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Photodynamic inactivation of *Escherichia coli* – Correlation of singlet oxygen kinetics and phototoxicity

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ABSTRACT

Photodynamic inactivation (PDI) of bacteria may play a major role in facing the challenge of the ever expanding antibiotic resistances. Here we report about the direct correlation of singlet oxygen luminescence kinetics and phototoxicity in *E. coli* cell suspension under PDI using the widely applied cationic photosensitizer TMPyP. Through direct access to the microenvironment, the time resolved investigation of singlet oxygen luminescence plays a key role in understanding the photosensitization mechanism and inactivation pathway. Using the homemade set-up for highly sensitive time resolved singlet oxygen luminescence detection, we show that the cationic TMPyP is localized predominantly outside the bacterial cells but in their immediate vicinity prior to photodynamic inactivation. Throughout following light exposure, a clear change in singlet oxygen kinetics indicates a redistribution of photosensitizer molecules to at least one additional microenvironment. We found the signal kinetics mirrored in cell viability measurements of equally treated samples from same overnight cultures conducted in parallel: A significant drop in cell viability of the illuminated samples and stationary viability of dark controls. Thus, for the system investigated in this work – a Gram-negative model bacteria and a well-known PS for its PDI – singlet oxygen kinetics correlates with phototoxicity. This finding suggests that it is well possible to evaluate PDI efficiency directly via time resolved singlet oxygen detection.

1. Introduction

The ever-expanding worldwide rise in antibiotic resistant bacterial pathogens has made the development of new anti-bacterial strategies an inevitable challenge. Over the last decades, a promising alternative for non-antibiotic killing of bacteria has emerged from enhancements of photodynamic therapy – the photodynamic inactivation (PDI). It reveals a broad spectrum of applications, e.g. localized infections treatment, surface disinfection and wastewater sterilization [1–6]. Its main advantages over other antibacterial methods are a high efficiency towards inactivation of multi-resistant bacterial strains independent of already existing resistance patterns, e.g. the widely spread MRSA [7,8], a low mutagenic potential [9,10] and vast possibilities of application adaption via different photosensitizing agents, as well as surface functionalization [11–13].

PDI is based on the photodynamic effect combining three components: a photoactive dye (photosensitizer, short PS), visible light for its excitation and molecular oxygen, which – throughout the (Type II) photosensitization [14–16] – is activated to highly reactive singlet oxygen ($^1O_2$), leading directly or through generation of secondary radicals to finally cytotoxic oxidation of cell components. Apparently, photosensitizers known to be capable of effectively inactivating Gram-positive are harmless towards Gram-negative bacteria due to fundamental differences in cell wall structures. The outer cell wall of Gram-negative bacteria contents an additional layer of dense negatively charged lipopolysaccharides and lipoproteins presenting an effective barrier for photodynamic action, thus, additional strategies for cell wall permeability enhancement were considered inevitable for a long time [17,18]. Nonetheless the development of cationic photosensitizers demonstrated an effective inactivation of bacteria without additional pretreatment of the cell wall, among them the tetra-cationic porphyrin TMPyP being one of the most prominent agents [14,19–27]. Due to its four-fold cationic nature it is considered a potent PS for the inactivation of Gram-negative bacteria as the positive charge may provide a better affinity of the PS to the complex outer cell wall and especially its polyanionic lipopolysaccharide layer [17,18,22,28–31]. Nowadays its efficiency in PDI is well documented with up to 7log10 reductions in cell count for typical PS concentrations of 1–10 μM [1,20,28,32–34].

Although the high potentials of antimicrobial photodynamic inactivation are well-known, the exact mechanisms and interactions of PS and target microorganisms are not completely understood [35]. Especially the PS localization prior to photodynamic action is subject of

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controversial discussion [19,20]. In this work, we conduct a non-invasive investigation of TMPyP localization in cell suspensions with the model Gram-negative bacteria *E. coli* by monitoring the signal kinetics of the extremely low near-infrared $^1O_2$ luminescence. As against stationary measurements, a time-resolved detection gives access to the processes involved in $^1O_2$ generation, diffusion and deactivation [36]. Of special interest in heterogeneous and complex environments, this provides additional information on PS, quencher and oxygen interactions. By monitoring the $^1O_2$ signal kinetics, it is possible to gain direct insight to its microenvironment and thus following the inactivation pathway.

Finally, the superior signal quality presented in this work, allows a qualitative correlation of microenvironment and photodynamic inhibition.

2. Materials and Methods

2.1. Singlet Oxygen Generation and its Time-Resolved Detection

The meso-tetra-substituted cationic porphyrin 5,10,15,20-Tetrakis (1-methyl-4-pyridinio)porphyrin tetra(*p*-toluenesulfonate), short TMPyP, used as photosensitizing agent in this work was purchased from Sigma-Aldrich Co. LLC., Product Number 323497. For phototoxicity and singlet oxygen luminescence experiments a PS concentration of 5 µM in water has been used. Typical porphyrin concentrations for PDI investigations range from 1 to 10 µM [33].

