Prevention of ventilation-associated pneumonia applying a functionalized endotracheal tube and photodynamic therapy: a proof of principle with an artificial intubation model

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Research Article

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Abstract

Background

Biofilm in medical devices occurs with bacterial adherence through virulence factors by a favorable environment for their proliferation. The transfer of microbial cells from biofilm in endotracheal tube to lungs increases the chances of developing severe infections. This study aimed to optimize antimicrobial photodynamic therapy (PDT) by applying a curcumin-functionalized endotracheal tube in an artificial respiratory system avoiding bacterial and their dispersion in the respiratory system, adding a mechanical ventilator.

Methods

This model was built containing three components representing oropharynx, trachea, and lungs. ET-curc was inserted into the system's trachea followed by biofilm formation.

Results

Microbial migration from *Staphylococcus aureus* and Methicillin-resistant *S. aureus* (MRSA) biofilms to the left and right lungs were evaluated with and without mechanical ventilation. PDT was applied to ET-curc using a laser (450nm) and resulted in a total bacterial inactivation, avoiding microbial flow relationships from the upper to the lower air system. The effects indicated high effectiveness in bacterial biofilm inactivation and, consequently, in the prevention of their colonization in lungs.

Conclusion

The results indicated that PDT can be an excellent alternative to prevent the spread of infectious lung diseases by multidrug-resistant microorganisms in patients under mechanical ventilation and provide conditions for starting animal model experiments.

1. Introduction

Lung (LG) related infections have increased in importance and cost in recent years. The prolonged hospitalization times have amplified the problem [1–3]. The reported mortality is 26–74% higher than other nosocomial infections in Intensive Care Units ICU [4]. Anti-pathogen exposure methods are widely described to prevent LG infections. However, most organisms found in clinical isolates of LG infections are genetically different to environmental isolates [3]. Patients using mechanical ventilators have increased 7 to 47% chances of developing Ventilator-associated pneumonia (VAP) [4], which occurs from 2 to 5 days after endotracheal intubation and mechanical ventilation [1, 2]. The intubation procedure is
usually necessary in cases of surgery or emergency, such as respiratory failure, cardiorespiratory arrest, hypoventilation, and respiratory diseases [5, 6].

Routine cleaning in mechanical ventilation is difficult to perform, and only broncho-aspiration is feasible, which increases the possibility of contamination of the lower airways. The recommended preventive measures for avoid VAP are: elevation of 45° the headboard, closed endotracheal suction, subglottic drainage, and decontamination of the oropharynx (ORO) and digestive tract. In addition, antibiotics are prescribed with the same goal of preventing or decreasing the percentage of infections. However, studies have shown that this procedure may not be as efficient against methicillin-resistant \( S. aureus \) (MRSA) [7, 8]. \( Staphylococcus aureus \) is a gram-positive opportunistic bacterium and one of the most frequently isolated species from intubated patients diagnosed with VAP [4].

Medical devices introduced in patients such as endotracheal tubes (ETs) increase the chances of contamination. ETs can contribute to the fixation, colonization, and development of pathogenic bacteria on their surfaces, creating favorable conditions for developing microbial biofilms, as were reported in 84% of ETs removed from intubated patients [8]. Biofilm is a strategic way for microorganisms to protect themselves against external factors such as temperature, pH and antimicrobial agents [9]. The extracellular matrix is formed and reaches 90% of the biofilm biomass, providing protection and nutrition [9], making it up to 100,000 times more resistant to antibiotic agents than planktonic cells [10]. Furthermore, mechanical ventilator connected to the patient will also assist in microbial transport by gas flow, which facilitates the formation and maintenance of biofilms in ETs. The respirator is considered a renewable source of oropharyngeal bacteria and water droplets on the tube [11] and LG.

Proof of principles studies on biofilms colonizing ETs under mechanical ventilation are hard to perform and realistic simulations of ET environments are rare. In this study, we constructed an artificial respiratory system to provide proof of principles that the infection inhibition demonstrated in a previous publication [12] is relevant for clinical settings. Here we describe the development of an artificial model for testing the functionalized ETs with curcumin molecules (ET-curc), in order to demonstrate that the photodynamic action prevent both biofilm formation in the tube and bacterial migration to the lower part of the ventilated respiratory system. This proof of principle is a fundamental and necessary step to progress from \textit{in vitro} tests and to animal models and, finally, to clinical studies.

