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Original Contribution

# Photodynamic biofilm inactivation by SAPYR—An exclusive singlet oxygen photosensitizer

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ABSTRACT

Prevention and control of biofilm-growing microorganisms are serious problems in public health due to increasing resistances of some pathogens against antimicrobial drugs and the potential of these microorganisms to cause severe infections in patients. Therefore, alternative approaches that are capable of killing pathogens are needed to supplement standard treatment modalities. One alternative is the photodynamic inactivation of bacteria (PIB). The lethal effect of PIB is based on the principle that visible light activates a photosensitizer, leading to the formation of reactive oxygen species, e.g., singlet oxygen, which induces phototoxicity immediately during illumination. SAPYR is a new generation of photosensitizers. Based on a 7-perinaphthenone structure, it shows a singlet oxygen quantum yield $\Phi_\Delta$ of 99% and is water soluble and photostable. Moreover, it contains a positive charge for good adherence to cell walls of pathogens. In this study, the PIB properties of SAPYR were investigated against monospecies and polyspecies biofilms formed in vitro by oral key pathogens. SAPYR showed a dual mechanism of action against biofilms: (I) it disrupts the structure of the biofilm even without illumination; (II) when irradiated, it inactivates bacteria in a polymicrobial biofilm after one single treatment with an efficacy of ≥99.99%. These results encourage further investigation on the potential of PIB using SAPYR for the treatment of localized infectious diseases.



## Introduction

Successful inactivation of biofilm-growing bacteria has gained increasing importance because, according to the NIH, biofilm-associated diseases are causative for more than 80% of all human infections [1]. Bacteria embedded in biofilms have completely different properties from their planktonic counterparts, e.g., an enhanced tolerance against antimicrobial agents [2]. It has been shown that the concentrations of disinfectants and antibiotics needed to kill biofilms were about 100 to 1000 times higher than the concentrations necessary to eradicate the equal quantity of planktonic bacteria [3,4]. Therefore, evaluation of the efficacy of a given antimicrobial agent using biofilm models seems to be more appropriate than the currently used MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) assays where planktonic cultures are used [3–5].

However, due to the extended application of antimicrobials used for combating biofilm-associated infections, resistances of pathogens against antibiotics and antiseptics, e.g., triclosan [6] and chlorhexidine [7], are increasing, especially in methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant *Enterococcus* (VRE) strains which are known to cause severe nosocomial infections all over the world [8].

Consequently, effective alternatives are required for an efficient inactivation of biofilm-growing bacteria. A new option for combating biofilms is the photodynamic inactivation of bacteria (PIB) [9,10]. PIB consists of the irradiation of a per se nontoxic photosensitizer (PS) by

*Abbreviations: AN*, *Actinomyces naeslundii*; BA, Blood agar; CFU, Colony-forming units; Con A, Concanavalin A; CS, Complete saliva medium; *EF*, *Enterococcus faecalis*; EPS, Extracellular polymeric substance; *FN*, *Fusobacterium nucleatum*; $H_2O_2$, Hydrogen peroxide; HO•, Hydroxyl radical; ICG, Indocyanine green; MHA, Mueller-Hinton-agar; OD, Optical density; PBS, Phosphate-buffered saline; PIB, Photodynamic inactivation of bacteria; PN, 7-perinaphthenone, phenalen-1-one; PNS, Perinaphthenone-2-sulfonic acid, phenalen-1-one-2-sulfonic acid; PS, Photosensitizer; ROS, Reactive oxygen species; $^1O_2$, Singlet oxygen; $\Phi_\Delta$, Singlet oxygen quantum yield; $O_2^{\bullet-}$, superoxide ion; TMPyP, 5,10,15,20-tetrakis(1-methyl-4-pyridino)-porphyrin tetra-(p-toluenesulfonate)

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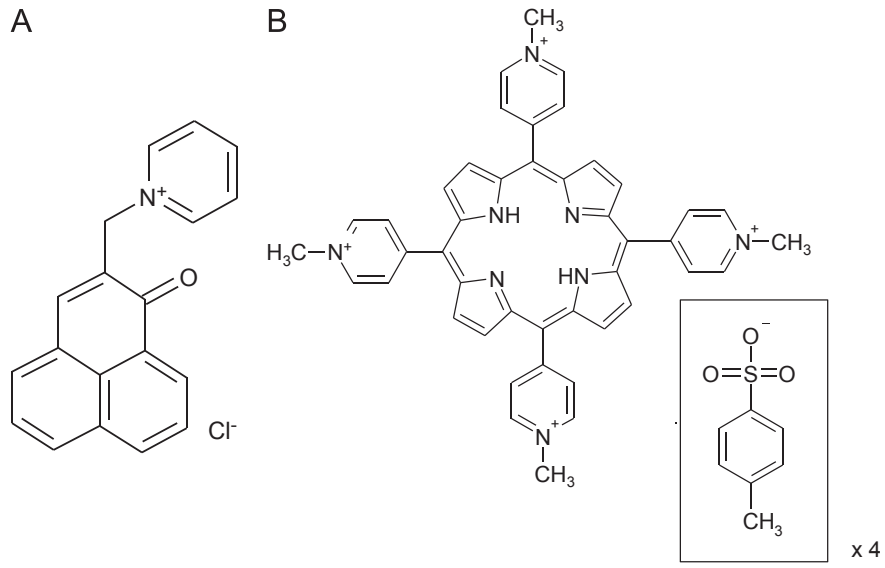


**Fig. 1.** Chemical structures of SAPYR and TMPyP. (A) Chemical structure of SAPYR containing a positively charged pyridinium-methyl substituent. (B) Chemical structure of TMPyP, a well-known reference PS and porphyrin derivative.

means of light of an appropriate wavelength. The absorption of light by the PS leads to a transition of the molecule to its triplet state. There are two general mechanisms of reaction to make the photosensitizer regain its original state: Type I mechanism means that charge is transferred to a substrate or to molecular oxygen via electron abstractions and redox reactions with emergence of reactive oxygen species (ROS) such as the superoxide ion ($O_2\bullet-$), hydrogen peroxide ($H_2O_2$), and the free hydroxyl radical (HO•). Here $O_2\bullet-$ is most likely generated by reaction of molecular oxygen with the one-electron reduction product of the PS [11,12]. Thereby only HO• which is formed via Fenton-like reactions involving $H_2O_2$ is able to induce the decomposition of proteins or lipids [13]. Type II mechanism means that energy (not charge) is transferred directly to molecular oxygen from which the highly reactive and selective singlet oxygen ($^1O_2$) is produced [11,12].

In general, to avoid development of resistances in pathogens, mechanisms acting as multitarget processes are needed in contrast to those of antibiotics which are very specific acting according to the so-called key-hole principle. PIB is such a multitarget process: to avoid any resistance toward the photodynamic process, no specific intracellular or extracellular localizations of a PS or specific targets for the oxidative burst mediated by PIB are in focus [14].

No significant development of resistance to PIB could be observed in microbial cells so far: Tavares et al. demonstrated that strains of *Vibrio fischeri* and *Escherichia coli* did not develop resistance to PIB after 10 repeated cycles of partial inactivation followed by regrowth [15]. Likewise, Giuliani et al. showed that strains of *S. aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* exhibited no decreased susceptibility to PIB even after 20 such cycles [16].

