. 6 % and a size of 190 nm (phosphate-buffered saline (PBS) at pH 7.4) could be obtained via our synthetic route (Table 1). The conjugates were measured by dynamic light scattering (DLS) in PBS at pH 7.4.

1.4.2 *Photosensitized inactivation of S. aureus*

S. aureus and MRSA are standard benchmarks in studying antibacterial activity.[154] MRSA started as a hospital-acquired infection, but has already evolved to a community infection.[154] As part of this thesis, we investigated the multivalency effect of different mannose loadings on porphyrin-hPG conjugates (with a fixed polymer size of 19.5 kDa, see 1.4.2).[149] Mannooligosaccharides are described in literature to bind to *S. aureus* and various bacteria, therefore, we choose this germ to investigate the multivalency effect further.[145] The zinc-porphyrin-hPG conjugates **5a-d** were tested in vitro on their photodynamic toxicity against the *S. aureus* bacterium and compared. To investigate the multivalency effect different hPG-core sizes were chosen starting from 3.7 to 100 kDa. The absolute number of the mannose groups as targeting units has been varied between approx. 14 and 527 (cf. Table 2). After evaporation of the solvent, the 100 kDa conjugate proved to be not soluble in water anymore, therefore for the experiments a small amount of DMSO was added as a solubilizer (see experimental part).

To suspensions of *S. aureus*, bacterial cells in PBS or PBS + 10 % horse serum the conjugates **5a-d** were added in amounts equal to 10 and 100 µM PS concentration, respectively. The samples were incubated for 30 min in the absence of light at 37 °C and afterward irradiated with white light. In control experiments, the effect of incubation with the photosensitizing agent alone (in the 100 µM concentration) without subsequent illumination on the *S. aureus* cultures was assessed (dark toxicity).

Table 2. Antibacterial toxicity of the synthesized porphyrin-mannose-hPG-conjugates **5a-d.**

Entry	Conjugate (mannose groups)	hPG-core size [kDa]	Dark toxicity	10 µM with light	100 µM with light
1	**5a (14)**	3.7	141 %	42 %	8.5 %
2	**5b (38)**	9.8	111 %	0.79 %	Complete eradication of bacteria
3	**5c (108)**	19.5	104 %	Complete eradication of bacteria	Complete eradication of bacteria
4	**5d (527)**	100	0 %	0 %	0 %

The results of the antibacterial photodynamic activity of the conjugates are shown in Figure 10 and summarized in Table 2.

Table 2 shows the antibacterial phototoxicity of the zinc PS **5a-d** against *S. aureus* in the concentrations of 10 and 100 µM in PBS, respectively. The conjugates **5a-c** with approx. 14 to 527 mannose units show an increasing activity at both concentrations in PBS. The conjugates **5c,d** carrying approx. 108 and 527 mannose units led to a complete eradication of the bacteria at both concentrations in PBS. With the addition of serum, the antibacterial phototoxicity of all conjugates vanished completely at 10 µM and 100 µM concentration. This has been discussed in the publication under 1.4.2 in this thesis.[149]

In PBS the increasing absolute number of mannose groups (a consequence of the increasing size of the polymer hPG-core) leads to a higher antibacterial photodynamic activity of the bigger conjugates. These new results of PS-hPG conjugates with different sizes reinforce the findings on the multivalent effect from our publication under 1.4.2[149] .

1.5 *Experimental Section*

Reagents:

Propargyl-α-D-mannopyranoside was purchased from ABCR. L-Ascorbic acid sodium salt (99 %) and DMF (99.8 %) extra dry over molecular sieves were purchased from Acros Organics. Methanol (≥ 99.8 %) and triethylamine (≥ 99 %) were purchased from Sigma Aldrich. DMSO ROTIDRY® (≤ 200 ppm H_2O) (≥ 99.5 %) was purchased from Roth. All these chemicals were used without further purification. D_2O (99.95 %) and DMSO-*d*$_6$ (99.8 %) were purchased from Deutero GmbH. (1R,8S,9s)-Bicyclo[6.1.0]non-4-yn-9-methyl (4-nitrophenyl) carbonate *endo*;[73d, 73e] hPG-azide-mannose protected[84b] and hPG-azide[84a] were prepared according to the literature or with slight modifications. All solvents were dry or distilled before use.

Thin-layer chromatography (TLC): TLC analysis was performed on Merck silica gel 60 F_{254} precoated aluminum sheets with fluorescence indicator F_{254}. Also, detection of the intrinsic tetrapyrrole fluorescence was carried out with UV light at 366 nm.

Dialysis: Dialysis (dialysis tubing benzoylated, avg. flat width 32 mm (1.27 in.), *Sigma Aldrich*) or (Spectra/Por® 7, Dialysis membranes made of reg. cellulose – molecular weight cut-off (MWCO) 50000, avg. flat width 34 mm, *Roth*) was performed in 1 or 2 L beakers and the solvents were changed 3 times over a period of 24 hours. The solvents used are given in the individual procedures.

