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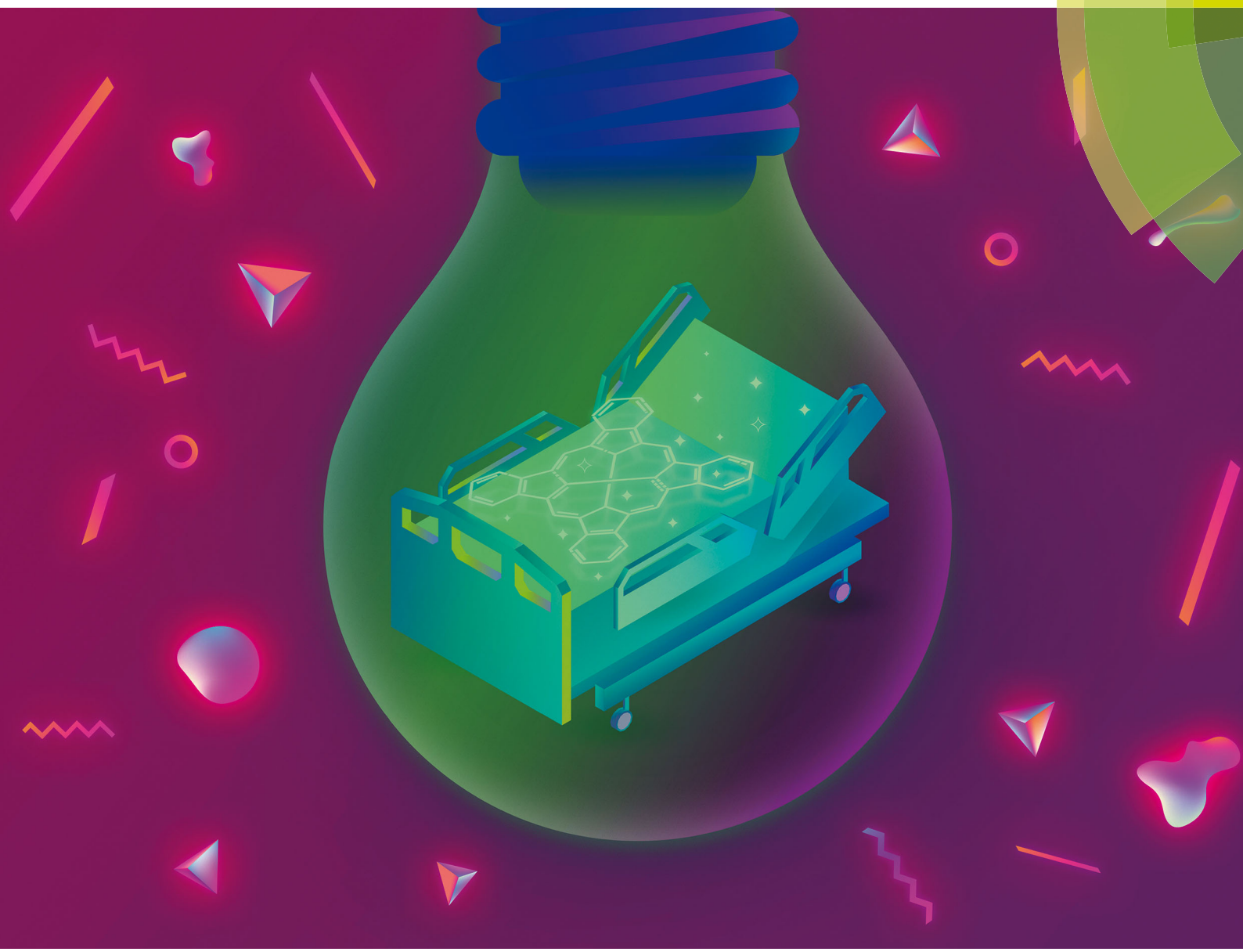

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PAPER

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Zinc phthalocyanine activated by conventional indoor light makes a highly efficient antimicrobial material from regular cellulose†

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Zn phthalocyanine with improved synthesis suitable for bulk production shows extremely high antimicrobial efficacies even under weak indoor light. The dye-impregnated cellulose material inactivates over 99.996% of drug-resistant *C. albicans*, *S. aureus* and *E. faecalis* in just one hour exposure with consumer-grade fluorescent lamps and diodes.