PIB efficacies against biofilms described for currently known PSs are largely varying and are dependent on experimental conditions; for example, using methylene blue, Fontana et al. demonstrated a reduction of only 30% on a polymicrobial biofilm whereas Kishen et al. achieved a killing efficacy of 5 $\log_{10}$ steps against an *Enterococcus faecalis* monospecies biofilm [17,18]. Moreover, it is important to note that the quantum yield of ROS generated by a PS directly affects the efficacy of PIB [19]. In order to optimize PIB with respect to type II mechanism of action, we developed SAPYR—a new generation of photosensitizers, based on a 7-perinaphthenone structure. In contrast to currently known PSs, it shows a $^1O_2$ quantum yield of $\Phi_\Delta = 0.99$, therefore reacting almost quantitatively according to type II mechanism.

The object of the present study was to evaluate the antibacterial photodynamic properties of this new photosensitizer SAPYR against biofilms. Since oral diseases, e.g., periodontitis, are a superior field for application of PIB-mediated topical killing of bacteria [9], biofilms were formed by oral key pathogens. PIB efficacy with SAPYR was tested against monospecies biofilms of *Enterococcus faecalis* and *Actinomyces naeslundii* and a polymicrobial biofilm containing *A. naeslundii*, *Fusobacterium nucleatum*, and *Enterococcus faecalis*, representing an early colonizer, a bridge bacterium, and a species with increased resistance against antimicrobial agents found in each phase of biofilm formation [20–22].

## Materials and methods

### *Photosensitizers and light source*

The new photosensitizer SAPYR (2-((4-pyridinyl)methyl)-1*H*-phenalen-1-one chloride) is a water-soluble dye based on a 7-perinaphthenone (phenalen-1-one, PN) structure (Fig. 1A). It was synthetized in the Department of Organic Chemistry (University of Regensburg, Germany) according to patent No. WO/2012/113860 [23]. The photosensitizer was prepared with a purity of >99%, controlled by NMR and HPLC-MS. The porphyrin-based TMPyP (5,10,15,20-tetrakis(1-methyl-4-pyridino)-porphyrin tetra-(*p*-toluenesulfonate), purity: 97%; Sigma-Aldrich, St. Louis, MO) was used as control PS for antibacterial efficacy (Fig. 1B).

In all PIB experiments SAPYR ($\lambda_{max}$ 360–410 nm with $\varepsilon$>9000 $(\text{mol cm})^{-1}$) and TMPyP ($\lambda_{max}$ 422 nm with $\varepsilon$>220000 $(\text{mol cm})^{-1}$) were irradiated for 120 s with a hand-held dental light-curing unit (Bluephase C8, Ivolar Vivadent, Schaan, Liechtenstein) with an output intensity of $1360 \pm 30$ mW/cm$^2$ in "high power" mode. Absorption spectra of both PSs and the emission spectrum of the light source are shown in Fig. 2. The intensity of light reaching the probes was $600 \pm 15$ mW/cm² because irradiation was from above, obtaining a distance of 0.9 cm due to the height of the wells. Intensities were measured with a thermal low-power sensor (Nova 30 A-P-SH, Ophir-Spiricon, North Logan, UT) and the emission spectrum of the light source was recorded by means of a monochromator with a CCD detection system (SPEX 232, HORIBA Jobin Yvon, Longjumeau Cedex, France).

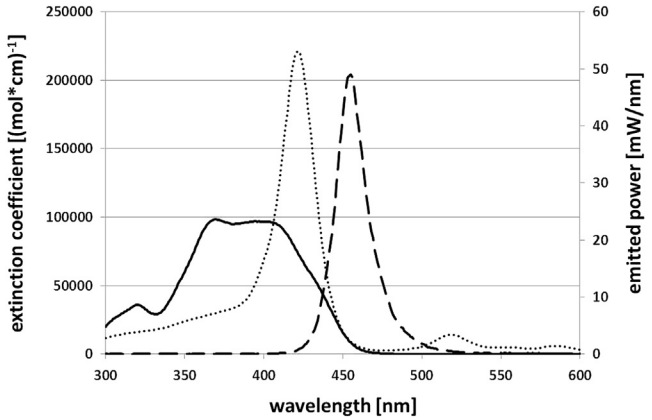


**Fig. 2.** Characteristic absorption spectra of SAPYR and TMPyP and emission spectrum of the light source. The *x*-axis shows the wavelength in nm, the left *y*-axis the extinction coefficients of both PS (solid line: SAPYR magnified by factor 10; dotted line: TMPyP), and the right *y*-axis shows the emitted power of the light source (dashed line: Bluephase C8).

### *Photophysical characterization of SAPYR*

The $^1O_2$ quantum yield $\Phi_\Delta$ of SAPYR was determined using the water-soluble photosensitizer PNS (perinaphthenone-2-sulfonic acid, phenalen-1-one-2-sulfonic acid; $\Phi_\Delta = 1.03 \pm 0.10$; synthesized according to Nonell et al. with a purity of 99% in the Department of Organic Chemistry, University of Regensburg, Germany) [24] and TMPyP ($\Phi_\Delta = 0.74$) [25] as reference PSs. Therefore, the absorbed energies of SAPYR (10 μM), of PNS (12 μM), and of TMPyP (10 μM) were compared with their emitted singlet oxygen luminescence at 1270 nm, as described earlier [26]. The $^1O_2$ luminescence was detected in a time-resolved manner using a nitrogen-cooled photomultiplier (R5509-42, Hamamatsu Photonics, Hamamatsu, Japan) and an ultrafast multiscaler (P7889, FAST ComTec, Oberhaching, Germany). For spectral resolution, luminescence was detected at wavelengths ranging from 1150 to 1400 nm using interference filters in front of the photomultiplier. SAPYR and PNS were excited at 410 nm and TMPyP at 455 nm by a tunable laser system (NT 242-SH/SFG, Ekspla, Vilnius, Lithuania) to ensure a similar rate of absorption for all PSs, whereby different excitation powers ranging from 9 to 100 mW were used. In addition to this, the quenching ability of the triplet state of the PS or of $^1O_2$ was investigated by using the methodology described by Baier et al. [27]. The absorption spectra of SAPYR at different concentrations (1 μM to 1 mM) were measured by means of a photospectrometer (DU 640, Beckman Coulter, Brea, CA) in order to investigate the dimerization of SAPYR. The same spectrometer was used to examine the photostability of SAPYR expending an irradiation energy up to 120 J.

### *Biofilm formation*

Three reference strains, *Enterococcus faecalis* (EF; ATCC 29212), *A. naeslundii* (AN; T14V), and *F. nucleatum* (FN; ATCC 10953) were used in this study. Biofilms were formed in 96-well polystyrene culture plates (Corning Costar, Corning, NY). The medium used was the complete saliva (CS) described by Pratten et al., modified by adding 1% sucrose and applying $N_2$ up to $O_2 = 0\%$ [28]. EF and AN were incubated aerobically in BHI broth (Sigma-Aldrich, St. Louis, MO), FN anaerobically in PYG broth (manufactured according to DSMZ-protocol: medium 104) at 37 °C on a shaker as overnight cultures to obtain bacteria in the static growth phase. Afterward suspensions were harvested by centrifugation (1700 *g*, 5 min; Megafuge 1.0, Heraeus Sepatech, Osterode, Germany) and resuspended in sterile phosphate-buffered saline (PBS: Sigma-Aldrich, St. Louis, MO) in order to yield an optical density (OD) of 0.13, measured at 600 nm by means of a photospectrometer and corresponding to a bacterial count of $10^4$ to $10^5$ bacteria/ml. Subsequently, suspensions were pelletized again and resuspended in CS so that they could be used for biofilm formation.

