AUS DEM LEHRSTUHL
FÜR ZAHNERHALTUNG UND PARODONTOLOGIE
PROF. DR. WOLFGANG BUCHALLA
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

Der Einfluss der Anzahl absorbierter Photonen auf die photodynamische Inaktivierung von Bakterien im Biofilm und anhand eines Zahnmodells *in vitro*

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Zahnmedizin

der Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Andreas Pummer

2016

AUS DEM LEHRSTUHL
FÜR ZAHNERHALTUNG UND PARODONTOLOGIE
PROF. DR. WOLFGANG BUCHALLA
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

Der Einfluss der Anzahl absorbierter Photonen auf die photodynamische Inaktivierung von Bakterien im Biofilm und anhand eines Zahnmodells *in vitro*

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Zahnmedizin

der Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Andreas Pummer

2016

Dekan:	Prof. Dr. Dr. Torsten E. Reichert
1. Berichterstatter:	Prof. Dr. Wolfgang Buchalla
2. Berichterstatter:	PD. Dr. Tim Maisch

Tag der mündlichen Prüfung: 08.12.2016

Meiner Familie in Liebe und Dankbarkeit

Inhaltsverzeichnis

Vorwort

Bestandteil vorliegender Dissertation sind zwei Originalpublikationen, welche in der Fachzeitschrift *Frontiers in Microbiology* erschienen sind. Die vorliegende Arbeit ist entsprechend dieser beiden Publikationen in zwei Teile gegliedert, wobei beide Teile gleichermaßen jeweils in eine Zusammenfassung und einen Ausblick bezüglich zukünftiger Forschungsmöglichkeiten gegliedert sind sowie den Abdruck der englischsprachigen Originalpublikation enthalten.

In der ersten Arbeit wird anhand der photodynamischen Inaktivierung von Monospezies-Biofilmen eine Formel vorgestellt, welche es erlaubt, die Zahl der von einem bestimmten Photosensibilisator pro Sekunde absorbierten Photonen zu berechnen. Im zweiten Teil erfolgt die Anwendung der im ersten Teil vorgestellten Methodik zur Angleichung der Belichtungsparameter anhand eines Modells bestehend aus extrahierten humanen Zähnen und simulierten zahnumgebenden Geweben aus Kunststoff.

Nachfolgend aufgeführt die Titel der beiden Publikationen:

Fabian Cieplik, **Andreas Pummer**, Johannes Regensburger, Karl-Anton Hiller, Andreas Späth, Laura Tabenski, Wolfgang Buchalla, Tim Maisch:

The impact of absorbed photons on antimcrobial efficacy.

Front. Microbiol. 2015; 6: 706

(Impact Factor 2015: 4,165)

Fabian Cieplik*, **Andreas Pummer***, Christoph Leibl, Johannes Regensburger, Gottfried Schmalz, Wolfgang Buchalla, Karl-Anton Hiller, Tim Maisch:

Photodynamic inactivation of root canal bacteria by light-activation through human dental hard and surrounding soft tissue.

Front. Microbiol. 2016; 7: 929

(Impact Factor 2015: 4,165)

(* = gleichberechtigte Erstautorschaft)

Teil I: Der Einfluss absorbierter Photonen auf die antimikrobielle photodynamische Wirksamkeit

Zusammenfassung

In einer kürzlich erschienenen Stellungnahme erklärten die "Centers for Disease Control and Prevention (CDC) of the United States of America", dass antibiotikaresistente Bakterien allein in den USA als Ursache für mindestens zwei Millionen Krankheits- und 23 000 Todesfälle pro Jahr anzusehen sind [1]. Als Reaktion darauf veröffentlichte das Weiße Haus im September 2014 ein Strategiedokument, in welchem der Anstieg antibiotikaresistenter Stämme als ernsthafte Bedrohung für die öffentliche Gesundheit und die gesamte Wirtschaft erklärt wird. Als einige der Ziele, welche in diesem Dokument ausdrücklich erwähnt werden, sind der nachhaltige Umgang mit Antibiotika sowie die Beschleunigung der Grundlagen- und Zweckforschung und die Entwicklung neuartiger Strategien zur Bekämpfung bakterieller Resistenzen zu nennen [2].

Speziell auf dem Gebiet der Zahnheilkunde sollte es ein vorrangiges Ziel sein, die Anwendung lokaler und systemischer Antibiose weitgehend zu vermeiden. So ist es z.B. zur Behandlung von schweren Formen der Parodontitis heute üblich, eine Kombination aus Amoxicillin (500mg) und Metronidazol (400mg) als Zusatz zur konventionellen mechanischen Therapie (scaling and root planing) einzusetzen (so genannter „Van Winkelhoff – Cocktail“) [3,4], was zunehmend kritisch gesehen wird [5]. Darüber hinaus werden Zahnärzte vermehrt mit Erkrankungen parodontaler oder

endodontaler Genese konfrontiert, die durch antibiotikaresistente Pathogene verursacht werden [6,7].

Angesichts dessen stellen alternative Therapieverfahren wie die photodynamische Inaktivierung von Bakterien (PIB) eine vielversprechende Möglichkeit dar. PIB besteht aus der Applikation eines an sich nicht-toxischen Moleküls, dem sog. Photosensibilisator (PS), und dessen darauffolgender Bestrahlung mit sichtbarem Licht geeigneter Wellenlänge in Anwesenheit von molekularem Sauerstoff. Die Absorption des Lichts durch den PS bewirkt dessen Anhebung auf das Triplett-Energieniveau, von welchem zwei Möglichkeiten bestehen, wieder in den Grundzustand zu gelangen: beim Typ-I-Mechanismus wird Ladung auf ein Substrat oder auf molekularen Sauerstoff übertragen, wobei reaktive Sauerstoffspezies (ROS) wie Wasserstoffperoxid (H_2O_2), Sauerstoffradikale wie das Superoxid Anion ($O_2^{\bullet -}$) sowie Hydroxylradikale ($HO^{\bullet}$) gebildet werden. Im Gegensatz dazu wird beim Typ-II-Mechanismus Energie direkt auf molekularen Sauerstoff übertragen, wobei der hochreaktive Singulettsauerstoff ($^1O_2$) gebildet wird [8,9]. Das Verhältnis der beiden PIB-Mechanismen ist für jeden PS unterschiedlich, wobei die Singulettsauerstoff-Quantenausbeute Φ_Δ den Anteil des Typ-II-Mechanismus beschreibt [10].

Aktuell werden in der Zahnmedizin hauptsächlich PS klinisch angewandt, welche strukturell den Phenothiazinen zuzuordnen sind [11]. Methylenblau, welches als erster klinisch eingesetzter Farbstoff aus dieser Gruppe anzusehen ist, wurde Ende des 19. Jahrhundert im Zuge einer florierenden deutschen Textilindustrie entwickelt [12]. MB besteht aus einem Dreiring-π-System mit angehängter auxochromer Seitengruppe und ist einfach positiv geladen. Es zeigt hohe Absorptionswerte im roten Spektralbereich (λ_{abs}=600-680 nm) [13]; seine Singulett-Sauerstoff Quantenausbeute Φ_Δ liegt bei 0,52 [14].

Kürzlich stellte unsere Arbeitsgruppe SAPYR als Vertreter einer neuen Klasse von PS vor, basierend auf einer Phenalen-1-one-Struktur mit positiv geladenem Methyl-Pyridinium-Substituenten. SAPYR absorbiert Licht im ultravioletten bis blauen Wellenlängenbereich (λ_{abs}=360-420 nm) und weist eine Singulett-Sauerstoff Quantenausbeute von Φ_{Δ}=0.99 auf, was bedeutet, dass dieser PS nahezu vollständig gemäß dem Typ-II-Mechanismus reagiert [15,16]. SAPYR zeigt ein ausgezeichnetes desinfizierendes Potenzial nicht nur gegen Bakterien in Suspension [16], sondern auch gegen solche, die in Mono- oder Polyspezies-Biofilmen organisiert sind [15].

Trotz der bereits sehr vielversprechenden Ergebnisse früherer Studien scheint es vor einer klinischen Anwendung sinnvoll, die antibakterielle photodynamische Effektivität von SAPYR direkt mit der eines klinischen Standards, wie zum Beispiel MB, zu vergleichen. Aufgrund verschiedener Absorptioncharakteristika dieser PS müssen jeweils passende Lichtquellen verwendet werden. Daher ist es nötig, die Bestrahlungsparameter derart anzugleichen, dass ein direkter Vergleich der jeweiligen antibakteriellen photodynamischen Effektivität möglich wird.

In der vorliegenden Arbeit wurde deswegen die antibakterielle PIB-Effektivität von SAPYR mit der von MB bezüglich der Inaktivierung von Monospezies-Biofilmen verglichen. Dabei wurden zwei Methoden zur Anpassung der Bestrahlungsparameter gewählt: im ersten Fall wurden die Lichtdosen, welche durch die entsprechenden Lichtquellen appliziert werden, angepasst; im zweiten Fall hingegen wurde die Zahl der Photonen, welche von den PS bei Bestrahlung mit der entsprechenden Lichtquelle absorbiert werden, unter Anwendung der folgenden dafür entwickelten Formel abgestimmt:

$$\text{absorbierte Photonen/Sekunde} = \sum_{\lambda} (1 - 10^{-\varepsilon(\lambda) \cdot c \cdot d}) \cdot P_{em}(\lambda) \cdot \frac{\lambda}{c_0 \cdot h}$$

In die Berechnung der Zahl der von einem PS pro Sekunde absorbierten Photonen gehen der Extinktionskoeffizient ε des PS sowie dessen Konzentration c (hier 100 µM) und die Schichtstärke d der Lösung (hier 1,3 mm) ein. Außerdem gehen die von der Lichtquelle emittierte Wellenlänge λ sowie die Strahlungsleistung P bei einer bestimmten Wellenlänge λ (P(λ)) mit ein. Zuletzt benutzt man noch die physikalischen Konstanten der Lichtgeschwindigkeit (c_0) sowie des Planck'schen Wirkungsquantums (h).

Mit Hilfe der in dieser Arbeit präsentierten Formel zur Berechnung der von einem bestimmten PS pro Sekunde absorbierten Photonen kann die Effektivität verschiedener photodynamisch-aktiver Systeme unter Einbeziehung der jeweiligen spektralen Charakteristika von PS und Lichtquelle verglichen werden; dies erscheint notwendig, da gemäß dem Prinzip der Photodynamik ein PS-Molekül genau dann in seinen angeregten Zustand gelangt, wenn es ein Photon absorbiert.

In der vorliegenden Arbeit wurde die antimikrobielle Effektivität des Phenalen-1-one PS SAPYR mit der des klinischen Standards MB auf Monospezies-Biofilme von *Enterococcus faecalis* und *Actinomyces naeslundii* verglichen. Im Falle von SAPYR zeigte sich bei einer applizierten Lichtdosis von 30 J/cm², was $6{,}78 \times 10^{18}$ absorbierter Photonen pro Sekunde entspricht, für beide Mikroorganismen ein desinfizierender Effekt (Reduktion um 5,1 $\log_{10}$ -Stufen bzw. 6,5 $\log_{10}$ -Stufen jeweils für *E. faecalis* und *A. naeslundii*); MB wies im Vergleich zu SAPYR bei Gleichsetzung der Lichtdosis (30 J/cm²) eine geringere antibakterielle Wirkung auf (Reduktion um 3,4 $\log_{10}$ -Stufen und 4,2 $\log_{10}$ -Stufen jeweils gegen *E. faecalis* und *A. naeslundii*). Nach Anpassung

der Zahl der absorbierten Photonen (Anwendung der Formel; 6,78x10^{18} absorbierte Photonen pro Sekunde), zeigte sich bei MB kein antimikrobieller photodynamischer Effekt (siehe Abbildung 1).

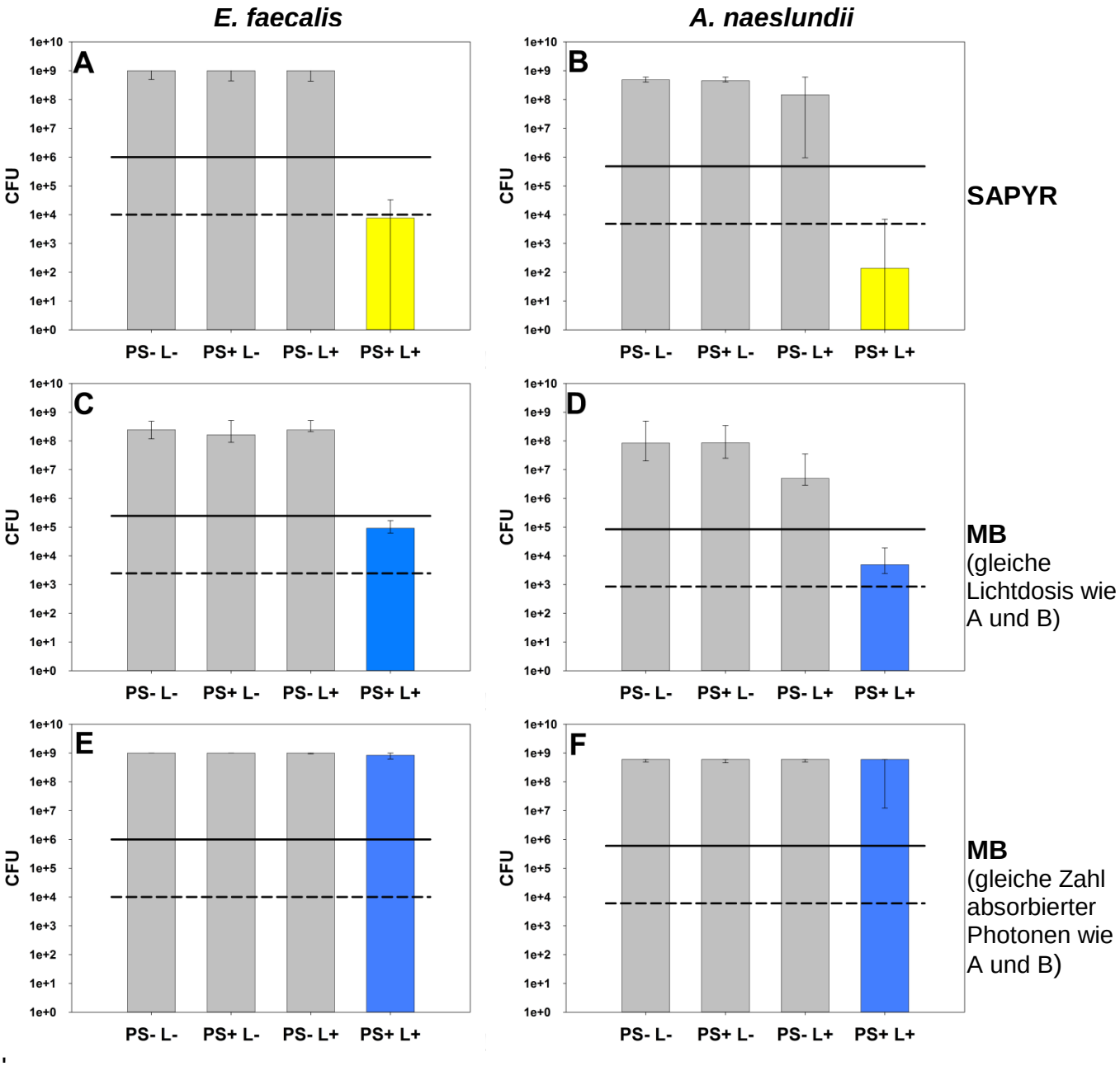

Abbildung 1: Photodynamische Inaktivierung von *E. faecalis* und *A. naeslundii* Monospezies-Biofilmen mit SAPYR und MB

Photodynamische Inaktivierung von *E. faecalis* und *A. naeslundii* Monospezies-Biofilmen mit beiden PS-Lichtquellen-Systemen. Alle PIB-Versuche in dieser Grafik sind als CFU-Mediane mit 25%- und 75%-Quantilen dargestellt. Die durchgezeichneten bzw. gestrichelten Linien

stellen jeweils CFU-Reduktionen um 3 log_{10}- bzw. 5 log_{10}-Stufen im Vergleich zur unbehandelten Kontrollgruppe PS-L- dar.

(A) PIB von *E. faecalis* Monospezies-Biofilmen mit SAPYR (Zahl der pro Sekunde absorbierten Photonen: $6{,}78x10^{18}$; Lichtdosis: 30 J/cm²): Die PIB-Gruppe PS+L+ (gelb) zeigt eine Reduktion von 5,1 log_{10}-Stufen im Vergleich zur unbehandelten Kontrollgruppe PS-L-.

(B) PIB von *A. naeslundii* Monospezies-Biofilmen mit SAPYR (Zahl der pro Sekunde absorbierten Photonen: $6{,}78x10^{18}$; Lichtdosis: 30 J/cm²): Die PIB-Gruppe PS+L+ (gelb) zeigt eine Reduktion von 6,5 log_{10}-Stufen im Vergleich zur unbehandelten Kontrollgruppe PS-L-. Die Lichtkontrolle PS-L+ zeigt eine Reduktion von 0,5 log_{10}-Stufen im Vergleich zur unbehandelten Kontrollgruppe PS-L-.

(C) PIB von *E. faecalis* Monospezies-Biofilmen mit MB (angepasste Lichtdosis von 30 J/cm², Zahl der pro Sekunde absorbierten Photonen $56{,}5x10^{8}$): Die PIB-Gruppe PS+L+ (blau) zeigt eine Reduktion von 3,4 log_{10}-Stufen im Vergleich zur unbehandelten Kontrollgruppe PS-L-.

(D) PIB von *A. naeslundii* Monospezies-Biofilmen mit MB (angepasste Lichtdosis von 30 J/cm; Zahl der pro Sekunde absorbierten Photonen $56{,}5x10^{8}$): Die PIB-Gruppe PS+L+ (blau) zeigt eine Reduktion von 4,2 log_{10}-Stufen im Vergleich zur unbehandelten Kontrollgruppe PS-L-. Die Lichtkontrolle PS-L+ zeigt eine Reduktion von 1,2 log_{10}-Stufen im Vergleich zur unbehandelten Kontrollgruppe PS-L-.

(E) PIB von *E. faecalis* Monospezies-Biofilmen mit MB (angepasste Zahl absorbierter Photonen pro Sekunde: $6{,}78x10^{18}$ Lichtdosis 3,6 J/cm²): Die PIB-Gruppe zeigt keine Reduktion im Vergleich zur unbehandelten Kontrollgruppe PS-L-.

(F) PIB von *A. naeslundii* Monospezies-Biofilmen bei Verwendung von MB (angepasste Zahl absorbierter Photonen pro Sekunde: $6{,}78x10^{18}$; Lichtdosis 3,6 J/cm²): PIB-Gruppe zeigt keine Reduktion im Vergleich zur unbehandelten Kontrollgruppe PS-L-.

Es ist daher sinnvoll, die Zahl der absorbierten Photonen in künftige Arbeiten zum Vergleich von verschiedenen PS miteinzubeziehen, da sich beträchtliche Unterschiede in der antibakteriellen photodynamischen Effektivität der PS einstellen, je nachdem ob man die Zahl der absorbierten Photonen oder nur die Lichtdosen verschiedener PS anpasst.

Ausblick

In dieser Arbeit konnte gezeigt werden, dass es beim Vergleich verschiedener PS sinnvoll ist, nicht nur die applizierten Lichtdosen, sondern die spektralen Charakteristika der verwendeten Lichtquelle wie auch des PS zu berücksichtigen. Dadurch kann das Potenzial von PS, Mikroorganismen zu inaktivieren, genauer beurteilt werden.