NMR spectroscopy: ^{1}H and ^{13}C spectra were recorded on *Bruker BioSpin* AVANCE700 (^{1}H NMR: 700 MHz, ^{13}C NMR: 176 MHz) instruments. D_2O and DMSO-*d*$_6$ were used as deuterated solvents. Chemical shifts δ are given in ppm relative to tetramethylsilane (TMS) as an internal standard or relative to the resonance of the solvent (^{1}H NMR: D_2O: δ = 4.79 ppm and DMSO-*d*$_6$: δ = 2.50 ppm. ^{13}C NMR: DMSO-*d*$_6$: δ = 39.52 ppm. All spectra were recorded at rt. Abbreviations for the signals: bs (broad singlet) and m (multiplet).

In vitro biological studies:

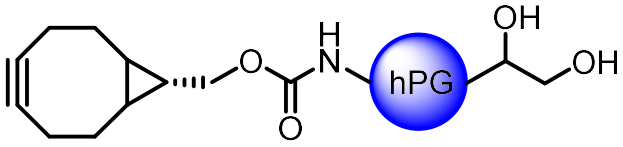
Bacterial testing: The organism studied was a typical member of the wound microflora; *S. aureus* DSM 11729, Gram-positive. Cultured bacterial cells were suspended in sterile PBS, or sterile PBS supplemented with 10 % sterile horse blood serum. The final OD (Optical Density) at 600 nm, l cm in all cases was 0.015. The bacterial suspensions were placed into sterile black well plates with clear bottoms. Concentrations of PS used in the study were 100 µM and 10 µM, respectively.

After an incubation time of 30 minutes at rt, the samples were exposed to white light, and power set to 0.5 W, and an irradiation time of 85 s. For compound **15** (conjugate with free-base porphyrin) a diode laser with irradiation at 652 nm (Ceralas PDT 652, CeramOptec GmbH) was used. With the irradiation time, the resulting energy fluence is about 100 J/cm². Control plates contained no PS and were not exposed to white light. The control samples for dark toxicity are only exposed to PS without any illumination.

After irradiation, the samples were removed, suspended again in the culture media and the bacteria suspensions were inoculated on agar plates using a jet spiral plater (Eddy Jet, IUL Instruments GmbH, Königswinter, Germany). After overnight culture, the colonies on the plates were counted using an automatic colony counter (Countermat Flash, IUL Instruments GmbH, Königswinter, Germany) to determine the number of colony-forming units (CFU/ml).

DLS: DLS measurements were conducted using a Zetasizer Nano-ZS (*Malvern Instruments*, Ltd, UK) with integrated 50 mW laser, λ = 532 nm. The solvents and temperatures are given in the individual procedures.

Cyclooctin-hPG$_{5.0}$ with 5 % cyclooctin



In a 10 mL flask with magnetic stirrer $hPG_{5.0}[NH_2]_4$ (122 mg, 24.4 µmol, 97.6 µmol amine groups) was dissolved in anhydrous DMF (4.0 mL). Triethylamine (99 %, 4.09 µL, 2.96 mg, 29.0 µmol) and (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate *endo* (47.4 mg, 150 µmol) were added to the stirring solution. The solution was stirred for 16 h at rt. The crude product was purified by dialysis (methanol) to obtain the product cyclooctin-$hPG_{5.0}$ with 5 % cyclooctin (133 mg, 23.4 µmol, 93.6 µmol cyclooctin, 96 % yield, quant. conversion).

Cyclooctin-$hPG_{5.0}$ with 5 % cyclooctin:

^{1}H NMR (700 MHz, D_2O, δ): 4.22–4.09 (m, OC*H*$_2$-bicyclo), 4.05–3.07 (m, hPG-backbone), 2.31–2.14 (m, 2,3,6,7-bicyclo), 1.62–1.48 (m, bicyclo), 1.42–1.24 (m, bicyclo), 0.99–0.88 (m, bicyclo), 0.85–0.76 ppm (m, bicyclo). ^{13}C NMR (176 MHz, acetone-*d*$_6$, δ): 100.14, 79.65, 79.43, 78.19, 77.96, 72.16, 70.86, 70.70, 70.42, 69.20, 68.88, 62.61, 60.75, 28.73, 20.90, 19.83, 17.34 ppm.

Preparation of polyglycerol nanogels by nanoprecipitation:

$hPG_{5.0}$-4-CycloOct (7.5 mg, 1.32 µmol) and $hPG_{5.0}[N_3]_7$ (5.6 mg, 1.12 µmol) were dissolved separately in methanol (7.5 mL). The solutions were cooled down to 4 °C, mixed and added quickly to magnetically stirred DCM (300 mL). After 2 h excess $hPG_{3.7}[N_3]_{33}$ (52.5 mg, 11.7 µmol) was added. After 16 h the reaction was quenched with azidopropanol (100 µL, 110 mg, 1.08 mmol). Precipitated PG nanoparticles were obtained as dispersions, and the particle size was determined by DLS (Intensity: 140 nm). The nanogels were purified by dialysis (acetone). To the dialyzed solution DMSO was added (1.5 mL), and the remaining acetone was evaporated to obtain the $nPG[N_3]$ nanogels 6 in DMSO for further reactions.

