Bactericidal Effect of a Photoresponsive Carbon Monoxide-Releasing Nonwoven against *Staphylococcus aureus* Biofilms

Mareike Klinger-Strobel,^{a,b} Steve Gläser,^c Oliwia Makarewicz,^{a,b} Ralf Wyrwa,^d Jürgen Weisser,^d Mathias W. Pletz,^{a,b} Alexander Schiller^c

Center for Infectious Diseases and Infection Control, Jena University Hospital, Jena, Germany^a; Center for Sepsis Control and Care, Jena University Hospital, Jena, Germany^b; Friedrich Schiller University Jena, Institute for Inorganic and Analytical Chemistry, Jena, Germany^c; Innovent e.V., Biomaterials Department, Jena, Germany^d

Staphylococcus aureus is a leading pathogen in skin and skin structure infections, including surgical and traumatic infections that are associated with biofilm formation. Because biofilm formation is accompanied by high phenotypic resistance of the embedded bacteria, they are almost impossible to eradicate by conventional antibiotics. Therefore, alternative therapeutic strategies are of high interest. We generated nanostructured hybrid nonwovens via the electrospinning of a photoresponsive carbon monoxide (CO)-releasing molecule [CORM-1, $\text{Mn}_2(\text{CO})_{10}$] and the polymer polylactide. This nonwoven showed a CO-induced antimicrobial activity that was sufficient to reduce the biofilm-embedded bacteria by 70% after photostimulation at 405 nm. The released CO increased the concentration of reactive oxygen species (ROS) in the biofilms, suggesting that in addition to inhibiting the electron transport chain, ROS might play a role in the antimicrobial activity of CORMs on *S. aureus*. The nonwoven showed increased cytotoxicity on eukaryotic cells after longer exposure, most probably due to the released lactic acid, that might be acceptable for local and short-time treatments. Therefore, CO-releasing nonwovens might be a promising local antimicrobial therapy against biofilm-associated skin wound infections.

Staphylococcus aureus, both methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA), is the leading pathogen in skin and skin structure infections (SSSIs), including surgical and traumatic infections (1, 2). *S. aureus* has developed a highly effective mechanism to invade tissues and evade the immune system by biofilm formation and intracellular persistence. This can result in chronic and recurring SSSI, particularly in patients with comorbidities such as diabetes mellitus (e.g., diabetic foot syndrome). Nonradicable *S. aureus* SSSI can be the focus of *S. aureus* bacteremia, which has a mortality of up to 30% (3). In the United States, community-acquired MRSA has emerged as one of the leading causes of community-acquired SSSI (2).

Biofilms are sessile matrix-embedded microbial communities that colonize nearly all artificial and natural surfaces. They are thought to be present in more than 80% of all nosocomial bacterial infections (4). Compared to their planktonic counterparts, bacteria embedded in a biofilm benefit from the protective properties of the matrix, conveying up to 1,000-fold-higher resistance to antibiotics (5). The enhanced phenotypic resistance (correctly termed tolerance) of biofilms is caused mainly by decreased growth rates and metabolically inactive persister cells (a protected phenotypic state) (6). Most antibiotics inhibit metabolic processes in the cell, such as replication (e.g., fluoroquinolones), transcription (e.g., rifampin) or translation (e.g., aminoglycosides and macrolides), and therefore they become ineffective in these persister cells. Altered microenvironments (7) and enzymes hydrolyzing or modifying the antimicrobials that are enriched within the matrix are additional mechanisms that reduce antimicrobial susceptibility (e.g., to aminoglycosides and β -lactams) (8). Thus, under therapeutically feasible antibiotic concentrations, biofilms remain robust, leading to treatment failure and recurring bacteremia. As a consequence, the only practical measure to help patients with life-threatening biofilm-associated infections is to surgically remove the implant or infected body part. Therefore,

alternative therapeutic strategies enabling the eradication of the accrued biofilms would greatly improve patient care.

We focused on an alternative strategy that is not affected by common mechanisms of resistance, including phenotypic resistance instilled by biofilm formation: carbon monoxide (CO). CO is a toxin that inhibits the key enzymes of the essential electron transport chain, such as cytochromes or cytochrome *c* oxidase, leading to cell dieback (9, 10). CO is a small “toxic” molecule that is known to have a role in numerous physiological processes (11). It is endogenously produced via the catabolism of heme by heme oxygenases I and II (12). CO is a gasotransmitter and is a ubiquitous ligand that interacts with metalloproteins such as those bearing heme moieties (12, 13). Its affinity to hemoglobin is approximately 200-fold higher than that of O_2 , leading to hypoxia. However, CO in metered concentrations (controlled dose) has been recognized as a therapeutic agent with antihypertensive, anti-inflammatory, and cell-protective effects (12, 14, 15). CO has been well studied in anoxia-reoxygenation and ischemia-reperfusion models and has advanced to phase II trials for the treatment of several clinical entities (16). Safety trials demonstrated that CO inhalation at levels up to 100 ppm for 2 h, 500 ppm for 1 h, or even

Received 28 March 2016 Returned for modification 12 April 2016

Accepted 17 April 2016

Accepted manuscript posted online 25 April 2016

Citation Klinger-Strobel M, Gläser S, Makarewicz O, Wyrwa R, Weisser J, Pletz MW, Schiller A. 2016. Bactericidal effect of a photoresponsive carbon monoxide-releasing nonwoven against *Staphylococcus aureus* biofilms. *Antimicrob Agents Chemother* 60:4037–4046. doi:10.1128/AAC.00703-16.

Address correspondence to Mathias W. Pletz, Mathias.Pletz@med.uni-jena.de, or Alexander Schiller, alexander.schiller@uni-jena.de.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00703-16>.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

400 to 1,000 ppm for approximately 1 h is without adverse effect and does not elevate carboxyhemoglobin (COHb) levels above those seen in heavy smokers (17).

We used electrospun poly(L-lactide-co-DL-lactide) nonwovens with noncovalently embedded CO-releasing molecule 1 (CORM-1) [$\text{Mn}_2(\text{CO})_{10}$] to release CO via irradiation with light of 405 nm. Mature *S. aureus* biofilms were exposed to this treatment *in vitro*, and the effectiveness of the biofilm eradication was determined by confocal laser scanning microscopy. Our results showed that the controlled release of CO effectively reduced the bacteria within mature *S. aureus* biofilms.

MATERIALS AND METHODS

Chemicals. Chemicals and solvents were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany) and VWR International GmbH (Darmstadt, Germany). Crystalline $\text{Mn}_2(\text{CO})_{10}$ (CORM-1) was obtained from ABCR GmbH & Co. KG (Karlsruhe, Germany). Chloroform (CHCl_3) was purchased from Fisher Scientific GmbH (Schwerte, Germany) and used without further purification. Poly(L-lactide-co-DL-lactide) 70/30 (PLA) (Resomer LR 708) was purchased from Boehringer Ingelheim Pharma GmbH & Co. KG (Ingelheim am Rhein, Germany). A weight-average molecular weight of 800,000 g/mol was determined for the polymer by gel permeation chromatography using CHCl_3 as the solvent and polystyrene as the external standard.