2. Material and methods

2.1. Reagents

Cesium carbonate and all the solvents were acquired from Sigma-Aldrich, FluoroChem or Kasvi and used without further purifications. The photosensitizer curcumin \((1E,6E)-1,7\text{-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione}\) with 95% purity was supplied by PDT Pharma® (Brazil). The PVC-based endotracheal tube (ET) (Solidor®) is commercially available. Dimethyl sulfoxide (DMSO) was acquired from Sigma-Andrich.
2.2. ET Functionalization

Following our methodology [12], curcumin immobilization onto ET surface was performed by reacting curcumin with the ET surface in the presence of a base. ET (11,8 g) was placed inside a container (A) filled with a curcumin and Cs$_2$CO$_3$ solution in DMSO with the desired concentration (Table 2). For each experimental condition, two ETs were immersed for 1 and 4 hours at 25°C, respectively. After each reaction time, the resulting tube (ET-curc) was removed from container A to container B and extensible washed with DMSO (ca. 500 mL) to remove molecules of curcumin that weren’t bound to the tube. After, ET-curc was washed with ethanol in container C (ca. 500 mL) (Figure S1 A-C respectively). Solvent samples, both used in the washing process, were analyzed through UV-Vis spectroscopy and the process followed until the complete disappearance of the absorption band at 421 nm (Abs$_{max}$ of curcumin), considered criterium for removing all excess molecules. Finally, the tubes were dried and kept in dark at room temperature.

2.3. Curcumin Quantification on ET-curc using UV-Vis

The direct curcumin quantification bonded to ET-curc was performed by UV-Vis analysis on a Hitachi U-2010 spectrophotometer with double-stranded quartz cells (1 cm optical path) using tetrahydrofuran (THF) as solvent. For each section of ET-curc small pieces of the tube were cut, weighted (ca. 100 mg), dissolved in THF (10 mL) and the UV-Vis spectra were collected. The stock solution was diluted as needed to assure compliance with Beer-Lambert law. The curcumin quantity (mg) per gram of ET-curc was determined using curcumin molar absorption coefficient (59359 M$^{-1}$cm$^{-1}$ in THF), obtained through a calibration curve (Figure S2).

2.4. Fluorescence confocal microscopy

Inverted laser scanning confocal fluorescence microscopy (Zeiss – LSM780, Zeiss, Jena, Germany) was used to assess curcumin distribution and penetration on ET-curc surface. The emission is made by an Argon laser (Ar$^+$) emitting at 458 nm operating in continuous mode (cw) and image acquisition interval was fixed through optical filters in the region of 470–700 nm. Samples were 1 cm long of ET-curc pieces. Three-dimensional images approximately 1 mm depth (on the Z axis) were obtained by 10 slices of 100 µm.

2.5. Microorganism Preparation

A pre-inoculum was prepared with *Staphylococcus aureus* (ATCC 25925) and an isolated clinical MRSA. One mL of frozen bacteria was added to 15 mL of a Falcon with 9 mL of Brain Heart Infusion (BHI) broth. The solution was kept in a shaker incubator at 150 rpm overnight at 37°C. The bacterial solution was centrifuged, supernatant was discarded, and the cells were washed twice with phosphate-buffered saline (PBS), pH 7.4.

2.6. Respiratory system
The respiratory system was built based on previously described work [13]. In brief, the system simulates natural and forced transport of microorganisms from oral cavity through the trachea and finally to lungs. The model constructed (Fig. 1A and 1B) consists of three parts representing the LGs, trachea and oral cavity (ORO). Two peristaltic pumps below ORO box are used to introduce bacterial inoculum to ORO, and a second pump to maintain the liquid circulation in the system during the experiments. ET or ET-curc are introduced in the system through the ORO box, where the proximal end remained in the ORO box, and the distal edge was inside the trachea (simulated by a larger PVC tube), closer to the right lung (RL). From ORO box, microdroplets of a solution carrying microorganisms can migrate through this tube getting to be deposited in the ETs or migrate to the LG. Teflon connectors were customized and designed to be placed in each opening of the boxes (LGs and ORO) to connect silicone tubes in the boxes to the pumps. An Arduino system was programmed for controlling the frequency of the contaminated solution and maintaining the LGs temperature at 37°C, kept by a water bath reservoir surrounding them. The system was completely sealed to avoid external communication and exchange of contaminants. The three reservoirs (LGs and ORO boxes) have been sterilized with 70% alcohol and irradiated with an ultraviolet lamp placed inside the microbiology flow for 30 minutes. Silicone tubes and connectors were autoclaved before and after the experiments. Peristaltic pumps were cleaned with 70% alcohol circulating for 5 minutes. Excess alcohol was removed with autoclaved water circulating. The whole system operates continuously as an independent mode. A mechanical ventilator (homemade) promotes the forced airflow from ORO to LGs through the ETs.