Die von uns entwickelte Formel unterstreicht darüber hinaus die Wichtigkeit der Bestrahlung mit Licht einer geeigneten Emissionswellenlänge, welche eine breite Überlappung mit den Maxima des Absorptionsspektrums eines PS zeigt. Eine effiziente photodynamische Inaktivierung von Mikroorganismen kann nur bei suffizienter Aktivierung des jeweiligen PS erfolgen.

In der hier vorgestellten Arbeit fand die von uns entwickelte Formel Anwendung bei der PIB von Bakterien *in vitro*. Nachdem die Anwendung dieser Formel unter diesen Bedingungen zum Vergleich der Effektivität verschiedener PS etabliert werden konnte, soll als nächster Schritt die Anwendung der Formel in einem *ex vivo* Zahnmodell erfolgen. Ein derartiges *ex vivo* Modell zum Einsatz in der Endodontie unter Vergleich der Effektivität verschiedener PS bei gleicher Zahl absorbierter Photonen wird nachfolgend im zweiten Teil dieser Arbeit vorgestellt.

Publikation

Fabian Cieplik, Andreas Pummer, Johannes Regensburger, Karl-Anton Hiller, Andreas Späth, Laura Tabenski, Wolfgang Buchalla, Tim Maisch:

The impact of absorbed photons on antimcrobial efficacy.

Front. Microbiol. 2015; 6: 706

(Impact Factor 2015: 4,165)

frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 July 2015
doi: 10.3389/fmicb.2015.00706

The impact of absorbed photons on antimicrobial photodynamic efficacy

Fabian Cieplik[1], Andreas Pummer[1], Johannes Regensburger[2], Karl-Anton Hiller[1], Andreas Späth[3], Laura Tabenski[1], Wolfgang Buchalla[1] and Tim Maisch[2]*

[1] Department of Conservative Dentistry and Periodontology, University Medical Center Regensburg, Regensburg, Germany, [2] Department of Dermatology, University Medical Center Regensburg, Regensburg, Germany, [3] Department of Organic Chemistry, University of Regensburg, Regensburg, Germany

OPEN ACCESS

Edited by:
Rustam Aminov, Technical University of Denmark, Denmark

Reviewed by:
Ali Al-Ahmad, Universitätsklinikum Freiburg, Germany
Daniela Vecchio, Massachusetts General Hospital, USA
Heinrich Walt, University Hospital Zürich, Switzerland
Joanna Nakonieczna, Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk, Poland

***Correspondence:**
Fabian Cieplik, Department of Conservative Dentistry and Periodontology, University Medical Center Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany
fabian.cieplik@ukr.de

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *02 April 2015*
Accepted: *26 June 2015*
Published: *15 July 2015*

Citation:
Cieplik F, Pummer A, Regensburger J, Hiller K-A, Späth A, Tabenski L, Buchalla W and Maisch T (2015) The impact of absorbed photons on antimicrobial photodynamic efficacy. Front. Microbiol. 6:706. doi: 10.3389/fmicb.2015.00706

Due to increasing resistance of pathogens toward standard antimicrobial procedures, alternative approaches that are capable of inactivating pathogens are necessary in support of regular modalities. In this instance, the photodynamic inactivation of bacteria (PIB) may be a promising alternative. For clinical application of PIB it is essential to ensure appropriate comparison of given photosensitizer (PS)-light source systems, which is complicated by distinct absorption and emission characteristics of given PS and their corresponding light sources, respectively. Consequently, in the present study two strategies for adjustment of irradiation parameters were evaluated: (i) matching energy doses applied by respective light sources (common practice) and (ii) by development and application of a formula for adjusting the numbers of photons absorbed by PS upon irradiation by their corresponding light sources. Since according to the photodynamic principle one PS molecule is excited by the absorption of one photon, this formula allows comparison of photodynamic efficacy of distinct PS per excited molecule. In light of this, the antimicrobial photodynamic efficacy of recently developed PS SAPYR was compared to that of clinical standard PS Methylene Blue (MB) regarding inactivation of monospecies biofilms formed by *Enterococcus faecalis* and *Actinomyces naeslundii* whereby evaluating both adjustment strategies. PIB with SAPYR exhibited CFU-reductions of 5.1 $\log_{10}$ and 6.5 $\log_{10}$ against *E. faecalis* and *A. naeslundii*, respectively, which is declared as a disinfectant efficacy. In contrast, the effect of PIB with MB was smaller when the applied energy dose was adjusted compared to SAPYR (CFU-reductions of 3.4 $\log_{10}$ and 4.2 $\log_{10}$ against *E. faecalis* and *A. naeslundii*), or there was even no effect at all when the number of absorbed photons was adjusted compared to SAPYR. Since adjusting the numbers of absorbed photons is the more precise and adequate method from a photophysical point of view, this strategy should be considered in further studies when antimicrobial efficacy rates of distinct PS-light source systems are compared.

Keywords: photodynamic, absorbed photons, biofilm, SAPYR, Methylene Blue, antimicrobial resistance

Introduction

In a current assessment the Centers for Disease Control and Prevention of the United States of America stated that antibiotic-resistant bacteria are causative for at least two million illnesses and 23,000 deaths per year in the U.S. alone (Centers for Disease Control and Prevention, 2013). Consequently, in September 2014, the White House published a strategy paper where the rise of antibiotic-resistant bacteria was declared as a serious threat to public health and economy. Some of the goals outlined in this strategy paper were to promote judicious use of antibiotics and to accelerate basic and applied research on and development of novel therapeutic tools for combating bacterial resistance (The White House, 2014).

In particular in the field of dentistry, it should be a major goal to moderate the application of systemic antibiotics. For example, it is common practice to deliver Amoxicillin and Metronidazol combined for 1 week in addition to conventional scaling and root planing for treatment of severe forms of chronic periodontitis (Winkelhoff et al., 1996; Zandbergen et al., 2013), which is critically judged more and more nowadays (Preus et al., 2014). Furthermore, dentists are increasingly faced with situations, where they have to deal with refractory progressions of periodontal or endodontic infections caused by antibiotic-resistant pathogens (Al-Ahmad et al., 2014; Rams et al., 2014).

In light of this, a promising alternative may be the photodynamic inactivation of bacteria (PIB). PIB is an antimicrobial approach consisting of the application of a *per se* non-toxic molecule, the so-called photosensitizer (PS), and subsequent irradiation with visible light of an adequate wavelength in the presence of molecular oxygen. The absorption of light by the PS molecule transfers it to its energized triplet state, from which there are two mechanisms to regain its ground state: In type I mechanism, charge is transferred to a substrate or to molecular oxygen with emergence of reactive oxygen species (ROS) like superoxide anions ($O_2-\bullet$), free hydroxyl radicals (HO•) and hydrogen peroxide (H_2O_2). In contrast, in type II mechanism, energy is transferred directly to molecular oxygen, whereby highly reactive singlet oxygen ($^1O_2$) is generated (Wainwright, 1998; Schweitzer and Schmidt, 2003). The proportion of both PIB mechanisms is unique for each PS with the singlet oxygen quantum yield Φ_Δ describing the proportion of type II mechanism (Maisch et al., 2007).

Currently, in dental practice predominantly PS based on a phenothiazinium structure are clinically used like Methylene Blue (MB) (Gursoy et al., 2013). MB has a strong absorption in the red spectral region ($\lambda_{abs} = 600-680$ nm) (Felgenträger et al., 2013) and its singlet oxygen quantum yield Φ_Δ is 0.52 (Wilkinson et al., 1993). However, results with MB regarding inactivation of biofilms *in vitro* are quite conflicting (Cieplik et al., 2014b) and clinical trials on PIB with MB as a supportive modality for treatment of periodontitis suggest that there are no long-term positive effects (Sgolastra et al., 2013). In contrast, SAPYR has recently been introduced by our group as a new class of PS based on a phenalen-1-one structure with a positively charged pyridinium-methyl substituent and has shown pronounced antimicrobial efficacy against planktonic bacteria (Späth et al., 2014) as well as biofilms (Cieplik et al., 2013). SAPYR absorbs in a range from UV-A to the blue spectral region ($\lambda_{abs} = 360-420$ nm) and exhibits a singlet oxygen quantum yield $\Phi_\Delta = 0.99$, thus reacting virtually exclusively according to type II mechanism (Cieplik et al., 2013; Späth et al., 2014).

For appropriate comparison of antimicrobial photodynamic efficacy rates of given PS-light source systems, it is essential to adjust irradiation parameters, which is complexed by distinct absorption and emission characteristics of given PS and their corresponding light sources. Hereby, it is common practice to match the energy doses applied by the respective light sources. In this instance, emission and absorption characteristics are mostly left without consideration. However, – according to the photodynamic principle – one PS molecule is excited by the absorption of one photon, wherefore the numbers of photons absorbed by PS upon irradiation by their corresponding light sources should be calculated allowing comparison of photodynamic efficacy rates of distinct PS per excited molecule.

Aim of the present study was to evaluate two strategies for adjustment of irradiation parameters: (I) adjusting the energy doses applied by the respective light sources irrespective of spectral properties and (II) by development and application of a formula for calculating the numbers of absorbed photons for each PS upon irradiation by its corresponding light source. Applying these strategies, SAPYR and MB were exemplarily compared regarding inactivation of monospecies biofilms formed by oral key pathogens *E. faecalis* and *A. naeslundii*.

Materials and Methods

Photosensitizers and Light Sources

The phenalen-1-one PS SAPYR [2-((4-pyridinyl)methyl)-1H-phenalen-1-one chloride; **Figure 1A**] was synthetized at the Department of Organic Chemistry (University of Regensburg, Germany) with a purity of ≥ 99%, controlled by NMR and HPLC-MS, as described earlier (Cieplik et al., 2013; Späth et al., 2014). The phenothiazinium PS Methylene Blue [3,7-bis(dimethylamino)-phenothiazin-5-ium chloride; **Figure 1B**] was purchased from Sigma-Aldrich (St. Louis, MO) and used as received (purity ≥ 95%). Absorption spectra of SAPYR and MB were recorded by means of a photosprectrometer (DU 640, Beckman-Coulter, Krefeld, Germany).

For irradiation of SAPYR, a Waldmann PIB 3000 blue light source (λ_{em} = 380–600 nm) was used, whereas MB was irradiated with a Waldmann PDT 1200L red light source (λ_{em} =

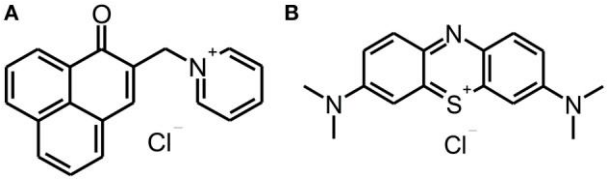

FIGURE 1 | Chemical structures of SAPYR and Methylene Blue. Chemical structures of phenalen-1-one PS SAPYR **(A)** and phenothiazinium PS Methylene Blue **(B)**.

580–750 nm) (both: Waldmann Medizintechnik, Villingen-Schwenningen, Germany). Emission spectra of the light sources were recorded by means of a monochromator with a CCD detection system (SPEX 232, HORIBA Jobin Yvon, Longjumeau Cedex, France). **Figure 2** shows the absorption spectra of both PS and the emission spectra of the corresponding light sources.

For PIB experiments, the light intensities obtained from both light sources at the level of the samples were measured with a thermal low-power sensor (Nova 30 A-P-SH, Ophir-Spiricon, North Logan, UT).

Calculation of Irradiation Parameters

For calculation of the numbers of absorbed photons, the extinction coefficients of the PS at distinct wavelengths [$\varepsilon(\lambda)$] and the spectral radiant power emitted by the respective light sources $P_{em}(\lambda)$ were measured. For determination of the spectral resolved absorption, the concentration of the PS (c, here: 100 μM) and the thickness of the solution (d, here: 1.3 mm) are necessary parameters. Moreover, the universal physical constant of the velocity of light (c_0) and the Planck constant (h) are required.

$$\text{absorbed photons/ second} = \sum_{\lambda} \left(1 - 10^{-\varepsilon(\lambda)\cdot c\cdot d}\right) \cdot P_{em}(\lambda) \cdot \frac{\lambda}{c_0 \cdot h}$$

$\varepsilon(\lambda)$	Extinction coefficient [(mol×cm)$^{-1}$]	Measured spectrally resolved by a photospectrometer
$P_{em}(\lambda)$	Spectral radiant power of the respective light source [mW/nm]	Measured spectrally resolved by a CCD detector system
c	Concentration of the PS [μM]	Here: 100 μM
d	Thickness of the solution [mm]	Here: 1.3 mm
c_0	Velocity of light [m/s]	299,792,458 m/s
h	Planck constant [J×s]	$6.62606957 \times 10^{-34}$ Js
λ	Wavelength [nm]	

First, irradiation parameters for SAPYR were determined to irradiation with PIB 3000 for 600 s at an irradiance at sample-level of 50 mW/cm^2, corresponding to an energy dose of 30 J/cm^2.

In order to compare antimicrobial photodynamic efficacy of SAPYR and MB at corresponding energy dose, irradiation of MB was with PDT 1200L at 50 mW/cm^2 for 600 s.

For comparing antimicrobial photodynamic efficacy of both PS at matching numbers of absorbed photons, the constants, the experimental data and the measured values were inserted in the formula outlined above, resulting in a number of 1.13×10^{16} photons/second absorbed by SAPYR for irradiation with PIB 3000 (irradiance at sample-level: 50 mW/cm^2). Likewise, a number of 3.75×10^{16} photons/second absorbed by MB for irradiation with PDT 1200L (irradiance at sample-level: 20 mW/cm^2) was calculated. Consequently, in order to adjust the numbers of absorbed photons for both PS, a 3.32 times longer irradiation period was necessary for SAPYR with PIB 3000 compared to MB with PDT 1200L. Therefore, irradiation periods were determined to be 600 s for SAPYR with PIB 3000 (energy dose: 30 J/cm^2) and 181 s for MB with PDT 1200L (energy dose: 3.6 J/cm^2).

Biofilm Formation

Enterococcus faecalis ATCC 29212 and *Actinomyces naeslundii* T14V were used as model organisms in this study. Monospecies biofilms were cultured, as it has been described earlier (Cieplik et al., 2013). Biofilm formation was in 96-well polystyrene culture plates (Corning Costar®, Corning, NY) with the complete saliva medium (CS) described by Pratten et al. (1998), modified by adding 1% sucrose and applying N_2 up to O_2 = 0% in order to ensure anaerobic medium conditions (Tabenski et al., 2014). *E. faecalis* and *A. naeslundii* were incubated under aerobic conditions in Brain Heart Infusion broth (BHI; Sigma-Aldrich, St. Louis, MO) at 37°C on an orbital shaker as overnight-cultures in order to obtain bacteria in the static growth phase. Afterwards, suspensions were harvested by centrifugation (1700 g, 5 min; Megafuge 1.0, Heraeus Sepatech, Osterode, Germany) and re-suspended in sterile phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) yielding an optical density (OD) of 0.1, which was measured at 600 nm by means of a photospectrometer (SPECORD 50 PLUS, Analytik Jena, Jena, Germany) and corresponds to a bacterial count of 10^4 to 10^5 bacterial cells per ml. Subsequently, bacterial suspensions were pelletized again and re-suspended in CS so that they could be used for biofilm formation. Wells were inoculated with 200 μl of these *E. faecalis* or *A. naeslundii* suspensions and incubated aerobically at 37°C for 72 h, while the medium was substituted every 24 h.

Confirmation of Extracellular Polymeric Substance (EPS)

The presence of EPS in *A. naeslundii* and *E. faecalis* monospecies biofilms was verified by a lectinsorbent assay (ELLA), based on the manual described by Leriche et al. (2000). Concanavalin A (Con A) was chosen as a lectin, since it binds to the most common monosaccharide-residues ($_D$-glucose and $_D$-mannose) in the EPS of bacterial biofilms (Sutherland, 1990).

Peroxidase-labeled Con A (Sigma-Aldrich, St. Louis, MO) was diluted in PBS containing 0.05% (vol/vol) Tween 20 (Merck, Darmstadt, Germany) (diluting buffer; DB) obtaining final concentrations of 10.0 and 12.5 μg/ml of Con A. *E. faecalis* and *A. naeslundii* monospecies biofilms were grown as described above in 96-well plates yielding 8 wells in a row colonized with biofilm for each sample. After 72 h, medium was removed and biofilms were washed carefully with PBS to remove non-adherent bacteria. 200 μl of the Con A solution at concentrations of 10.0 μg/ml (*A. naeslundii*, *E. faecalis*) or 12.5 μg/ml (*E. faecalis*), respectively, were added to the first of the eight wells in a row and 100 μl DB to the remaining wells. Consequently, serial half dilutions were performed by transferring 100 μl from the previous to the proximate well. Wells incubated with CS medium for 72 h were submitted to the same procedure and used as a blank. Well plates were incubated in the dark for 60 min at room temperature to allow lectin-binding. Afterwards, peroxidase-labeled Con A solutions were removed from the wells by inverting the plates. Subsequently, the biofilms were washed triply with DB to remove unbound lectin and peroxidase-labeled

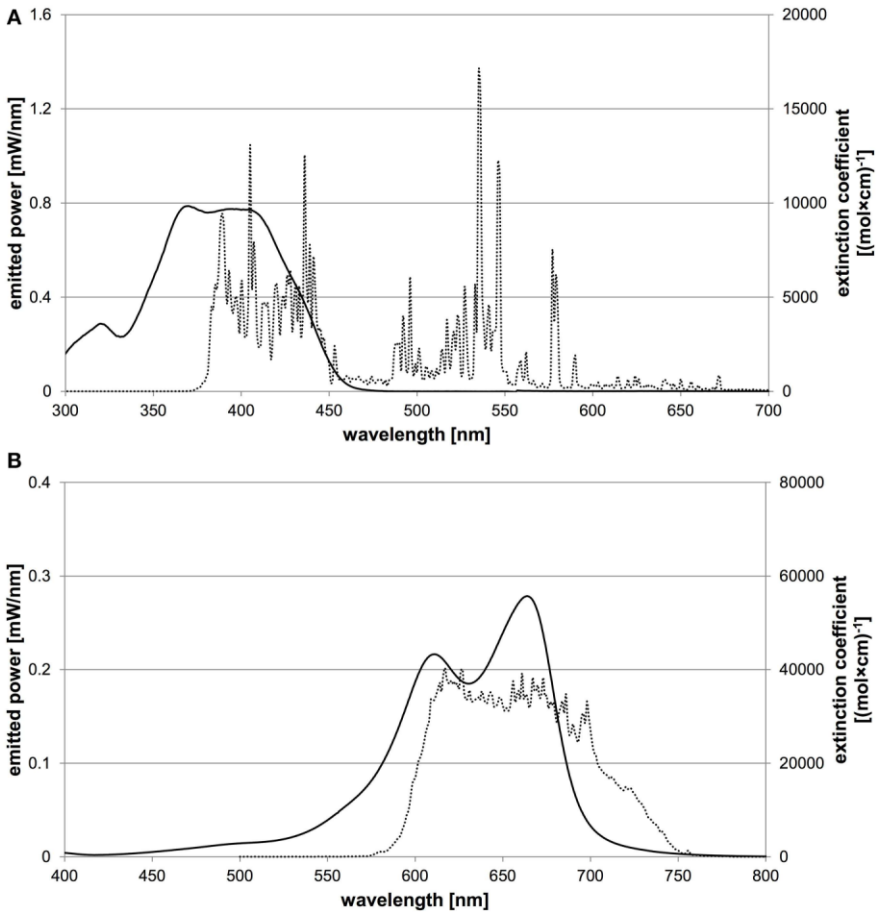

FIGURE 2 | Characteristic absorption spectra of both PS and emission spectra of corresponding light sources. The abscissa shows the wavelength in nm, the left ordinate the spectral radiant power of the light source and the right ordinate the extinction coefficient of the PS. **(A)** Characteristic absorption spectrum of SAPYR (solid line) and emission spectrum of Waldmann PIB 3000 (dotted line). **(B)** Characteristic absorption spectrum of MB (solid line) and emission spectrum of Waldmann PDT 1200L (dotted line).