Functionalization of the polyglycerol nanogels with mannose:

Propargyl α-D-mannopyranoside (123 µL, 0.46 M in H_2O, 56.4 µmol) was added to $nPG[N_3]$ in DMSO (1.5 mL) from the previous step. L-ascorbic acid sodium salt (22.4 µL, 0.50 mM in H_2O, 11.3 µmol) and copper(II) sulfate pentahydrate (14.1 µL, 0.40 M in H_2O, 5.64 µmol) were added to the stirring solution. The solution was stirred at 60 °C for 12 h. Afterwards the reaction mixture was stirred for 6 h at rt. The crude product was purified by dialysis (H_2O) for 3 d. To

the dialyzed solution DMSO-d_6 was added (1 mL), and the remaining H_2O was evaporated to obtain the nPG[Mannose] 7 nanogels in DMSO-d_6 for further reactions and NMR spectroscopy.

^{1}H NMR (700 MHz, DMSO-d_6, δ): 8.23–7.79 (m, C=C*H*), 5.36–3.18 ppm (m, hPG-backbone + Man + Cyclo); ^{13}C NMR (176 MHz, DMSO-d_6, δ): 143.97, 124.09, 99.39, 78.56, 78.39, 74.53, 73.33, 73.20, 73.03, 71.82, 71.37, 71.17, 71.06, 71.02, 70.94, 70.62, 70.12, 69.80, 69.45, 69.24, 69.09, 67.43, 63.55, 61.72, 60.90, 60.69, 60.58, 59.49, 53.92, 51.63, 51.36, 51.22 ppm.

Functionalization of the polyglycerol-mannose nanogels with porphyrin:

{5,10,15-tris(3-hydroxyphenyl)-20-[4-(prop-2-yn-1-ylamino)-tetrafluorophenyl]porphyrinato}-zinc(II) 2 (17.2 mg, 20.2 µmol) was added to nPG[Mannose] in DMSO (1.5 mL) from the previous step. L-ascorbic acid sodium salt (8.01 µL, 0.50 M in H_2O, 4.04 µmol) and copper(II) sulfate pentahydrate (5.05 µL, 0.40 M in H_2O, 2.02 µmol) were added to the stirring solution. The solution was stirred at 60 °C for 12 h. Afterwards the reaction solution was stirred for 6 h at rt. The crude product was purified by dialysis (DMSO) for 3 d, followed by dialysis (H_2O) for 3 d. To one third of the dialyzed solution DMSO-d_6 was added (1 mL) and the remaining H_2O was evaporated to obtain the nPG[Porphyrin-Mannose] 8 nanogels in DMSO-d_6 for NMR spectroscopy.

^{1}H NMR (700 MHz, DMSO-d_6, δ): 9.83–9.62 (bs, porphyrin β-pyrrole-*H*), 8.97–7.03 (m, Ar + porphyrin/Man triazole-*H*), 5.14–2.89 ppm (m, hPG-backbone + Cyclo +porphyrin-CH_2 + Man); ^{13}C NMR (176 MHz, DMSO-d_6, δ): 155.89, 150.11, 149.97, 149.69, 149.46, 147.54, 146.17, 144.16, 137.74, 136.46, 133.08, 132.26, 131.99, 130.77, 127.84, 126.26, 122.33, 121.07, 115.02, 102.69, 99.40, 78.47, 74.48, 73.30, 73.05, 71.37, 71.07, 70.65, 69.33, 67.44, 63.59, 61.72, 60.90, 60.70, 60.58, 59.42, 53.92, 51.36 ppm.

1.6 *Conclusion and Outlook*

This thesis consists of four different parts of the synthesis and investigation of various porphyrins containing different types of linkers for the connection to substrates, conjugates of these porphyrins with hPG in nanoparticles and nanogels, hPG-porphyrin conjugates additionally loaded with mannose and investigation of their antibacterial activity, and cleavable porphyrin-hPG-conjugates:

A) The reaction of pentafluorophenyl-substituted A_3B-porphyrins with amines has been used to obtain diverse reactive groups which are useful for the further functionalization and/or conjugation to different substrates and/or materials. With this method, porphyrins with alkenyl-, alkynyl-, amino-, azido-, cyclooctyne-, epoxide-, hydroxyl- and maleimido-groups have been obtained. The conjugation of an alkynyl porphyrin to an hPG-azide via the CuAAC is shown. For a possible application in the PDT the photocytotoxicity of porphyrin-hPG-conjugates has been demonstrated against human epidermoid carcinoma A-253 and squamous carcinoma CAL-27 cells. To allow cleavage of the porphyrins from a carrier system, examples of porphyrins containing disulfide (for reductive cleavage) and acetal (for pH-triggered cleavage) linkers are presented.