1. Introduction

Pathogens of various taxonomies have become resistant rapidly even to novel antibiotics and have claimed millions of lives annually, not to mention the burden of trillions of US dollars to the global economy.[1] Global mobility has resulted in the spread of, *e.g.*, the Severe Acute Respiratory Syndrome coronavirus (SARS),[2] the multidrug-resistant bacterium *S. aureus* (MRSA),[3] and more recently the *C. auris* fungus.[4] Newly arriving pathogen species can be the carriers of new antibiotic-resistant genes that, together with growing resistance to commonly prescribed antimicrobial drugs, can result in new generations of "superbugs".[5–8] The existence of pathogens resistant to "last-resort" antibiotics, *i.e.* colistin, is a cause for alarm.[9] The circumscription of recently emerging *Candida auris* is another global health threat that needs to be tackled opportunely.[4,10,11] Notwithstanding the somewhat efficacy of conventional antiseptics and disinfectants, the high volatility of their components drastically limits the desired long-term effects.[12] Therefore, preventive methods that are easy to apply and capable of targeting simultaneously several classes of pathogens without offering them a possibility to develop resistance are of utmost importance.

Self-disinfecting materials (SDMs) such as fabrics, films, and coatings can play a vital role in preventing the transmission of pathogens from, *e.g.*, hospital surfaces to patients, and from patient to patient. Ideally, self-sterilizing surfaces should be non-specific to pathogens, easy-to-make, scalable and robust. To this end, materials utilizing principles of photodynamic therapy represent a promising approach to combat pathogenic species, including antibiotic-resistant ones.[13–15] Photodynamic antimicrobial chemotherapy (PACT) relies on the cytotoxic action of reactive oxygen species (ROS), such as singlet oxygen and superoxide radicals, generated by a photosensitizer (PS) upon light excitation.[16,17] The absence of any antimicrobial resistance mechanisms towards ROSs, spatiotemporal precision, controllability and non-invasive properties make PACT a method of choice in hampering the evolution of drug resistant bacteria and controlling the spread of existing ones. Of ultimate importance, photodynamic inactivation does not induce resistivity in microorganisms.[18–20]

Incorporation of photosensitizers (metal nanoparticles and/or small organic dyes, like phenothiazines) into polymers has already provided a potent class of antimicrobial surfaces.[14,15] In particular, SDMs made of polysiloxane with methylene blue and gold nanoparticles resulted in more than 3.5 $\log_{10}$ reduction of methicillin-resistant *S. aureus* after 5 min of exposure to 250 mW laser light (660 nm).[21] Notwithstanding their efficacy, such systems require either tricky covalent linkage or labour intensive "swell–encapsulation–shrink" methods to load dyes/nanoparticles into polymer matrixes. Metal nanoparticles tend to be condition- and pathogen-specific, while phenothiazines are prone to photobleaching, which together with the other above-mentioned limitations profoundly hinders their applicability.[22–25]

Porphyrinoids can be an excellent option to abrogate pathogenic microorganisms *via* a PACT mechanism.[26] In particular, cationic metallated phthalocyanines exhibit significant phototoxicity because of their high yield of ROS generation.[27,28] Broad absorption bands together with high molar absorption coefficients and long triplet state lifetimes allow phthalocyanines to generate high ROS concentrations even while using low intensity light.

Therefore, over 99% eradication efficiencies of pathogens have been achieved under cheap LED source illumination,

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white light or even sunlight instead of expensive laser setups.[29–31] Although the hydrophobicity of phthalocyanines is an issue, their strong resistance against photobleaching makes them good candidates for PACT-based materials with prolonged life durability.[30]

2. Experimental

2.1. Materials and methods

Reagents and solvents were purchased from TCI Europe, Sigma Aldrich Co. or VWR and were used without further purifications unless otherwise mentioned. NMR spectra were recorded using a JEOL JNM-ECZ500R 500 MHz spectrometer using TMS as an internal standard. HRMS measurements were performed with a Waters LCT Premier XE ESI-TOF benchtop mass spectrometer. Lock-mass correction (leucine enkephalin as a reference compound), centering and calibration were applied to raw data to obtain accurate mass. Paper samples containing 80 and 160 mg m^{-2} of **3** were prepared as described earlier.[29] An OSRAM Star PAR16 80 W 575 lm GU10 diode lamp was used to generate 270 lux emittance. Illumination power density was measured with TKA-PKM(09) apparatus that allows monitoring luminous emittance (E, lux) in the visible spectral range (380–760 nm), percent flicker (PF, %) and luminance (L, cd m^{-2}) with the overlapping method of extended self-luminous objects (self-luminous objects with an extended spatial distribution) in the visible range of the spectrum (380–760 nm).