Con A was visualized by adding 100 µl ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Sigma-Aldrich, St. Louis, MO]. After maintaining in the dark for 15 min, OD was measured at 405 nm with a microplate reader (EMax® Precision Microplate Reader, Molecular Devices, Biberach, Germany). Blanks were substracted.

Photodynamic Inactivation of Bacteria

After a cultivation period of 72 h, biofilms were washed twice with sterile PBS to remove non-adherent bacteria and were incubated either with 50 µl of the respective PS at a concentration of 100 µM (experimental group PS+L+: *PIB*; control group PS+L-: *treatment with PS only*) or with 50 µl PBS (control group PS-L-: *untreated control*; control group PS-L+: *treatment with light only*) for 25 min in the dark. Then the samples were irradiated either for 600 s (SAPYR, MB) or for 181 s (MB) (groups PS+L+ and PS-L+) or maintained in the dark during the same period (groups PS+L- and PS-L-).

Immediately afterwards PS or PBS was carefully removed and each biofilm was brought to suspension with 200 µl of PBS by multiple up-and-down-pipetting and transferred to a 1.5 ml Eppendorf tube. These were placed in an ultrasonic water-bath chamber (35 kHz; Qualilab USR30H, Merck Eurolab, Bruchsal, Germany) for 5 min and then vigorously vortexed (REAX top, Heidolph Instruments, Schwabach, Germany) for 5 s for separation of aggregated bacteria. Serial tenfold dilutions (10^{-2} to 10^{-7}) were prepared in BHI-broth and aliquots (3 × 20 µl) were plated on Mueller-Hinton-agar (*E. faecalis*) or blood agar (*A. naeslundii*) plates, respectively, according to the methodology described by Miles et al. (1938). Plates were incubated at 37°C for 24 h (*E. faecalis*) or 48 h (*A. naeslundii*). Afterwards, colony forming units (CFU) were counted.

Data Analysis

For ELLA experiments (data from 12 independent samples), OD at 405 nm was plotted versus the logarithm of Con A concentrations. Values were fitted in a dose-response curve including 95% confidence intervals using Table Curve 2D (Systat Software Inc., San Jose, CA).

All results of PIB experiments are graphically shown as medians, including 25 and 75% quantiles 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. Horizontal solid and dashed lines in the figures depict reductions of 3 and 5 $\log_{10}$ steps CFU respectively, compared to untreated control groups PS-L-. Medians on these lines exhibit inactivation efficacy rates of 99.9% (3 $\log_{10}$) or 99.999% (5 $\log_{10}$). According to the infection control guidelines, this is declared as biologically relevant antimicrobial activity or disinfectant effect, respectively (Boyce and Pittet, 2002).

Results

Confirmation of Extracellular Polymeric Substance (EPS)

D-glucose and D-mannose residues in the EPS of *E. faecalis* and *A. naeslundii* monospecies biofilms were detected with ELLA using Con A as a lectin. Plotting the OD values at 405 nm versus the logarithm of the concentration of Con A added, resulted in dose-response curves for biofilms of *E. faecalis* ($r^2 = 0.872$) and *A. naeslundii* ($r^2 = 0.957$) (**Figure 3**). Consequently, increasing concentrations of Con A result in heightened OD values, which affirms the presence of binding sites for Con A and therefore the presence of EPS containing D-glucose and D-mannose residues. Non-specific binding of Con A to carbohydrates in CS medium can be neglected, since blank values did not show any dose-response curves (data not shown).

Photodynamic Inactivation of Bacteria

SAPYR and MB were evaluated against *E. faecalis* and *A. naeslundii* monospecies biofilms. Irradiation parameters for SAPYR were determined to irradiation with PIB 3000 for 600 s obtaining irradiance at sample-level of 50 mW/cm^2, which corresponds to an energy dose of 30 J/cm^2 and a number of 6.78×10^{18} absorbed photons. Under these conditions, SAPYR revealed an inactivation efficacy of 5.1 $\log_{10}$ steps against *E. faecalis* (**Figure 4A**) and 6.5 $\log_{10}$ steps against *A. naeslundii* (**Figure 4B**).

For comparing MB at adjusted energy dose compared to SAPYR (30 J/cm^2; number of absorbed photons: 56.5×10^{18}), MB was irradiated with PDT 1200L for 600 s at an irradiance of 50 mW/cm^2 reaching the samples, too. Here, CFU of *E. faecalis* were reduced by 3.4 $\log_{10}$ steps (**Figure 4C**) and CFU of *A. naeslundii* by 4.2 $\log_{10}$ steps (**Figure 4D**).

In contrast, for comparing MB at adjusted numbers of absorbed photons compared to SAPYR (6.78×10^{18}; energy dose: 3.6 J/cm^2), MB was irradiated with PDT 1200L for 181 s at an irradiance at sample-level of 20 mW/cm^2, which resulted in no reduction of CFU-median against both, *E. faecalis* (**Figure 4E**) and *A. naeslundii* (**Figure 4F**).

In all cases there was no reduction of CFU after treatment with PS only (PS+L−). Treatment with light only (PS−L+) had no effect on CFU of *E. faecalis*. In the case of *A. naeslundii*, CFU were diminished slightly by 0.5 $\log_{10}$ step when irradiated with Waldmann PIB 3000 (50 mW/cm^2; 30 J/cm^2) and by 1.2 $\log_{10}$ steps when irradiated with Waldmann PDT 1200L at 30 J/cm^2. In contrast, irradiation with PDT 1200L at 3.6 J/cm^2 had no effect on CFU of *A. naeslundii*.

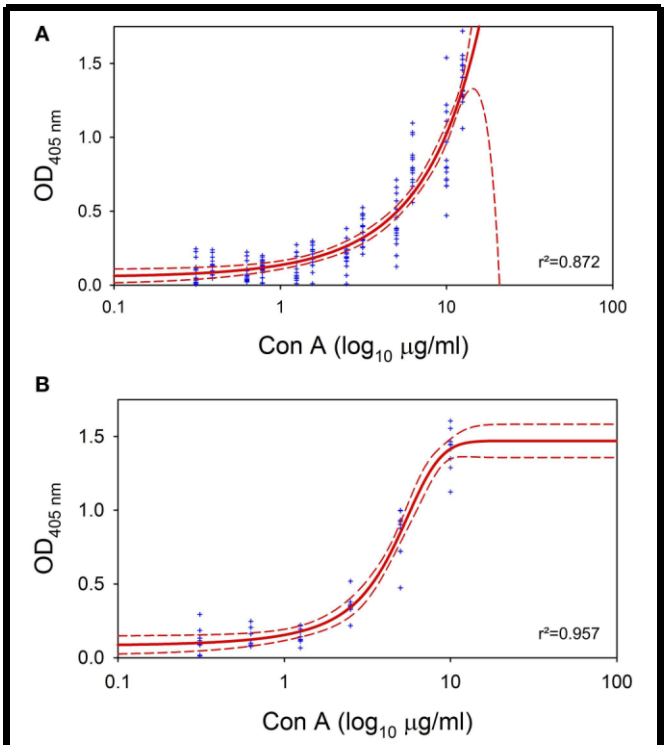

FIGURE 3 | Enzyme-linked lectinsorbent assay (ELLA). ELLA applied on 72 h *E. faecalis* and *A. naeslundii* monospecies biofilms for confirmation of EPS. Blue dots represent original measured values, red solid line represents the fit of the sigmoideal curve and red dashed lines depict 95% confidence intervals. r^2 denotes the correlation coefficient. **(A)** ELLA on *E. faecalis* monospecies biofilm. **(B)** ELLA on *A. naeslundii* monospecies biofilm.

Discussion

Aim of the present study was to compare the antimicrobial photodynamic efficacy of given PS by using two strategies for adjustment of irradiation parameters. Since different PS exhibit distinct absorption characteristics, distinct light sources have to be used and their irradiation parameters have to be adjusted in order to ensure appropriate comparison of antimicrobial photodynamic efficacy. Usually, for adjustment of irradiation parameters only the energy doses applied by the respective light sources are matched. In this instance neither the molar extinction coefficients of a given PS nor the energy per wavelength emitted by the respective light source are factored in. Furthermore, the number of emitted photons of a given light source not only depends on the power but also on the wavelength of the emitted photons. In this study, a formula is presented considering all these aspects in order to calculate the numbers of photons, which are absorbed per second by a given PS when irradiated by its corresponding light source:

$$\text{absorbed photons/ second} = \sum_{\lambda}(1 - 10^{-\varepsilon(\lambda)\cdot c\cdot d}) \cdot P_{em}(\lambda) \cdot \frac{\lambda}{c_0 \cdot h}$$

For a certain wavelength λ_0, $\frac{c_0 \cdot h}{\lambda_0}$ describes the energy of a single photon. Its reciprocal $\frac{\lambda_0}{c_0 \cdot h}$ is multiplied by the spectral radiant power $P_{em}(\lambda_0)$ to obtain the number of emitted photons per second. The absorption of the PS is expressed by the factor $(1 - 10^{-\varepsilon(\lambda_0) \cdot c \cdot d})$. The sum is calculated for wavelengths ranging from 300 to 800 nm in 1-nm-steps.

Depending on the mechanism of photodynamic action and based on the Jablonski diagram (Baier et al., 2007; Maisch et al.,

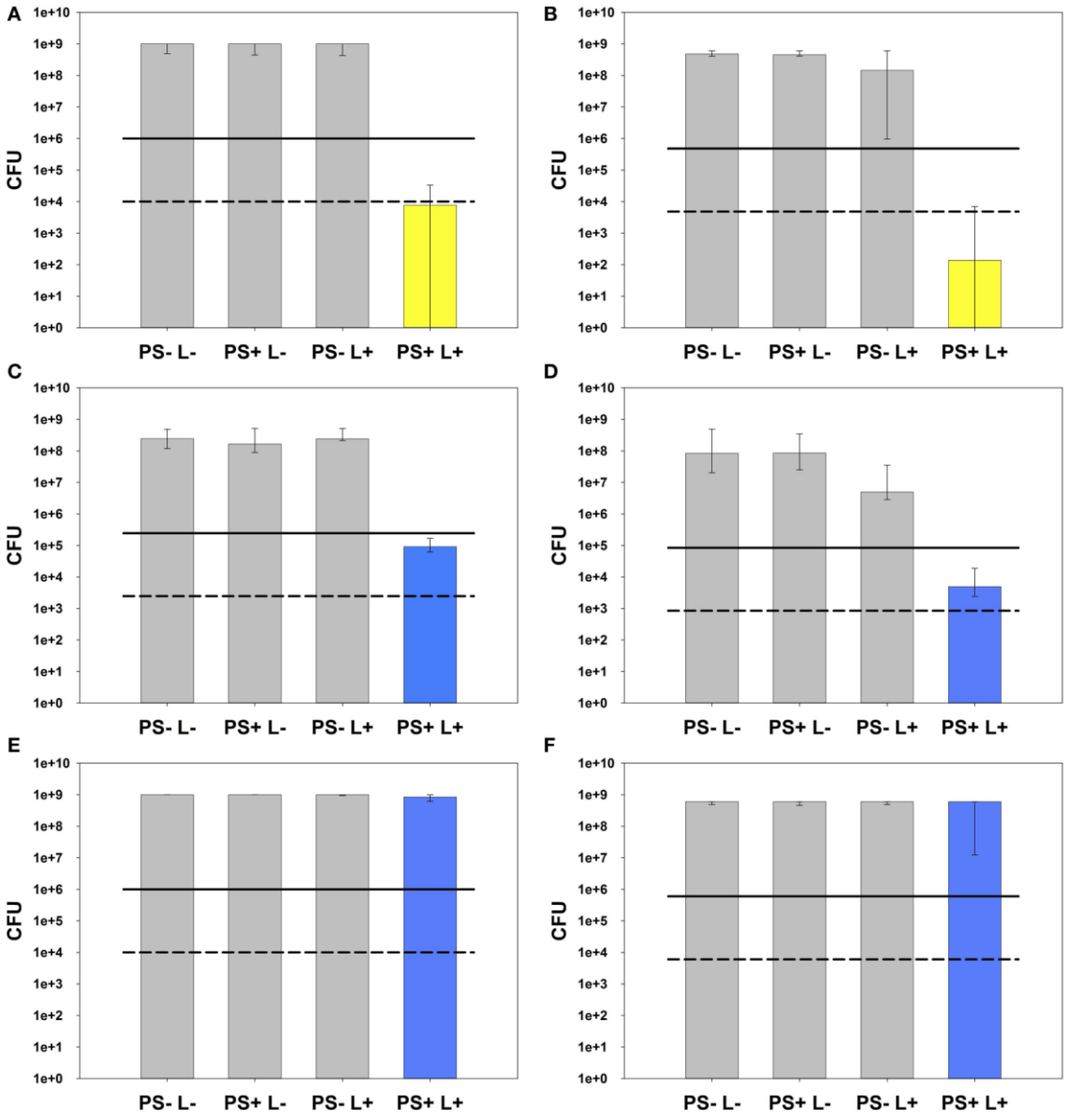

FIGURE 4 | Photodynamic inactivation of AN and EF monospecies biofilms. All PIB results are shown as CFU medians with 25 and 75% quantiles depicted on a $\log_{10}$ scaled ordinate. Medians on or below the solid and dashed lines represent CFU reductions of ≥ 3 $\log_{10}$ and ≥ 5 $\log_{10}$ steps, respectively, compared to untreated control groups PS-L-. **(A)** PIB against *E. faecalis* monospecies biofilm using SAPYR (number of absorbed photons: 6.78×10^{18}; energy dose: 30 J/cm^2): PIB group PS+L+ (yellow) shows a reduction by 5.1 $\log_{10}$ steps CFU. **(B)** PIB against *A. naeslundii* monospecies biofilm using SAPYR (number of absorbed photons: 6.78×10^{18}; energy dose: 30 J/cm^2): PIB group PS+L+ (yellow) shows a reduction by 6.5 $\log_{10}$ steps CFU. PS-L+ group shows a reduction by 0.5 $\log_{10}$ steps CFU. **(C)** PIB against *E. faecalis* monospecies biofilm using MB (adjusted energy dose: 30 J/cm^2; corresponding number of absorbed photons: 56.5×10^{18}): PIB group PS+L+ (blue) shows a reduction by 3.4 $\log_{10}$ steps CFU. **(D)** PIB against *A. naeslundii* monospecies biofilm using MB (adjusted energy dose: 30 J/cm^2; corresponding number of absorbed photons: 56.5×10^{18}): PIB group PS+L+ (blue) shows a reduction by 4.2 $\log_{10}$ steps CFU. PS-L+ group shows a reduction by 1.2 $\log_{10}$ steps CFU. **(E)** PIB against *E. faecalis* monospecies biofilm using MB (adjusted number of absorbed photons: 6.78×10^{18}; corresponding energy dose: 3.6 J/cm^2): PIB group PS+L+ (blue) shows no reduction of CFU. **(F)** PIB against *A. naeslundii* monospecies biofilm using MB (adjusted number of absorbed photons: 6.78×10^{18}; corresponding energy dose: 3.6 J/cm^2): PIB group PS+L+ (blue) shows no reduction of CFU.

2007), it is plausible that one PS molecule is excited by the absorption of one photon, whereby the ground state of the PS is transferred to its singlet state. By intersystem crossing the energy can change over to an excited sensitized triplet state, which can now generate singlet oxygen molecules.

So, adjusting the numbers of absorbed photons of distinct PS-light source systems ensures equal numbers of activated PS-molecules, thus allowing comparison of the antimicrobial photodynamic efficacy rates per excited PS-molecule. Therefore, distinct photodynamic antimicrobial efficacy despite adjusted numbers of absorbed photons may not be caused by photophysical properties but rather by PS-inherent features like mechanism of action (type I or type II; singlet oxygen quantum yield), attachment, uptake, intracellular localization etc.

For experimental validation of this formula, we exemplarily compared antimicrobial photodynamic efficacy rates of phenalen-1-one PS SAPYR to that of clinical standard PS MB (adjusted either by energy doses or by numbers of absorbed photons) regarding inactivation of monospecies biofilms formed by oral key pathogens *E. faecalis* and *A. naeslundii*. According to the general definition of a biofilm it is crucial to verify the presence of extracellular polymeric substance (EPS) before using the term "biofilm" for attached bacteria (Donlan and Costerton, 2002). With respect to the biofilms cultured according the protocol used in the present study, the presence of EPS has already been shown earlier by fluorescence microscopy using fluorescence-labeled Con A (Cieplik et al., 2013). Con A is a lectin that specifically binds to D-glucose and D-mannose residues, which are the saccharide components most frequently encountered in bacterial EPS (Sutherland, 1990). In this study an enzyme-linked lectinsorbent assay (ELLA) modified after the manual published by Leriche et al. (2000) was conducted, whereby peroxidase-labeled Con A was used as a lectin. Increasing concentrations of Con A resulted in heightened OD values whereby rendering clear dose-response curves, which demonstrates the existence of binding sites for Con A. Consequently the presence of EPS containing D-glucose and D-mannose residues was confirmed (**Figure 3**).

Irradiation parameters for SAPYR were designated and for irradiation of MB either the energy dose applied by the respective light sources or the numbers of absorbed photons were adjusted compared to SAPYR. For the latter, we inserted the constants, the experimental data and the measured values in the formula presented above in order to calculate the numbers of photons, which were absorbed per second by each PS when irradiated by its corresponding light source.

Under these conditions, SAPYR was able to inactivate monospecies biofilms of *E. faecalis* by 5.1 $\log_{10}$ steps of CFU, which is defined as a disinfectant effect (Boyce and Pittet, 2002). Biofilms of *A. naeslundii* could even be eradicated by 6.5 $\log_{10}$ steps. When comparing the inactivation efficacy of MB at adjusted energy dose, inactivation efficacy of MB was approximately 2 $\log_{10}$ steps inferior (*E. faecalis*: 3.4 $\log_{10}$; *A. naeslundii*: 4.2 $\log_{10}$). When evaluating MB at adjusted numbers of absorbed photons, MB exhibited no CFU-reduction at all. Due to this pronounced difference in the efficacy rates of MB according to whether energy dose was adjusted or the numbers of absorbed photons, adjusting the numbers of absorbed photons has to be considered in further studies since reliable comparison of given PS and corresponding light sources is essential for further optimization of PIB efficacy.

Despite adjusted numbers of absorbed photons, there were distinct antimicrobial photodynamic efficacy rates of SAPYR and MB in this study, which may be explained by PS-inherent aspects like mentioned above: Since there is no remarkable difference in molecular dimension between SAPYR and MB (272.3 and 284.4 g/mol without counterions, respectively), the extent of steric hindrance for penetration through EPS may be similar and unlikely accounts for the observed difference in the PIB efficacy. As both, SAPYR and MB, are single positively charged, electrostatic interactions with negatively charged EPS molecules should be similar, too. Though, it is known that SAPYR may act like a tenside due to its chemical structure comprising the combination of a hydrophilic pyridinium unit and a large hydrophobic tail (Cieplik et al., 2013). Consequently, this tenside-like character of SAPYR may facilitate disruption of the EPS and penetration of this PS throughout the biofilm, thus enhancing PIB efficacy. On the other hand, phenothiazinium salts like MB are known to be substrates for efflux pumps in a variety of bacterial species (Tegos and Hamblin, 2006), which may reduce antimicrobial photodynamic efficacy. However, this may be overcome by combined administration of MB with efflux pump inhibitors (Kishen et al., 2010).