B) The multivalency effect has been investigated using hPG loaded with zinc porphyrins as PS and a systematically varied number of mannose units as targeting groups. These conjugates have been tested on their antibacterial photodynamic activity. hPG-azide with a core MW of 19.5 kDa was functionalized with approx. 14 groups of the PS using the CuAAC. Subsequently, the conjugates have been further functionalized with systematically different loadings of mannose in the range of approx. 20 to 110 units. For the conjugates with approx. 58-110 mannose groups with this method the water insoluble zinc-porphyrin have been transformed to water soluble formulation. For these conjugates, the antibacterial phototoxicity against *S. aureus* was investigated in PBS. Conjugates with approx. 69 to 110 mannose groups showed a significantly higher antibacterial activity, whereas conjugates with approx. 20 to 58 groups exhibited no activity at all. These in vitro tests showed promising results shedding light on the multivalency effect in combination with PDT. With the addition of serum, the antibacterial activity is completely quenched. We investigated this behavior with fluorescence studies performed with BSA suggesting protein-conjugate association as a possible cause for the loss of antibacterial activity. This behavior will be investigated further in the future to circumvent the loss of activity. Possibly different PS or different targeting groups can avoid this loss in

activity. Another approach can be a different formulation of the PS (e.g. inside the nanoparticle with subsequent release).

C) Another insight into multivalency effect came from the preparation of hPG with systematically different MW loaded with zinc porphyrins as PS and mannose groups as targeting functionality which have been tested on their antibacterial photodynamic activity. hPG-azides with a core MW between 3.7 and 100 kDa have been functionalized with approx. 4 to 108 groups of the PS [[5,10,15-tris(3-hydroxyphenyl)-20-[4-(prop-2-yn-1-ylamino)tetrafluorophenyl]porphyrinato]]-zinc(II) using the CuAAC. Additionally, polyglycerol based nanogel (nPG) with azide functionalities have been synthesized using a modified inverse nanoprecipitation. With this new method, it was possible to obtain nPGs with excess azide functionalities on the surface. The nPG-azides were further transformed to the porphyrin-nPG-conjugates via CuAAC. Subsequently, the conjugates have been further functionalized with mannose in the range of approx. 14 to 527 units. With this method the complete water insoluble zinc-porphyrin could be transformed into water-soluble formulations. Investigation of the antibacterial phototoxicity of these conjugates against *S. aureus* has revealed that conjugates with approx. 14 to 108 mannose groups exhibit an increasing antibacterial activity, whereas conjugates with approx. 108 to 527 groups exhibit a complete eradication of the bacteria. It has been shown that the increasing size of the porphyrin-mannose-hPG conjugates has a direct effect of antibacterial phototoxicity. With the results of the conjugates with a fixed polymer size and systematically varied mannose loadings from part B these in vitro tests show another aspect of the multivalency effect in combination with PDT.

D) Porphyrin-hPG-conjugates connected via reductive and acid-cleavable linkers have been synthesized for application in PDT using CuAAC and SPAAC. Employing S_NAr, a pentafluorophenyl substituted porphyrin has been functionalized with 1,6-diaminohexane and cystamine. In the next step, these porphyrins have been further reacted with propiolic acid or bicycle[6.1.0]non-4-yn-9-ylmethyl(4-nitrophenyl)carbonate. Porphyrin-hPG-conjugates with hPG-azide and/or hPG with acid-labile benzacetal linkers have been obtained using CuAAC or SPAAC and the previously synthesized porphyrins. With this method, the final porphyrin-hPG conjugates with acidic and/or reductive cleavable linkers were synthesized. The successful cleavage of the PS from these conjugates has been shown in different release studies. To illustrate the possible application of these conjugates selected compounds have been tested for their phototoxicity in four tumor cell lines and on the typical Gram-positive germ *S. aureus*. Future research should focus on the synthesis of alternative cleavable linker types, which should

allow release under different conditions. As well, the addition of specific targeting groups is an interesting field.

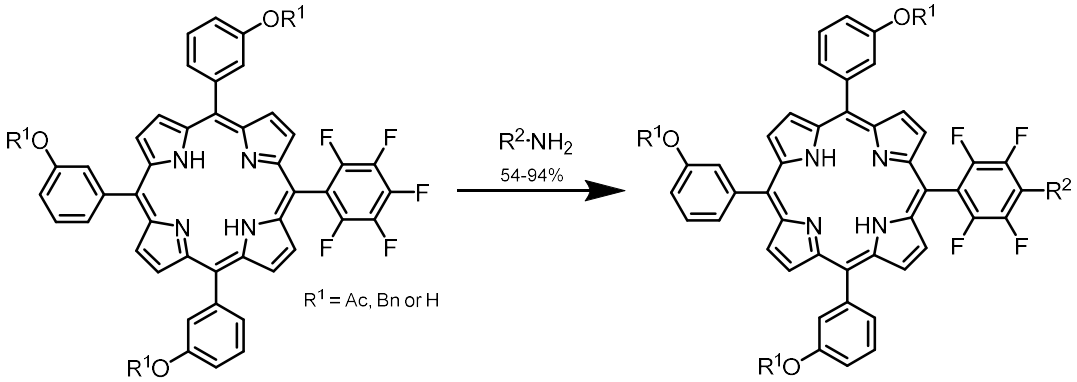
In conclusion various porphyrins containing different types linkers could be synthesized. Selected porphyrins could then be conjugated to hPG and hPG loaded with mannose. The photocytotoxicity of the conjugates was assessed in cellular assays against several tumor cell lines as well as in assays against the typical Gram-positive germ *S. aureus*. The PDT activity of the conjugates in selected tumor cell lines could be shown. Moreover, their antibacterial activity, specifically of the conjugates decorated with mannose units, could be proven, and a multivalency effect for these targeted aPDT nanoparticles could be shown. Finally, cleavable porphyrin-hPG-conjugates were synthesized, their biological activity assessed and the release of the PS could be demonstrated.