For monospecies biofilms, wells were inoculated with 200 μl of EF or AN suspensions (OD = 0.13) and incubated aerobically. For polyspecies biofilms, 80 μl of each suspension of AN, EF and FN (OD = 0.13) was consecutively pipetted into the wells. For anaerobic incubation, a box for anaerobes containing anaer-generators (bioMérieux, Marcy-l'Etoile, France) was used. For duospecies biofilm, wells were inoculated with 240 μl of AN suspension and 10 μl of EF suspension (OD = 0.13) and were incubated aerobically. In all cases biofilms were incubated at 37 °C for 72 h; the medium was substituted every 24 h.

### *Visualization of extracellular polymeric substance (EPS)*

The existence of an EPS was shown by multichannel fluorescence microscopy. A Texas Red-conjugated concanavalin A (Molecular Probes, Eugene, OR) was used as staining dye for EPS. Concanavalin A (Con A) is a lectin that binds to D-glucose and D-mannose residues which are very common carbohydrates in the EPS of bacterial biofilms [29]. The corresponding bright-field image of the biofilm was colorized green to enhance contrast using Image-Pro Plus 5.0 (Media Cybernetics, Rockville, MD) as an imaging software. Biofilms were formed as described above and washed twice with PBS to remove nonadherent bacteria. Subsequently, samples were incubated for 30 min with Con A (100 μg/ml). Then biofilms were washed twice with PBS and were visualized with a multichannel fluorescence microscope (AXIO Imager.Z1 with ApoTome, Carl Zeiss Microscopy, Göttingen, Germany) using an appropriate filter set for excitation and emission of Texas Red-conjugated Con A ($\lambda_{ex}$ 595 nm/$\lambda_{em}$ 615 nm).

### *Photodynamic inactivation of bacteria*

In preliminary experiments, PIB was done on bacterial suspensions using different concentrations of SAPYR. A 100 μM SAPYR achieved a photodynamic killing efficacy of ≥7 $\log_{10}$ steps against planktonic EF and AN; therefore, 100 μM was used as concentration against biofilm-grown bacteria. Different incubation periods were examined in EF monospecies biofilms before the period of 60 min was chosen; inactivation of EF-CFU was ≥1 $\log_{10}$ step for 10 min and ≥2 $\log_{10}$ steps for 30 min incubation of SAPYR.

After a cultivation period of 72 h, biofilms were washed twice with sterile PBS to remove nonadherent bacteria and were incubated either with 100 μl of 100 μM PS (groups PS+L+, PIB and PS+L-, treatment with PS only) or with 100 μl PBS (PS-L+, treatment with light only; and PS-L-, untreated control) for 60 min in the dark. Then the samples were irradiated for 120 s (PS+L+, PS-L+) or maintained in the dark during the same period (PS+L-, PS-L-). The intensity of the light source reaching the probes (600 mW/cm$^2$) and the period of irradiation (120 s) were constant for all PIB experiments. Immediately afterward PS or PBS was carefully removed and each biofilm was brought to suspension with 200 μl of PBS and transferred to an Eppendorf tube. These were placed in an ultrasonic water-bath chamber (Qualilab USR30H, Merck Eurolab, Bruchsal, Germany) obtaining a frequency of 35 kHz for 5 min and vortexed (REAX top, Heidolph Instruments, Schwabach, Germany) for 5 s to separate aggregated bacteria. Then serial 10-fold dilutions ($10^{-2}$ to $10^{-7}$) were prepared in BHI broth and aliquots (3 × 20 μl) were plated on agar plates, as described earlier [30]. Agar plates were incubated aerobically for 24 h (EF) or 48 h (AN) at 37 °C; then colony-forming units (CFU) were counted.

For CFU detection in monospecies biofilms, blood agar (BA) was used for AN whereas Mueller-Hinton Agar (MHA) was used for EF. Since there was no growth of AN on MHA, this agar was used to detect EF in polyspecies and duospecies biofilms. In contrast to this, as both species can be cultured on BA, some adjustments were necessary for detecting AN in polyspecies biofilms. In PS+L+ group, AN-CFU were countable on BA; distinction against EF was made optically; and veracity of collected samples was periodically proved by MALDI-TOF mass spectroscopy (Institute of Medical Microbiology and Hygiene, University Hospital Regensburg, Germany). In untreated control groups of polyspecies biofilms, it was not possible to distinguish the colony forms of EF and AN neither in undiluted nor in higher diluted samples due to the faster growth of EF; however, it was apparent that the amount of AN-CFU reached at least the level of AN monospecies biofilms. So we had to define an amount of CFU for the PS-L- group. Conservative values of $10^8$ to $10^9$ CFU were chosen in analogy with CFU levels of PS-L- group in AN monospecies biofilms.

Reduction of FN was not investigated since PIB treatment was not possible under anaerobic conditions; thus, results of PIB against this obligate anaerobe would have been distorted and killing by PIB would have been indiscernible from killing by exposure to aerobiosis. Yet, FN played an important role as bridge bacterium in polyspecies biofilm formation [21].

For eliminating the securing effect of an intact EPS, PIB was also done in AN monospecies biofilms which were disrupted by bringing to suspension with 100 μl PBS and by placing in an ultrasonic water-bath chamber for 5 min and vortexing for 5 s. Afterward each sample was mixed with 100 μl PS obtaining a final PS concentration of 100 μM (groups PS+L+ and PS+L-) or with 100 μl PBS (PS-L- and PS-L+). The procedure was continued as described above.

### *Measurement of extracellular polysaccharides*

The content of extracellular polysaccharides in our biofilms was assessed as an index for the amount of EPS. A recently published manual based on the DuBois method was used to compare the total carbohydrate content of our biofilms [31,32]. Glucose in concentrations from 10 to 50 μg/ml was used as standard sugar for calibration.

EF and AN monospecies biofilms and polyspecies biofilms were formed as described. After 72 h, biofilms were washed with PBS to remove excess medium. Then the samples were brought to suspension with 200 μl PBS, transferred to Eppendorf tubes, placed in an ultrasonic water-bath chamber for 5 min, and vortexed for 5 s. Thirty microliters of these suspensions or 30 μl PBS only (blank) was mixed with 30 μl of 5% phenol and 150 μl of concentrated sulfuric acid in 96-well plates. After 30 min OD was measured at 490 nm with a microplate reader (EMax Precision Microplate Reader, Molecular Devices, Biberach, Germany).

### *Biofilm detachment*

Detachment of parts of the biofilm following PIB was detected by a biofilm formation assay [33]. EF monospecies biofilms were formed and PIB was done with SAPYR as described (groups PS+L+, PS+L-, PS-L+, PS-L-). Subsequently PS or PBS was removed and 25 μl of 1% Crystal Violet (Merck, Darmstadt, Germany) was added to each well. Samples were incubated at room temperature for 15 min in the dark and washed triply with $H_2O$ to remove excess stain. Then $2 \times 200$ μl of 95% ethanol was added to each stained sample to dissolve the bound Crystal Violet. This suspension was transferred to an Eppendorf tube and $H_2O$ was added to a total volume of 1 ml. After vortexing for 10 s, 200 μl of each sample was transferred to a new 96-well plate and the absorbance was measured with a microplate reader.