Since $^1O_2$ is known to be the main ROS in the mechanism of PIB (Maisch et al., 2007), the higher killing efficacy of SAPYR may also be attributed to its substantially higher $^1O_2$ quantum yield compared to MB (0.99 vs. 0.52). Consequently, SAPYR generates considerably more $^1O_2$ than MB, supporting its higher killing efficacy. In addition, MB is a metachromatic compound that forms dimers or higher aggregates at increasing PS concentrations, which tend to react mainly according to type I mechanism (Usacheva et al., 2003; Núñez et al., 2015).

In a former study, SAPYR was evaluated against monospecies and polyspecies biofilms formed by *E. faecalis*, *A. naeslundii,* and *Fusobacterium nucleatum* and demonstrated pronounced antimicrobial photodynamic efficacy rates ($\geq$ 4 $\log_{10}$ steps of CFU reduction in the polyspecies biofilm) (Cieplik et al., 2013). The porphyrin derivative TMPyP, which was used as a control PS, had no effect at all, although it is described as efficient PS for inactivation of biofilms (Di Poto et al., 2009; Collins et al., 2010; Cieplik et al., 2014b). However, the emission of the light source, which was used in this study for irradiation of SAPYR and TMPyP (blue light emitting dental light-curing unit Bluephase C8, Ivoclar-Vivadent, Schaan, LIE; $\lambda_{em} = 460 \pm 20$ nm), exhibited only partial overlap with the absorption of SAPYR. Consequently, the overall absorbed energy of TMPyP was about 6.4 times higher compared with the absorbed energy of SAPYR and the absolute yield for $^1O_2$ generation was 4.8 times higher for TMPyP (Cieplik et al., 2013). In contrast, here a light source was employed with an emission spectrum adapted to the absorption of SAPYR (Waldmann PIB 3000). Likewise, for irradiation of MB a light source was chosen with an appropriate emission spectrum for the absorption of MB (Waldmann PDT 1200L).

When comparing the PIB efficacy rates of SAPYR here to those of the former study, *A. naeslundii* was inactivated more efficiently here ($\geq$ 6 $\log_{10}$ compared to $\geq$ 2 $\log_{10}$), whereas inactivation

rates against *EF* are identical ($\geq$ 5 $\log_{10}$ both) (Cieplik et al., 2013). Since the concentration of SAPYR was identical in both studies (100 μM), the optimized emission spectrum of the Waldmann PIB 3000 may be causative for this. In addition, due to the better overlap of the emission of PIB 3000 with the absorption of SAPYR, the total treatment time for PIB with SAPYR could be reduced from 62 min (60 min incubation followed 2 min irradiation) to 35 min (25 min incubation followed by 10 min irradiation). Besides that, the irradiance at sample-level could be strikingly decreased from 600 mW/cm^2 to 20 mW/cm^2. This is an important aspect for clinical application, since high-power light-curing units (like Bluephase C8) may cause damage to oral soft tissues due to heat generation (Spranley et al., 2012).

Apart from that, it was found that irradiation of *A. naeslundii* with blue or red light at an energy dose of 30 J/cm^2 without any PS led to a slight reduction of CFU (Waldmann PIB 3000: 0.5 $\log_{10}$; Waldmann PDT 1200L: 1.2 $\log_{10}$). This finding is in contrast to the former study, where no light toxicity was observed when *A. naeslundii* biofilms were irradiated with blue light derived from Bluephase C8 (Cieplik et al., 2013). However, this may be explained by different emission spectra of these light sources and a prolonged period of irradiation (600 s compared to 120 s) in the present study. An effect of heat can be excluded because this light toxicity could only be observed with monospecies biofilms of *A. naeslundii* but not *E. faecalis*. Therefore, the presence of endogenous substances in *A. naeslundii* (e.g. porphyrins) that can act as PS when irradiated with blue or red light may be accounted for that occurrence like it has already been demonstrated for some other oral pathogens like *Aggregatibacter actinomycetemcomitans* (Cieplik et al., 2014a) or *Porphyromonas gingivalis* and *Prevotella* spp. (Soukos et al., 2005). These endogenous PS may only be formed when *A. naeslundii* cells are in a sessile biofilm-state because no light toxicity could be observed when planktonic cultures of *A. naeslundii* were irradiated with PIB 3000 for the same period (data not shown). This is conceivable due the large amount of genetic diversity, which is generated when bacteria grow in a biofilm, in contrast to the homogenous populations of genetically identical cells, which are produced by growth of bacteria in planktonic cultures (Boles et al., 2004; Kolter and Greenberg, 2006). However, further studies are needed for understanding this auto-photosensitization process in sessile *A. naeslundii* cells.

Taken together, the results of this study provide evidence that PIB with SAPYR may be a promising approach for inactivation of biofilms, whereas PIB with MB is ineffective in this regard. However, before any clinical application of SAPYR, its toxicity against host cells has to be examined in subsequent studies. Furthermore, the capability of blue light to penetrate dental hard and gingival tissues has to be investigated for proper light-activation of SAPYR like it has already been shown for red light and MB (Ronay et al., 2013). In this regard, PIB with a combined administration of two PS (e.g. SAPYR and MB) might provide a synergistic strategy to enhance the antimicrobial photodynamic effect in more complex systems like the oral cavity (Acedo et al., 2014). In this instance irradiation with broadband light (≈400–650 nm) would be necessary for sufficient activation of both PS.

In general, a PS should feature high binding affinity for microorganisms (positive charge for adherence to negatively charged bacterial cell walls) (Alves et al., 2009) and low chemical toxicity and mutagenicity (Soukos and Goodson, 2011). In addition, photostability during irradiation is a critical point (no formation of toxic by-products upon irradiation) (Engel et al., 2008) and the respective combination of PS and light source has to be coordinated to yield an appropriate system for good PIB efficacy.

The data of this study clearly demonstrates that adjusting the numbers of absorbed photons is crucial for comparing antimicrobial photodynamic efficacy rates of distinct PS. This is essential for further development and/or optimization of both, PS and light sources.

Conclusion

In this study, a formula is presented for calculation of the number of photons absorbed by a given PS upon irradiation by its corresponding light source. In this way, appropriate comparison of PIB efficacy of given PS-light source systems is ensured because according to the photodynamic principle one PS molecule can only be excited by the absorption of one photon.

For experimental validation of this formula, antimicrobial photodynamic efficacy of the phenalen-1-one PS SAPYR was exemplarily compared to that of clinical standard PS Methylene Blue regarding inactivation of monospecies biofilms formed by *E. faecalis* and *A. naeslundii*. PIB with SAPYR demonstrated a disinfectant efficacy on both pathogens whereas Methylene Blue exhibited a smaller effect when energy dose was adjusted compared to SAPYR. In contrast, when the number of absorbed photons was adjusted compared to SAPYR, no effect could be observed at all. Due to the pronounced differences of the photodynamic efficacy of MB when either energy dose or numbers of absorbed photons were adjusted compared to SAPYR, calculation of the numbers of absorbed photons has to be considered for further studies when the antimicrobial photodynamic efficacy rates of distinct PS are compared. Besides that, the high efficacy of photodynamic inactivation exhibited by SAPYR encourages further research on PIB with this PS as an approach for treatment and control of oral biofilm infections.

Acknowledgments

Isabelle Tabenski is gratefully acknowledged for helpful discussions. FC thanks for financial support from the University Medical Center Regensburg (ReForm A program) and for a travel grant from the German Society of Periodontology (DG PARO). JR was funded by a grant of the German Research Foundation (DFG-RE-3323/2-1). Publication of this work was supported by the German Research Foundation (DFG) within the funding program Open Access Publishing.

References

Acedo, P., Stockert, J. C., Cañete, M., and Villanueva, A. (2014). Two combined photosensitizers: a goal for more effective photodynamic therapy of cancer. *Cell Death Dis.* 5, e1122. doi: 10.1038/cddis.2014.77

Al-Ahmad, A., Ameen, H., Pelz, K., Karygianni, L., Wittmer, A., Anderson, A. C., et al. (2014). Antibiotic resistance and capacity for biofilm formation of different bacteria isolated from endodontic infections associated with root-filled teeth. *J. Endod.* 40, 223–230. doi: 10.1016/j.joen.2013.07.023

Alves, E., Costa, L., Carvalho, C. M., Tomé, J. P., Faustino, M. A., Neves, M. G., et al. (2009). Charge effect on the photoinactivation of Gram-negative and Gram-positive bacteria by cationic meso-substituted porphyrins. *BMC Microbiol.* 9:70. doi: 10.1186/1471-2180-9-70

Baier, J., Fuß, T., Pöllmann, C., Wiesmann, C., Pindl, K., Engl, R., et al. (2007). Theoretical and experimental analysis of the luminescence signal of singlet oxygen for different photosensitizers. *J. Photochem. Photobiol. B Biol.* 87, 163–173. doi: 10.1016/j.jphotobiol.2007.02.006

Boles, B. R., Thoendel, M., and Singh, P. K. (2004). Self-generated diversity produces "insurance effects" in biofilm communities. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16630–16635. doi: 10.1073/pnas.0407460101

Boyce, J. M., and Pittet, D. (2002). Guideline for hand hygiene in health-care settings: recommendations of the healthcare infection control practices advisory committee and the HICPAC/SHEA/APIC/IDSA hand hygiene task force. *Infect. Control Hosp. Epidemiol.* 23, 3–40. doi: 10.1086/503164

Centers for Disease Control and Prevention. (2013). *Antibiotic resistance threats in the United States, 2013*. Available online at: http://cdc.gov

Cieplik, F., Späth, A., Leibl, C., Gollmer, A., Regensburger, J., Tabenski, L., et al. (2014a). Blue light kills *Aggregatibacter actinomycetemcomitans* due to its endogenous photosensitizers. *Clin. Oral Investig.* 18, 1763–1769. doi: 10.1007/s00784-013-1151-8

Cieplik, F., Späth, A., Regensburger, J., Gollmer, A., Tabenski, L., Hiller, K.-A., et al. (2013). Photodynamic biofilm inactivation by SAPYR-An exclusive singlet oxygen photosensitizer. *Free Radic. Biol. Med.* 65, 477–487. doi: 10.1016/j.freeradbiomed.2013.07.031

Cieplik, F., Tabenski, L., Buchalla, W., and Maisch, T. (2014b). Antimicrobial photodynamic therapy for inactivation of biofilms formed by oral key pathogens. *Front. Microbiol.* 5:405. doi: 10.3389/fmicb.2014.00405

Collins, T. L., Markus, E. A., Hassett, D. J., and Robinson, J. B. (2010). The effect of a cationic porphyrin on *Pseudomonas aeruginosa* biofilms. *Curr. Microbiol.* 61, 411–416. doi: 10.1007/s00284-010-9629-y

Di Poto, A., Sbarra, M. S., Provenza, G., Visai, L., and Speziale, P. (2009). The effect of photodynamic treatment combined with antibiotic action or host defence mechanisms on Staphylococcus aureus biofilms. *Biomaterials* 30, 3158–3166. doi: 10.1016/j.biomaterials.2009.02.038

Donlan, R. M., and Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15, 167–193. doi: 10.1128/CMR.15.2.167-193.2002

Engel, E., Schraml, R., Maisch, T., Kobuch, K., König, B., Szeimies, R.-M., et al. (2008). Light-induced decomposition of indocyanine green. *Invest. Ophthalmol. Vis. Sci.* 49, 1777–1783. doi: 10.1167/iovs.07-0911

Felgenträger, A., Maisch, T., Dobler, D., and Späth, A. (2013). Hydrogen bond acceptors and additional cationic charges in methylene blue derivatives: photophysics and antimicrobial efficiency. *Biomed. Res. Int.* 2013:482167. doi: 10.1155/2013/482167

Gursoy, H., Ozcakir-Tomruk, C., Tanalp, J., and Yilmaz, S. (2013). Photodynamic therapy in dentistry: a literature review. *Clin. Oral Investig.* 17, 1113–1125. doi: 10.1007/s00784-012-0845-7

Kishen, A., Upadya, M., Tegos, G. P., and Hamblin, M. R. (2010). Efflux pump inhibitor potentiates antimicrobial photodynamic inactivation of *Enterococcus faecalis* biofilm. *Photochem. Photobiol.* 86, 1343–1349. doi: 10.1111/j.1751-1097.2010.00792.x

Kolter, R., and Greenberg, E. P. (2006). Microbial sciences: the superficial life of microbes. *Nature* 441, 300–302. doi: 10.1038/441300a

Leriche, V., Sibille, P., and Carpentier, B. (2000). Use of an enzyme-linked lectinsorbent assay to monitor the shift in polysaccharide composition in bacterial biofilms. *Appl. Environ. Microbiol.* 66, 1851–1856. doi: 10.1128/AEM.66.5.1851-1856.2000

Maisch, T., Baier, J., Franz, B., Maier, M., Landthaler, M., Szeimies, R.-M., et al. (2007). The role of singlet oxygen and oxygen concentration in photodynamic inactivation of bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 104, 7223–7228. doi: 10.1073/pnas.0611328104

Miles, A. A., Misra, S. S., and Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *J. Hyg. (London)* 38, 732–749. doi: 10.1017/S002217240001158X

Núñez, S. C., Yoshimura, T. M., Ribeiro, M. S., Junqueira, H. C., Maciel, C., Coutinho-Neto, M. D., et al. (2015). Urea enhances the photodynamic efficiency of methylene blue. *J. Photochem. Photobiol. B Biol.* doi: 10.1016/j.jphotobiol.2015.03.018. [Epub ahead of print].

Pratten, J., Wills, K., Barnett, P., and Wilson, M. (1998). *In vitro* studies of the effect of antiseptic-containing mouthwashes on the formation and viability of *Streptococcus sanguis* biofilms. *J. Appl. Microbiol.* 84, 1149–1155. doi: 10.1046/j.1365-2672.1998.00462.x

Preus, H. R., Scheie, A. A., and Baelum, V. (2014). Letter to the editor: Re: the clinical effect of scaling and root planing and the concomitant administration of systemic amoxicillin and metronidazole: a systematic review; Re: Effectiveness of systemic amoxicillin/metronidazole as adjunctive therapy to scaling and root planing in the treatment of chronic periodontitis: a systematic review and meta-analysis; Re: effectiveness of systemic amoxicillin/metronidazole as an adjunctive therapy to full-mouth scaling and root planing in the treatment of aggressive periodontitis: a systematic review and meta-analysis. *J. Periodontol.* 85, 374–384. doi: 10.1902/jop.2014.130379

Rams, T. E., Degener, J. E., and Winkelhoff, A. J. (2014). Antibiotic resistance in human chronic periodontitis microbiota. *J. Periodontol.* 85, 160–169. doi: 10.1902/jop.2013.130142

Ronay, V., Buchalla, W., Sahrmann, P., Attin, T., and Schmidlin, P. R. (2013). *In vitro* evaluation of the oxidation efficacy of transgingival photodynamic therapy. *Acta Odontol. Scand.* 71, 1216–1220. doi: 10.3109/00016357.2012.757639

Schweitzer, C., and Schmidt, R. (2003). Physical mechanisms of generation and deactivation of singlet oxygen. *Chem. Rev.* 103, 1685–1757. doi: 10.1021/cr010371d

Sgolastra, F., Petrucci, A., Severino, M., Graziani, F., Gatto, R., and Monaco, A. (2013). Adjunctive photodynamic therapy to non-surgical treatment of chronic periodontitis: a systematic review and meta-analysis. *J. Clin. Periodontol.* 40, 514–526. doi: 10.1111/jcpe.12094

Soukos, N. S., and Goodson, J. M. (2011). Photodynamic therapy in the control of oral biofilms. *Periodontol. 2000* 55, 143–166. doi: 10.1111/j.1600-0757.2010.00346.x

Soukos, N. S., Som, S., Abernethy, A. D., Ruggiero, K., Dunham, J., Lee, C., et al. (2005). Phototargeting oral black-pigmented bacteria. *Antimicrob. Agents Chemother.* 49, 1391–1396. doi: 10.1128/AAC.49.4.1391-1396.2005

Späth, A., Leibl, C., Cieplik, F., Lehner, K., Regensburger, J., Hiller, K.-A., et al. (2014). Improving photodynamic inactivation of bacteria in dentistry: highly effective and fast killing of oral key pathogens with novel tooth-colored type-II photosensitizers. *J. Med. Chem.* 57, 5157–5168. doi: 10.1021/jm4019492

Spranley, T. J., Winkler, M., Dagate, J., Oncale, D., and Strother, E. (2012). Curing light burns. *Gen. Dent.* 60, e210–e214.

Sutherland, I. W. (1990). *Biotechnology of Microbial Exopolysaccharides*. Cambridge: Cambridge University Press.

Tabenski, L., Maisch, T., Santarelli, F., Hiller, K.-A., and Schmalz, G. (2014). Individual growth detection of bacterial species in an *in vitro* oral polymicrobial biofilm model. *Arch. Microbiol.* 196, 819–828. doi: 10.1007/s00203-014-1021-z

Tegos, G. P., and Hamblin, M. R. (2006). Phenothiazinium antimicrobial photosensitizers are substrates of bacterial multidrug resistance pumps. *Antimicrob. Agents Chemother.* 50, 196–203. doi: 10.1128/AAC.50.1.196-203.2006

The White House. (2014). *National Strategy for Combating Antibiotic-resistant Bacteria*. Available online at: http://www.whitehouse.gov/sites/default/files/docs/carb_national_strategy.pdf

Usacheva, M. N., Teichert, M. C., and Biel, M. A. (2003). The role of the methylene blue and toluidine blue monomers and dimers in the photoinactivation of bacteria. *J. Photochem. Photobiol. B Biol.* 71, 87–98. doi: 10.1016/j.jphotobiol.2003.06.002

Wainwright, M. (1998). Photodynamic antimicrobial chemotherapy (PACT). *J. Antimicrob. Chemother.* 42, 13–28. doi: 10.1093/jac/42.1.13

Wilkinson, F., Helman, W. P., and Ross, A. B. (1993). Quantum yields for the photosensitized formation of the lowest electronically excited singlet state of molecular oxygen in solution. *J. Phys. Chem. Ref. Data* 22, 113–262. doi: 10.1063/1.555934

Winkelhoff, A. J. V., Rams, T. E., and Slots, J. (1996). Systemic antibiotic therapy in periodontics. *Periodontol. 2000* 10, 45–78. doi: 10.1111/j.1600-0757.1996.tb00068.x

Zandbergen, D., Slot, D. E., Cobb, C. M., and Van der Weijden, F. A. (2013). The clinical effect of scaling and root planing and the concomitant administration of systemic amoxicillin and metronidazole: a systematic review. *J. Periodontol.* 84, 332–351. doi: 10.1902/jop.2012.120040

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Cieplik, Pummer, Regensburger, Hiller, Späth, Tabenski, Buchalla and Maisch. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Teil II: Photodynamische Inaktivierung von Bakterien im Wurzelkanal bei Belichtung durch humanes Zahnhartgewebe sowie simulierte zahnumgebende Gewebe

Zusammenfassung

Nachdem im ersten Teil die Formel zur Anpassung der Belichtungsparameter bezüglich der Zahl der absorbierten Photonen etabliert wurde, war es das Ziel des zweiten Teils der Doktorarbeit, zwei PS unter Anwendung dieser Formel anhand eines angewandten Modells zu vergleichen. Hierfür wurde ein Modell hergestellt, welches die Situation, wie sie im Rahmen einer Wurzelkanalbehandlung auftritt, nachahmen sollte. Dieses bestand aus humanen Zähnen mitsamt simulierten zahnumgebenden Geweben.