Manufacture of the nonwovens. The concept of embedding CORMs in electrospun nonwovens was described by Bohlender et al. (18). For use in the cell assay with confocal microscopy, we controlled the thickness of the nonwovens. Briefly, the electrospinning process was carried out using the commercial electrospinning apparatus E-Spintronic (Erich Huber GmbH, Maisach, Germany) under dim light at 23°C and an air humidity of 32%. The integrated infusion pump was mounted with a 10-ml plastic syringe connected with a 35-cm polytetrafluoroethylene (PTFE) tube (Intra Special Catheters GmbH, Rehlingen-Siersburg, Germany) to the nozzle, a stainless-steel straight-end hollow needle (0.4 mm). A ground-glass mirror (30 by 30 mm) was used as a collector plate for collecting the electrospun nanofibers. The distance between the needle tip, the collector was maintained at 21 cm, and the voltage applied to the needle was set to 20 kV. The spinning solution was composed of 80 mg PLA and 20 mg of CORM-1 (20% [wt/wt] CORM-1-PLA20) in 4.9 g of chloroform. The blank nonwoven PLA was produced without the addition of CORM-1. For the preparation of the polymer solution, the PLA was dissolved first. The carbonyl complex was then added in darkness, and the mixture was stirred for 30 min at room temperature. The polymer solution was fed at a constant rate of 1.5 ml/h. The size of the electrospun nonwoven fleece obtained from 0.5 ml of polymer solution was approximately 30 cm², with a yellowish color due to the $\text{Mn}_2(\text{CO})_{10}$ (19); the color of the PLA was white.

Analyses of the nonwovens. The structure of the fibers was analyzed by scanning electron microscopy (SEM) with a Supra 55VP (Carl Zeiss AG, Jena, Germany) field-emission scanning electron microscope, as described previously (18).

The elemental composition was confirmed by energy-dispersive X-ray spectroscopy (EDX) using the SEM equipped with an EDX system [Quantax with Si(Li)-detector; Bruker Nano GmbH, Berlin, Germany], as described previously (18).

The content of CORM-1 incorporated into the nonwoven was verified by UV-visible spectroscopy. Briefly, free CORM-1 and the CORM-1-PLA20 nonwoven containing an equal incipient amount of CORM-1 were dissolved in oxygen-free chloroform, and the absorbances at 343 nm were compared using a Specord S600 instrument (Analytik Jena AG, Jena, Germany). Additionally, the overall manganese contents of three CORM-1-PLA20 samples were measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES) on a Varian 725 ES instrument (Agilent Technologies, Santa Clara, California, USA). For this, samples of the

CORM-1-PLA20 were weighed and dissolved in 1.5 ml of concentrated sulfuric acid and 1 ml of hydrogen peroxide. This solution was stirred for 5 min. After cooling to room temperature, the probes were diluted in deionized water (total volume, 25 ml), and the amount of manganese in the acidic aqueous solution was measured. Calibration was performed with the Merck IV multielement standard from Merck KGaA, Darmstadt, Germany.

CO release kinetics. The CO release kinetics were measured under both dry and wet conditions. The irradiation was performed at 10 mW/cm² and was controlled by a PM100USB power meter combined with a photodiode (S120VC; Thorlabs GmbH, Germany). The distance between the light source and the samples was fixed at 4.5 cm.

The CO release under dry conditions was performed in a desiccator and was monitored with a PAC7000 electrochemical CO detector (Drägerwerk AG & Co. KGaA, Lübeck, Germany) (20). In the desiccator, an open quartz cuvette containing a small piece of CORM-1-PLA20 was placed. The amount of CO released was calculated using the ideal gas equation ($pV = nRT$) and expressed as $\mu\text{mol}/\text{mg}$ of nonwoven. The experiments were repeated four times.

Quantification of the CO release under wet conditions was performed as described previously (21). The myoglobin assay was adapted for heterogeneous probes in Mueller-Hinton (MH) medium. Small pieces of CORM-1-PLA20 were fixed on a paper clip and placed into a quartz cuvette sealed with a rubber septum. Then, 1.5 ml reduced horse heart myoglobin (Mb) and 1.5 ml MH medium were added. The CO release from the nonwoven was induced via irradiation with light (405 nm, 10 mW/cm²) for 5 min. After the irradiation was finished, the UV-visible spectra (300 to 800 nm) were taken. The changes in the Mb-CO content were plotted as differences in absorbance at 540 nm, considering the specific molar extinction coefficient ($15.4 \text{ mM}^{-1} \text{ cm}^{-1}$) as a function of time.

The evaluation of the maximum released CO yield and the half time of the release was performed by fitting the plots using a four-parameter equation (equation 1) (for the dry condition) or a first-order exponential equation (equation 2) (for the myoglobin assay):

$$Y = Y_{\min} + \{[Y_{\max} - Y_{\min}] / [1 + 10^{(t_{50}-x)^n}]\} \quad (1)$$

$$Y = Y_{\max} (1 - e^{-Kx}) \quad (2)$$

where Y is the measured CO content in ppm, x is the time ordinate, $Y_{\min}$ represents the concentration signal at $x = 0$, $Y_{\max}$ represents the fluorescence signal at infinite time, and n is the scope. The half time (t_{50}) corresponds to the time when Y equals $0.5 \cdot Y_{\max}$ in equation 1; in equation 2, t_{50} is achieved when x equals $\ln 2/K$.

The presence of manganese-bound CO in the nonwoven before and after irradiation was verified by infrared (IR) spectroscopy using an Alpha FT-IR or Vertex 70 FT-IR instrument (Bruker Optik GmbH, Ettlingen, Germany), as described previously (18).

Strains and culture conditions. We used the MRSA strain ATCC 43300 (LGC Standards GmbH, Wesel, Germany). All media were purchased from Carl Roth GmbH (Karlsruhe, Germany). Stock culture was stored at -80°C in 10% (vol/vol) glycerol. A loop of frozen bacteria was struck on lysogenic broth (LB) agar (1.5%) plates and incubated overnight at 37°C. A single colony of the MRSA strain was cultured in MH medium overnight at 37°C under rotation.

To encourage biofilm formation, the cultures were freshly inoculated into MH medium by a 1:100 dilution. The CORM-1-PLA20 and PLA (blank) nonwovens were cut into 80-mm² (8- by 10-mm²) pieces and subsequently disinfected with 70% (vol/vol) ethanol and equilibrated in MH medium for 15 min. The pieces of nonwovens were placed onto the bottoms (22) of X-well tissue culture chambers (on cover glass II) (Sarstedt AG & Co, Nürnbrecht, Germany), and 300 μl of the bacterial suspension was added. Biofilms were grown at 37°C without shaking for 48 h.

CO treatment of the biofilms. The biofilms grown on the CORM-1-PLA20 and PLA nonwovens were irradiated at 405 nm for 5 min, as described above. Biofilms grown without the nonwovens were used as controls to determine the effect of the irradiation. The irradiation was

performed at 10 mW/cm². The biofilms were stained for 15 min in the dark using the Live&Dead cell viability assay (Thermo Fischer Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol at 40 min after treatment.

The effect of the CO release on the vital biofilms was recorded with an inverse confocal laser scanning microscopy (CLSM) 510 system (Carl Zeiss AG, Jena, Germany) by excitation at 490 nm using the Ar laser line and a 40× objective. Areas of approximately 100 μm (x) by 100 μm (y) were screened at the focus level near basal biofilm layers at the green (522-nm) and red (635-nm) channels, respectively. Due to bleaching effects, the images were taken from different positions between 60- and 135-min intervals. Because of the known toxic effects of the staining dye propidium iodide (23), longer observation was inappropriate; therefore, an additional set of biofilms was incubated up to 24 h (1,440 min) after irradiation and subsequently stained and analyzed by CLSM. The biofilm data were visualized with ZEN 9.0 software (Carl Zeiss AG, Jena, Germany). The two-dimensional (2D) images of the green and red channels from each experiment were exported as bitmap images for further analysis using noncommercial in-house software (54). Briefly, the green- and red-stained bacterial cells of the scanned biofilm layer were identified and counted by a novel adaptive algorithm based on local gray-scale intensities in both channels. The dead bacteria directly corresponded to the red-stained cells, whereas the viable bacteria were quantified by the algorithm as the difference between the green signals and those overlaid by red signals. The experiments were repeated at least three times.