### *Bacterial viability in supernatants*

Viability of detached bacteria was assessed following PIB with SAPYR. Therefore monospecies biofilms of EF were grown and PIB was performed as described (groups PS+L+, PS+L-, PS-L+, PS-L-). PS or PBS was then removed and biofilms were washed triply with 200 μl PBS. Afterward this supernatant was transferred to an Eppendorf cup and it was proceeded as described above in PIB experiments. Serial 10-fold dilutions were prepared and were plated on MHA. CFU were counted after incubation for 24 h at 37 °C.

### *Data treatment and statistical analysis*

All results are shown as medians, including 25 and 75% quartiles and were calculated using SPSS for Windows, version 20 (SPSS Inc., Chicago, IL) from the values of at least six independent experiments, each performed in duplicate. Significance analyses were performed using nonparametric Mann-Whitney tests, followed by applying the error-rates method ($\alpha = 0.05$). In PIB experiments, horizontal solid and dashed lines represent reductions of 3 and 5 $\log_{10}$ steps CFU respectively, compared with untreated control groups PS-L-. Medians on or below these lines exhibit an efficacy of killing of bacteria of 99.9% (3 $\log_{10}$) or 99.999% (5 $\log_{10}$). According to the guidelines of hand hygiene, this is declared as biologically relevant antimicrobial activity or disinfectant effect [34].

## Results

### *Photophysical characterization of SAPYR*

For visualization of the $^1O_2$ luminescence of SAPYR (Fig. 1A) and TMPyP (Fig. 1B), three-dimensional plots were generated as a combination of time-resolved and spectrally resolved measurements at the same absorbed energies (350 mJ per measurement) (Fig. 3A, SAPYR; B, TMPyP). For SAPYR, a $^1O_2$ quantum yield of $\Phi_\Delta = 0.99 \pm 0.05$ could be determined; this is clearly higher compared with TMPyP ($\Phi_\Delta = 0.74$) [25]. For concentrations from 1 to 200 μM, a $^1O_2$ decay time of $3.5 \pm 0.2$ μs and a triplet decay time of SAPYR of $2.3 \pm 0.2$ μs were measured; so no quenching ability of $^1O_2$ or of the triplet state of SAPYR could be observed. Moreover, no dimerization of the SAPYR solutions could be detected. In order to investigate the $^1O_2$ generation of SAPYR and therefore its photostability, the $^1O_2$ luminescence was measured every minute over a period of 20 min under continuous irradiation (100 mW) (Fig. 4). Here a slight decrease of $^1O_2$ luminescence generated by SAPYR could be observed compared with TMPyP. The $^1O_2$ luminescence and absorption of SAPYR decreased by ≤20% when irradiated at 410 nm with 120 J for 20 min; during this process approximately 20 J of the applied energy was absorbed. The $^1O_2$ luminescence and absorption of TMPyP decreased by ≤2% with virtually the same absorbed energy at 455 nm. However, the $^1O_2$ luminescence of SAPYR still exceeded the $^1O_2$ luminescence of TMPyP after 20 min of continuous irradiation.

For further comparison of both PSs, the overall energies absorbed $E_{abs}$ for SAPYR and TMPyP were calculated as follows:

$$E_{abs} = \sum_{\lambda} A \times E_{\lambda} = \sum_{\lambda} \left(1 - 10^{-\varepsilon(\lambda) c d}\right) \times E_{\lambda}$$

where $A$ is absorption of PS, $E_\lambda$ is emitted energy of light source (Bluephase C8) after irradiation for 120 s, $\varepsilon$ is extinction coefficient, $c$ is concentration of PS, $d$ is thickness of PS solution.

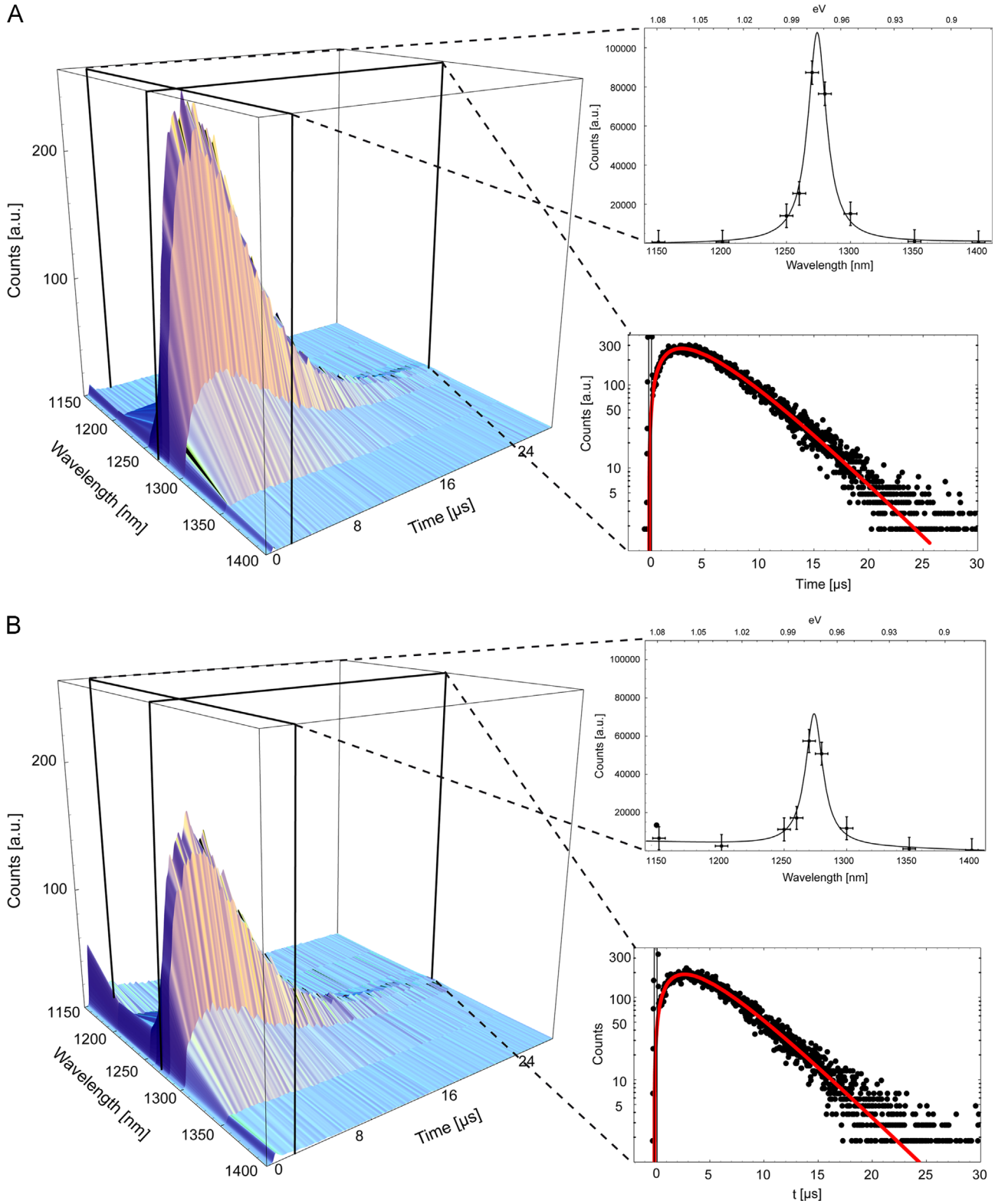


**Fig. 3.** 3D plots of the $^1O_2$ luminescence of SAPYR and TMPyP. (A) Three-dimensional plot (left) generated by combination of time-resolved and spectrally resolved singlet oxygen signals (right) of SAPYR. (B) Three-dimensional plot (left) generated by combination of time-resolved and spectrally resolved $^1O_2$ signals (right) of TMPyP.