2.6.1. Respiratory system contamination

In order to introduce microorganisms into the device, first system components were sterilized as described and ET or ET-curc were placed inside the PVC tube (Trachea) (Fig. 1). The Oropharynx box (ORO box) was contaminated with a S. aureus inoculum solution (10^7 CFU/mL of concentration) in 250 mL of PBS in a beaker (Inoculum). The beaker (inoculum) was connected to a silicone tube in the first peristaltic pump (left). Each LG was filled with 300 mL of PBS, and the ORO box was filled with 200 mL of sterile PBS. After establishing the connections along the whole system, the pumps were activated by an Arduino system programmed to inject the inoculum solution for ten seconds every ten minutes directly to the Oro box. During all the procedures, the temperature was maintained at 37°C in the LGs and the trachea. The temperature produced water vapor around the ET and reached the LGs. One, 2, 3, and 7 days were the periods tested for biofilm formation in ET. Three samples were collected of each LG and the ORO box in each experimental day, diluted and plated. At the end of each experiment, contaminated PBS from the LGs and ORO box were discarded, silicon tubes and connectors were removed and autoclaved, and ET/ET-curc were separated for dilution and plating. The peristaltic pumps were sterilized by circulating alcohol 70%. Tubes were cut in 2 cm, and each piece was added to a Falcon with 20 mL of PBS to remove biofilm cells developed, inner and outer, by one minute stirring. Finally, each group was diluted and plated for CFU/mL quantification.

2.6.2. Mechanical ventilation in the circulating system
A mechanical ventilator (MV) operates with a mechanized chamber built in our Support Laboratory. MV was coupled to a proximal edge system and simulated mechanical ventilation, such as in ICU. The studies using MV were performed to compare the effect of the free and mechanical ventilation applied in a contaminating system to analyze the difference in the infection dynamics in control ET and functionalized ET-curc applying PDT and the number of CFU/mL in the three reservoirs: ORO box and LGs.

2.6.3. Photodynamic inactivation in the respiratory system

The system was assembled, sterilized, maintained at 37°C, ET-curc was added, inoculum with $1 \times 10^7$ CFU/mL was pumped and the system started to circulate. The irradiation system (details in previous studies) composed of a cylindrical and diffuse optical fiber coupled to a laser (450 nm − 140 mW/cm²), placed inside ET-curc [12]. Each irradiation period was maintained for two hours. The tube received 100 J/cm$^{-2}$ illumination dose per cycle of illumination. The parameters studied in the PDT were the curcumin concentration and the number of light doses (1–4 doses). Each experimental group was performed in quadruplicate. The mean and standard error of each group were calculated for CFU/mL. Table 1 describes each irradiation time in the experiment varying the number of light doses.

<table>
<thead>
<tr>
<th>Number of light doses</th>
<th>2h</th>
<th>6h</th>
<th>18h</th>
<th>24h</th>
<th>Total delivered energy (J/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>2D</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>3D</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>4D</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>400</td>
</tr>
</tbody>
</table>

2.7. Statistical Analysis

Data were calculated as mean and SD (standard deviation among experiments). Two-group comparisons were performed by Student’s t-test and One-way ANOVA followed by Tukey's test. Two-tailed p-values < 0.05 were considered statistically significant. Analyzes were performed using the Origin software® academic license granted by the University of São Paulo (USP). Principal Component Analysis (PCA) was performed using the free software GNU Octave® version 5.2.0.