In der endodontischen Behandlung ist heutzutage die mechanische Entfernung jeglichen Gewebes und die Formgebung der Wurzelkanäle mit verschiedenen rotierenden Nickel-Titan-Feilen sowie die Spülung mit antimikrobiellen oder gewebeauflösenden Spüllösungen wie z.B. Chlorhexidin, Natriumhypochlorit oder Chelatverbindungen wie EDTA und Zitronensäure Stand der Technik [17]. Außerdem finden Medikamente, welche aus Calciumhydroxid [18] oder Antibiotika-Glucocorticoid-Kombinationen (z.B. Ledermix oder Odontopaste) [19] bestehen, Anwendung. Diese werden in die entsprechenden Kanäle eingebracht und verbleiben zwischen den einzelnen Terminen in diesen. Neben ihren antimikrobiellen Eigenschaften besitzen die meisten dieser Agenzien diverse Nachteile wie die mögliche Induktion bakterieller Resistenzen [20], das Risiko von

Medikamentenunverträglichkeiten, einer mangelnden Biokompatibilität [21,22], einer ungewissen Wirkdauer [23], der Inaktivierung durch organische Komponenten [24] oder von Zahnverfärbungen [25]. Des Weiteren können zurückbleibende, resistente Bakterien bei endodontischen Infektionen zu refraktären Verläufen führen [26,27].

Als unterstützende Maßnahme für die Desinfektion des Wurzelkanalsystems könnte die photodynamische Inaktivierung von Bakterien (PIB) dienen [28-30]. *In vitro* konnten für die PIB bereits vielversprechende Ergebnisse nachgewiesen werden, sowohl was die Inaktivierung von planktonischen Bakterien [31,32] als auch von Pilzen [33] oder von Mikroorganismen, welche in Biofilmen organisiert sind, betrifft [33-36]. Diese Ergebnisse können allerdings nicht direkt auf eine klinische Anwendung hin übertragen werden. Um eine Brücke zwischen Grundlagen- und klinischer Forschung zu schaffen, sollten daher realitätsnähere *ex-vivo*-Modelle genutzt werden, welche die Situation in der menschlichen Mundhöhle besser nachahmen.

Hierbei stellen extrahierte humane Zähne eine Möglichkeit dar, indem diese entweder zunächst autoklaviert und dann *in vitro* kontrolliert infiziert werden oder aber indem die bereits vorhandene Mikroflora des Wurzelkanalsystems dieser Zähne genutzt wird [37, 38]. Ng *et al.* untersuchten beispielsweise die Effektivität der PIB anhand *ex vivo* infizierter humaner Zähne [37]. Frisch extrahierte Zähne mit einer Pulpanekrose wurden entweder einem chemomechanischen Debridement mit 6% NaOCl (CMD) oder einem CMD mit anschließender PIB unterzogen. Als PS wurde Methylenblau bei einer Konzentration von 50 µg/ml benutzt; hierbei wurde eine Bestrahlungsdauer von 5 Minuten bei Benutzung eines Diodenlasers, welcher an einen Lichtwellenleiter mit einem Durchmesser von 250 µm angeschlossen wurde (Abgabeleistung: 1 W; zentrale Wellenlänge: 665 nm; Lichtdosis: 30 J/cm²), wodurch eine 360 Grad Bestrahlung innerhalb des Wurzelkanals möglich war, gewählt. Es zeigte sich in dieser Arbeit, dass CMD gefolgt von PIB mit 86,5% bakterienfreier Wurzelkanäle signifikant höhere Werte

bezüglich der Inaktivierung von Bakterien im Vergleich zur alleinigen CMD zeigte, wo sich nur 49% der Kanäle bakterienfrei zeigten [37].

In einer weiteren Arbeit derselben Arbeitsgruppe etablierten Fimple *et al.* ein Modell einer Wurzelkanalinfektion [38]. Im Labor wurden aus einwurzeligen menschlichen Zähnen Wurzelkanalmodelle vorbereitet, autoklaviert und schließlich für 3 Tage mit *Actinomyces israelii*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* und *Prevotella intermedia* inkubiert. Nach diesem Vorgang wurde MB in einer Konzentration von 67 µM in die Kanäle gegeben, wobei man eine Inkubationszeit von 10 Minuten gefolgt von einer Bestrahlungszeit von 5 Minuten, aufgeteilt in zwei Intervalle à 2,5 Minuten mit dazwischenliegender Pause von 2,5 Minuten, wählte; es wurde der gleiche Diodenlaser wie in der vorherigen Arbeit verwendet (Abgabeleistung: 1 W; zentrale Wellenlänge: 665 nm; Lichtdosis: 30 J/cm²), welcher wiederum an eine optische Faser, welche eine 360 Grad Lichterverteilung ermöglichte, gekoppelt wurde. Die Abnahme der zählbaren CFU betrug 80% [38].

Allen diesen Experimenten ist allerdings gemeinsam, dass die Bestrahlung des PS stets vom Inneren des Wurzelkanals aus erfolgte. Garcez *et al.* unterstrichen daher den Vorteil eines optischen Diffusors für PIB beim Einsatz in der Endodontie und betonten die Wichtigkeit eines intrakanalären Bestrahlungsinstruments für die adäquate Lichtaktivierung eines bestimmten PS [39].

Allerdings ist bisher keine Literatur über die Möglichkeit der Aktivierung eines PS bei Bestrahlung von außen durch humane Zahnhartgewebe und die zahnumgebenden Gewebe bekannt; eine derartige Form der Bestrahlung würde die Handhabung der Lichtquellen bei der Anwendung der PIB beim Einsatz in der Endodontie deutlich erleichtern.

Daher waren die Ziele der folgenden Arbeit zum einen die Entwicklung eines humanen Zahnmodells für die Einordnung der PIB bei intrakanalärer Applikation des PS und

dessen Lichtaktivierung von außerhalb des Zahnes und zum anderen die Untersuchung, ob der durch rotes Licht aktivierte PS Methylenblau (MB) und der durch blaues Licht aktivierte PS TMPyP durch menschlichen Zahnschmelz, Dentin und nachgebildetes Knochen- und Ginigvagewebe hindurch aktiviert werden können. Hierbei wurde die antibakterielle Wirksamkeit der PIB gegen das mit vielen endodontischen Infektionen vergesellschaftete Bakterium *Enterococcus faecalis* untersucht.

Es wurde ein Zahnmodell hergestellt, bestehend aus einem humanen Prämolaren sowie zwei Molaren in einem Kunststoffblock, dessen Zusammensetzung die optischen Eigenschaften eines Schweinekiefers simuliert [40] (siehe Abbildung 2). Der distale Wurzelkanal des ersten Molaren wurde erweitert, so dass ein Glasröhrchen mit 2 mm Außendurchmesser eingeführt werden konnte. Im Rahmen der Versuchsdurchführung wurde dieses Glasröhrchen mit PS und planktonischen Kulturen von *E. faecalis* in der stationären Wachstumsphase befüllt. Beide Photosensibilisatoren (10 µM) wurden für 120 Sekunden bestrahlt, wobei je nach Absorptionseigenschaften blaues (BlueV; 20 mWcm²; λ_{em} = 400-460nm) bzw. rotes Licht (PDT 1200L; 37,8 mW/cm²; λ_{em}=570-680nm; beide: Waldmann Medizintechnik, Villingen-Schwenningen, D) verwendet wurde. Es wurden drei verschiedene Versuchsgruppen definiert: Bestrahlung des Glasröhrchens allein (G), Bestrahlung des Glasröhrchens im Zahn (GT) sowie im Zahn mit simulierten zahnumgebenden Geweben (GTM). Nach Versuchsdurchführung erfolgte die Bestimmung der koloniebildenden Einheiten (CFU).

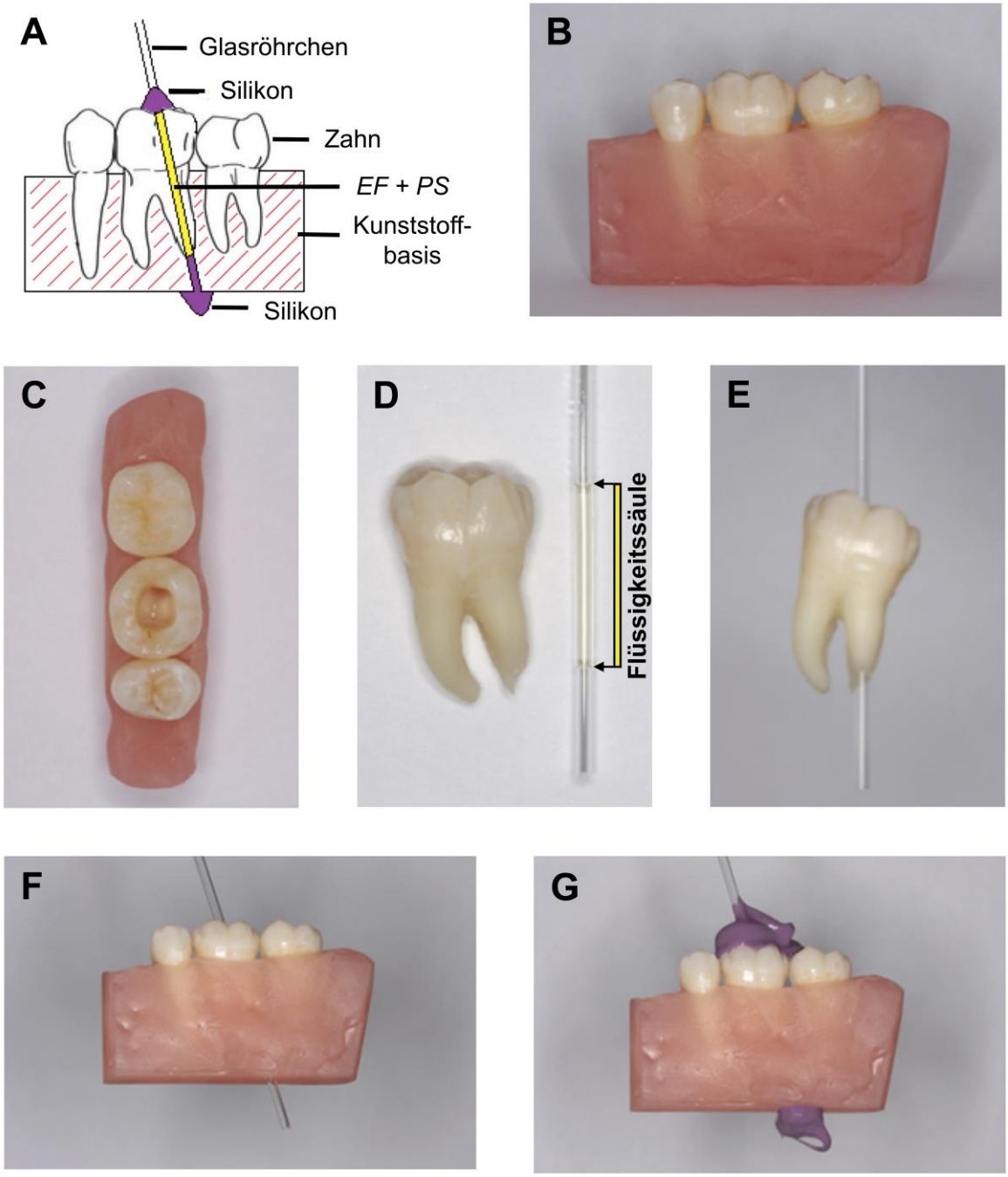

Abbildung 2: Zahnmodell für die Beurteilung der Effektivität der PIB bei Bestrahlung durch humanes Zahnhartgewebe sowie simulierte zahnumgebende Gewebe

A: Schematische Darstellung des Zahnmodells: ein humaner Prämolar sowie ein erster und zweiter Molar wurden unter Approximalkontakt in einer Kunststoffbasis eingebettet. Der distale Wurzelkanal nimmt ein Glasröhrchen auf, welches den PS sowie *E. faecalis* enthält, und ist dort mit zahnärztlichem Abformmaterial auf Silikonbasis fixiert.

B-F: Fotografische Darstellungen des Zahnmodells: ein menschlicher Prämolar sowie ein erster und zweiter Molar wurden unter Approximalkontakt in eine Kunststoffbasis eingebettet **(B)**. Das Pulpenkavum des ersten Molaren wurde eröffnet und der distale Wurzelkanal

erweitert **(C)**. PS + *E. faecalis* wurden in ein Glasröhrchen eingebracht, wobei die Flüssigkeitssäule an die Zahnlänge des ersten Molaren angepasst wurde. **(D**; G-Gruppe). Das Glasröhrchen wurde in den ersten Molaren **(E**; GT-Gruppe) oder in das Zahnmodell eingebracht (**F**; GTM-Gruppe) und mit zahnärztlichem Abformmaterial auf Silikonbasis fixiert.

TMPyP führte in allen Versuchsgruppen (G, GT, GTM) zu einer Reduktion von 6,5 $\log_{10}$-Stufen. MB bewirkte bei Bestrahlung des Glasrohrs allein (G) eine Reduktion von 7,2 $\log_{10}$-Stufen, wohingegen bei Bestrahlung des Glasrohrs im Zahn (GT) bzw. im Zahnmodell (GTM) eine Reduktion von 5,8 $\log_{10}$-Stufen erzielt wurde (siehe Abbildung 3).

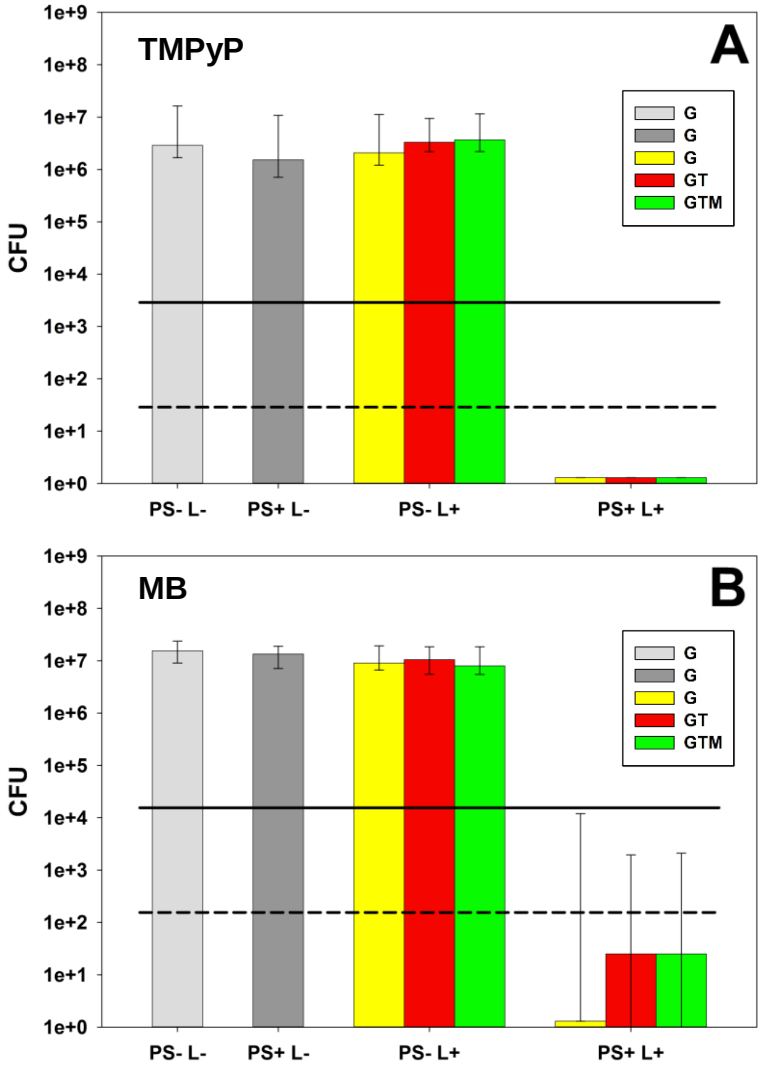

Abbildung 3: Photodynamische Inaktivierung im Zahnmodell

Photodynamische Inaktivierung von Bakterien mit beiden PS-Lichtquellen-Systemen gegen E. faecalis in allen drei Versuchsgruppen des Zahnmodells: Glasröhrchen alleine (G), Glasröhrchen im Einzelzahn (GT) oder im Zahnmodell (GTM). Alle PIB-Versuche in dieser Grafik sind als CFU-Mediane mit 25%- und 75%-Quantilen dargestellt. Die durchgezeichnete bzw. gestrichelte Linie stellen jeweils eine Reduktion der CFU um 3 $\log_{10}$- bzw. 5 $\log_{10}$-Stufen im Vergleich zur unbehandelten Kontrollgruppe PS-L- dar. In allen Fällen war keine Reduktion der CFU mit PS (PS+L-) oder Licht alleine (PS-L+) zu beobachten.

A: PIB mit TMPyP und PIB 3000. Die PS+L+-Gruppe weist eine Inaktivierung von 6,5 $\log_{10}$-Stufen auf und befindet sich unterhalb der Nachweisgrenze für alle drei Versuchsgruppen (G; GT; GTM)

B: PIB mit MB und PDT 1200L. Die PS+L+-Gruppe zeigt eine Inaktivierung von 7,2 $\log_{10}$-Stufen und befindet sich unterhalb der Nachweisgrenze für die Bestrahlung des Glasrohrs allein (G); bei Bestrahlung im Zahn (GT) bzw. Im Zahnmodell (GTM) zeigt sich eine Reduktion der KBE von 5.8 $\log_{10}$-Stufen.

Ausblick

Es werden in Zukunft weitere Studien zu dieser Fragestellung notwendig sein, um PIB hinsichtlich eines Einsatzes in der Endodontie bei Lichtapplikation von außerhalb des Zahnes besser einordnen zu können. Einen wichtigen Schritt im Zuge dieser Arbeiten sollte die Etablierung eines Biofilms bakteriellen Ursprungs im Wurzelkanalsystem mit anschließender PIB darstellen, um bessere Rückschlüsse auf die Situation *in vivo* ziehen zu können, da bekannt ist, dass Bakterien im Wurzelkanal in Form von Biofilmen organisiert sind. Erstrebenswert wäre hierbei sicherlich die Etablierung von Polyspezies-Biofilmen, in welchen fakultativ oder strikt anaerobe Bakterien vorhanden sind, da in geschlossenen Wurzelkanälen diese Form der Bakterien *in vivo* überwiegt [41].

Bei Bestätigung der in dieser Arbeit erzielten Ergebnisse könnte die Bestrahlung durch das Zahnhartgewebe hindurch, welche den Einsatz leistungsfähigerer Apparaturen ermöglicht als bei einer Bestrahlung innerhalb des Wurzelkanals, zu einer Ergänzung für die heute vorherrschenden Desinfektionsverfahren des Kanalsystems beitragen. Das Ziel einer vollständigen Desinfektion vor Obturation des Kanalsystems in nur einer Sitzung wäre dadurch in Reichweite gerückt.

Publikation

Fabian Cieplik*, Andreas Pummer*, Christoph Leibl, Johannes Regensburger, Gottfried Schmalz, Wolfgang Buchalla, Karl-Anton Hiller, Tim Maisch:

Photodynamic inactivation of root canal bacteria by light-activation through human dental hard and sorrounding soft tissue.