Antibiotic treatment of biofilms. The biofilms were grown without the nonwovens and washed as described above. Subsequently, the biofilms were treated for 24 h with daptomycin (100 mg/liter supplemented with 50 mg/liter calcium chloride), rifampin (16 mg/liter), or colistin sulfate (16 mg/liter) in fresh MH medium. After treatment, the biofilms were washed, stained, and analyzed by CLSM as described above. The chosen concentrations correspond to the recommended daily dosages of the respective antibiotics.

Quantification of CFU and SCVs. The MRSA biofilms were grown and CO treated as described above. The biofilms were carefully washed with 300 μl of phosphate-buffered saline (PBS) two times and resuspended in 100 μl of PBS by sonication. The cell suspension was serially diluted and spread on Columbia blood agar (BD Diagnostics, Heidelberg, Germany). The colonies were manually counted after 48 h at 35°C to quantify the CFU/ml, including the small-colony variants (SCVs). Thereby, the SCVs were identified as pinpoint colonies that were nonhemolytic and nonpigmented. The experiments were performed in triplicate.

Measurement of ROS. A previously described fluorometric assay (24) was adapted for the *S. aureus* biofilms to determine the accumulation of reactive oxygen species (ROS). Biofilms grown in 24-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) on the nonwoven (CORM-1-PLA20 and a PLA) were exposed to 405-nm radiation for 5 min. Afterwards, the biofilms were incubated in 10 μM 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich Chemie GmbH, Munich, Germany) solution for 15 min. The fluorescence emission at 535 nm was measured by applying excitation at 485 nm using an Infinite M200 Pro fluorescence reader (Tecan Trading AG, Männedorf, Swiss) and corrected for background fluorescence (medium and DCFH-DA with and without nonwovens, respectively). ROS were quantified for each condition at time points 0 (before irradiation) and 15 min, 60 min, 90 min, and 135 min after irradiation. A control experiment was performed without irradiation under similar conditions. The experiments were repeated three times.

Assay of CO-mediated cytotoxicity on eukaryotic cells. Mouse 3T3 fibroblasts were harvested from a preculture and suspended in Dulbecco modified Eagle medium (DMEM) cell culture medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from Biochrom GmbH, Berlin, Germany). Cells were seeded into 8-well tissue culture chambers (Sarstedt AG & Co, Nümbrecht, Germany) containing 80-mm² pieces CORM-1-PLA20 or pure PLA and

empty wells at a density of approximately 2.5×10^4 cells/cm². The cells were grown in the dark for 24 h at 37°C under a 5% carbon dioxide humid atmosphere.

The cells were irradiated at 405 nm for 5 min; the conditions were similar to those for the CO treatment of biofilms. In one series of samples, the cells were cultured for a further 2 h at 37°C, followed by live/dead staining, and in a second series, they were cultured for 72 h and stained. For this, the medium was changed to a staining solution containing fluorescein diacetate and GelRed (Biotium Inc., Hayward, CA, USA) in PBS. The viable cells (green fluorescent) and nuclei from dead cells (red fluorescent) were visualized by inverse fluorescence microscopy (Observer Z1m; Carl Zeiss AG, Jena, Germany) equipped with a 10× objective EC plan-neofluar and LED illumination of 470 nm and 530 nm. Fluorescence micrographs were taken as z-stack (15-μm z-stack interval) using an AxioCam HRC microscope camera and filter sets 10 and 14. Extended field-of-depth images were created from z-stacks using ZEN 2012 (blue edition) software (Carl Zeiss AG, Jena, Germany). The percentage of dead cells was calculated after counting up to 10 images per treatment, supported by ImagePro software (Media Cybernetics Inc., Rockville, MD, USA).

The experiment was replicated a second time independently. The results after 72 h were statistically analyzed, as the percentage of dead cells varied at this time.

Data analysis and statistics. The data were analyzed and graphically visualized using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). The statistical analysis of the treatment effects was performed by two-way analysis of variance (ANOVA) and Bonferroni posttests (two-tailed, 95% confidence intervals) comparing the corresponding pairs of controls to the treatments. Significance was assumed at a *P* value of ≤0.05.

RESULTS

Characteristics of the nonwovens. Nonwovens from biocompatible polymers, such as polylactide, are well suited for having cells grown on them (18). In this study, confocal microscopy was used to investigate live/dead-stained MRSA biofilms after photoactivated CO treatment. Therefore, the manufacture of thin nonwovens (approximately 25 μm) was necessary. These thin nonwovens were characterized by infrared (IR) spectroscopy, elemental analysis (EDX), and scanning electron microscopy (SEM) and showed results similar to those published by Bohlender et al. (approximately 50 μm) (18). The average fiber diameters of the CORM-1-PLA20 and PLA nonwovens were both approximately 1.3 to 2.1 μm (Fig. 1A). In contrast to PLA, the CORM-1-PLA20 fibers showed porosity caused by the release of CO during the electrospinning process. The presence of manganese in the fibers was confirmed by the presence of Mn-specific peaks in EDX analysis (see Fig. S1 in the supplemental material).

The incorporation efficiency was determined by UV-visible spectroscopy (see Fig. S2 in the supplemental material) by comparing the CO release from corresponding amount of free CORM-1 and the nonwovens, considering the initial 20% of CORM-1 used for the manufacture of the nonwovens. The amount of CO released from CORM-1-PLA after irradiation at 405 nm indicated that 77.3% was successfully incorporated into the nonwoven, corresponding to a concentration of only 15.4%. Using the alternative ICP-OES method, an average content of 12.4% (wt/wt) CORM-1 in the CORM-1-PLA20 nonwovens was recovered, corresponding to an incorporation efficiency of 62.3% (see Table S1 in the supplemental material). These results are in agreement with those published by Bohlender et al. (18). Assuming that 10 molecules of CO are bound in one CORM-1 complex,

FIG 1 Characteristics of the CORM-1-PLA20 nonwoven. (A) SEM images of CORM-1-PLA20 at two different magnifications. (B) CO release kinetics of the CORM-1-PLA20 nonwoven exposed to 405 nm under dry conditions. Concentrations were determined in a sealed beaker at 10-s intervals for 75 min, but only the first 30 min are shown. (C) CO release kinetics of the CORM-1-PLA20 nonwoven exposed to 405-nm radiation, measured in a heterogeneous myoglobin assay in MH medium. Concentrations of Mb-CO were determined at 540-nm absorption, considering the specific molar extinction coefficient (15.4 mM^{-1}). The black dotted lines in panel B and symbols in panel C represent the CO release profiles of different nonwoven pieces and the solid gray lines the respective fitting curves, with black solid lines indicating the averaged fitting curves. The gray box indicates 5 min of irradiation time.

a maximum concentration of 3 µmol CO/mg nonwoven can be estimated.

The CO release was measured under dry ($n = 4$) and wet ($n = 3$) conditions using 80-mm^2 pieces of the CORM-1-PLA20 nonwoven. Because the spinning procedure generates a nonhomogeneous nonwoven, we determined the masses of 10 pieces. The masses of the pieces varied from 0.229 mg to 0.765 mg, and the average mass was $0.509 \pm 0.197 \text{ mg}$.