The concentration of both PSs was 100 μM and the thickness of the PS solutions was 3 mm. Following this equation an overall absorbed energy for SAPYR is calculated as 12.5 J, for TMPyP as 80 J, which means that the total absorbed energy of TMPyP was 6.4 times higher than that of SAPYR.

For determination of the absolute quantum yields of singlet oxygen, both absorbed energies must be multiplied by the respective singlet oxygen quantum yields (0.99 for SAPYR and 0.74 for TMPyP). These results showed that the absolute singlet oxygen quantum yield of TMPyP was 4.8 times higher than that of SAPYR.

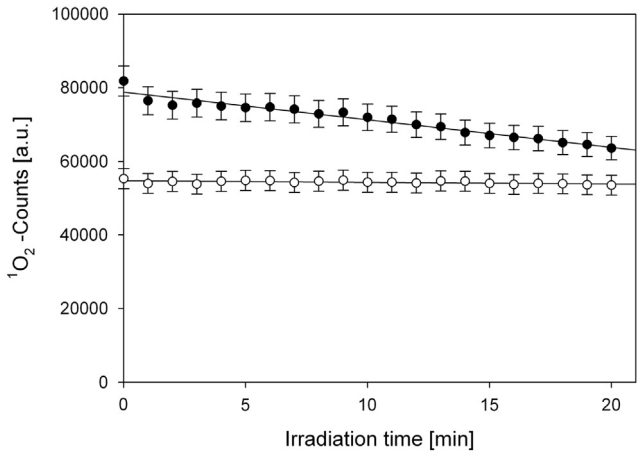


**Fig. 4.** $^1O_2$ luminescence of SAPYR and TMPyP under long-term irradiation: $^1O_2$ luminescence generated by SAPYR (filled dots) and TMPyP (open dots) under continuous irradiation for 20 min (100 mW) at 410 and 455 nm, respectively.

*Visualization of extracellular polymeric substance*

EPS was visualized by staining with a Texas Red-conjugated Con A to confirm a mature biofilm. Fig. 5 shows an image of a stained polyspecies biofilm containing a diffuse red-colored structure, where Con A binds to, confirming the existence of an EPS.

*Photodynamic inactivation of bacteria*

SAPYR was evaluated against EF and AN in monospecies and polyspecies biofilms. In all experiments the light intensity reaching the samples (600 mW/cm$^2$) and the period of irradiation (120 s) were constant. In EF monospecies biofilms TMPyP served as control PS for PIB efficacy under identical experimental conditions.

Here EF exhibited negligible reduction of CFU ($\leq$1 $\log_{10}$ step) when TMPyP was used (Fig. 6A) whereas SAPYR revealed a high inactivation efficacy of $\geq$5 $\log_{10}$ steps CFU (Fig. 6B). In polyspecies biofilms, SAPYR reduced CFU of EF by $\geq$5 $\log_{10}$ steps as well (Fig. 6C). In AN monospecies biofilms, CFU of AN were reduced by $\geq$2 $\log_{10}$ steps after irradiation with SAPYR. When AN was detected in polyspecies biofilms, the decrease of CFU was $\geq$4 $\log_{10}$ steps (Fig. 6D). Moreover, biofilms containing EF and AN were formed with a 20-fold amount of AN cells at the start of biofilm culture in order to confirm the securing effect of an AN-like EPS composition. In these duospecies biofilms, CFU of EF were reduced by $\geq$4 $\log_{10}$ steps (Fig. 6E) and therefore $\approx$1 $\log_{10}$ step less than in EF monospecies biofilms, affirming the hypothesis of a protective effect for EF by an AN-like EPS. For further confirmation that EPS might act as a shelter for bacteria in a biofilm, PIB was performed with bacteria from a disrupted 72 h AN monospecies biofilm. Here PIB resulted in an eradication of AN CFU by $\geq$8 $\log_{10}$ steps (Fig. 6F). In all cases there was no reduction of CFU after treatment with PS or light only.

*Measurement of extracellular polysaccharides*

The total carbohydrate content of monospecies and polyspecies biofilms was chemically assessed to obtain an index for the amount of EPS. Results showed a significant difference in OD median ($P$=0) of AN monospecies biofilms in comparison to EF monospecies biofilms and polyspecies biofilms (Fig. 7A). The calibration curve was obtained with glucose as standard and showed clear linearity ($r^2$=0.997) (Fig. 7B).

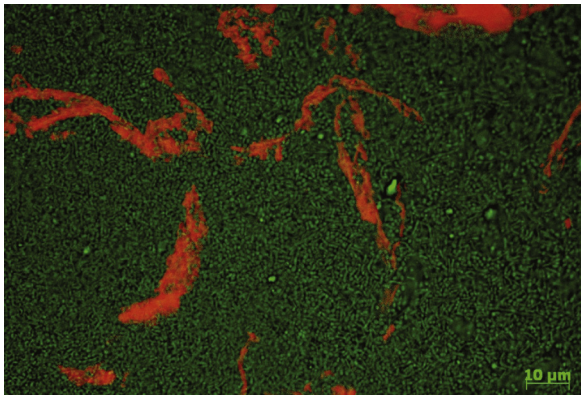


**Fig. 5.** Visualization of EPS. Polyspecies biofilm stained with Con A (red) for EPS, visualized by multichannel fluorescence microscopy. The corresponding bright-field image was colored green to enhance contrast using an imaging software. Presence of EPS is demonstrated by the red-colored area.

*Biofilm detachment*

The ability of SAPYR to detach parts of the biofilm was analyzed in EF monospecies biofilms which were used as a model. The results (Fig. 8A) show a significant decrease of OD median ($P$=0) in experimental groups that were incubated with SAPYR, regardless of whether the samples were illuminated or not (groups PS+L+ and PS+L-), compared with the controls that were incubated with PBS (PS-L- and PS-L+). This means that incubation with SAPYR leads to detachment of parts of the biofilm.

*Bacterial viability in supernatants*

Supernatants of EF monospecies biofilms were assessed for viable bacteria following PIB with SAPYR to demonstrate viability or death of detaching bacteria. CFU were $\leq$10 in the photodynamic treated group PS+L+. In contrast to this, CFU of control groups (PS-L-, PS-L+, PS+L-) were $\geq$10$^8$, indicating that bacteria that detach following PIB are not viable (Fig. 8B).

## Discussion

In the present study we evaluated a new photosensitizer SAPYR which is based on a 7-perinaphthenone structure against EF and AN in monospecies and polyspecies biofilms.