Future research could be directed to introduction of new and more specific targeting groups. Specifically designed antibodies allow a direct targeting of particular cellular receptors. With different targeting moieties it should be possible to expand the use of conjugates synthesized to different cell and bacteria strains. Another important field of research is the use of other cleavable linker moieties. This could be e.g. photo-cleavable linkers which are of great interest for the PDT. In this case irradiation at two different wavelengths in subsequent order would allow the cleavage of the PS followed by irradiation at another wavelength to induce photocytotoxicity. Also, the use of other more potent PSs could be investigated. The design of porphyrins capable of two-photon absorption would allow PDT with longer wavelength and therefore irradiation deeper into the tissue, which would allow treating more invasive tumors. A first step could be the transformation of the existing porphyrin PSs into the corresponding chlorins, which are known to be more efficient PS due to their higher extinction coefficients in the bathochromic region of their absorption spectra.

1.7 *Kurzzusammenfassung*

Die vorliegende Dissertation umfasst vier separate welche zum Teil aufeinander aufbauen. Darunter fällt die Synthese und Funktionalisierung diverser Porphyrine für die zukünftige Verlinkung mit Substraten, Porphyrin-hPG Konjugate mit systematisch variierter Mannose-Beladung, Porphyrin-hPG Konjugate funktionalisiert mit Mannose mit systematisch variierter Polymergröße und Porphyrin-hPG Konjugate, in welchen die Porphyrine über spaltbare Linker mit dem Polymer verbunden sind:

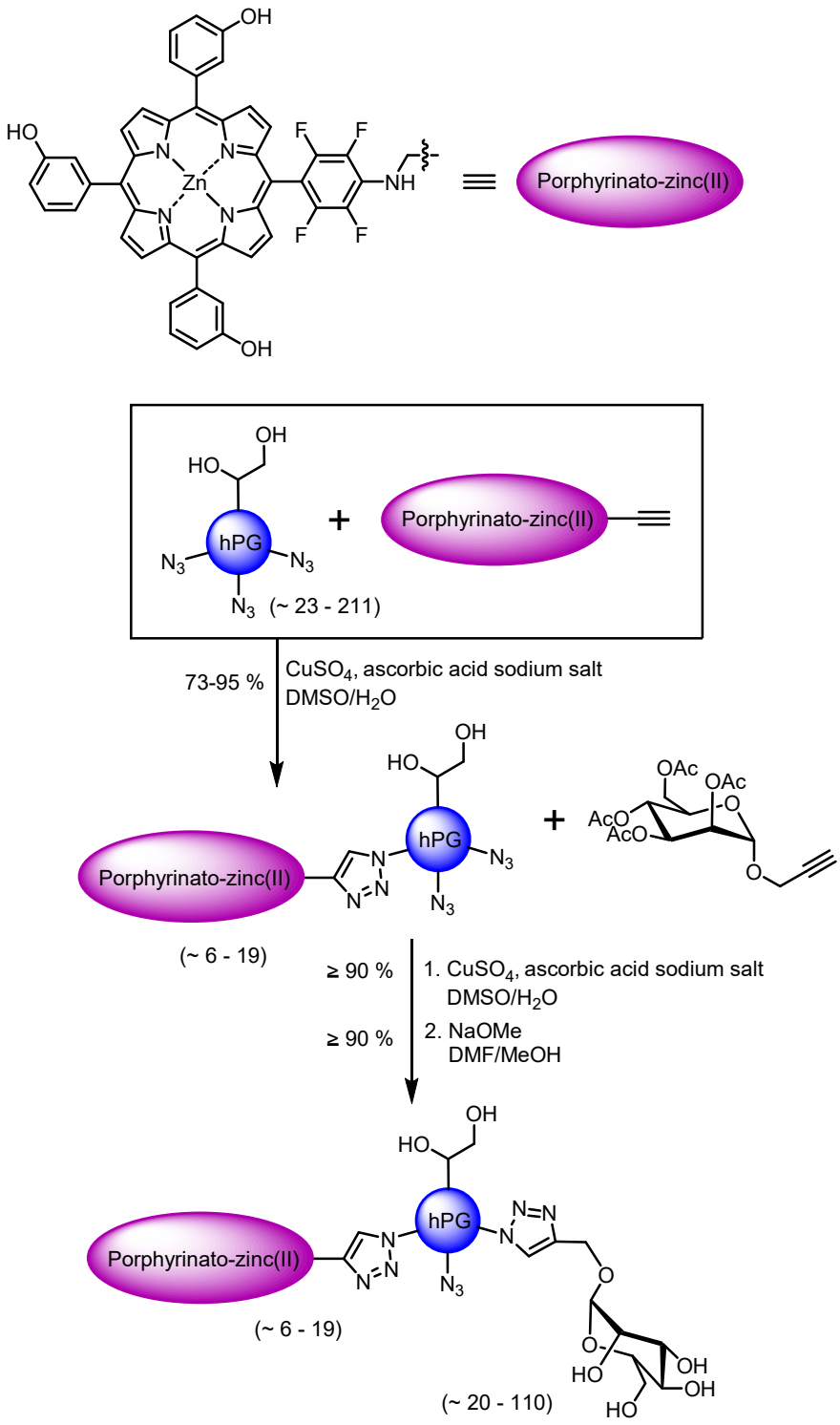
1. Die Reaktion von Aminen mit Pentafluorphenyl-substituierten A_3B-Porphyrinen wurde benutzt um verschiedene reaktive Gruppen für die zukünftige weitere Funktionalisierung und/oder Konjugation dieser Porphyrine an andere Substrate oder Materialien einzuführen. Porphyrine mit Alkenyl, Alkinyl, Amino, Azido, Epoxid, Hydroxyl und Maleimido-Gruppen konnten so synthetisiert werden.