Under dry conditions, 5 min of irradiation at 405 nm was sufficient to release the whole CO content from the CORM-1-PLA20. This was confirmed by IR spectroscopy through the absence of the CO-specific absorption band at wavenumber $2,100 \text{ cm}^{-1}$ (see Fig. S3 in the supplemental material). The released CO content, determined as parts (released CO molecules) per million (air molecules in the desiccator, 570 ml), varied from 71.8 ppm (1.6 µmol CO/mg nonwoven) to 127.4 ppm (2.9 µmol/mg) (Fig. 1B). The average concentration was $92.0 \pm 24.4 \text{ ppm/mg}$ nonwoven, corresponding to $2.1 \pm 0.6 \text{ µmol CO/mg}$ nonwoven, indicating that the CORM-1 incorporation into the CORM-1-PLA20 nonwoven was approximately 68%, consistent with previous results (18). The half time, i.e., the time to release 50% of the CO, was $2.6 \pm 0.5 \text{ min}$ on average.

Under wet conditions, 5 min of irradiation at 405 nm also induced a release of CO that was measured as the time-dependent increase of the Mb-CO concentration. The half time was $12.6 \pm 6.21 \text{ min}$ on average, and saturation was achieved 60 min after irradiation at $7.5 \pm 1.2 \text{ µM CO-bound myoglobin}$ (Fig. 1C).

Reduction of MRSA biofilms. MRSA biofilms grown on the nonwovens or on glass were exposed to 405-nm radiation for 5 min, followed by live/dead staining. The amounts of viable and

dead cells within the biofilms were determined between 60 min and 135 min at intervals and after 24 h after CO treatment (Fig. 2). A preliminary test revealed that within 60 min after CO exposure, any changes were not microscopically visible (data not shown).

The amount of viable bacteria within the biofilms grown on glass upon exposure to 405-nm radiation was not significantly smaller than that in the nonirradiated biofilms (Fig. 3A and B, black solid lines). This is in accordance with previous studies showing that the bactericidal effects of blue light at 405 nm are expected upon longer exposure and that 5 min of irradiation has no observed impact on cell viability (25). Similarly, no significant changes were observed for biofilms grown on PLA without and after irradiation (Fig. 3, black dotted lines). The amount of viable cells was also not significantly reduced by the CORM-1-PLA20 nonwoven without irradiation (Fig. 3B, gray solid line) compared to biofilms grown on the PLA nonwoven or glass, indicating that the CO toxin was not unintentionally released under the experimental conditions. On the other hand, the CO-treated biofilms (grown on CORM-1-PLA20 and exposed to 405 nm) (Fig. 3A, gray line) showed a high proportion of dead bacteria and strongly reduced viable cell counts after 90 min and 135 min compared to the nonirradiated biofilms grown on CORM-1-PLA20 (Fig. 3B, gray line). The viable cells were reduced by $68.7\% \pm 16.8\%$ on average after 135 min (Fig. 3B, gray line). The effect of CO was even stronger after 24 h after irradiation, being reduced by up to $79.63\% \pm 24.5\%$. The differences between the experiments (high standard deviation) were most probably caused by the different contents of CO due to a nonhomogeneous distribution in the nonwoven, as observed in the release kinetics (18).

The microscopically observed effects were confirmed by deter-

FIG 2 Live/dead-stained MRSA biofilms 60 min and 135 min after irradiation at 405 nm. Biofilms were grown on glass (control), PLA, or CORM-1-PLA20. Viable bacteria are shown in green and dead bacteria in red. The CLSM images were taken close (2- μ m to 4- μ m distance) to the glass or nonwoven surface. n.i., no irradiation.

mination of the CFU/ml on agar plates before irradiation and after 60 and 135 min and 24 h (Fig. 3C). The viable cells were determined as CFU/ml on agar plates and were related to time point zero (100%), because this method does not identify the dead cells and because the viable cells of the biofilms before the irradiation represented nearly 100%. The CFU/ml of the samples grown on glass and PLA increased after 60 and 135 min after irradiation and dropped after 24 h to $78\% \pm 17\%$ and $72\% \pm 6\%$, respectively, whereas the CFU/ml of the samples grown on CORM-1-PLA20 decreased in a time-dependent manner to $20\% \pm 1.5\%$ after 24 h after irradiation. The increased CFU/ml for the glass and PLA samples can be explained by the difficulties in the washing step, as planktonic bacteria cannot be completely removed from the biofilms.

Comparison of effectiveness with other antibiotics. The effect of CO on biofilms was compared to the effects of daptomycin and rifampin, both known to be active against biofilms of *S. aureus* (26), and colistin. The biofilms were treated with the antibiotics for 24 h, whereas CO-treated biofilms were grown for 24 h after exposure in MH medium (Table 1). As expected, colistin led to a slightly reduced but not significantly different number of the viable cells compared to those in the untreated biofilms, because *S. aureus* exhibits an intrinsic resistance to colistin. Rifampin caused a reduction of the viable cells by nearly 50% at a concentration of 16 mg/liter. Daptomycin at 100 mg/liter showed the highest antibiofilm activity by reducing the viable MRSA cells by approximately 80%. This effect was comparable to that of the CO treatment with CORM-1-PLA20.

SCV formation. To examine whether CO treatment with CORM-1-PLA20 caused any morphological variants known as small-colony variant (SCVs), the biofilm-embedded bacteria were resuspended and spread on blood agar plates. The morphology of the colonies was assessed after growth for 24 h and 48 h. The percent-

age of SCVs after CO treatment (CORM-1-PLA20) was 40.6% on average and was similar to those in the control group without nonwoven (46.7%) and biofilms grown on PLA (45.5%), indicating that CO treatment had no impact on the formation of SCVs.

ROS formation. To examine whether irradiation and CO release induce the formation of ROS, irradiated and nonirradiated biofilms were stained with DCFH-DA, whose fluorescence is enhanced by ROS. The changes in the fluorescence intensity were recorded for 135 min at intervals (Fig. 3D and E).

Nonirradiated biofilms that were grown on glass and PLA showed similar curve slopes, with slightly decreasing concentrations of ROS during the measurement. Although there was no significance ($P > 0.5$) because of the scattering of the measurements, a slightly increased ROS amount was detected for biofilms grown on CORM-1-PLA20.

Comparing the biofilms that were not exposed (Fig. 3E) and those that were exposed (Fig. 3D) to 405-nm radiation, a visible increase in the ROS concentration after irradiation at 405 nm was observed for all samples. These differences were highly significant ($P < 0.001$) for biofilms on glass (without nonwoven), indicating that irradiation at 405 nm might also contribute to ROS formation. Irradiated biofilms on PLA showed a lower concentration of ROS than those on glass, suggesting that the PLA nonwoven has a light-protective property. Irradiated biofilms on CORM-1-PLA20 (Fig. 3D, gray line) showed the highest concentration of ROS, which was significantly different ($P < 0.01$) from the corresponding concentrations in nonirradiated biofilms (Fig. 3E, gray line) and irradiated biofilms on PLA (Fig. 3D, black dotted line). Because of the light-protective effect of the PLA nonwoven background, a comparison of the ROS amounts in the irradiated biofilms on glass and those on CORM-1-PLA remained inadequate.

FIG 3 Changes in the biofilms in response to irradiation at 405 nm and CO release. Biofilms were grown in the presence (CORM-1-PLA20) or absence (PLA) of CORM-1 or without the nonwoven (glass). (A and B) Quantification of viable cells after irradiation at 405 nm (A) and without irradiation (B). The viable cells are given in relation (percent) to the total cell number (viable plus dead). (C) Quantification of the CFU/mL on agar plates. All CFU/mL were given in relation (percent) to the corresponding biofilms before irradiation. (D and E) Quantification of ROS in biofilms after irradiation at 405 nm (D) and without irradiation (E). ROS were measured indirectly with the fluorescent indicator DCFH-DA.