Oxidative stress, mostly generated by ROS such as superoxide ($O_2\bullet^-$), hydrogen peroxide ($H_2O_2$), and the free hydroxyl radical (HO•), is ubiquitous in bacterial life [35]. Thus, bacteria express superoxide dismutases (SOD) as a defense mechanism; these SOD enzymes, catalases, and peroxidases are able to catalyze the dismutation of ROS to $H_2O$ and molecular oxygen [36]. The ROS generated by type I mechanism of the photodynamic processes are similar to those produced in the bacterial oxygen metabolism or by neutrophils and macrophages [37]. Therefore, these defense mechanisms could be effective against PIB-mediated oxidative death of bacteria. Karavolos et al. demonstrated that expression of SOD increased in *S. aureus* after treatment with oxidative stress-generating agents [38]. Furthermore SOD activity of *S. aureus* was increased after PIB with Protoporphyrin IX, but only in PIB-susceptible strains [39].

However, these defense mechanisms may also be overcome by exalted generation of ROS of type I mechanism of action. This has been shown for several functionalized fullerenes which act mostly via type I mechanism and were found to be efficient PSs for PIB [40].

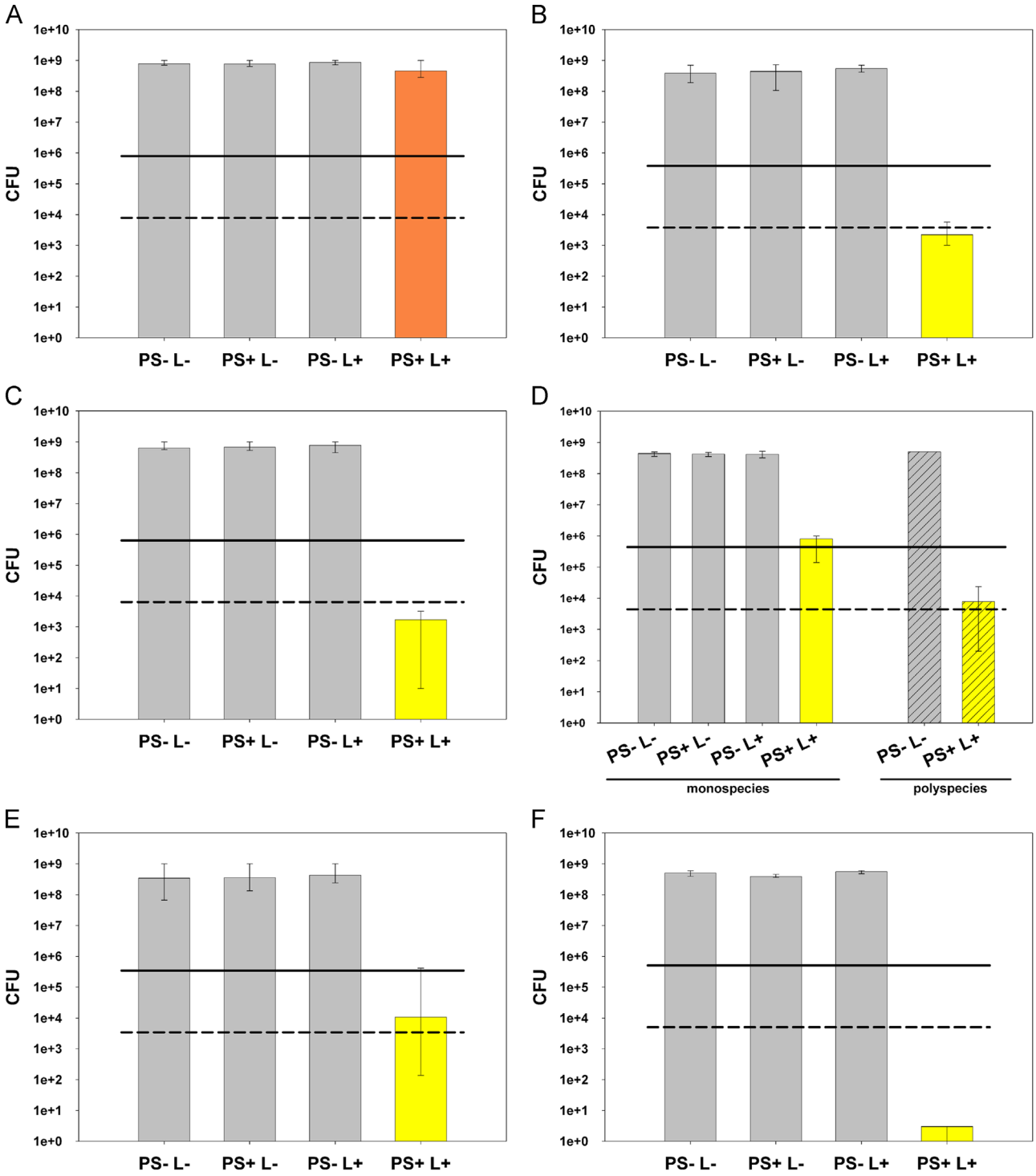


**Fig. 6.** Photodynamic biofilm inactivation. (A–F) All following PIB experiments in this figure are shown as CFU medians with 25 and 75% quartiles. Solid and dashed lines show a CFU reduction of ≥3 and ≥5 $\log_{10}$ steps, respectively. In detail: (A) PIB in EF monospecies biofilm with TMPyP: PS+L+ group (orange) shows no reduction of CFU. (B) PIB in EF monospecies biofilm with SAPYR: CFU of PS+L+ group (yellow) reduced by ≥5 $\log_{10}$ steps. (C) PIB in polyspecies biofilm with SAPYR, detection of EF: CFU of PS+L+ group (yellow) reduced by ≥5 $\log_{10}$ steps. (D) Open bars left: PIB in AN monospecies biofilm with SAPYR: CFU of PS+L+ group (yellow) reduced by ≥2 $\log_{10}$ steps. Hatched bars right: PIB in polyspecies biofilm with SAPYR, detection of AN: CFU of PS+L+ group (yellow) reduced by ≥4 $\log_{10}$ steps. (E) PIB in duospecies biofilm with SAPYR, detection of EF: PS+L+ group (yellow) shows a reduction ≥ 4 $\log_{10}$ steps CFU. (F) PIB with bacteria from a disrupted *AN* biofilm with SAPYR: PS+L+ group (yellow) shows a reduction by ≥8 $\log_{10}$ steps CFU.

The proportion of PIB mechanisms of reaction is different for each PS: hereby the $^1O_2$ quantum yield $\Phi_\Delta$ describes the amount of type II reaction. Currently used PSs such as methylene blue ($\Phi_\Delta$=0.52) or TMPyP ($\Phi_\Delta$=0.74) have a $\Phi_\Delta$ in a range of approximately 0.4 to 0.8 [25] so that a substantial amount of type I mechanism remains. Thus, to minimize up-regulation of stress-regulated genes on oxidative stress signals induced by PIB, we developed a new type of photosensitizer—SAPYR—for PIB regarding type II mechanism of action only. The precursor molecules of SAPYR, 7-perinaphthenone (phenalen-1-one; PN) and perinaphthenone-