2.2. Synthesis of compounds and PACT paper preparation

The compounds 2,3-dicyanophenyl trifluoromethanesulfonate and 4-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl)pyridine were synthesized according to the literature procedure.[32,33] Pyridine boronate ester (0.726 g, 3.78 mmol), triflate phthalonitrile (1.0 g, 3.78 mmol), $PdCl_2$(dppf)·DCM (0.092 g, 0.1134 mmol) and K_3PO_4 (2.4 g, 11.31 mmol) were dissolved in a 1 : 1 mixture of water and toluene (40 ml). The reaction mixture was stirred at room temperature and saturated by bubbling with argon for 15 minutes. After saturation, the mixture was heated with vigorous stirring at 90 °C for 2 h under argon. The reaction mixture was cooled to room temperature, extracted with $CHCl_3$, washed with brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to yield a crude product. Re-precipitating the crude product from a 9 : 1 mixture of $CHCl_3$/hexane yielded 0.603 g of brown solid as pure product, yield 77%. The analytical data (NMR, MS, *etc.*) were identical to previously reported values.[29] Further synthesis of phthalocyanine **2** and its cationic form **3** was performed as reported in our earlier paper.[29]

A stock solution was prepared by dissolving 1.0 mg of the dye in 200 μl of water. For PACT paper with 80 mg m^{-2} dye load, 20 μl of this stock solution was diluted in 1 ml of water and taken into a Petri dish. Filter paper (Qualitative Grade 413 from VWR, 3.5 cm × 3.5 cm) was soaked into the aqueous solution for approximately 5 minutes. The paper became evenly light green in colour, leaving behind transparent water, indicating complete loading of the dye on the paper. Similarly, to prepare 160 mg m^{-2} dye load paper, 40 μl of stock solution in 1 ml of water was used to impregnate 3.5 cm × 3.5 cm filter paper. The papers were dried in air for 24 h protected from direct sunlight before use. The papers were stored at r.t. in the dark. The 6 mm disks used for inactivation experiments were cut with a hole punch. The preparation process is illustrated in the ESI,† page S6.

2.3. Cell culture

Clinical isolates of *Staphylococcus aureus* strain 88 (MRSA), *Enterococcus faecalis* 583 (vancomycin-resistant) and *Candida albicans* 604M, resistant to fluconazole, were used for the study. The microorganisms were kept at −75 °C in casein–soymeal–peptone broth with 10–15% glycerol. Before the experiment a bacterial strain was activated from cryoconservation by seeding it onto CASO Agar medium (''Sifin'', Germany) and incubated at 35 ± 2 °C for 18–20 hours. *Candida albicans* 604M was seeded onto Sabouraud Dextrose Agar medium (Becton Dickinson, Germany) and incubated at 35 ± 2 °C for 48 hours.

Overnight bacterial strains were seeded onto Mueller–Hinton Broth (''Sifin'', Germany) and adjusted to have 0.5 McFarland standard turbidity. Microorganism suspension turbidity was determined by using a DEN-1 (densitometer (suspension turbidity detector) BioSan, Riga, Latvia).

The microorganisms were incubated further at 35 ± 2 °C to obtain 1 McF optical turbidity. Then, they were precipitated by centrifugation and washed with phosphate buffered saline (PBS). The cell densities were adjusted with PBS to reach 0.5 McF and were equal to *ca.* 1.5 × 10^8 CFU per ml (cells per ml).

To perform an experiment with *Candida albicans* 604M, individual colonies were collected from Sabouraud Agarose medium, suspended in PBS, pH 7.2–7.6 (Eco Service, Russia), precipitated by centrifugation and washed twice with PBS. The cell density was adjusted in PBS solution to reach 3 McF turbidity and was equal to 3 × 10^7 CFU per ml.