CO toxicity on eukaryotic cells. Cytotoxicity was investigated using the 3T3 fibroblast cell line, which was cultured directly on chamber slides or on CORM-1-PLA20 and exposed to 405 nm for 5 min. Images were taken after live/dead staining at 2 and 72 h after irradiation. Compared to that of the control cell cultures, the cell growth seemed to be unaffected by the nonwovens. After 2 h, no visible increase in dead cells (red) was visible for all treatments (see Fig. S4 in the supplemental material). On the other hand, at 72 h after irradiation, the amount of dead cells was elevated to an average of $22.2\% \pm 3.8\%$ for CORM-1-PLA20 and to $13.4 \pm 2.8\%$ for PLA20, whereas the control maintained an average of $2.8\% \pm 0.5\%$. Statistical analysis by two-way ANOVA for this time point revealed no significant difference for the irradiation factor ($P = 0.908$) but significant differences for the material factor ($P <$

0.001). Multiple-comparison (Bonferroni) procedures identified CORM-1-PLA20 versus control and CORM-1-PLA20 versus PLA as being significantly different. Also, upon comparing the corresponding pairs of controls to the treatments, the elevated cytotoxicity values for the nonwovens did not depend primarily on irradiation, as no statistically significant difference was found between the irradiated and nonirradiated samples (Fig. 4), al-

TABLE 1 Effects of treatment with daptomycin, rifampin, and colistin for 24 h in comparison to the CORM-1-PLA20 treatment on the amount of viable MRSA cells in biofilms

Treatment	% viable cells (mean $\pm$ SD) ^a	<i>P</i> value
Untreated control	96.3 $\pm$ 5.9	
CORM-1-PLA20 ^b	20.4 $\pm$ 24.3	<0.0001
Daptomycin	21.2 $\pm$ 2.6	<0.0001
Rifampin	49.6 $\pm$ 4.3	<0.0001
Colistin	87.4 $\pm$ 10.1	>0.05

^a Percentage of viable cells in relation to the total cell number (viable plus dead cells).

^b The CO was released for 5 min by irradiation at 405 nm, and the biofilms were subsequently grown for 24 h without irradiation.

FIG 4 Evaluation of the cytotoxicities of the nonwovens on mouse 3T3 fibroblasts. Live and dead cells were separately counted from images, and the percentage of dead cells was determined. Statistics were performed using two-way ANOVA and Bonferroni posttests (two-tailed, 95% confidence intervals), comparing the corresponding pairs of controls to the treatments. The error bars indicate the standard deviation (SD).

though significant differences ($P < 0.05$) between PLA and CORM-1-PLA20 for irradiated cells indicated an impact of CO on the increased cell death after 72 h.

DISCUSSION

The main hurdle for the clinical use of CO in aqueous compartments such as body fluids or tissues is its relatively low solubility (1 mM at 20°C and 0.8 mM at 37°C). For the controlled delivery and release of the toxic gas in aqueous environments, carbon monoxide-releasing molecules (CORMs) have proven to be the most promising for therapeutic usage (27, 28). Transition metal carbonyl complexes are the most prominent CORMs because they can be triggered via light, solvent exchange on the metal coordination sphere, and enzymes (29, 30). Macromolecular systems have also been exploited as CORM carriers to adapt CO release kinetics and retain toxic metabolites after CO release (27). Bactericidal effects on various planktonic bacterial species, including *S. aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, were demonstrated for some soluble CORMs, such as CORM-2 [$\text{Ru}_2(\text{CO})_6\text{Cl}_4$], CORM-3 [$\text{Ru}_2(\text{CO})_3\text{Cl}(\text{glycinate})$], ALF021 [$\text{Mn}(\text{CO})_5\text{Br}$], and ALF062 [$\text{Mo}(\text{CO})_5\text{Br}(\text{N}(\text{C}_2\text{H}_5)_4)$] (10, 31–34). Besides inhibition of the respiratory chain, CORMs seem to also target other proteins, because the bactericidal effects were found under aerobic and anaerobic conditions (33, 35). CORMs are also known to induce oxidative stress (36), but the effect depends on the CORM and bacterial species (35). CORM-2 was shown to inhibit the biofilm formation by *P. aeruginosa* (37), but it increased biofilm formation in *E. coli* (38, 39). However, the effects of CORMs embedded in nonwovens on mature biofilms have not yet been examined.

Methicillin-resistant *S. aureus* (MRSA) is the most common nosocomial biofilm-forming pathogen, causing diverse SSSIs with limited treatment options (40). Moreover, in some regions of the United States, community-acquired SSSIs are predominantly caused by a specific MRSA clone (USA 300) (41). In this study, we investigated whether MRSA biofilms can be reduced or eradicated by controlled CO treatment using a polymeric hybrid nonwoven containing the photoactive CORM-1.

We used a direct microscopic method where live and dead cells were visualized by specific fluorescent staining and confirmed the results by counting the CFU on agar plates. Although the results were comparable, the data obtained by the second method showed higher variation, mainly because the washing step was more complicated for the biofilms grown on the loosely placed nonwoven. Additionally, there was a declining trend in the CFU/ml in all samples after 72 h of growth. This effect was not observed by the microscopic method for the untreated samples. Most probably this was caused by the so-called viable-but-nonculturable cells (VBNC), as observed for different species with reduced metabolism (42).

The MRSA biofilms were grown *in vitro* in MH medium, a rich medium with peptones and beef infusion as carbon sources, because it is recommended for antimicrobial testing by international standards and more adequately matches the environment of human compartments. The mature biofilms were exposed to CO by photostimulated release from the nonwoven at 405 nm. In a previous study, we demonstrated that CO is released from CORM-1-PLA20 more rapidly at 365 nm (UV-A) than at 480 nm (18). Because exposure to UV-A is associated with DNA damage that can cause skin cancer (43), we decided to use 405 nm to induce the

CO release. This is the lowest wavelength above UV-A and can be achieved by cost-effective LED sources.

The CORM-1-PLA20 nonwoven exhibited a thickness of approximately 25 μm , and 5 min of irradiation at 405 nm was sufficient to completely release $2.1 \pm 0.6 \mu\text{mol}$ of CO per mg nonwoven under dry conditions. The photoactivated release under wet conditions in MH medium (Fig. 1C, myoglobin assay) was approximately 5 times slower, but the whole content of CO could be released after 5 min of photostimulation, and saturation was achieved within 60 min. The delay in saturation was in accordance with the first visible killing effects on biofilms, which were achieved 60 min after photoactivation and increased within 135 min. Considering the mass of the nonwovens (on average 0.5 mg) and the medium volume (300 μl) used per X-well, the theoretical maximal CO concentration to which the biofilms were exposed was $3.5 \pm 1.1 \text{ mM}$. The CO solubility in water is 0.8 mM at 37°C. The composition of the medium might influence the solubility of CO, but the solubility in water suggested that the effective CO concentration that was sufficient to kill up to 86% of the MRSA biofilms was 0.8 mM rather than 3.5 mM.

The bactericidal and biofilm-inhibitory effects of CORMs depend on the bacterial species and growth conditions as well as the type and concentration of the specific CORM. For nonphotoactive CORMs, it was demonstrated that at a concentration of 50 μM , ruthenium-containing CORMs (CORM-2 and CORM-3) were more effective than manganese-based CORMs (ALF021 and ALF062), killing up to 80% of planktonic bacteria within 30 min in a minimal medium (33). Biofilm formation by *P. aeruginosa* was also reduced by 100 μM CORM-2 in minimal medium containing glucose, whereas CORM-2 showed no effect in a rich medium with peptones as a carbon source (LB) (37). In *E. coli*, 250 μM CORM-2 exhibited an opposite effect, increasing biofilm formation 1.6-fold in LB (38). In all these studies, the CORMs were used as soluble additives, releasing CO upon acceptor contacts. Thus, the effective CO concentration remains unclear, even if up to 1.5 mM CO could be released from 250 μM CORM-2. In contrast, the CO bound in CORM-1-PLA20 was completely released within a short time frame, achieving a saturated concentration. We hypothesize that the biofilm-killing effect of CORM-1-PLA20 is mainly due to the photoinduced release of a high dose of CO that is not achieved by the soluble CORMs.