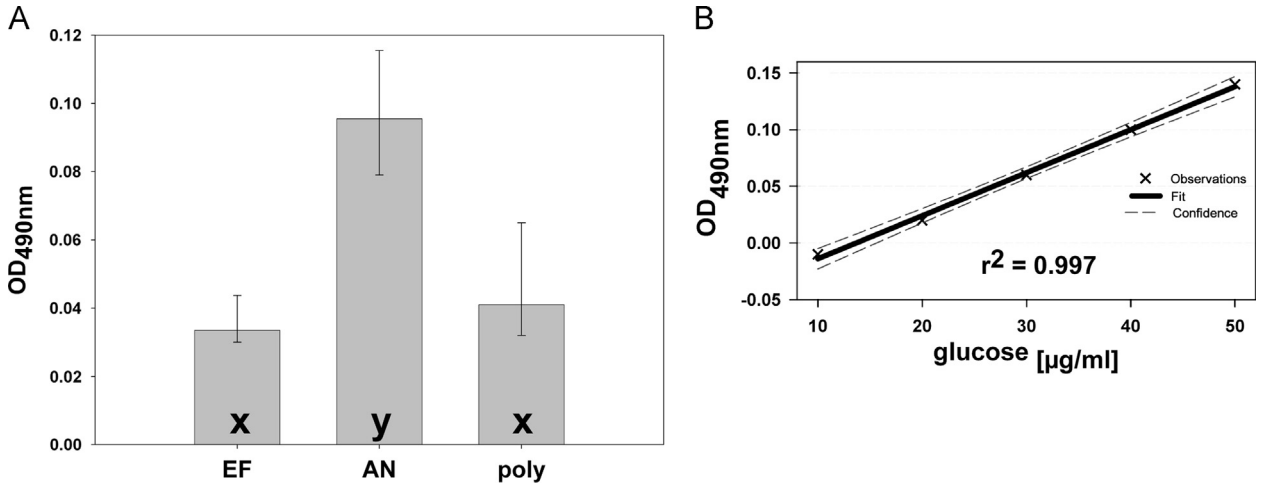


**Fig. 7.** Measurement of extracellular polysaccharides. (A) OD medians with 25 and 75% quartiles of total carbohydrate concentration in *AN* monospecies (AN), EF monospecies (EF), and polyspecies (poly) biofilms. Groups with different lower letters *x* or *y* were found significantly different ($P=0$ for each pair). (B) Standard calibration curve with glucose concentrations from 10 to 50 μg/ml which was used for ensuring linearity ($r^2=0.997$).

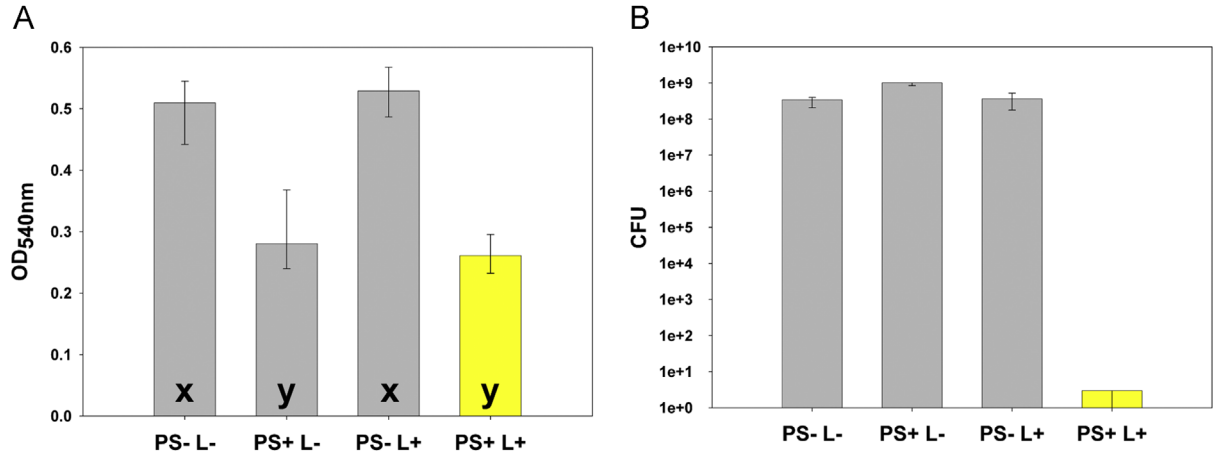


**Fig. 8.** Biofilm detachment following PIB and bacterial viability in supernatants. (A) Biofilm detachment following PIB with SAPYR, EF monospecies biofilm: OD medians with 25 and 75% quartiles representing the amount of biofilm that stayed attached. Groups incubated with SAPYR (PS+) exhibit significantly more detachment compared to groups incubated with PBS (PS-). Groups with different lower letters x or y were found significantly different ($P=0$ for each pair). (B) Bacterial viability in supernatants following PIB with SAPYR: EF monospecies biofilm: CFU medians with 25 and 75% quartiles. There were ≤10 CFU detectable in PS+L+ group (yellow) compared to $\geq 10^8$ in control groups, indicating that bacteria that detach following PIB are not viable.

2-sulfonic acid (phenalen-1-one-2-sulfonic acid; PNS), are the only currently known PSs with $\Phi_\Delta \approx 1$, therefore reacting nearly quantitative in accordance to type II mechanism [24]. The drawbacks of PN are its insolubility in water showing a relatively low photostability in ethanol [41,42] and the lack of a positive charge, whereas PNS is water soluble and photostable but has a negative charge [24]. However, positively charged photosensitizers are essential for good electrostatic adherence to or penetration through the cell walls of pathogens [43]. Therefore we introduced a pyridinium-methyl substituent in the neighboring position to the carbonyl group in PN to develop SAPYR (2-((4-pyridinyl) methyl)-1*H*-phenalen-1-one chloride), eliminating the drawbacks of PN and PNS but conserving the high $^1O_2$ quantum yield $\Phi_\Delta = 0.99$. Consequently, in contrast to the oxygen radicals generated by type I mechanism, $^1O_2$ cannot be quenched by oxidative stress enzymes since $^1O_2$ is no oxygen radical but energized molecular oxygen (+0.98 eV = $+1.57 \times 10^{-19}$ J).

Moreover, the water-soluble SAPYR showed adequate photostability. So neither any quenching ability of both $^1O_2$ and the triplet state of SAPYR nor any dimerization of SAPYR molecules could be observed. However, the generation of $^1O_2$ by SAPYR decreased slightly on long-term irradiation for 20 min. In contrast to this, the irradiation period in PIB experiments for inactivation of biofilms was 120 s. Within this time period, under our experimental conditions for biofilm inactivation, an eventual photoinstability of SAPYR does not have a significant impact on $^1O_2$ generation since the decrease in $^1O_2$ luminescence was less than 5% within the first 2 min of irradiation which is within experimental accuracy. Furthermore, the generation of $^1O_2$ by SAPYR still exceeded the generation of $^1O_2$ by TMPyP distinctly when both were irradiated long term for up to 20 min.

Here PIB with SAPYR was able to inactivate EF in both monospecies and polyspecies biofilms by at least 5 $\log_{10}$ steps of CFU; this is defined as a disinfectant effect [34]. TMPyP, also referred to as TMP, was used as control for evaluation of SAPYR since it is described as an efficient PS against planktonic [44] and sessile bacteria [45,46]. In this study, light intensity and irradiation period were constant for all PIB experiments with SAPYR and TMPyP. However, it must be considered that due to the distinct absorption spectra of both PSs the overall absorbed energy of TMPyP was about 6.4 times higher compared with the absorbed energy of SAPYR. Thus, the absolute yield for $^1O_2$ generation was 4.8 times higher for TMPyP under the given conditions. Nevertheless, even so TMPyP had no effect against EF monospecies biofilms; there

was no reduction of CFU. The difference in molecular structure possibly accounts for this difference in PIB efficacy against bacterial biofilms. TMPyP has a larger molecular structure compared with SAPYR (682.2 and 272.3 g/mol without counterions, respectively); therefore, the penetration of TMPyP through EPS may be hindered by steric reasons since permeability of diffusion decreases with increasing biomass [47]. TMPyP also contains four positive charges per molecule while SAPYR is positively single charged; thus, stronger electrostatic interactions with biofilm components like negatively charged EPS molecules may delay drug diffusion [48,49]. As a result, the generation of $^1O_2$ induced by PIB with TMPyP may occur only at the outer area of the biofilm which leads to a marginal effect of biofilm killing. For further enhancement of PIB efficacy with SAPYR, a light source must be developed with an emission spectrum optimized for SAPYR since the one used here must be regarded as much more suitable for TMPyP than for SAPYR. However, this blue-light emitting lamp was selected because it is a commonly used light source in dental practice for photopolymerization of resins.