3. Results

3.1. Process optimization for the photoactive curcumin-endotracheal tube preparation
Regarding the ETs functionalization, we highlight that most endotracheal tubes used in clinic are prepared from polyvinyl chloride (PVC). The chlorine atoms presence in ET structure allows for its functionalization through nucleophilic substitution reactions. We recently demonstrated that with this synthetic strategy enables covalent binding of curcumin derivatives to the surface of ETs made of PVC [12]. However, before performing in vivo and clinical tests, studies on the scale-up of this process, optimization of functionalization and curcumin quantification on ET-curc surface are needed. This work addresses these issues.

Initially, high curcumin and base amounts were employed (4.9 mg/mL and 24.0 mg/mL of Cs$_2$CO$_3$; Table 2) [12]. Here, we optimized reaction parameters (curcumin, base concentrations and reaction time) (Fig. 2) using milder reaction conditions. Table 2 presents the results.

First, two ET-curc were prepared using a curcumin concentration of 0.4 g/L and 3.1 g/L of Cs$_2$CO$_3$, one with 1 hour reaction time and the other with 4 hours, ET-curc 1R and 2R respectively. After, the ET-curcs were dried for 2 days in the dark at room temperature. The studies pursued with the development of a new direct quantification approach using ET-curc 1R. First, small pieces from ET-curc 1R upper section were cut and a solid-state UV-Vis spectrum was recorded and compared with the UV-Vis spectrum of the curcumin in a THF solution, Fig. 3. The spectra clearly shows that covalent binding of curcumin does not compromise the chromophore, because curcumin attached to ET-curc preserves its spectral properties. However, this methodology does not allow the quantitative determination of the curcumin due to signal saturation at higher curcumin concentrations and heterogeneity of the ET-curc solid sample.

To overcome this limitation an alternative methodology for curcumin quantification on ET-curc was developed. Small pieces taken from the same ET-curc section (top) were cut, weighted, and dissolved in THF (10 mL). As selected example, the UV-Vis spectrum of ET-curc 1R was recorded and is presented in Fig. 3, red line. The quantitative curcumin determination of ET-curc functionalized under different reaction conditions was carried out using a curcumin calibration curve in THF (Figure S2) and the results are presented in Table 2.

From this quantification procedure, 0.05 and 0.14 mg of curcumin/g of ET-curc were obtained for 1- and 4-hours reaction time (Table 2, ET-curc 1R and 2R, respectively). These results indicated that the amount of curcumin bound to ET is critically dependent on the reaction time and consequently the following experiments were carried out over four hours. Then, aiming to find milder reaction conditions for pilot scale industrial ET functionalization the curcumin effect (2.5 and 3.1 g/L) and Cs$_2$CO$_3$ (0.8 and 3.1 g/L) concentrations was evaluated and tubes 3R, 4R and 5R were prepared. Under these conditions, no significant differences of immobilized curcumin amount were obtained (0.22–0.25 mg/g). Therefore, we selected the combination: 2.5 g/L of curcumin with 0.8 g/L of Cs$_2$CO$_3$, as the ideal conditions for preparation of the tubes ET-curc 5R used in the following experiments.
Table 2
Reaction conditions used in the curcumin immobilization process and the final amount of incorporated curcumin in the ET-curc. a)

<table>
<thead>
<tr>
<th>ET-curc</th>
<th>Curcumin [g/L]</th>
<th>Cs$_2$CO$_3$ [g/L]</th>
<th>Time (h)</th>
<th>[Curc] on ET-curc mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R</td>
<td>0.4</td>
<td>3.1</td>
<td>1</td>
<td>0.05</td>
</tr>
<tr>
<td>2R</td>
<td>0.4</td>
<td>3.1</td>
<td>4</td>
<td>0.14</td>
</tr>
<tr>
<td>3R</td>
<td>2.5</td>
<td>3.1</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>4R</td>
<td>3.1</td>
<td>0.8</td>
<td>4</td>
<td>0.23</td>
</tr>
<tr>
<td>5R</td>
<td>2.5</td>
<td>0.8</td>
<td>4</td>
<td>0.22</td>
</tr>
<tr>
<td>6 Conditions from [12]</td>
<td>4.9</td>
<td>24.0</td>
<td>4</td>
<td>0.70</td>
</tr>
</tbody>
</table>

a) DMSO solutions, 25°C.

It is known that under clinical practice, long-term intubated patients can develop infections resulting from microorganisms that grow in the inner and outer endotracheal tube surfaces, being the cuff area one of the most critical regions (Fig. 4 detail C).