2.4. Photoinactivation experimental conditions

Each 6 mm disk of the paper impregnated with zinc phthalocyanine **3** was placed into a Petri dish (40 mm diameter) and covered with 25 μl of cell suspension of each microorganism suspended in PBS. The calculated infection dose was equal to *ca.* 3.7 × 10^6 ($\log_{10}$ 6.5) for bacterial strains and *ca.* 7.5 × 10^5 ($\log_{10}$ 5.87) for *Candida albicans*.

The experiment included the following experimental variants:

1. Control measurements under 4000 lux illumination;
2. Dark toxicity control measurements;
3. Photoinactivation of microorganisms under 270 lux illumination;
4. Photoinactivation of microorganisms under 4000 lux illumination.

Illumination of the samples was performed for either 30 or 60 minutes. The distance between the illumination source and the PACT paper disk was kept constant (14 cm) to get 4000 lux. Office light was utilized to obtain a luminous emittance of 270 lux. The black background below the Petri dish was implemented

to avoid the diffusely-reflecting effect on the results of the experiments.

A 2–3 lux illumination was provided at the end of all experimental variants. A series of dilutions was prepared having already the paper disk inside the Petri dish. Ten-fold dilutions were already performed in vials. An aliquot of 0.1 ml of the corresponding dilution was introduced into a Petri dish 90 mm in diameter. All Petri dishes, including the first one with the paper disk, were filled with selective media: for *S. aureus* – BD™ Mannitol Salt Agar (BD, France), for *Enterococcus faecalis* – BBL™ Enterococcosel™ Agar (BD, France), for *Candida albicans* – BBL Sabouraud Dextrose Agar with Chloramphenicol (BD, France), and were left to harden the medium. They were incubated for 24–96 hours at 35 ± 2 °C.

Each experimental variant was reproduced on different days in triplicate.

Microorganisms' maximal CFU values were achieved after 72 hours of incubation for bacterial strains and after 96 hours for *Candida*. A further increase in CFU values wasn't observed. Each experimental variant was analyzed in terms of CFU average values in Petri dishes, recalculated with respect to dilution and converted into $\log_{10}$ values ($\log_{10}$ CFU per ml).

2.5. Statistical analysis

SPPS (SPSS: An IBM Company, USA) was employed for statistical analysis. Figures were plotted by using OriginPro 2018 software (OriginLab Corp., USA).

Briefly, testing for normality distribution for repeated measurements or for intergroup comparison in terms of $\log_{10}$ CFU per ml values was performed by using Shapiro–Wilk's test. The average mean (M) and standard deviation (SD) values were used to analyze the data with normal distribution. ANOVA dispersion analysis with *a posteriori* tests for multiple comparison by using Tukey's criterion for repeated measurements was utilized to compare the values of $\log_{10}$ CFU per ml with respect to both luminous emittance and exposure time. Testing of statistical hypotheses was performed by using a critical significance level of 95%, *i.e.* the difference was statistically significant when $p < 0.05$. Figures represent average mean values, medians, and standard mean squared errors.

Evaluation of the difference between controls and experimental groups was carried out by using the Mann–Whitney U-test when the distribution of the values was different from normal (see the ESI†). Hence, the median values were used for graphical representation of the data.