Zum Beispiel wurde ein Alkinyl-substituiertes Porphyrin mittels CuAAC an hPG-Azid als einem möglichen Trägersystem für die PDT konjugiert. Die Photocytotoxizität von ausgewählten Porphyrinen wie auch von den Porphyrin-hPG-Konjugaten wurde in Zell Assays mit zwei Tumorzellkulturen untersucht. Für verschiedene biomedizinische Anwendungen ist eine Freisetzung der aktiven Substanz und/oder des Fluoreszenzfarbstoffes erwünscht. Deswegen wurden zusätzlich A_3B-Porphyrine synthetisiert, welche spaltbare Linker enthalten, speziell Disulfid (spaltbar unter reduktiven Bedingungen) und Acetal Linker (spaltbar unter sauren pH Bedingungen).

2. In diesem Teil der Arbeit wurde die antibakterielle photodynamische Aktivität von hPG beladen mit Zink Porphyrin als PS und Mannose Gruppen untersucht. hPG mit einer molekularen Masse MW von 19,5 kDa wurde mittels der CuAAC mit ~14 Molekülen des PS {5,10,15-Tris(3-hydroxyphenyl)-20-[4-(prop-2-yn-1-ylamino)tetrafluorphenyl]porphyrinato}Zink(II) funktionalisiert. Diese Nanopartikel

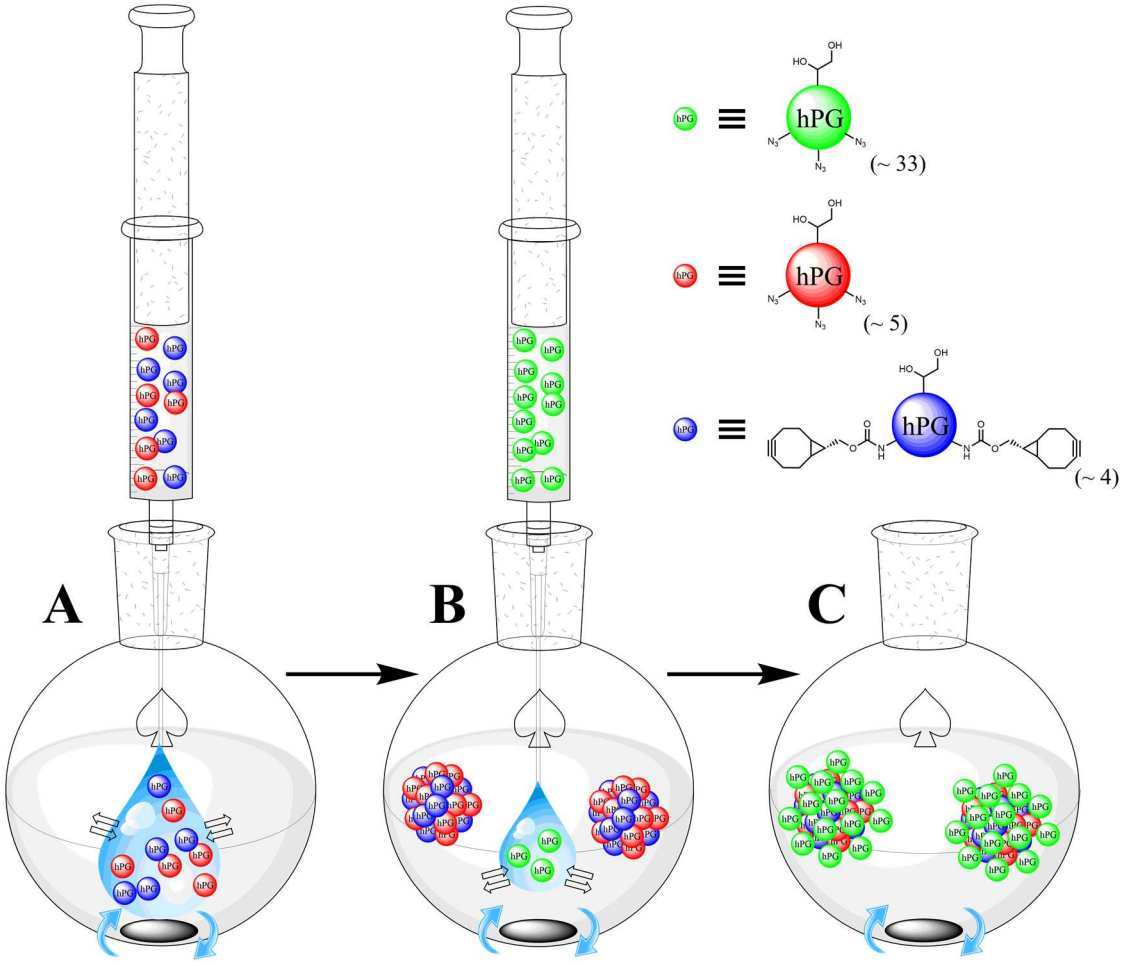
Konjugate wurden weiterhin systematisch mit verschiedenen Beladungen von Mannose (zwischen ~20 und 110) Gruppen funktionalisiert. Mit höheren Mannose Beladungen (zwischen ~58 und 110 Gruppen) konnte der wasserunlösliche Zink-Porphyrin-PS in eine wasserlösliche Form überführt werden. Anschließend wurde die antibakterielle Phototoxizität der Konjugate gegen *S. aureus* (als typischer Gram positiver Keim) in PBS untersucht. Es zeigte sich, dass Konjugate mit ~70 bis 110 Mannose Einheiten eine