The released CO effectively killed bacterial cells within the MRSA biofilms. The effect was comparable to that of daptomycin, which exhibited the most promising biofilm-eradicating activity compared to that of other antibiotics in *in vitro* studies (26). Moreover, after a short exposure (only 5 min) to CO, a long-term effect was observed, and it was even stronger after 24 h. The post-antibiotic effect on bacterial growth was previously observed for conventional antibiotics with different species (44) but was usually limited to few hours. This indicates that CO triggers a long-term cascade of processes resulting in gradually increasing cell damage and cell death.

The inhibition of the respiratory chain by CO has been shown to be the main toxic mechanism in various species, but it also induces the formation of reactive oxygen species (ROS) as a result of the oxidative stress response (36). Moreover, the transition metals in CORMs have been shown to trigger the formation of hydroxyl radicals from water oxygen in the presence of CO ligands (35, 36). ROS have been shown to play a role in the bactericidal mechanism in *E. coli* (36) but not in *P. aeruginosa* (37). The pres-

ent study indicates that ROS are most likely involved in the process of killing of biofilm-embedded *S. aureus*, as the concentration of ROS was strongly increased after the photostimulated CO release compared to that for the PLA control. It must be mentioned that irradiation at 405 nm substantially induced ROS formation also in the absence of the nonwoven and that this effect was reduced by the nonwoven, most likely due to its light-absorbing properties. We found a weak and time-dependent increase in the ROS concentration in nonirradiated biofilms on CORM-1–PLA20; this might be caused by the transition metal manganese, but more likely it was a result of the CO release caused by the fluorescence excitation at 485 nm during the experiment.

S. aureus, both MRSA and MSSA, is known to form SCVs under stress conditions that allow intracellular persistence in host cells or biofilms (45, 46) that are tolerant to antibiotic therapy and evade host defenses (47). SCVs are deficient in electron transport, and therefore we verified whether CO might have an impaired activity against this specific phenotype or even enhances the formation of SCVs. In our study we did not find any significant differences in the SCV amounts in CO-treated and untreated specimens, demonstrating that CO treatment does not increase the SCV formation, as is known for other bactericidal antibiotics (48). The ratio between CFU/ml and SCVs was constant even when the CFU/ml dropped after CO treatment, suggesting that CO might be effective against both normal cells and against SCVs. Because the mechanism of action of CO is of a more general nature, it might even succeed in killing the persisting and metabolically inactive cells in a biofilm. This capacity should be further investigated in detail.

The bactericidal properties of photoactivated CORM-1 against MRSA biofilms suggest that CO-releasing nonwovens might be potential wound dressings for the antimicrobial treatment of human pathogens in accessible sites, such as in traumatic and surgical SSSIs or diabetic foot infections. We observed some cytotoxicity in eukaryotic cells grown for few days on the nonwovens, which was caused mainly by the nonwovens and was slightly increased for CORM-1–PLA20. The contribution of CO to the cytotoxicity was low, especially in the short time period after CO release, indicating an impeded accessibility of CO to the mitochondrial membrane-located and CO-sensitive respiratory enzymes (10). Cytotoxic effects of the PLA-based nonwoven were observed occasionally with longer exposure times but rarely within 24 h of exposure. A possible cause is the accumulation of released lactate (49). For applications such as wound dressings this should be considered, and short exposure to the nonwoven would be preferred to avoid cytotoxic effects of the PLA on the tissue. Although the cytotoxic effect of the CO *in vitro* remains acceptable, it should be further investigated in an *in vitro* model. Interestingly, it was shown that macrophages sense and kill bacteria through carbon monoxide-dependent inflammasome activation (50); thus, CO treatment may be even more effective *in vivo*.

Electrospun nonwovens with embedded drugs have been used for decades to treat various superficial infections, e.g., as wound dressings. As early as the early 1980s, hydroxypropyl cellulose strips that contained chlorhexidine or tetracycline in low doses were developed (51), and more recently, the antibiotic metronidazole spun in PLA fibers was shown to be effective in the treatment of periodontal diseases (52). Electrospun nonwovens based on chitosan and PLA without antibiotics are effective against planktonic solutions of *S. aureus* and *E. coli* and were suggested as

promising wound dressings. The antibacterial efficacy was attributed to ionic interactions between the protonated amino groups of chitosan or its quaternized derivatives and the negatively charged surface of the bacteria, which lead to a loss of membrane permeability, cell leakage, and cell death (53). The efficacy of these chitosan-PLA nonwovens against biofilms was not tested, but one can speculate that the negatively charged surface of the biofilm matrix would absorb and inactivate most of the positive residues of chitosan. Thus, it remains unclear whether any activity can be expected from this pure nonwoven against mature biofilms. It is also unlikely that biofilms can be effectively eradicated by the nonwoven-embedded antibiotics because of the strongly increased antibiotic tolerance of biofilms. Thus, nonwovens that release reactive, noncharged, and small diffusible compounds, such as CO or NO, targeting essential cell processes might be more promising as bactericidal wound dressings. Coupled to a light trigger, as in CORM-1–PLA20, nonwovens allow a controlled local release.

Considering the release kinetics, a CORM-1–PLA20 wound dressing of 5 cm by 5 cm (25 cm²) would release approximately 32.5 μ mol of CO. Depending on the volume to which the CO will be liberated, the concentration can vary, causing different side effects. However, when used as bactericidal wound dressing, only a local and temporary limited exposure to CO has to be applied. This high local CO concentration without systemic toxicity can be obtained by covering a potential CO-containing nonwoven wound dressing with a gas-impermeable light-transmitting material.

Our work presents the first proof that the repeated use of a CORM, which is feasible in medical routine, is able to eliminate MRSA in biofilms. Repeated short-time use of a potential CORM wound dressing covered with a gas-impermeable light-transmitting sheet may increase local CO concentrations and simultaneously decrease systemic toxicity. Now, animal studies for the use of this CORM in wound infections are needed to provide evidence of the presented effects in real *in vivo* situations. However, the antimicrobial effects might even be exceeded, since CO enhances the sensing and killing of bacteria by macrophages.

ACKNOWLEDGMENT

We thank Martina Schweder at Innovent for the SEM investigations.

FUNDING INFORMATION

This work, including the efforts of Mareike Klinger-Strobel and Mathias W. Pletz, was funded by Deutsche Forschungsgemeinschaft (DFG) (PL320/3-1). This work, including the efforts of Steve Glaeser and Alexander Schiller, was funded by Deutsche Forschungsgemeinschaft (DFG) (number SCHI 1175/2-2). This work, including the efforts of Alexander Schiller, was funded by Deutsche Forschungsgemeinschaft (DFG) (SCHI 1175/4-1 and SCHI 1175/5-1). This work, including the efforts of Mareike Klinger-Strobel and Oliwia Makarewicz, was funded by Bundesministerium für Bildung und Forschung (BMBF) under grant number 01EO1002. This work, including the efforts of Ralf Wyrwa and Juergen Weisser, was funded by Bundesministerium für Wirtschaft und Energie (BMWi) (MF130124).