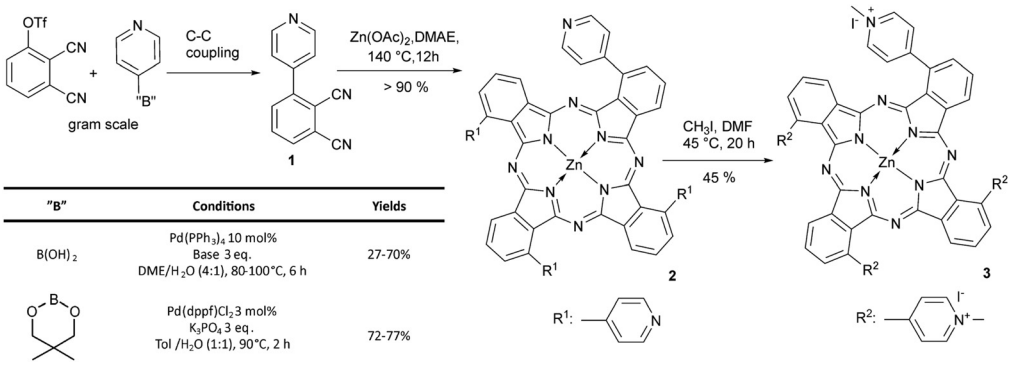
3. Results and discussion

We have recently reported the synthesis of pharmacologically relevant tetrapyridinyl phthalocyanine **2** and its water soluble cationic form **3** and shown their activity against Gram-negative microbes *E. coli* and *A. baylyi* under LED light.[29,30] Moving a step forward, in the present work we report an unusually high efficacy of a self-disinfecting material based on tetracation **3** against drug-resistant Gram-positive bacteria and fungi. Of exceptional importance, efficient antimicrobial activities of this material are achieved even under weak indoor light of 270 lux.

While appropriate for milligram scale synthesis, the previously reported procedure had a bottleneck of the Suzuki–Miyaura coupling between triflate phthalonitrile and 4-pyridyl boronic species.[29] Unreproducible yields were obtained in the gram scale preparation of **1**. It should be noted that a general method for the Pd-catalysed C–C coupling of pyridyl boronic derivatives remains undeveloped, despite previous methods reported for 2-heterocyclic boronates and aryl bromides.[34,35] In order to overcome this inconsistency and to reliably obtain reasonable yields during the preparation of pyridylphthalonitrile **1**, we screened different boronic components (acid and ester), Pd catalysts ($Pd(PPh_3)_4$ and $Pd(dppf)Cl_2$), bases (K_2CO_3, Na_2CO_3, Cs_2CO_3 and K_3PO_4), solvents and reaction temperatures. Surprisingly, different batches of $Pd(PPh_3)_4$ produced inconsistently variable yields. A closer examination by NMR and IR revealed that they contained remarkably different proportions of phosphine and oxide ligands with no clear dependence on the reaction yield (see the ESI†). However, by modifying the previously reported procedure, *i.e.* by reducing the amount of $Pd(dppf)Cl_2$ to 3 mol%, and heating the reaction mixture at 90 °C for 2 hours in a 1 : 1 toluene:water mixture and with K_3PO_4 as base, we were able to obtain the desired compound **1** in 72–77% yield after purification. Importantly, saturation of the reaction mixture by bubbling it with argon for 15 minutes prior to heating was essential for reproducibility and improved yields in gram scale reactions. Condensation of **1** in zinc acetate/dimethylaminoethanol provided metallated complex **2** in excellent yield, which was further methylated to provide tetracationic phthalocyanine **3** for antimicrobial studies (Scheme 1).

In our quest towards self-disinfecting surfaces, zinc phthalocyanine **3** was incorporated into cellulose matrix (PACT paper). Cellulose is a material of choice due to its biocompatibility, physical stability, high loading capacity for cationic molecules, low-cost and flexibility.[22,36–39] Unlike polysiloxane or polyurethane matrix, this paper doesn't block the interaction between the PS triplet state and molecular oxygen because of its porous structure that in general facilitates the formation of ROS.[40]

The impregnation of cellulose with phthalocyanine **3** was remarkably simple and efficient. Qualitative Grade 413 filter paper (VWR) was immersed into aqueous solutions of **3** for 10 minutes; the concentration of phthalocyanine was calculated according to the paper area to obtain 80 or 160 mg m^{-2} dye loading. In a few minutes, the deeply coloured solution became colourless, while all the dye was adsorbed onto the paper, forming a visually even colouring (see the ESI†). After drying in air at room temperature, the desired PACT paper was ready to be used in antimicrobial testing. The strong interaction between the positively charged photosensitizer and cellulose fibres makes the PACT paper resistant to leaching and bleaching. Hence, this method is extremely simple and versatile since dye loading can be easily controlled.[30] A unique



Scheme 1 Synthetic route for the synthesis of pyridine substituted phthalocyanine zinc complex **3**.