Front Microbiol. 2016; 7: 929

(Impact Factor 2015: 4,165)

(* = gleichberechtigte Erstautorschaft)

frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 June 2016
doi: 10.3389/fmicb.2016.00929

Photodynamic Inactivation of Root Canal Bacteria by Light Activation through Human Dental Hard and Simulated Surrounding Tissue

Fabian Cieplik[1]†, Andreas Pummer[1†], Christoph Leibl[1,2], Johannes Regensburger[3], Gottfried Schmalz[1,4], Wolfgang Buchalla[1], Karl-Anton Hiller[1] and Tim Maisch[3]*

[1] Department of Conservative Dentistry and Periodontology, University Medical Center Regensburg, Regensburg, Germany, [2] Private Practice, Geiselhöring, Germany, [3] Department of Dermatology, University Medical Center Regensburg, Regensburg, Germany, [4] Department of Preventive, Restorative and Pediatric Dentistry, School of Dental Medicine, University of Bern, Bern, Switzerland

Introduction: Photodynamic inactivation of bacteria (PIB) may be a supportive antimicrobial approach for use in endodontics, but sufficient activation of photosensitizers (PS) in root canals is a critical point. Therefore, aim of this study was to evaluate the ability of PS absorbing blue (TMPyP) or red light (Methylene Blue; MB) for light activation through human dental hard and simulated surrounding tissue to inactivate root canal bacteria.

Methods: A tooth model was fabricated with a human premolar and two molars in an acrylic resin bloc simulating the optical properties of a porcine jaw. The distal root canal of the first molar was enlarged to insert a glass tube (external diameter 2 mm) containing PS and stationary-phase *Enterococcus faecalis*. Both PS (10 μM) were irradiated for 120 s with BlueV (20 mW/cm^2; λ_{em} = 400–460 nm) or PDT 1200L (37.8 mW/cm^2; λ_{em} = 570–680 nm; both: Waldmann Medizintechnik), respectively. Irradiation parameters ensured identical numbers of photons absorbed by each PS. Three setups were chosen: irradiating the glass pipette only (G), the glass pipette inside the single tooth without (GT) and with (GTM) simulated surrounding tissues. Colony forming units (CFU) were evaluated. Transmission measurements of the buccal halves of hemisected mandibular first molars were performed by means of a photospectrometer.

Results: PIB with both PS led to reduction by ≥ 5 $\log_{10}$ of *E. faecalis* CFU for each setup. From transmission measurements, a threshold wavelength λ_{th} for allowing an amount of light transmission for sufficient activation of PS was determined to be 430 nm.

Conclusion: This study can be seen as proof of principle that light activation of given intra-canal PS from outside a tooth may be possible at wavelengths ≥ 430 nm, facilitating clinical application of PIB in endodontics.

Keywords: photodynamic, *Enterococcus faecalis*, endodontics, optical fiber, transmission

OPEN ACCESS

Edited by:
Octavio Luiz Franco, Universidade Catolica de Brasilia, Brazil

Reviewed by:
Marco Rinaldo Oggioni, University of Leicester, UK
Taia Maria Berto Rezende, Universidade Católica de Brasília, Brazil

****Correspondence:***
Fabian Cieplik
fabian.cieplik@ukr.de

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *23 March 2016*
Accepted: *31 May 2016*
Published: *15 June 2016*

Citation:
Cieplik F, Pummer A, Leibl C, Regensburger J, Schmalz G, Buchalla W, Hiller K-A and Maisch T (2016) Photodynamic Inactivation of Root Canal Bacteria by Light Activation through Human Dental Hard and Simulated Surrounding Tissue. Front. Microbiol. 7:929. doi: 10.3389/fmicb.2016.00929

INTRODUCTION

It is well established that effective disinfection of the root canal system is crucial for success of endodontic treatment. State-of-the-art procedures involve mechanical debridement and intra-canal irrigation with antimicrobial, tissue dissolving or chelating agents (such as chlorhexidine, sodium hypochlorite or EDTA; Zehnder, 2006). Different medicaments as inter-appointment

dressings are used for enhancing disinfection and dissolution of pulpal soft tissue or suppressing inflammation, e.g., based on calcium hydroxide (Mohammadi et al., 2012) or as combined corticosteroid-antibiotic pastes (e.g., Ledermix, Odontopaste; (Athanassiadis et al., 2007). However, most of these substances exhibit evident disadvantages, such as potential development of resistances in bacteria (Arias and Murray, 2009), risk of drug hypersensitivity (Kawashima et al., 2009), or biocompatibility problems (Schmalz, 2014), vague duration of action (Hecker et al., 2013), inactivation by organic compounds (Abouassi et al., 2014), or esthetic limitations due to staining of teeth (Kim et al., 2000). In addition, the presence of resistant pathogens may cause refractory processes of endodontic infections (Al-Ahmad et al., 2014; Łysakowska et al., 2015).

Consequently, alternative approaches as supportive tools for antimicrobial application in endodontics are a current research focus with the photodynamic inactivation of bacteria (PIB) being one option (Gursoy et al., 2013; Chrepa et al., 2014; Cieplik et al., 2014). The bactericidal effect of PIB is based on the excitation of a *per se* non-toxic dye, the so-called photosensitizer (PS), by light of an appropriate wavelength. Upon irradiation, the PS molecule is converted into an excited state from where there are two reaction mechanisms: type I mechanism describes the transfer of charge to a substrate or molecular oxygen resulting in reactive oxygen species (ROS) such as hydroxyl radicals and superoxide ions. Type II mechanism describes the direct transfer of energy to molecular oxygen with generation of the highly reactive singlet oxygen ($^1O_2$;Wainwright, 1998; Schweitzer and Schmidt, 2003).

PIB has already shown promising results *in vitro*, regarding inactivation of planktonic cultures of bacteria (Maisch et al., 2009; Späth et al., 2014) as wells as yeasts (Gonzales et al., 2013) or microorganisms embedded in biofilms (Cieplik et al., 2013, 2015; Gonzales et al., 2013; Voos et al., 2014). However, for bridging the gap between basic and clinical research more realistic *ex vivo* test models are necessary in order to better mimic the situation in the human oral cavity. Here, extracted human teeth are commonly utilized, either by making use of the potentially existing root canal micro flora of those teeth (Ng et al., 2011) or artificially infected after an autoclavation process (Foschi et al., 2007; Fimple et al., 2008).

For example, Ng et al. (2011) studied the effect of PIB on infected human teeth *ex vivo*. Freshly extracted teeth with pulpal necrosis received either conventional chemo-mechanical debridement (CMD) with 6% NaOCl or CMD followed by PIB. Methylene Blue (MB) was used as a PS at a concentration of 50 μg/ml. Root canals were incubated with MB for 5 min, dried with paper points and irradiated for 5 min with a diode laser. This was coupled to a 250-μm diameter optical fiber (output power: 1 W; central wavelength: 665 nm; light dose: 30 J/cm^2) allowing light distribution at 360° within the root canal. CMD followed by PIB showed a significantly higher efficacy in inactivation of bacteria with 86.5% bacteria-free root canals compared to 49% by CMD alone (Ng et al., 2011).

Fimple et al. (2008) prepared root canal specimens from freshly extracted single-rooted human teeth, autoclaved and incubated them with *Actinomyces israelii, Fusobacterium nucleatum, Porphyromonas gingivalis,* and *Prevotella intermedia* for 3 days. After that period canals were filled with MB (67 μM) as PS and incubated for 10 min. Subsequently, excess MB solution was removed and the canals were irradiated for 5 min (2×2.5 min intermitted by 2.5 min break) with the diode laser as described above. PIB achieved up to 80% reduction of CFU counts (Fimple et al., 2008).

However, all these experimental setups have in common that the PS were activated from inside the root canal. Consequently, Garcez et al. (2013) ascertained the benefit of an optical diffusor for endodontic photodynamic therapy and reasoned the importance of an intra-canal irradiation for appropriate light activation of a given PS. However, to the best of our knowledge, there has not been published any study so far on the activation of a given PS from outside the tooth through human dental hard and surrounding tissues, which would facilitate the handling of light sources for application of PIB in endodontics.

Consequently, the aim of the present study was to evaluate PIB for light activation from outside the tooth through human dental hard and simulated surrounding tissue regarding inactivation of the endodontic key pathogen *Enterococcus faecalis* using PS activated by red light (Methylene Blue) and blue light (TMPyP). For this purpose, an applied *in vitro* tooth model was established to evaluate the antimicrobial photodynamic efficacy of given PS.

MATERIALS AND METHODS

Ethics Statement

All human teeth used in this study were donated after informed consent according to a protocol approved by an appropriate review board at the University of Regensburg.

Bacterial Culture

E. faecalis (ATCC 29212) was grown overnight at 37°C in Brain Heart Infusion broth (BHI; Sigma–Aldrich, St. Louis, MO, USA) on an orbital shaker. After reaching the stationary phase of growth, cultures were harvested by centrifugation (2500 rpm for 5 min; Megafuge 1.0, Heraeus Sepatech, Osterode, Germany), washed with phosphate-buffered saline (PBS; Sigma–Aldrich, St. Louis, MO, USA) and resuspended in PBS yielding an optical density (OD) of 0.06 measured at 600 nm by means of a photospectrometer (SPECORD® 50 PLUS, Analytik Jena, Jena, Germany). These suspensions were used for PIB experiments.

Photosensitizers and Light Sources

TMPyP [5,10,15,20-tetrakis(1-methyl-4-pyridinium)-porphyrin tetra-(p-toluenesulfonate)] and Methylene Blue [MB; 3,7-bis(dimethylamino)-phenothiazin-5-ium chloride] were used as PS in this study. Both were obtained from a commercial supplier (Sigma–Aldrich, St. Louis, MO, USA; purity > 99%) and were used as received. All PS suspensions were freshly prepared and stored in the dark at 4°C prior to use.

The blue light emitting prototype BlueV was used for irradiation of TMPyP, whereas the red light emitting and commercially available light source PDT 1200L (both Waldmann, Villingen-Schwenningen, Germany) was used for irradiation of MB. Characteristic absorption spectra of both PS were measured

using a photospectrometer (SPECORD® 50 PLUS, Analytik Jena AG, Jena, Germany). Emission spectra of the corresponding light sources were recorded using a monochromator with CCD detection system (SPEX 232, HORIBA Jobin Yvon, Longjumeau Cedex, France). Spectra are shown in **Figure 1**.

For comparing the biological activity of different PS-light source systems independently from the spectral properties of the respective PS and their corresponding light sources, we calculated identical irradiation parameters by adjusting the numbers of absorbed photons for each PS light source system (Cieplik et al., 2015) to a number of 1.06×10^{16} photons per second (TMPyP: BlueV, 20 mW/cm^2, 120 s, 2.4 J/cm^2; MB: PDT 1200L, 37.8 mW/cm^2, 120 s; 4.54 J/cm^2).

Tooth Model

The tooth model was fabricated with a human premolar, a first and second human molar (freshly extracted permanent teeth), which were examined visually as well as by radiographs after cleansing to exclude any signs of tooth damage such as caries or fractured cusps. A schematic design of the tooth model is shown in **Figure 2A**.

The teeth were embedded into acrylic resin (Paladur®, Heraeus Kulzer, Hanau, Germany) under proximal contacts in order to simulate a mandible lateral teeth area of the third quadrant (**Figure 2B**). A mixture of 76% pink and 24% clear Paladur was used, which has been shown to mimic the transmission of a porcine jaw (buccal resin thickness: 2.9–3.1 mm) (Hiller et al., 2013). All three teeth were removable from the resin base.

The pulp cavity of the first molar was accessed by means of an Endo Access Bur (Dentsply Maillefer, Ballaigues, Suisse, Switzerland) and an airotor (EXPERTtorque E680 L, KaVo, Biberach/Riß, Germany) and its pulp was removed with endodontic files (Pro Taper For Hand Use, Dentsply Maillefer, Ballaigues, Suisse, Switzerland; **Figure 2C**). The distal root canal was instrumented cylindrically to a diameter of 2 mm (Dremel Multi 395, Dremel, Racine, WI, USA) for inserting a glass tube (external diameter: 2 mm; Glass Pasteur Pipette 230 mm length, VWR International, Radnor, PA, USA) containing *E. faecalis* and PS (**Figures 2D,E**). With the same tool, a canal was drilled through the resin bloc to accommodate for the glass-tube extending the root apex (**Figure 2F**). The glass tube was fixed in the single tooth or the tooth model with silicone for dental impression (Silagum-Mono; DMG Chemisch-Pharmazeutische Fabrik, Hamburg, Germany; **Figure 2G**). The bottom and the top part of the glass tube were covered by the silicone, too. In addition, a shadow mask made of black cardboard was used for the experiments in order to ensure that light for activation of the PS must have penetrated through the tooth model instead of being refracted by or transmitted through the top and the bottom of the glass tube.

Photodynamic Inactivation of Bacteria

About 10 µl of *E. faecalis* suspension was mixed with either 10 µl TMPyP (10 µM) or MB (10 µM) in 96-well microtiter plates (Corning Costar®, Corning, NY, USA). This suspension (20 µl) was transferred into a glass tube obtaining final PS concentrations of 10 µM (**Figure 2D**), whereby the fill level was adjusted to the tooth length of the first molar. Fixing of the glass tube in the tooth or the tooth model was done with silicone for dental impression (Silagum-Mono).

Three different experimental setups were chosen: irradiating the glass tube containing *E. faecalis* and PS only (G; **Figure 2D**), the glass tube placed in the single first molar (GT; **Figure 2E**) or the whole tooth model (GTM; **Figure 2G**). Irradiation was for 120 s from the buccal side in all cases (using PDT 1200L for MB and BlueV for TMPyP; **Figure 2F**). Controls were neither sensitized with PS nor irradiated (PS–L–) or

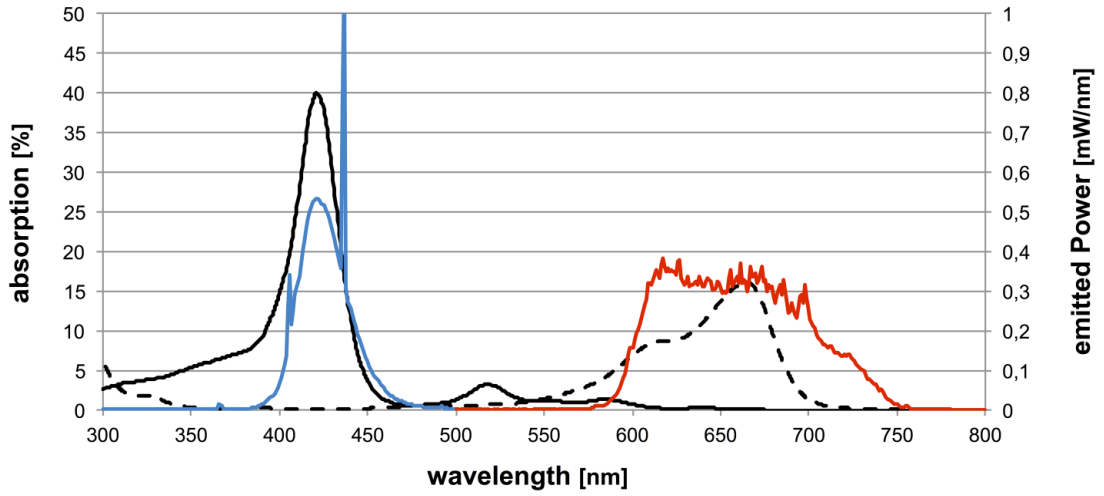

FIGURE 1 | Characteristic absorption spectra of both PS and emission spectra of corresponding light sources. The abscissa shows the wavelength in nanometers, the left ordinate the absorption of the PS (%) and the right ordinate the emitted power of the light sources (mW/nm): characteristic absorption spectrum of TMPyP (solid black line) and emission spectrum of Waldmann Blue V (blue line) as well as characteristic absorption spectrum of MB (dashed black line) and emission spectrum of Waldmann PDT 1200L (red line).

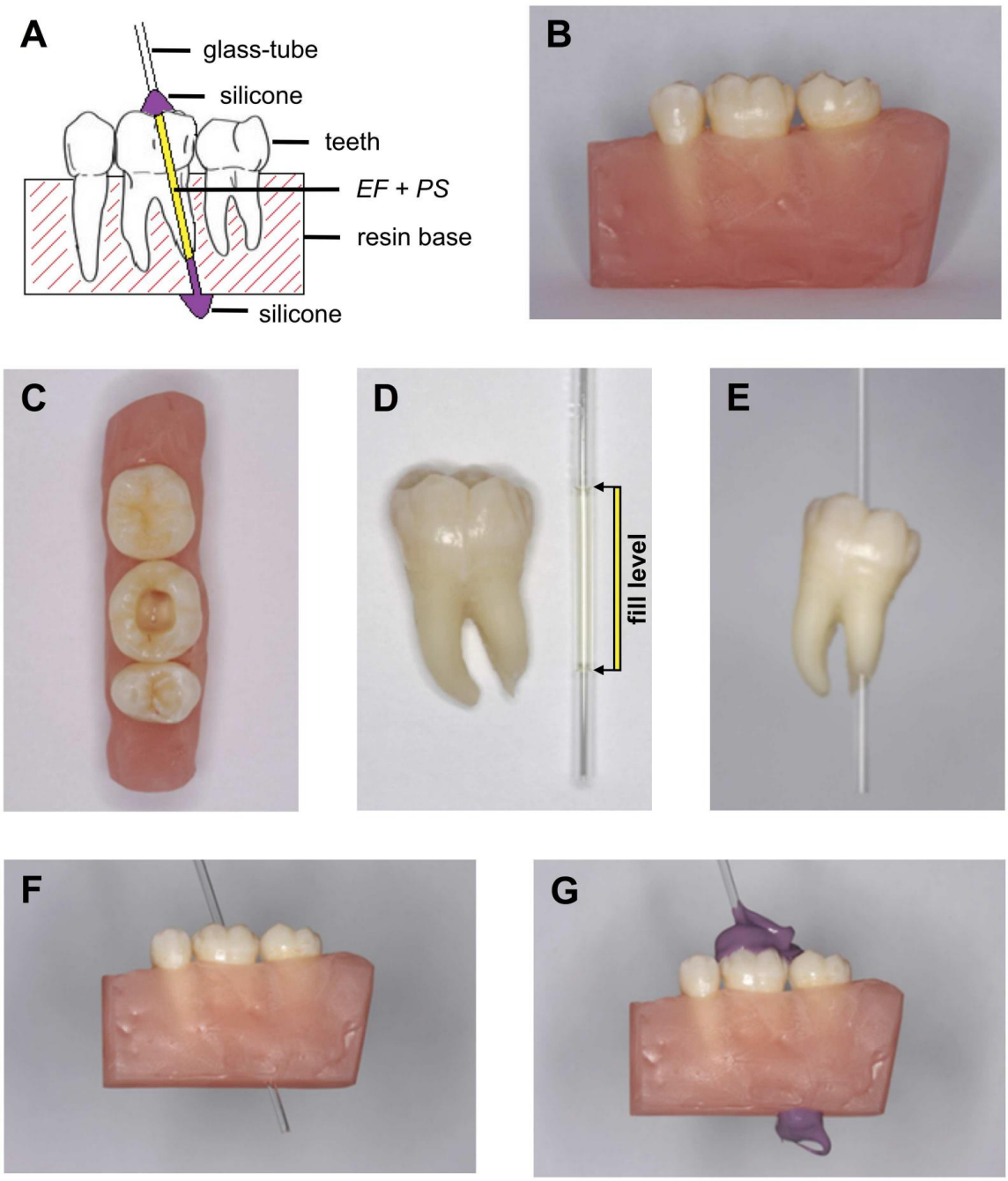

FIGURE 2 | Tooth model. Tooth model for evaluation of PIB for light activation through human dental hard and simulated surrounding tissue: **(A)** scheme of the tooth model: a human premolar, first and second molar under proximal contacts in a resin base. The glass tube containing PS + *E. faecalis* is placed in the distal root canal of the first molar and fixed with silicone dental impression material. **(B–F)** Photographic documentation of the tooth model: A human premolar, first molar, and second molar were embedded in a resin base under proximal contacts (**B**). The pulp cavity of the first molar was accessed and the distal root canal was extended (**C**). PS + *E. faecalis* were placed in a glass tube whereby the fill level was adjusted to the tooth length of the first molar (**D**; G setup). The glass tube was placed in the first molar (**E**; GT setup) or in the tooth model (**F**; GTM setup) and fixed with silicone for dental impression (**G**).

were incubated with the PS only (PS+L–) or were exposed to light only (PS–L+). After irradiation, the bacterial suspension was completely removed from the glass tube. Serial tenfold dilutions (10^{-2}–10^{-7}) were prepared in BHI broth and aliquots ($3 \times 20\,\mu L$) were plated on Mueller–Hinton agar plates (provided by the Institute of Medical Microbiology and Hygiene, University Medical Center Regensburg, Regensburg, Germany), as described earlier (Miles et al., 1938). Plates were incubated aerobically

for 24 h at 37°C. Later, colony forming units (CFUs) were counted.