REFERENCES

1. Livermore DM, Mushtaq S, Warner M, James D, Kearns A, Woodford N. 2015. Pathogens of skin and skin-structure infections in the UK and their susceptibility to antibiotics, including cefazolin. *J Antimicrob Chemother* 70:2844–2853. <http://dx.doi.org/10.1093/jac/dkv179>.
2. Pardos de la Gandara M, Raygoza Garay JA, Mwangi M, Tobin JN, Tsang A, Khalida C, D'Orazio B, Kost RG, Leinberger-Jabari A, Coffran C, Evering TH, Collier BS, Balachandra S, Urban T, Parola C, Salvato S,

- Jenks N, Wu D, Burgess R, Chung M, de Lencastre H, Tomasz A. 2015. Molecular types of methicillin-resistant *Staphylococcus aureus* and methicillin-sensitive *S. aureus* strains causing skin and soft tissue infections and nasal colonization, identified in community health centers in New York City. *J Clin Microbiol* 53:2648–2658. <http://dx.doi.org/10.1128/JCM.00591-15>.
3. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev* 28:603–661. <http://dx.doi.org/10.1128/CMR.00134-14>.
  4. Romling U, Balsalobre C. 2012. Biofilm infections, their resilience to therapy and innovative treatment strategies. *J Intern Med* 272:541–561. <http://dx.doi.org/10.1111/joim.12004>.
  5. Cosgrove SE, Kaye KS, Eliopoulos GM, Carmeli Y. 2002. Health and economic outcomes of the emergence of third-generation cephalosporin resistance in *Enterobacter* species. *Arch Intern Med* 162:185–190. <http://dx.doi.org/10.1001/archinte.162.2.185>.
  6. Lewis K. 2001. Riddle of biofilm resistance. *Antimicrob Agents Chemother* 45:999–1007. <http://dx.doi.org/10.1128/AAC.45.4.999-1007.2001>.
  7. Walters MC, III, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* 47:317–323. <http://dx.doi.org/10.1128/AAC.47.1.317-323.2003>.
  8. Anderl JN, Franklin MJ, Stewart PS. 2000. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* 44:1818–1824. <http://dx.doi.org/10.1128/AAC.44.7.1818-1824.2000>.
  9. Zacharia VM, Shiloh MU. 2012. Effect of carbon monoxide on *Mycobacterium tuberculosis* pathogenesis. *Med Gas Res* 2:30. <http://dx.doi.org/10.1186/2045-9912-2-30>.
  10. Desmard M, Davidge KS, Bouvet O, Morin D, Roux D, Foresti R, Ricard JD, Denamur E, Poole RK, Montravers P, Motterlini R, Boczkowski J. 2009. A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against *Pseudomonas aeruginosa* and improves survival in an animal model of bacteraemia. *FASEB J* 23:1023–1031. <http://dx.doi.org/10.1096/fj.08-122804>.
  11. Gonzales MA, Mascharak PK. 2014. Photoactive metal carbonyl complexes as potential agents for targeted CO delivery. *J Inorg Biochem* 133:127–135. <http://dx.doi.org/10.1016/j.jinorgbio.2013.10.015>.
  12. Motterlini R, Otterbein LE. 2010. The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov* 9:728–743. <http://dx.doi.org/10.1038/nrd3228>.
  13. Spiro TG, Ibrahim M, Wasbotten I. 2008. CO, NO, and O₂ as vibrational probes of heme protein active sites. In Gosh A (ed), *The smallest biomolecules: diatomics and their interactions with heme proteins*, vol 1. Elsevier Science, Philadelphia, PA.
  14. Ryter SW, Otterbein LE. 2004. Carbon monoxide in biology and medicine. *Bioessays* 26:270–280. <http://dx.doi.org/10.1002/bies.20005>.
  15. Ryter SW, Choi AM. 2013. Carbon monoxide: present and future indications for a medical gas. *Korean J Intern Med* 28:123–140. <http://dx.doi.org/10.3904/kjim.2013.28.2.123>.
  16. Knauer M, Vangala S, Haslip M, Lee PJ. 2013. Therapeutic applications of carbon monoxide. *Oxid Med Cell Longev* 2013:360815. <http://dx.doi.org/10.1155/2013/360815>.
  17. Mayr FB, Spiel A, Leitner J, Marsik C, Germann P, Ullrich R, Wagner O, Jilma B. 2005. Effects of carbon monoxide inhalation during experimental endotoxemia in humans. *Am J Respir Crit Care Med* 171:354–360. <http://dx.doi.org/10.1164/rccm.200404-446OC>.
  18. Bohlender C, Gläser S, Klein M, Weisser J, Thein S, Neugebauer U, Popp J, Wyrwa R, Schiller A. 2014. Light-triggered CO release from nanoporous non-wovens. *J Mater Chem B* 2:1454–1463. <http://dx.doi.org/10.1039/c3tb21649g>.
  19. Schiller A, Schnabelrauch M, Altmann C, Wyrwa R. August 2013. Kohlenstoffmonoxid freisetzende Materialien und deren Verwendung. Patent DE 10 2012 004 132 A1.
  20. Hasegawa U, van der Vlies AJ, Simeoni E, Wandrey C, Hubbell JA. 2010. Carbon monoxide-releasing micelles for immunotherapy. *J Am Chem Soc* 132:18273–18280. <http://dx.doi.org/10.1021/ja1075025>.
  21. Atkin AJ, Lynam JM, Moulton BE, Sawle P, Motterlini R, Boyle NM, Pryce MT, Fairlamb IJ. 2011. Modification of the deoxy-myoglobin/carbonmonoxy-myoglobin UV-vis assay for reliable determination of CO-release rates from organometallic carbonyl complexes. *Dalton Trans* 40:5755–5761. <http://dx.doi.org/10.1039/c0dt01809k>.
  22. Sobke A, Klinger M, Hermann B, Sachse S, Nietzsche S, Makarewicz O, Keller PM, Pfister W, Straube E. 2012. The urinary antibiotic 5-nitro-8-hydroxyquinoline (Nitroxoline) reduces the formation and induces the dispersal of *Pseudomonas aeruginosa* biofilms by chelation of iron and zinc. *Antimicrob Agents Chemother* 56:6021–6025. <http://dx.doi.org/10.1128/AAC.01484-12>.
  23. Riccardi C, Nicoletti I. 2006. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc* 1:1458–1461. <http://dx.doi.org/10.1038/nprot.2006.238>.
  24. Vandenbosch D, Braeckmans K, Nelis HJ, Coenye T. 2010. Fungicidal activity of miconazole against *Candida* spp. biofilms. *J Antimicrob Chemother* 65:694–700. <http://dx.doi.org/10.1093/jac/dkq019>.
  25. Maclean M, MacGregor SJ, Anderson JG, Woolsey G. 2009. Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array. *Appl Environ Microbiol* 75:1932–1937. <http://dx.doi.org/10.1128/AEM.01892-08>.
  26. Raad I, Hanna H, Jiang Y, Dvorak T, Reitzel R, Chaiban G, Sherertz R, Hachem R. 2007. Comparative activities of daptomycin, linezolid, and tigecycline against catheter-related methicillin-resistant *Staphylococcus bacteremic* isolates embedded in biofilm. *Antimicrob Agents Chemother* 51:1656–1660. <http://dx.doi.org/10.1128/AAC.00350-06>.
  27. Heinemann SH, Hoshi T, Westerhausen M, Schiller A. 2014. Carbon monoxide—physiology, detection and controlled release. *Chem Commun (Camb)* 50:3644–3660. <http://dx.doi.org/10.1039/c3cc49196j>.
  28. Garcia-Gallego S, Bernardes GJ. 2014. Carbon-monoxide-releasing molecules for the delivery of therapeutic CO in vivo. *Angew Chem Int Ed Engl* 53:9712–9721. <http://dx.doi.org/10.1002/anie.201311225>.
  29. Schatzschneider U. 2015. Novel lead structures and activation mechanisms for CO-releasing molecules (CORMs). *Br J Pharmacol* 172:1638–1650. <http://dx.doi.org/10.1111/bph.12688>.
  30. Mede R, Loret-Velasquez VP, Klein M, Gorls H, Schmitt M, Gessner G, Heinemann SH, Popp J, Westerhausen M. 2015. Carbon monoxide release properties and molecular structures of phenylthiolatomanganese(I) carbonyl complexes of the type [(OC)4Mn(mu-S-aryl)]₂. *Dalton Trans* 44:3020–3033. <http://dx.doi.org/10.1039/C4DT03567D>.
  31. Motterlini R, Mann BE, Foresti R. 2005. Therapeutic applications of carbon monoxide-releasing molecules. *Expert Opin Invest Drugs* 14:1305–1318. <http://dx.doi.org/10.1517/13543784.14.11.1305>.
  32. Wilson JL, Jesse HE, Hughes B, Lund V, Naylor K, Davidge KS, Cook GM, Mann BE, Poole RK. 2013. Ru(CO)₃Cl(glycinate) (CORM-3): a carbon monoxide-releasing molecule with broad-spectrum antimicrobial and photosensitive activities against respiration and cation transport in *Escherichia coli*. *Antioxid Redox Signal* 19:497–509. <http://dx.doi.org/10.1089/ars.2012.4784>.
  33. Nobre LS, Seixas JD, Romao CC, Saraiva LM. 2007. Antimicrobial action of carbon monoxide-releasing compounds. *Antimicrob Agents Chemother* 51:4303–4307. <http://dx.doi.org/10.1128/AAC.00802-07>.
  34. Davidge KS, Sanguinetti G, Yee CH, Cox AG, McLeod CW, Monk CE, Mann BE, Motterlini R, Poole RK. 2009. Carbon monoxide-releasing antibacterial molecules target respiration and global transcriptional regulators. *J Biol Chem* 284:4516–4524. <http://dx.doi.org/10.1074/jbc.M808210200>.
  35. Tavares AF, Nobre LS, Saraiva LM. 2012. A role for reactive oxygen species in the antibacterial properties of carbon monoxide-releasing molecules. *FEMS Microbiol Lett* 336:1–10. <http://dx.doi.org/10.1111/j.1574-6968.2012.02633.x>.
  36. Tavares AF, Teixeira M, Romao CC, Seixas JD, Nobre LS, Saraiva LM. 2011. Reactive oxygen species mediate bactericidal killing elicited by carbon monoxide-releasing molecules. *J Biol Chem* 286:26708–26717. <http://dx.doi.org/10.1074/jbc.M111.255752>.
  37. Murray TS, Okegbe C, Gao Y, Kazmierczak BI, Motterlini R, Dietrich LE, Bruscia EM. 2012. The carbon monoxide releasing molecule CORM-2 attenuates *Pseudomonas aeruginosa* biofilm formation. *PLoS One* 7:e35499. <http://dx.doi.org/10.1371/journal.pone.0035499>.
  38. Nobre LS, Al-Shahrour F, Dopazo J, Saraiva LM. 2009. Exploring the antimicrobial action of a carbon monoxide-releasing compound through whole-genome transcription profiling of *Escherichia coli*. *Microbiology* 155:813–824. <http://dx.doi.org/10.1099/mic.0.023911-0>.
  39. Nguyen D, Nguyen TK, Rice SA, Boyer C. 2015. CO-releasing polymers exert antimicrobial activity. *Biomacromolecules* 16:2776–2786. <http://dx.doi.org/10.1021/acs.biomac.5b00716>.