advantage of this system over other photodynamic materials is that the PACT surface can be easily renewed just by soaking or spraying it with a fresh aqueous dye solution, which is of vital importance for practical applications. Moreover, as previously published, 64 h illumination with sunlight intensity bleaches out the PACT paper only by 13% and does not change its activity. The PACT paper was further examined against clinical isolates of antibiotic-resistant pathogens *Staphylococcus aureus* strain 88 (MRSA), vancomycin-resistant *Enterococcus faecalis* 583 (VRE) and *Candida albicans* 604M resistant to fluconazole. MRSA and VRE belong to the ESKAPE group of pathogens and take a leadership in the list of bacteria causing severe Healthcare Associated Infections (HAIs).[41] MRSA is the cause of skin and soft tissue infections, nosocomial pneumonia and bacteraemia.[42] VRE is mostly associated with HAIs, but public healthcare recently expressed concerns about wide spreading of VRE elsewhere.[43] Moreover, VRE strains are the source of vancomycin-resistant plasmids, which were detected already in MRSA strains.[44] *C. albicans* is a saprophyte fungus that can, however, cause various infections of any organ and tissue when the immune status is compromised.[4,45] The calculated infection dose was equal to either *ca.* 3.7×10^6 colony-forming units per ml (CFU per ml) (*ca.* $\log_{10} 6.5$) for bacterial strains or *ca.* 7.5×10^5 CFU per ml (*ca.* $\log 5.87$) for *C. albicans*.

Modern photodynamic agents suffer from the drawback of requiring intense light irradiation to be effective.[21,46] In contrast, we performed our photoinactivation experiments at light intensities typical of indoor living/office space: 270 lux or 4000 lux (40 or 586 μW cm^{-2}) light intensities with 30 min or 60 min exposure time. The value of 270 lux is so small that it falls outside the normal sensitivity of common (laser) light power meters, which justifies the use of lux units throughout this study. Additionally, lux is a well known unit for construction/architecture/work safety bodies and allows connecting the efficacy of photodynamic antimicrobial agents with demands of light levels in living spaces. In our study, a fluorescent lamp was utilized to obtain a luminous emittance of 270 lux, while a LED lamp was used to generate 4000 lux light intensity.

Control experiments on non-dyed papers showed that such low intensity light has no effect on the studied microorganisms regardless of the exposure time, and the same values were obtained for the viability of microorganisms. Dark controls with 80 mg m^{-2} PACT paper showed no difference with respect to non-dyed paper samples. Therefore, all control values were combined into one mean value (see the ESI†).

Under light irradiation, a very strong antibacterial effect was observed for the dyed papers (Fig. 1). Indoor light 270 lux on 80 mg m^{-2} paper resulted in a significant reduction of MRSA 88. The values of 3.5 $\log_{10}$ and 4.8 $\log_{10}$ CFU per ml reduction of cell viability with respect to control ($\log_{10} 6.2$) were obtained for 30 and 60 min irradiation, respectively. An increase in luminous emittance up to 4000 lux led to the total eradication of the cell population after 30 minutes of illumination (Fig. 1a). The antimicrobial efficiency against the VRE 583 strain under

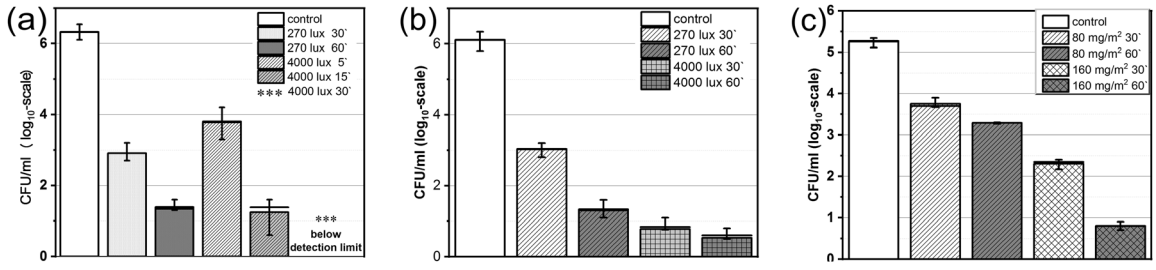


Fig. 1 Reduction in CFU per ml values (log 10-scale) of (a) methicillin-resistant *S. aureus* 88, (b) vancomycin-resistant *E. faecalis* 583, and (c) fluconazole-resistant *C. albicans* 604M under different illumination regimes with respect to control (30′ and 60′ mean 30 and 60 min exposure times, respectively). Studies with MRSA and VRE were performed by using 80 mg m^{-2} PACT paper only. *C. albicans* 604M experiments were performed under 4000 lux illumination.