SAPYR also had a high killing efficacy against AN in polyspecies biofilms (4 $\log_{10}$ steps reduction at least). In contrast to this, inactivation of AN was far smaller in AN monospecies biofilms where only a reduction of at least 2 $\log_{10}$ steps could be achieved. At first glance, this seems to be unexpected since polyspecies biofilms are normally more resistant against antimicrobials than their monospecies analogues [50,51]. However, many studies suggest that the amount of EPS in a biofilm influences the antimicrobial effect of PIB: Inactivation of bacteria occurred mostly in the exterior stratum of the biofilm whereas bacteria in the innermost regions stayed alive due to insufficient penetration of PS throughout EPS [51,52]. Likewise Zaura-Arite et al. showed by confocal microscopy that treatment with 0.2% chlorhexidine decreased viability of bacteria only in the outer layer of in situ grown dental plaque but the middle and inner layers were not affected [53]. In order to determine whether EPS is responsible for the enhanced survival rate of AN in its monospecies biofilms, e.g., by retarding penetration or by exclusion of a given PS within the biofilm, a duospecies biofilm of EF and AN was formed with the 20-fold amount of AN cells at the start of biofilm, assuming that through this AN would become prevalent in EPS composition. Therefore EF should be inactivated not as effectively by PIB in these duospecies biofilms than in EF monospecies and polyspecies biofilms due to an EPS composition similar to AN monospecies biofilms. CFU of EF were reduced tendentially less (≈1 $\log_{10}$ step) by PIB than in EF monospecies and polyspecies biofilms, affirming this hypothesis and indicating that there is a protective effect for EF when it is embedded in an EPS structure with AN dominating biofilm formation and EPS composition. PIB also was done with bacteria disrupted from a mature AN monospecies biofilm to eliminate this protective effect of EPS, anticipating that AN is inactivated more effectively. Results showed an eradication of AN-CFU by more than 8 $\log_{10}$ steps, confirming that the EPS may be responsible for smaller PIB killing rates in AN monospecies biofilms.

For further confirmation of EPS influence on PIB-mediated killing we analyzed EPS of our biofilms by comparing their carbohydrate component. The range of our OD values measured is quite a low area (≈0.03–0.15) but since our standard calibration curve showed clear linearity ($r^2$=0.997), the OD values measured must be regarded as assured. Our results revealed that the AN monospecies biofilm has a significant higher amount of carbohydrates than the other two biofilms. It can be assumed that a higher total carbohydrate content refers to a more mature EPS and furthermore to a better protection of the bacteria in the biofilm against penetration of PSs, explaining the results of PIB experiments. Similarly the EPS composition of the EF monospecies biofilm cannot virtually differ from the polyspecies biofilm since both have a similar total carbohydrate content; this can explain why there was only a negligible difference in PIB efficacy in both the EF monospecies and polyspecies biofilms (reduction of ≥5 $\log_{10}$ steps CFU each).

It is well known that biofilms are able to release planktonic bacteria and disperse them into their environment for colonization of new surfaces [5,54,55]. Detachment of microorganisms can be due to exposure to stress from mechanical shear forces [56] or generated by biocides and antimicrobials [57]. Collins et al. described that PIB had a 2-fold effect against biofilms of P. aeruginosa: (I) inactivation of bacteria after PIB with TMPyP and (II) also detachment of parts of the biofilm caused by a disruption and alteration of biofilm structure [46].

We analyzed SAPYR for its detachment properties in EF monospecies biofilm by measuring the amount of biofilm that stayed attached to the surface after PIB with SAPYR. There was significant detachment of parts of the biofilm following PIB as well as following treatment with SAPYR alone without irradiation, indicating that SAPYR can act as a tenside. This may be due to its chemical structure which comprises the combination of hydrophilic pyridinium unit with a large hydrophobic tail; it can be compared to known tensides such as cetylpyridinium chloride whose quality of causing biofilm detachment has already been shown [58]. Consequently we assayed the viability of detached bacteria in order to show if bacteria surviving PIB can disseminate and resettle on new surfaces. Our findings show that this can be excluded: following PIB there was CFU ≤10 in detached supernatants. This confirms even more the high killing efficacy of SAPYR.

The total treatment period of 62 min (60 min incubation followed by 120 s irradiation) needed in this study seems to be a drawback of PIB with SAPYR. Nevertheless, this study focuses on inactivation of a polymicrobial biofilm and this must be taken into account. Thus, total treatment time of PIB using SAPYR must be compared with other antimicrobial approaches to polymicrobial biofilms. “Gold standards” for combating biofilms such as disinfectants and antibiotics must be considered. For example, treatment with 30% ethanol was needed for at least 2 h against biofilms containing *C. albicans* and *S. aureus* to eliminate detectable metabolic cell activity [59]. Similar results have been achieved using chlorhexidine on polymicrobial biofilms: Pratten et al. described that treatment for 60 min was needed against six-species biofilms using 0.2% chlorhexidine and killing rates ranged from 2 to 5 $\log_{10}$ steps, depending on the detected microorganism [60]. Sedlacek and Walker tested antibiotics in biofilms formed with bacteria from collected human saliva and grown up to 10 days. Antibiotic treatment for 48 h with different drugs at therapeutic level concentrations reduced viability only less than 1 $\log_{10}$ whereas reductions of up to 4 $\log_{10}$ steps could be obtained when concentrations were heightened distinctly over therapeutically achievable levels [61].

Overall these total treatment times described are in a similar range like PIB with SAPYR, receiving reduction rates mostly similar to those shown in the present study. Yet, it must be a major goal for further studies to reduce the incubation period while conserving the high killing efficacy.

However, before clinical application SAPYR must be investigated in further studies in order to exclude any harmfulness on human tissues. Moreover, an appropriate formulation for applying SAPYR must be designed so that bacteria on dermal and mucosal surfaces can be reached.

## Conclusion

In this study we present SAPYR as a new generation of photosensitizers. Based on 7-perinaphthenone structure, SAPYR exhibits

a singlet oxygen quantum yield of $\Phi_{\Delta}=0.99$, water solubility, photostability, and a high killing efficacy against monospecies and polyspecies biofilms. Therefore PIB with SAPYR offers an efficient approach to destroying oral key pathogens by means of an exclusive type II mechanism of photodynamic action. SAPYR has a dual mechanism of action against bacterial biofilms: (I) it can disrupt the structure of the biofilm; (II) it is able to kill bacteria with a pronounced efficiency. Overall the high efficacy of photodynamic inactivation of a polymicrobial biofilm exhibited by SAPYR encourages further improvement of handling modalities and testing of PIB as an attractive approach to prevention and control of biofilm-related infections.

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