40. Kourbatova EV, Halvosa JS, King MD, Ray SM, White N, Blumberg HM. 2005. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA 300 clone as a cause of health care-associated infections among patients with prosthetic joint infections. *Am J Infect Control* 33:385–391. <http://dx.doi.org/10.1016/j.ajic.2005.06.006>.
41. King MD, Humphrey BJ, Wang YF, Kourbatova EV, Ray SM, Blumberg HM. 2006. Emergence of community-acquired methicillin-resistant *Staphylococcus aureus* USA 300 clone as the predominant cause of skin and soft-tissue infections. *Ann Intern Med* 144:309–317. <http://dx.doi.org/10.7326/0003-4819-144-5-200603070-00005>.
42. Li L, Mendis N, Trigui H, Oliver JD, Faucher SP. 2014. The importance of the viable but non-culturable state in human bacterial pathogens. *Front Microbiol* 5:258. <http://dx.doi.org/10.3389/fmicb.2014.00258>.
43. Runger TM, Kappes UP. 2008. Mechanisms of mutation formation with long-wave ultraviolet light (UVA). *Photodermatol Photoimmunol Photomed* 24:2–10. <http://dx.doi.org/10.1111/j.1600-0781.2008.00319.x>.
44. Gudmundsson S, Vogelmann B, Craig WA. 1994. Decreased bactericidal activity during the period of the postantibiotic effect. *J Antimicrob Chemother* 34:921–930. <http://dx.doi.org/10.1093/jac/34.6.921>.
45. Balwit JM, van Langevelde P, Vann JM, Proctor RA. 1994. Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. *J Infect Dis* 170:1033–1037. <http://dx.doi.org/10.1093/infdis/170.4.1033>.
46. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, Peters G. 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* 4:295–305. <http://dx.doi.org/10.1038/nrmicro1384>.
47. Kahl B, Herrmann M, Everding AS, Koch HG, Becker K, Harms E, Proctor RA, Peters G. 1998. Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. *J Infect Dis* 177:1023–1029. <http://dx.doi.org/10.1086/515238>.
48. Melter O, Radojevic B. 2010. Small colony variants of *Staphylococcus aureus*—review. *Folia Microbiol (Praha)* 55:548–558. <http://dx.doi.org/10.1007/s12223-010-0089-3>.
49. Taylor MS, Daniels AU, Andriano KP, Heller J. 1994. Six bioabsorbable polymers: in vitro acute toxicity of accumulated degradation products. *J Appl Biomater* 5:151–157. <http://dx.doi.org/10.1002/jab.770050208>.
50. Wegiel B, Larsen R, Gallo D, Chin BY, Harris C, Mannam P, Kaczmarek E, Lee PJ, Zuckerbraun BS, Flavell R, Soares MP, Otterbein LE. 2014. Macrophages sense and kill bacteria through carbon monoxide-dependent inflammasome activation. *J Clin Invest* 124:4926–4940. <http://dx.doi.org/10.1172/JCI72853>.
51. Noguchi T, Izumizawa K, Fukuda M, Kitamura S, Suzuki Y, Ikura H. 1984. New method for local drug delivery using resorbable base material in periodontal therapy. *Bull Tokyo Med Dent Univ* 31:145–153.
52. Reise M, Wyrwa R, Muller U, Zylinski M, Volpel A, Schnabelrauch M, Berg A, Jandt KD, Watts DC, Sigusch BW. 2012. Release of metronidazole from electrospun poly(L-lactide-co-D/L-lactide) fibers for local periodontitis treatment. *Dent Mater* 28:179–188. <http://dx.doi.org/10.1016/j.dental.2011.12.006>.
53. Ignatova M, Manolova N, Markova N, Rashkov I. 2009. Electrospun non-woven nanofibrous hybrid mats based on chitosan and PLA for wound-dressing applications. *Macromol Biosci* 9:102–111. <http://dx.doi.org/10.1002/mabi.200800189>.
54. Klinger-Strobel M, Suesse H, Fischer D, Pletz MW, Makarewicz O. 2016. A novel computerized cell count algorithm for biofilm analysis. *PLoS One* 11:e0154937. <http://dx.doi.org/10.1371/journal.pone.0154937>.