signifikante antibakterielle Aktivität aufwiesen, wohingegen Konjugate mit ~20 bis 60

Einheiten nicht antibakteriell aktiv waren. Die Resultate geben einen Einblick in den Multivalenzeffekt in Kombination mit PDT. Bei Zugabe von Serum zu den Bakterienkulturen verschwand die antibakterielle Phototoxizität vollständig. Fluoreszenzstudien der Konjugate in Gegenwart von zunehmenden BSA Konzentrationen deuteten auf eine Protein-Konjugat-Assoziation als mögliche Ursache hin.

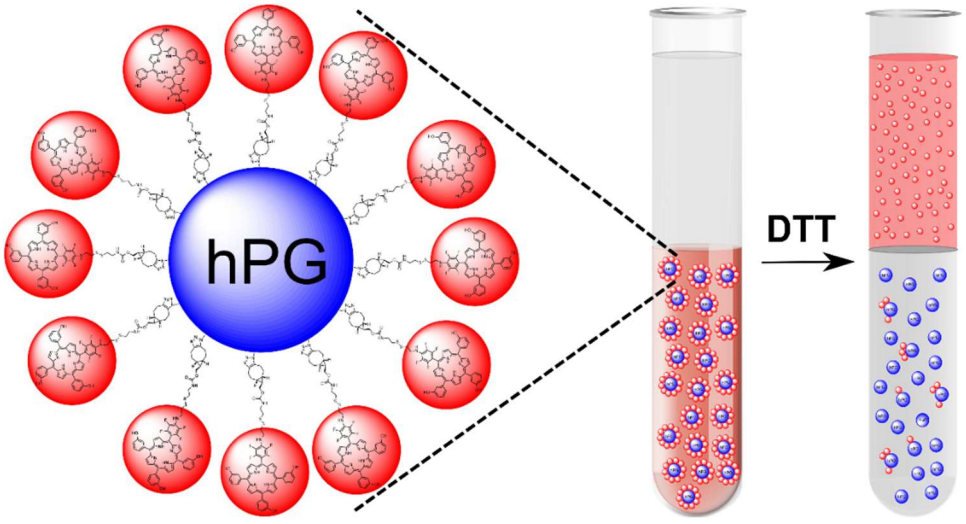
3. hPGs mit unterschiedlichen molekularen Massen bzw. Größen wurden mit Zink-Porphyrin (als PS) und Mannose Gruppen funktionalisiert. Diese Konjugate wurden anschließend auf ihre antibakterielle Phototoxizität untersucht. hPGs zwischen 3,7 und 100 kDa wurden mit ~4 bis ~108 Molekülen des PS {5,10,15-Tris(3-hydroxyphenyl)-20-[4-(prop-2-yn-1-ylamino)tetrafluorphenyl]porphyrinato}-Zink(II) mittels CuAAC funktionalisiert. Zusätzlich zu diesen Konjugate wurde ein Polyglycerol Nanogel mit Hilfe einer neuen inversen Nanofällung hergestellt. Diese modifizierte Technik erlaubt die Synthese von nPGs mit einem Überschuss von Aziden auf der Oberfläche.