Measurement of Transmission through Dental Hard Tissue

Five human mandibular first molars were chosen after visual and radiographic inspection in order to exclude any signs of tooth damage such as caries or fractured cusps in order to determine the transmission for wavelengths from 200 to 800 nm. Teeth were thoroughly cleansed and bisected in mesio-distal direction under water cooling by means of a rotating diamond saw (Wild-Heerbrugg, Aarau, Switzerland). While the oral half was discarded, the buccal one was kept in *aqua dest.* after removing any remaining pulpal tissue. Transmission was measured by means of a photospectrometer (SPECORD® 50 PLUS, Analytik Jena, Jena, Germany) with the light path directed from the buccal side toward the pulp chamber at a level ensuring transmission through enamel as well as dentine (diameter 3–4 mm).

Data Treatment

All PIB results are shown as medians, including 25 and 75% quartiles, which were calculated using SPSS for Windows, version 20 (SPSS Inc., Chicago, IL, USA), from the values of at least six independent experiments. Horizontal solid and dashed lines in PIB figures represent reductions of 3 and 5 $\log_{10}$ steps CFU, respectively, compared to untreated control groups (PS–L–). Medians on or below these lines represent PIB efficacy rates of 99.9% (3 $\log_{10}$) or 99.999% (5 $\log_{10}$) at least, which is declared as biologically relevant antimicrobial activity or disinfectant effect, respectively, according to infection control guidelines (Boyce and Pittet, 2002).

For transmission measurements (data from the buccal halves of five mandibular first molars), transmission was plotted versus wavelength. The resulting dose-response shaped curves were fitted to the best fit as calculated by TableCurve 2D (Systat Software Inc., San Jose, CA, USA). This fit was analyzed for the second derivative maximum, the point of maximal curvature, which was defined as threshold wavelength λ_{th}.

RESULTS

Photodynamic Inactivation of Bacteria

PIB with TMPyP inactivated *E. faecalis* by 6.5 $\log_{10}$ as compared to corresponding untreated control PS–L–, regardless whether the glass tube only (G) was irradiated or the glass tube inserted in the single tooth (GT) or the whole tooth model (GTM; **Figure 3A**). PIB with MB led to inactivation of 7.2 $\log_{10}$ as compared to corresponding untreated control PS–L– for irradiation in the glass tube only (G), whereas irradiation of the glass tube inside the single tooth (GT) or incorporated in the whole tooth model (GTM) revealed inactivation efficacy of 5.8 $\log_{10}$ (**Figure 3B**). Treatment with PS only (PS+L–) or light only (PS–L+) had no effect on CFU of *E. faecalis*.

Tooth Transmission Measurements

The best fit of the transmission values of the buccal halves of five human mandibular first molars as calculated by TableCurve 2D resulted in a four-parameter sigmoidal curve exhibiting $r^2 = 0.92$ (**Figure 4**). The threshold wavelength λ_{th} (second derivative maximum, the point of maximal curvature) was determined to be $\lambda_{th} =$ 430 nm exhibiting 0.84% transmission at this point.

DISCUSSION

The aim of this study was to evaluate the efficacy of PIB for light activation from outside the tooth through human dental hard and simulated surrounding tissue in a model approach for avoiding problems of intra-canal placement of the activating light source. For this purpose, a tooth model has been fabricated. The composition of the resin used for embedding the teeth was based on data from transmission measurements of a porcine mandible and comparing them to different formulations of acrylic resins (Hiller et al., 2013). Therefore, it may mimic the alveolar bone and gingival tissue around the teeth for the purpose of this study although the lack of blood flow has to be considered as a drawback. The teeth used for the model were thoroughly cleansed and stored moistly; autoclaving of the teeth was waived since autoclaving might have changed the photophysical properties of enamel and dentine and might have led to desiccation. The glass of the glass tube containing PS and *E. faecalis* caused a reduction of light transmission of approximately 10% (as specified by the manufacturer). However, using the glass tube was necessary, though, for being able to entirely retrieve the applied amount of PS and *E. faecalis* after PIB without any dilution effects caused by incomplete recovery. Furthermore, due to the glass tube, loss of bacteria or of PS by diffusion into dentinal tubules could also be excluded, by which reproducibility is improved. In this experimental setup, porphyrin derivative TMPyP and phenothiazinium derivative MB were used as PS since porphyrin and phenothiazinium dyes are widely used as PS in dental and dermatological clinical practice and for investigating whether there is a difference in efficacy between extra-dental activation by blue (TMPyP) and red light (MB) due to optical effects.

The light sources used in this study were chosen, as their emission spectra cover the absorption maxima of the respective PS. For ensuring reliable comparison of both PS-light source systems, the spectral emission of both light sources and the absorption spectra of both PS as well as the distribution of the emitted photons have to be considered and the numbers of photons absorbed by distinct PS have to be adjusted (Cieplik et al., 2015). In this way, irradiation of 10 μM TMPyP with BlueV for 120 s at 20 mW/cm^2 (light dose 4.54 J/cm^2) equals irradiation of 10 μM MB with PDT 1200L for 120 s at 37.8 mW/cm^2 (light dose: 2.4 J/cm^2) with a number of 1.06×10^{16} photons absorbed per second for each PS-light source system.

The Gram-positive, facultative aerobe *E. faecalis* was used as a model organism in this study since it is strongly associated with different forms of periradicular disease, primary endodontic infections as well as persistent infections after failed endodontic treatment (Stuart et al., 2006). A major characteristic of *E. faecalis*

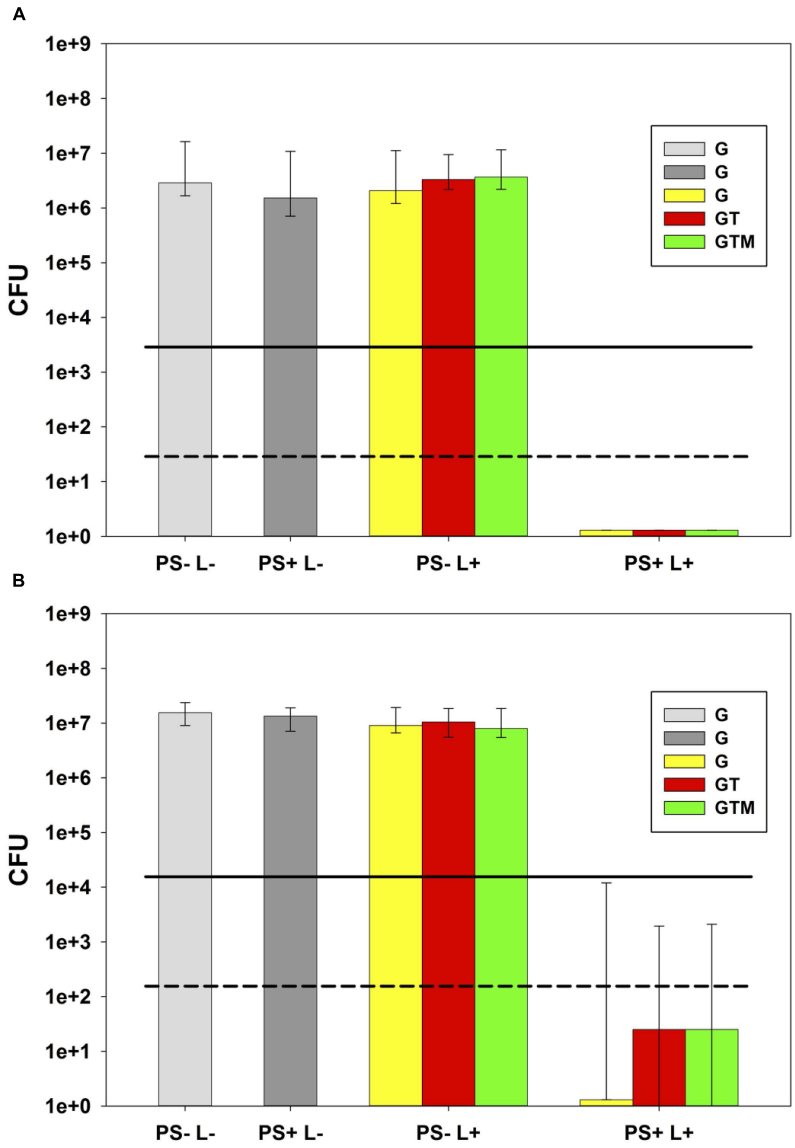

FIGURE 3 | Photodynamic inactivation of bacteria (PIB). Photodynamic inactivation of bacteria with both PS-light source systems against *E. faecalis* with light activation in all three setups of the tooth model: glass tube only (G), glass tube inserted in the single tooth (GT) or the whole tooth model (GTM). All PIB experiments in this figure are shown as Colony forming unit (CFU) medians with 25 and 75% quantiles ($n = 6$). Solid and dashed lines depict CFU reductions of ≥ 3 $\log_{10}$ or ≥ 5 $\log_{10}$ steps, respectively, related to untreated control groups PS–L–. In all cases, there was no CFU-reduction with PS (PS+L–) or light alone (PS–L+). **(A)** PIB with TMPyP and Blue V. PS+L+ groups show inactivation by 6.5 $\log_{10}$ below the detection limit regardless which set-up (G; GT; GTM) was applied. **(B)** PIB with MB and PDT 1200L. PS+L+ groups show inactivation by 7.2 $\log_{10}$ below the detection limit for irradiation of the glass tube only (G) and by 5.8 $\log_{10}$ for irradiation of the single tooth (GT) or the tooth model (GTM).

is its ability to withstand very high alkaline conditions (pH 11.5; Evans et al., 2002; McHugh et al., 2004), which elucidates the importance of alternative or supportive regimens like PIB for treatment of refractory endodontic infections.

Here, TMPyP as well as MB exhibited disinfecting efficacy with blue light activated TMPyP even being slightly more effective (bacterial reduction of 6.5 $\log_{10}$ steps) than MB (5.8 $\log_{10}$ steps) when light activated through dental tissues in the single tooth (GT) or the tooth model (GTM). Furthermore, for both PS there was virtually no difference between irradiating both PS in the glass tube only (G) or inserted in the single tooth (GT) or in the whole tooth model (GTM).

It is known that human teeth are able to transmit (red) light to the pulp, whereby enamel as well as dentine acts as light guides, albeit with anisotropic optical scattering (Odor et al., 1996; Kienle et al., 2002, 2006; Kienle and Hibst, 2006). When light hits on enamel prisms, which are arranged perpendicular to the surface, it is transmitted to the amelo-dentinal junction and guided toward the pulp along dentinal tubules (Odor et al., 1996; Kienle and Hibst, 2006). Hereby, light propagation in dentine is due

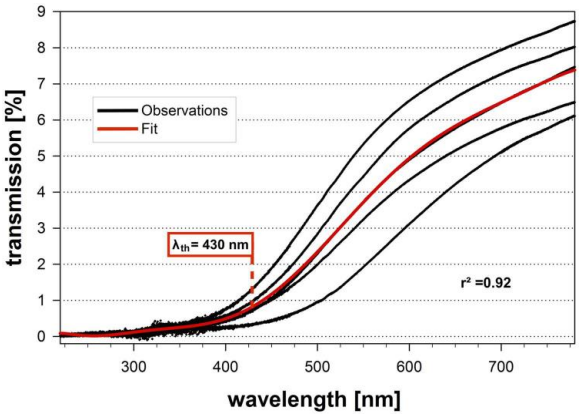

FIGURE 4 | Transmission measurements. Observed transmission measurements of the buccal halves of five human mandibular first molars in the range 200–800 nm (black dots). Red line depicts resulting four-parameter sigmoidal fit with $r^2 = 0.92$ as calculated by TableCurve 2D. 95% confidence intervals are depicted, but are overlayed by the line representing the best fit. The threshold wavelength λ_{th} (second derivative maximum, the point of maximal curvature) was determined to be $\lambda_{th} = 430$ nm exhibiting 0.84% transmission at this point.

to multiple scattering caused by the cylindrical microstructure, but not by total reflection as in optical fibers (Kienle et al., 2002, 2006; Kienle and Hibst, 2006), since optical fibers consist of a core with high refraction index and a cladding with low refraction-index, whereas in dentine the refraction index of the tubules is lower than of peritubular dentine (Kienle and Hibst, 2006). Light propagation in enamel is similar due to the prisms building a structure of aligned cylindrical scatterers (Kienle et al., 2006).

However, the similar efficacy rates for PS activation by blue and red light seem unexpected since it is well known that the penetration of light through tissue is dependent on its wavelength, with light from longer wavelengths penetrating tissue to a greater extent than light from shorter wavelengths (Hirmer et al., 2012). Therefore, we did transmission measurements of bisected human first mandibular molars and measured the transmission through their buccal halves. The resulting dose–response curves were fitted and the fit was analyzed for the point of maximal curvature (second derivative maximum), which was defined as threshold wavelength λ_{th} for the shortest wavelength that penetrates dental tissue proper enough for activating a given PS molecule and was found to be 430 nm. Although the transmission at this wavelength was 0.84% only, activation of TMPyP at the tested concentration of 10 μM was sufficient in the GTM setup for achieving a disinfecting efficacy. This may be explained due to an overlap of its Soret-band with this threshold wavelength. Furthermore, the measured transmission values likely underestimate the actual transmission through the tooth halves due to scattering effects in the light path inside the photospectrometer. Consequently, it may be possible to activate PS from outside the tooth through human dental hard and simulated surrounding tissue may be possible for wavelengths longer than 430 nm. Activating an intra-canal PS from outside the tooth may facilitate the use of PIB in endodontics, since, there are commonly known limitations for optical fibers, e.g., intense bendings of the root canal or the risk of a fracture of the optical fiber inside the root canal, therefore complicating endodontic application of PIB.

It may be a limitation of this study is that the efficacy of PIB was evaluated against planktonic cells, whereas in infected root canals bacteria are situated in a sessile biofilm mode. However, as this study was thought to be a proof of principle of extra-dental irradiation for PIB, we used planktonic bacteria due to their more simple culture and their greater sensitivity to most antimicrobial approaches for being able to measure small differences in antimicrobial efficacy. As this proof of principle was successful, in the next step the model has to be modified in order to investigate whether biofilms may also be inactivated by PIB with light activation through dental hard and simulated surrounding tissue.

CONCLUSION

The tooth model presented in this study allows evaluation of the antimicrobial photodynamic efficacy of given PS for light activation through human dental hard and simulated surrounding tissue. Under these conditions, the tested PS activated by either blue (TMPyP) or red light (Methylene Blue) resulted in inactivation rates of more than 5 $\log_{10}$ steps reduction of CFU, respectively. Transmission measurements of bisected human molars resulted in a threshold wavelength λ_{th} for allowing an amount of light transmission for appropriate activation of a PS was 430 nm. Therefore, it may be possible to activate PS absorbing at wavelength ranges above this threshold from outside the tooth. However, before transferring these results to the clinical situation there should be undertaken further tests on biofilms.

AUTHOR CONTRIBUTIONS

KAH, TM, FC, and GS conceived and designed the experiments. AP, CL, and JR performed the experiments. KAH, FC, TM, JR, AP, WB, and GS analyzed the data. FC, AP, TM, KAH, CL, GS, and WB wrote the paper.

FUNDING

Fabian Cieplik thanks for financial support from the University Medical Center Regensburg (ReForM A program) and for a travel grant from the German Association for Dental, Oral and Maxillofacial Medicine (DGZMK). Johannes Regensburger is funded by a grant of the German Research Foundation (DFG-RE-3323/2-1).

ACKNOWLEDGMENT

Anita Gollmer and Andreas Späth are gratefully acknowledged for their expert advice in photophysical or chemical issues, respectively.

REFERENCES

Abouassi, T., Hannig, C., Mahncke, K., Karygianni, L., Wolkewitz, M., Hellwig, E., et al. (2014). Does human saliva decrease the antimicrobial activity of chlorhexidine against oral bacteria? *BMC Res. Notes* 7:711. doi: 10.1186/1756-0500-7-711

Al-Ahmad, A., Ameen, H., Pelz, K., Karygianni, L., Wittmer, A., Anderson, A. C., et al. (2014). Antibiotic resistance and capacity for biofilm formation of different bacteria isolated from endodontic infections associated with root-filled teeth. *J. Endod.* 40, 223–230. doi: 10.1016/j.joen.2013.07.023

Arias, C. A., and Murray, B. E. (2009). Antibiotic-resistant bugs in the 21st century–a clinical super-challenge. *N. Engl. J. Med.* 360, 439–443. doi: 10.1056/NEJMp0804651

Athanassiadis, B., Abbott, P. V., and Walsh, L. J. (2007). The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics. *Aust. Dent. J.* 52, S64–S82. doi: 10.1111/j.1834-7819.2007.tb00527.x

Boyce, J. M., and Pittet, D. (2002). Guideline for hand hygiene in health-care settings: recommendations of the healthcare infection control practices advisory committee and the HICPAC/SHEA/APIC/IDSA hand hygiene task force. *Infect. Control Hosp. Epidemiol.* 23, 3–40. doi: 10.1086/503164

Chrepa, V., Kotsakis, G. A., Pagonis, T. C., and Hargreaves, K. M. (2014). The effect of photodynamic therapy in root canal disinfection: a systematic review. *J. Endod.* 40, 891–898. doi: 10.1016/j.joen.2014.03.005

Cieplik, F., Pummer, A., Regensburger, J., Hiller, K.-A., Späth, A., Tabenski, L., et al. (2015). The impact of absorbed photons on antimicrobial photodynamic efficacy. *Front. Microbiol.* 6:706. doi: 10.3389/fmicb.2015.00706

Cieplik, F., Späth, A., Regensburger, J., Gollmer, A., Tabenski, L., Hiller, K.-A., et al. (2013). Photodynamic biofilm inactivation by SAPYR-An exclusive singlet oxygen photosensitizer. *Free Radic. Biol. Med.* 65, 477–487. doi: 10.1016/j.freeradbiomed.2013.07.031

Cieplik, F., Tabenski, L., Buchalla, W., and Maisch, T. (2014). Antimicrobial photodynamic therapy for inactivation of biofilms formed by oral key pathogens. *Front. Microbiol.* 5:405. doi: 10.3389/fmicb.2014.00405

Evans, M., Davies, J. K., Sundqvist, G., and Figdor, D. (2002). Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *Int. Endod. J.* 35, 221–228. doi: 10.1046/j.1365-2591.2002.00504.x

Fimple, J. L., Fontana, C. R., Foschi, F., Ruggiero, K., Song, X., Pagonis, T. C., et al. (2008). Photodynamic treatment of endodontic polymicrobial infection in vitro. *J. Endod.* 34, 728–734. doi: 10.1016/j.joen.2008.03.011

Foschi, F., Fontana, C. R., Ruggiero, K., Riahi, R., Vera, A., Doukas, A. G., et al. (2007). Photodynamic inactivation of *Enterococcus faecalis* in dental root canals in vitro. *Lasers Surg. Med.* 39, 782–787. doi: 10.1002/lsm.20579

Garcez, A. S., Fregnani, E. R., Rodriguez, H. M., Núñez, S. C., Sabino, C. P., Suzuki, H., et al. (2013). The use of optical fiber in endodontic photodynamic therapy. Is it really relevant? *Lasers Med. Sci.* 28, 79–85. doi: 10.1007/s10103-012-1073-8

Gonzales, F. P., Felgenträger, A., Bäumler, W., and Maisch, T. (2013). Fungicidal photodynamic effect of a twofold positively charged porphyrin against *Candida albicans* planktonic cells and biofilms. *Future Microbiol.* 8, 785–797. doi: 10.2217/fmb.13.44

Gursoy, H., Ozcakir-Tomruk, C., Tanalp, J., and Yilmaz, S. (2013). Photodynamic therapy in dentistry: a literature review. *Clin. Oral Investig.* 17, 1113–1125. doi: 10.1007/s00784-012-0845-7

Hecker, S., Hiller, K.-A., Galler, K. M., Erb, S., Mader, T., and Schmalz, G. (2013). Establishment of an optimized ex vivo system for artificial root canal infection evaluated by use of sodium hypochlorite and the photodynamic therapy. *Int. Endod. J.* 46, 449–457. doi: 10.1111/iej.12010

Hiller, K.-A., Christa, T., Niklas, A., Danilov, S. N., Ganichev, S. D., and Schmalz, G. (2013). An in-vitro-model of a human jaw for testing optical properties. *J. Dent. Res.* 92, 3716.