270 lux was similarly high. The reduction of CFU per ml values with respect to the control ($\log_{10}$ 6.1) was as high as 4.88 and 5.4 $\log_{10}$ after 30 minutes and 60 minutes of treatment, respectively (Fig. 1b). However, 4000 lux illumination did not result in complete elimination of the microorganism; with either exposure time a small number of microbes survived (0.9 and 0.6 $\log_{10}$ CFU per ml for 30 and 60 min, respectively). The photoantimicrobial activity of 80 mg m^{-2} PACT paper using 270 lux illumination against the fungus *C. albicans* was lower. We did not observe significant differences between the control group and the experimental groups under 270 lux light applied for 30 or 60 minutes (p = 0.915 and 0.323, respectively). Treatment with 4000 lux for 30 and 60 minutes resulted in CFU value reduction by 2 and 1.6 $\log_{10}$ (Fig. 1c).

However, the paper sample with a 160 mg m^{-2} load of dye **3** under 4000 lux illumination deactivated *C. albicans* significantly, and only 2.3 and 0.8 $\log_{10}$ CFU per ml survived. Reduction of 2.97 and 4.4 $\log_{10}$ in cell viability ($p < 0.001$) was achieved after 30 and 60 minutes of light exposure, respectively (Fig. 1c). The control experiments were not dependent on the exposure time (p = 0.881); however, there was a profound effect for both 80 and 160 mg m^{-2} PACT paper samples with respect to the time of illumination ($p < 0.001$).

The direct comparison of PACT with existing antimicrobial coatings is always difficult, as there is no standardized procedure for measuring the efficacy of light-activated materials. In addition, commercial companies tend to mention just ''Tested according to ISO''. The procedures used in the present work are similar to ISO221961 (antimicrobial plastic surfaces and coatings), which typically gives 2 log reduction in 24 hours for silver-based paints. Another benchmark could be AATCC 100-2004 or EN ISO 20743 (antimicrobial textiles), which can give 5–6 log reduction in 1 hour, though with a load of silver/copper of *ca.* 10 g m^{-2}.[49]

It is also worth mentioning that the PACT papers used in this study were stored before use for different periods of time and still preserved their antimicrobial activity. Thus, the 80 mg m^{-2} paper used against MRSA 88 and VRE 583 was either 6 days, or 110 days, or 140 days old, and there was no observable difference in efficacy. The 80 mg m^{-2} paper used against *C. albicans* was 14 and 36 days old, and 160 mg m^{-2} PACT paper was 442 and 453 days old. The paper was stored at room temperature in a plastic bag, in a desk drawer.

4. Conclusion

In conclusion, a scalable and robust PACT material based on affordable components has been developed. We have already demonstrated its efficacy against drug-resistant Gram-negative strains, and with this work we extend its applicability to eradicate Gram-positive microorganisms and fungi of clinical isolates resistant to antibiotics. At light intensities and with light sources typical for indoor spaces (270 lux in offices, hospitals, and living rooms) PACT paper with 80 mg m^{-2} dye load is able to inactivate more than 99.998% of MRSA and VRE. A higher dye load (160 mg m^{-2}) allows inactivating 99.996% of *C. albicans* within an hour, though brighter 4000 lux light is beneficial.

Noteworthily, the ability of this self-disinfecting material to work efficiently under low intensity indoor light sources presents a considerable leap forward in the field of antimicrobial materials. The vast majority of known photosensitizers perform well only under strong laser or white light, and thus are suitable for therapeutic purposes or for smaller devices where the use of a focused light/laser beam of high power is feasible.[7,8,25,46–48] In contrast, our dye permits rendering self-disinfecting large areas, and requires no special equipment. This makes possible quick disinfection of surfaces in isolation wards, waiting rooms, field surgery tents, *etc.* High antimicrobial efficacy upon indoor light exposure, an ability to eradicate simultaneously fungi, Gram+, and Gram− bacteria, and a unique option for renewing and reapplying the coating as needed makes PACT material based on tetracationic phthalocyanine **3** the substance of choice for real life applications. It can be utilized to control the spreading of emerging infection diseases in hospitals or crowded areas such as airports, for disinfection of pathological waste, as a coating or treatment for personal protection items or clothes with disinfecting modalities upon indoor light illumination.

Conflicts of interest

There are no conflicts to declare.

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