The singlet oxygen ($^1O_2$) kinetics were recovered from time correlated multi photon counting (TCMPC) experiments using the highly sensitive set-up described in previous work published by Schlothauer, Hackbarth et al. [37,38]. Briefly, the samples are excited by a frequency-doubled diode pumped $Nd3^+$-YAG Laser (Vector 532–1000-20, 532 nm, 1000 mW at 20 kHz, Coherent, Germany) at a wavelength of 532 nm, used in single shot mode triggered at 12.207 kHz by the TCMPC module with a channel width of 20 ns and a total number of 4096 channels. For luminescence detection an H-10330-45 photomultiplier (Hamamatsu, Germany) is used – its rise time of 900 ps and the transit time spread of 300 ps may be neglected in the $^1O_2$ luminescence time domain. Wavelength selection is achieved by a 1270 nm interference filter (1270–70-B, BK-Interferenzoptik, Germany) with typical transmission of 80% and blocking outside the transmission range (70 nm FWHM) better than 10^5 from UV to > 1700 nm. A purpose-built sample chamber for 3 ml suspension cuvettes achieves high sealing from outside noise and allows the integration of a stirrer for cell suspension experiments.

In order to provide high statistical accuracy without strong affection of the examined systems, typical experiments were conducted with a measurement time of 120 s and an average power of 3.7 ± 0.1 mW (measured just before entrance of the excitation laser beam into the sample chamber with a LabMax/J 10MT-10 kHz, Coherent, Germany). Please note that the sample chamber shielding glass transmits approximately 70% of the incoming beam (3 mm of diameter), thus the resulting average power reaching the sample is reduced to 2.6 ± 0.1 mW, i.e. 0.21 ± 0.01 µJ per pulse. Based on the widely applied simplified biexponential function for singlet oxygen kinetics in homogenous solutions [19,25,37–44] a curve fitting model via the Levenberg-Marquardt algorithm was used to fit the registered $^1O_2$ luminescence intensity signal:

$$I_\Delta(t) = A\cdot\{\exp(-t/\tau_d) - \exp(-t/\tau_r)\} + O$$

Hereby A is a positive constant, O an offset implicating intrinsic set-up conditioned noise counts, τ_r and τ_d time constants related to signal rise and decay and determined by the slower and faster deactivation process respectively [41,45,46]. Both time constants have to be assigned to the singlet oxygen lifetime τ_Δ and the PS triplet (T_1) lifetime τ_T via additional examinations (e.g. transient absorption experiments for direct determination of τ_T [23,47,47,48]. In general, the typical singlet oxygen lifetime in aqueous solution is approximately 3.5 µs [43,49,50].

The highly sensitive set-up described above provides detection of signal kinetics within a conservative accuracy of 3% in homogenous solutions. The goodness of the fit is represented by the reduced (standardization by degrees of freedom) chi-squared-test (χ_{red}^2). If required, the fits of the data are supplemented with a plot of the corresponding counting rate weighted residuals (Pearson residulas). This allows a visual evaluation of the goodness of the corresponding fit.

2.2. Bacterial Strains and Preparation of Bacterial Cell Suspensions

For ease of comparison two strains of the same model Gram-negative bacteria *Escherichia coli* – the genetically modified already multi-resistant strain SURE 2 and a wild type strain (ATCC 25922) – were used in this work. Although being of the same species, preliminary experiments showed a different morphology (e.g. in terms of a 7-fold higher optical density of the SURE 2 for the same colony forming units count) and different response to environmental stress of the two strains, SURE 2 being the less robust strain. Thus we expected different behavior in terms of singlet oxygen kinetics and oxidative stress response allowing a juxtaposition of photophysical and biological phenomena. The SURE 2 strain was obtained from Stratagene, USA, product number 200152. The wild type was kindly provided by Angela Cunha, Dept. of Biology, University of Aveiro, Portugal.

The bacteria were grown at room temperature in 2.5 mg/l lysogeny broth (Luria/Miller) by shaking overnight (350 rpm). Kanamycin (5 mg/l), Merck KGaA, Germany, was added to the SURE 2 suspension in order to prevent any growth of other strains and SURE 2 without plasmid. For bacterial cell suspension experiments the overnight cultures were palleted by gentle centrifugation (9 min. at 800 g) and the supernatant was removed via decantation. The pellets were re-suspended in phosphate buffered saline (PBS), pH = 7.4 – Dullbecco's PBS, without Ca & Mg, Biochrom GmbH, Germany – to a concentration of 10^7 to 10^8 colony forming units (cfu) per ml, determined by absorption.

2.3. Phototoxicity Experiments

The photodynamic antibacterial efficacy of the tetracationic porphyrin TMPyP against the Gram-negative *E. coli* is well documented [1,2,29,51,52] and not subject of the subsequent investigations. The information we generate from the phototoxicity experiments is the chronological development of the viability of the *E. coli* during the photodynamic treatment. The goal is a further correlation with the corresponding singlet oxygen luminescence kinetics initiated by the same procedure. Thus, we chose a less time- and labor-consuming method for cell viability experiments compared to the standard colony counting method. In order to promote a time efficient viability determination parallel to singlet oxygen luminescence measurements we chose a photometric approach, namely the MTT viability assay adapted to *E. coli* viability [21,53]. The trade-off was a reduction in sensitivity with a maximum detection of $2\log_{10}$ in viability loss.

Phototoxicity experiments were performed at room temperature with a purpose-built white light LED-setup for sample irradiation (emission range from 400 to 800 nm) with a relatively low [33] fluence rate of 10 ± 5 mW/cm^2 [20], simultaneously using dark control samples in order to exclude any dark toxicity effects. Cell viability MTT tests were conducted on 96 well plates using a VICTOR^3 plate reader, PerkinElmer Inc., USA. The yellow tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is reduced by dehydrogenases to purple formazan crystals indicating the metabolic activity of the cells, thus allowing an indirect photometric viability measurement via formazan absorption. The exact procedure of the assay is described in Preuß et al. [20]. In this work, we slightly changed it to longer incubation times with the MTT reactant (130 min) and