Hirmer, M., Danilov, S. N., Giglberger, S., Putzger, J., Niklas, A., Jäger, A., et al. (2012). Spectroscopic Study of Human Teeth and Blood from Visible to Terahertz Frequencies for Clinical Diagnosis of Dental Pulp Vitality. *J Infrared Milli. Terahz. Waves* 33, 366–375. doi: 10.1007/s10762-012-9872-3

Kawashima, N., Wadachi, R., Suda, H., Yeng, T., and Parashos, P. (2009). Root canal medicaments. *Int. Dent. J.* 59, 5–11.

Kienle, A., Forster, F. K., Diebolder, R., and Hibst, R. (2002). Light propagation in dentin: influence of microstructure on anisotropy. *Phys. Med. Biol.* 48, N7–N14. doi: 10.1088/0031-9155/48/2/401

Kienle, A., and Hibst, R. (2006). Light guiding in biological tissue due to scattering. *Phys. Rev. Lett.* 97, 018104. doi: 10.1103/PhysRevLett.97.018104

Kienle, A., Michels, R., and Hibst, R. (2006). Magnification–a new look at a long-known optical property of dentin. *J. Dent. Res.* 85, 955–959. doi: 10.1177/154405910608501017

Kim, S. T., Abbott, P. V., and McGinley, P. (2000). The effects of Ledermix paste on discolouration of mature teeth. *Int. Endod. J.* 33, 227–232. doi: 10.1046/j.1365-2591.2000.00278.x

Łysakowska, M. E., Ciebiada-Adamiec, A., Sienkiewicz, M., Sokołowski, J., and Banaszek, K. (2015). The cultivable microbiota of primary and secondary infected root canals, their susceptibility to antibiotics and association with the signs and symptoms of infection. *Int. Endod. J.* 49, 422–430. doi: 10.1111/iej.12469

Maisch, T., Wagner, J., Papastamou, V., Nerl, H. J., Hiller, K.-A., Szeimies, R.-M., et al. (2009). Combination of 10% EDTA, Photosan, and a blue light hand-held photopolymerizer to inactivate leading oral bacteria in dentistry in vitro. *J. Appl. Microbiol.* 107, 1569–1578. doi: 10.1111/j.1365-2672.2009.04342.x

McHugh, C. P., Zhang, P., Michalek, S., and Eleazer, P. D. (2004). pH required to kill *Enterococcus faecalis* in vitro. *J. Endod.* 30, 218–219. doi: 10.1097/00004770-200404000-00008

Miles, A. A., Misra, S. S., and Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *J. Hyg. (Lond.)* 38, 732–749. doi: 10.1017/S002217240001158X

Mohammadi, Z., Shalavi, S., and Yazdizadeh, M. (2012). Antimicrobial activity of calcium hydroxide in endodontics: a review. *Chonnam Med. J.* 48, 133–140. doi: 10.4068/cmj.2012.48.3.133

Ng, R., Singh, F., Papamanou, D. A., Song, X., Patel, C., Holewa, C., et al. (2011). Endodontic photodynamic therapy ex vivo. *J. Endod.* 37, 217–222. doi: 10.1016/j.joen.2010.10.008

Odor, T. M., Watson, T. F., Pitt Ford, T. R., and McDonald, F. (1996). Pattern of transmission of laser light in teeth. *Int. Endod. J.* 29, 228–234. doi: 10.1111/j.1365-2591.1996.tb01374.x

Schmalz, G. (2014). Strategies to improve biocompatibility of dental materials. *Curr. Oral Health Rep.* 1, 222–231. doi: 10.1007/s40496-014-0028-5

Schweitzer, C., and Schmidt, R. (2003). Physical mechanisms of generation and deactivation of singlet oxygen. *Chem. Rev.* 103, 1685–1757. doi: 10.1021/cr010371d

Späth, A., Leibl, C., Cieplik, F., Lehner, K., Regensburger, J., Hiller, K.-A., et al. (2014). Improving photodynamic inactivation of bacteria in dentistry: highly effective and fast killing of oral key pathogens with novel tooth-colored type-ii photosensitizers. *J. Med. Chem.* 57, 5157–5168. doi: 10.1021/jm4019492

Stuart, C. H., Schwartz, S. A., Beeson, T. J., and Owatz, C. B. (2006). *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment. *J. Endod.* 32, 93–98. doi: 10.1016/j.joen.2005.10.049

Voos, A. C., Kranz, S., Tonndorf Martini, S., Voelpel, A., Sigusch, H., Staudte, H., et al. (2014). Photodynamic antimicrobial effect of safranine O on an ex vivo periodontal biofilm. *Lasers Surg. Med.* 46, 235–243. doi: 10.1002/lsm.22217

Wainwright, M. (1998). Photodynamic antimicrobial chemotherapy (PACT). *J. Antimicrob. Chemother.* 42, 13–28. doi: 10.1093/jac/42.1.13

Zehnder, M. (2006). Root canal irrigants. *J. Endod.* 32, 389–398. doi: 10.1016/j.joen.2005.09.014

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer TMBR and handling Editor declared their shared affiliation and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

Copyright © 2016 Cieplik, Pummer, Leibl, Regensburger, Schmalz, Buchalla, Hiller and Maisch. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Danksagung

Zuallererst bedanken möchte ich mich bei Herrn Prof. Dr. Wolfgang Buchalla für die freundliche Überlassung dieses interessanten Themas, die Anleitung zu wissenschaftlichem Arbeiten sowie die großzügige und fortwährende Unterstützung.

Mein ganz besonderer Dank gilt Herrn PD. Dr. Tim Maisch für die großartige Betreuung während der Versuchsdurchführungen sowie während des Verfassens der gesamten Doktorarbeit. Seine fachliche Kompetenz auf dem Gebiet der Mikrobiologie haben meine Arbeiten maßgeblich beeinflusst.

Herrn Dr. Anton Hiller gebührt mein besonderer Dank, nicht nur für die unermüdliche Unterstützung bei sämtlichen statistischen Auswertungen sowie beim Verfassen der Publikationen, sondern nicht zuletzt auch für unzählige sachbringende Konversationen bezüglich durchgeführter bzw. anstehender Versuche. Ganz besonders hervorheben möchte ich seine stete Geduld und Motivationsgabe.

Herrn Dr. Fabian Cieplik zu danken für das Einarbeiten in die praktischen Laborabläufe sowie für jegliche Hilfe sowohl bezüglich der Laborversuche als auch des Verfassens der Schriftstücke ist mir ein besonderes Anliegen. Durch sein offenes Ohr und seine Fachkompetenz geriet der Fortgang der Versuche zu keinster Zeit ins Wanken. Vielen Dank für deine unermüdliche Unterstützung!

Bei Herrn Dr. Johannes Regensburger möchte ich mich in besonderer Weise bedanken. Seine hilfreiche Unterstützung auf dem Gebiet der Physik haben diese Arbeit maßgeblich unterstützt.

Für die Synthese von SAPYR sowie für fortwährende Diskussionen auf dem Gebiet der Photochemie bedanken möchte ich mich bei Herrn Dr. Andreas Späth.

Darüber hinaus will ich allen nicht-namentlich genannten Mitarbeitern des H4-Laborteams Dank aussprechen. Die freundliche Aufnahme sowie unzählige Konversationen haben das Arbeiten im Labor zu einer wahren Freude gemacht. Ebenso gilt mein Dank den Mitgliedern des Labors der Zahnerhaltung und Parodontologie (Frau Sara Wennige, Herr Andreas Eidt, Frau Helga Ebensberger, Frau Claudia Waha), welche mir bei Bedarf stets hilfsbereit zur Seite standen.

Zuallerletzt gebührt meiner gesamten Familie, welche mich stets auf meinem Lebensweg unterstützt hat, Dank.

Lebenslauf

Persönliche Daten

Name:	Andreas Manfred Franz Pummer
Geburtsdatum:	06.12.1988
Geburtsort:	Straubing
Familienstand:	ledig
Eltern:	Manfred Pummer
	Maria Maier

Schulischer Werdegang

09/1995 – 07/1999	Grundschule Straubing-Ittling
09/1999 – 07/2008	Johannes-Turmair-Gymnasium Straubing
07/2008	allgemeine Hochschulreife

Studium

04/2010 – 07/2016	Studium der Zahnmedizin, Universität Regensburg
02/2011	Naturwissenschaftliche Vorprüfung
09/2013	Zahnärztliche Vorprüfung
02/2016 – 06/2016	Zahnärztliche Prüfung
07/2016	Approbation als Zahnarzt

Wissenschaftliche Tätigkeit

Publikationen

Fabian Cieplik, **Andreas Pummer**, Johannes Regensburger, Karl-Anton Hiller, Andreas Späth, Laura Tabenski, Wolfgang Buchalla, and Tim Maisch (2015): The impact of absorbed photons on antimcrobial efficacy.
Front. Microbiol. 2015; 6: 706

Fabian Cieplik*, **Andreas Pummer***, Christoph Leibl, Johannes Regensburger, Gottfried Schmalz, Wolfgang Buchalla, Karl-Anton Hiller, Tim Maisch

Photodynamic inactivation of root canal bacteria by light-activation through human dental hard and sorrounding soft tissue

Front. Microbiol. 2016; 7: 929

(* = gleichberechtigte Erstautorschaft)

Literaturverzeichnis

1 Centers for Disease Control and Prevention (2013): *Antibiotic resistance threats in the United States,2013 (http://cdc.gov). Available at: http://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf*

2. The White House: *National strategy for combating antibiotic-resistant bacteria (http.//whitehouse.gov). Available at https://www.whitehouse.gov/sites/default/files/docs/carb_national_strategy.pdf*

3. Winkelhoff AJV, Rams TE, Slots J (1996): *Systemic antibiotic therapy in periodontics. Periodontol 2000 10: 45–78.*

4. Zandbergen D, Slot DE, Cobb CM, Van der Weijden FA (2013): *The clinical effect of scaling and root planing and the concomitant administration of systemic amoxicillin and metronidazole: a systematic review. J Periodontol 84: 332–351.*

5. Preus HR; Scheie AA; Baelum V (2014): *Letter to the editor: Re: The clinical effect of scaling and root planing and the concomitant administration of systemic amoxicillin and metronidazole: a systematic review; Re: Effectiveness of systemic amoxicillin/metronidazole as adjunctive therapy to scaling and root planing in the treatment of chronic periodontitis: a systematic review and meta-analysis; Re: Effectiveness of systemic amoxicillin/metronidazole as an adjunctive therapy to full-mouth scaling and root planing in the treatment of aggressive periodontitis: a systematic review and meta-analysis. J Periodontol 85: 374–384.*

6. Rams TE; Degener JE; van Winkelhoff AJ (2014): *Antibiotic resistance in human chronic periodontitis microbiota. J Periodontol 85: 160–169.*

7 . Al-Ahmad A et al. (2014): *Antibiotic resistance and capacity for biofilm formation of different bacteria isolated from endodontic infections associated with root-filled teeth. J Endod 40: 223–230.*

8. Wainwright M (1998): *Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother 42: 13–28.*

9. Schweitzer C; Schmidt R (2003): *Physical mechanisms of generation and deactivation of singlet oxygen. Chem Rev 103: 1685–1757.*

10. Maisch T et al.(2007): *The role of singlet oxygen and oxygen concentration in photodynamic inactivation of bacteria. Proc Natl Acad Sci USA 17: 7223–7228.*

11. Gursoy H, Ozcakir-Tomruk C, Tanalp J, Yilmaz, S (2013): *Photodynamic therapy in dentistry: a literature review. Clin Oral Investig 17: 1113–1125.*

12. López-Muñoz F et al. (2005): *History of the Discovery and Clinical Introduction of Chlorpromazine. Ann Clin Psychiatry 17: 113–135.*

13. Felgenträger A, Maisch T, Dobler D, Späth A *(2013): Hydrogen bond acceptors and additional cationic charges in methylene blue derivatives: photophysics and antimicrobial efficiency. BioMed Res Int 2013: 482167.*

14. Wilkinson F, Helman WP, Ross AB (1993): *Quantum Yields for the Photosensitized Formation of the Lowest Electronically Excited Singlet State of Molecular Oxygen in Solution. J Phys Chem Ref Data 22: 113-262.*

15. Cieplik F et al. (2013): *Photodynamic biofilm inactivation by SAPYR-an exclusive singlet oxygen photosensitizer. Free Radic Biol Med 65: 477–487.*

16. Späth A et al. (2014): *Improving photodynamic inactivation of bacteria in dentistry: highly effective and fast killing of oral key pathogens with novel tooth-colored type-II photosensitizers. J Med Chem 57*: 5157–5168.

17. Zehnder M (2006*): Root canal irrigants. J Endod 32*:389–398.

18. Mohammadi Z, Shalavi S, Yazdizadeh M (2012): *Antimicrobial activity of calcium hydroxide in endodontics: a review. Chonnam Med J 48*:133–140.

19. Athanassiadis B, Abbott PV, Walsh LJ (2007*): The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics. Aust Dent J 52*:S64–82.

20. Arias CA, Murray BE (2009): *Antibiotic-resistant bugs in the 21st century--a clinical super-challenge. N Engl J Med 360*:439–443.

21. Kawashima N, Wadachi R, Suda H, Yeng T, Parashos P (2009): *Root canal medicaments. Int Dent J 59*:5–11.

22. Schmalz G (2014). *Strategies to Improve Biocompatibility of Dental Materials. Curr Oral Health Rep 1*:222–231.

23. Hecker S, Hiller K-A, Galler KM, Erb S, Mader T, Schmalz G (2013): *Establishment of an optimized ex vivo system for artificial root canal infection evaluated by use of sodium hypochlorite and the photodynamic therapy. Int Endod J 46*:449–457.

24. Abouassi T, Hannig C, Mahncke K, Karygianni L, Wolkewitz M, Hellwig E, Al-Ahmad A (2014): *Does human saliva decrease the antimicrobial activity of chlorhexidine against oral bacteria? BMC Res Notes 7*:711.

25. Kim ST, Abbott PV, McGinley P (2000): *The effects of Ledermix paste on discolouration of mature teeth. Int Endod J 33*:227–232.

26. Al-Ahmad A, Ameen H, Pelz K, Karygianni L, Wittmer A, Anderson AC, Spitzmüller B, Hellwig E (2014): *Antibiotic Resistance and Capacity for Biofilm Formation of Different Bacteria Isolated from Endodontic Infections Associated with Root-filled Teeth. J Endod 40*:223–230.

27. Łysakowska ME, Ciebiada-Adamiec A, Sienkiewicz M, Sokołowski J, Banaszek K (2015): *The cultivable microbiota of primary and secondary infected root canals, their susceptibility to antibiotics and association with the signs and symptoms of infection. Int Endod J* n/a–n/a.

28. Gursoy H, Ozcakir-Tomruk C, Tanalp J, Yilmaz S (2013): *Photodynamic therapy in dentistry: a literature review. Clin Oral Investig 17*:1113–1125.

29. Chrepa V, Kotsakis GA, Pagonis TC, Hargreaves KM (2014): *The effect of photodynamic therapy in root canal disinfection: a systematic review. J Endod 40*:891–898.

30. Cieplik F, Tabenski L, Buchalla W, Maisch T (2014): *Antimicrobial photodynamic therapy for inactivation of biofilms formed by oral key pathogens. Front Microbiol 5*:405.

31. Maisch T, Wagner J, Papastamou V, Nerl HJ, Hiller K-A, Szeimies R-M, Schmalz G (2009): *Combination of 10% EDTA, Photosan, and a blue light hand-held photopolymerizer to inactivate leading oral bacteria in dentistry in vitro. J Appl Microbiol 107*:1569–1578.

32. Späth A, Leibl C, Cieplik F, Lehner K, Regensburger J, Hiller K-A, Bäumler W, Schmalz G, Maisch T (2014): *Improving Photodynamic Inactivation of Bacteria in Dentistry: Highly Effective and Fast Killing of Oral Key Pathogens with Novel Tooth-Colored Type-II Photosensitizers. J Med Chem 57*:5157–5168.

33. Gonzales FP, Felgenträger A, Bäumler W, Maisch T (2013): *Fungicidal photodynamic effect of a twofold positively charged porphyrin against Candida albicans planktonic cells and biofilms. Future Microbiol 8*:785–797.

34. Cieplik F, Späth A, Regensburger J, Gollmer A, Tabenski L, Hiller K-A, Bäumler W, Maisch T, Schmalz G (2013): *Photodynamic biofilm inactivation by SAPYR-An exclusive singlet oxygen photosensitizer. Free Radic Biol Med 65*:477–487.

35. Voos AC, Kranz S, Tonndorf Martini S, Voelpel A, Sigusch H, Staudte H, Albrecht V, Sigusch BW (2014): *Photodynamic antimicrobial effect of safranine O on an ex vivo periodontal biofilm. Lasers Surg Med 46*:235–243.

36. Cieplik F, Pummer A, Regensburger J, Hiller K-A, Späth A, Tabenski L, Buchalla W, Maisch T (2015): *The impact of absorbed photons on antimicrobial photodynamic efficacy. Front Microbiol 6*:706.

37. Ng R, Singh F, Papamanou DA, Song X, Patel C, Holewa C, Patel N, Klepac-Ceraj V, Fontana CR, Kent R, Pagonis TC, Stashenko PP, Soukos NS (2011): *Endodontic photodynamic therapy ex vivo. J Endod 37*:217–222.

38. Fimple JL, Fontana CR, Foschi F, Ruggiero K, Song X, Pagonis TC, Tanner ACR, Kent R, Doukas AG, Stashenko PP, Soukos NS (2008): *Photodynamic treatment of endodontic polymicrobial infection in vitro. J Endod 34*:728–734.

39. Garcez AS, Fregnani ER, Rodriguez HM, Núñez SC, Sabino CP, Suzuki H, Ribeiro MS (2013): *The use of optical fiber in endodontic photodynamic therapy. Is it really relevant? Lasers Med Sci 28*:79–85.

40- Hiller, K.-A., Christa, T., Niklas, A., Danilov, S. N., Ganichev, S. D., Schmalz, G. (2013*). An In-Vitro-Model of a Human Jaw for Testing Optical Properties. J. Dent. Res.* 92, 3716.

41. Chávez de Paz LE, Bergenholtz G, Svensäter G.: *The effects of antimicrobials on endodontic biofilm bacteria. J. Endod 36*:70-7.