Die erhaltenen nPG-Azide wurden dann weiter benutzt um die Porphyrin-nPG Konjugate mittels CuAAC zu erhalten. Diese Nanopartikel wurden anschließend weiter mit zwischen ~14 und ~527 Mannose Einheiten (als Targeting Gruppen)

funktionalisiert. Auch mit dieser Methode konnte der wasserunlösliche Zink-Porphyrin-PS bei allen Konjugaten in eine wasserlösliche Formulierung überführt werden. Die antibakterielle Phototoxizität der Konjugate gegen Kulturen von *S. aureus* wurde in PBS untersucht. Es zeigte sich, dass Konjugate zwischen ~14 und ~108 Mannose Einheiten eine zunehmende antibakterielle Aktivität aufwiesen. Die Konjugate mit ~108 und ~527 Gruppen zeigten eine vollständige Auslöschung der Bakterien. Diese Resultate weisen einen weiteren Aspekt des Multivalenzeffektes hin: Der Einfluss unterschiedlicher molekularer Massen der Konjugaten in Kombination mit der PDT.

4. Dieser letzte Teil der Arbeit befasst sich mit hPG-Porphyrin-Konjugaten mit spaltbaren Linkern. Dafür wurde ein Syntheseweg für solche Porphyrin-hPG Konjugate mittels CuAAC und SPAAC entwickelt. Im ersten Schritt erfolgte eine Funktionalisierung von 10,15,20-Tris(3-hydroxyphenyl)-5-(2,3,4,5,6-pentafluorphenyl)porphyrin durch nukleophile aromatische Substitution mit 1,6-Diaminohexan bzw. Cystamin funktionalisiert. Die erhaltenen Porphyrine wurden weiter mit Propiolsäure oder Bicyclo[6.1.0]non-4-yn-9-ylmethyl(4-nitrophenyl)karbonat umgesetzt. Parallel wurden azid-funktionalisierte hPGs und hPGs mit säurelabilen Benzacetal Linkern synthetisiert und mit den Porphyrinen und Mannose mittels CuAAC und SPAAC verknüpft. Auf diese Weise konnten Porphyrin-hPG Konjugate mit sauer und/oder reduktiv spaltbaren Linkern erhalten werden. Die Abspaltung des PS vom Carrier konnte in verschiedenen Freisetzungsstudien gezeigt werden.



Um eine mögliche Anwendung für die PDT zu prüfen, wurde die Phototoxität von ausgewählten Konjugaten an verschiedenen Tumorzelllinien und Kulturen von *S. aureus* getestet. Hierbei erwiesen sich einige der Konjugate als gut wirksam gegen *S. aureus*, insbesondere eines der Konjugate mit einer Kombination aus sauer und reduktiv spaltbarem Linker.

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1.9 *Publications, patent applications and conference contributions*

Publications with peer review process

[1] D. Steinhilber, M. Witting, X. Zhang, **M. Staegemann**, W. Friess, S. Küchler, R. Haag
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[2] **M. H. Staegemann**, S. Gräfe, R. Haag, A. Wiehe
"*A toolset of functionalized porphyrins with different linker strategies for application in bioconjugation"*
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[3] **M. H. Staegemann**, B. Gitter, J. Dernedde, C. Kuehne, R. Haag, A. Wiehe
"*Mannose-Functionalized Hyperbranched Polyglycerol Loaded with Zinc Porphyrin: Investigation of the Multivalency Effect in Antibacterial Photodynamic Therapy"* *Chem. Eur. J.* **2017**, *23*, 3918-3930, DOI: 10.1002/chem.201605236.

[4] **M. H. Staegemann**, S. Gräfe, B. Gitter, K. Achazi, E. Quaas, R. Haag, A. Wiehe
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Biomacromolecules, **Article ASAP**, DOI: 10.1021/acs.biomac.7b01485.

Patents

M. Staegemann, R. Haag, A. Wiehe, S. Graefe, Susanna, B. Gitter, V. Albrecht
"Conjugates of porphyrinoid photosensitizers and glycerol-based polymers for photodynamic therapy"
Pat. Appl. (2017), EP 3210626 A1

Conferences

[5] *International Symposium on Medicinal Chemistry* 2012, **Berlin**, Germany.

[6] *ORCHEM* 2012, **Weimar**, Germany.

[7] *International Symposium of the Collaborative Research Center 765* 2012, **Berlin**, Germany

[8] *International Conference for Porphyrins and Phtalocyanines* 2014, **Istanbul**, Turkey.

[9] *International Symposium of the Collaborative Research Center 765* 2014, **Berlin**, Germany

Oral presentations

[10] **M. H. Staegemann**, A. Wiehe, R. Haag
"Conjugates of Porphyrins and Hyperbranched Polyglycerols for an Application in Photodynamic Therapy"
ORCHEM, 2012, **Weimar**, Germany.

Posterpresentations

[11] **M. H. Staegemann**, A. Wiehe, R. Haag
"Conjugates of Porphyrins and Hyperbranched Polyglycerols for an Application in Photodynamic Therapy"
ORCHEM, 2012, **Weimar**, Germany.

[12] **M. H. Staegemann**, A. Wiehe, R. Haag
"Conjugates of Porphyrins and Hyperbranched Polyglycerols for an Application in Photodynamic Therapy"
International Conference for Porphyrins and Phtalocyanines, 2014, **Istanbul**, Turkey.

1.10 *Curriculum vitae*

Der Lebenslauf ist aus Gründen des Datenschutzes nicht enthalten.