Thus, to evaluate the curcumin homogeneity distribution over the ET-curc, cross-sectional quantification of curcumin in three longitudinal parts (A, B and C) was carried out and the results are presented in Table 3. The values obtained for curcumin immobilized in each section are approximately the same (ca. 0.27 mg/g) which indicates a homogeneous curcumin distribution.

Table 3
Quantification of incorporated curcumin in different ET-curc positions as described in Fig. 4.

<table>
<thead>
<tr>
<th>Curcumin</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C]°mg/g</td>
<td>0.30 ± 0.02</td>
<td>0.27 ± 0.02</td>
<td>0.25 ± 0.03</td>
</tr>
</tbody>
</table>

a) Quantification by UV-Vis using a transversal section of ET-curc 5R areas. A; B and C.

Another relevant issue for ET-curc characterization is curcumin quantification in the inner and outer tube surfaces. The quantitative curcumin distribution over the outer and inner ET-curc surfaces was also determined and a mean value of 3.40 × 10$^{-4}$ mg/mm$^2$ of curcumin was obtained for the external surface of the tube (see details in SI, Figure S3). Additionally, the thermal stability of ET-curc 5R was assessed by thermogravimetric analysis (TG) and no significant differences from the pristine ET were observed. This analysis points out that, under the selected reaction conditions, the curcumin immobilization onto ET does not affect its thermal stability indicating maintenance of the physical proprieties (Figure S4).

3.2. Respiratory system characterization with ET.
According to the capacity of natural transport of bacteria using the system, the next step of the experiments was to evaluate photodynamic inactivation.

### 3.3. Photodynamic inactivation for illuminated ET-curc coupled to the system

In order to optimize the parameters to obtain an effective photodynamic disinfection in the respiratory system, the tubes tested were prepared according to the conditions described in Table 2: **4R** (0.8 g/L $\text{Ce}_2\text{CO}_3$ and 3.1 g/L curcumin) and **5R** (0.8 g/L $\text{Ce}_2\text{CO}_3$ and 2.5 g/L curcumin). The functionalized ET-curc was placed in the tracheal position and several light doses were tested. In the first set of experiments, mechanical ventilator was off. Figure 6A and B display the results for the measured colonization quantity along the tube position and in Orobox and Lungs applying one light dose (100 J/cm²). Figure 6C and D described results quantifying biofilm bacterial cells from the ET-curc **5R** irradiated with four light doses, and in Oro box and Lungs. The best parameters (ET-curc **5R** and four light doses per day) were applied for 7 days (Fig. 6E and F).

### 3.4. Mechanical ventilation coupled to the ET-device

The results above considered the infection transport promoted by natural movement of aerosols and droplets present in the system at 37 °C. To demonstrate the performance of our system under conditions closer to real conditions, a mechanical ventilation was coupled to promotes a forced motion. ET and ET-curc **5R** were tested here. The results obtained with forced air motion conditions are presented in Figs. 7A and B.

### 4. Discussion

The strategy to functionalize polymeric materials is relevant for avoid infections, specially when these materials are placed in the patient, such as catheters and endotracheal tubes. The optimization studies focusing on studying different curcumin concentrations immobilized in the tube were significant to understand the influence of the reaction time and how the curcumin concentration would influence in the physical properties of the ET and, after, in *in vitro*. Longer periods of reaction time are better for curcumin establishes a bound with the polymeric material and concentrations of: 3R, 4R and 5R resulted in similar effects of saturation, with no physical modification in the tube. According to these results, it was possible to introduce two different functionalized tubes in a respiratory system built to simulate an intubated patient.

This system was characterized by biofilm development on the tube without immobilization for seven days to understand how the biofilm would growth and how the contamination would spread on oral cavity, in the trachea and in the lungs. The results showed (even though not statistical different) a *S. aureus* cells migration from the upper (ORO box) to the distal end of the ET, located in the trachea in the end of the seven days. In 24 h, the bacterial cells reached both LGs and ET’s surface, presenting a more homogeneous adhesion of cells along the tube compared to the other periods longer tested. Therefore,
according to an increase in circulation time, the biofilm developed was more heterogeneous and irregular along the tube, resulting in more adhesion in the proximal part, the one closest to the Orobox. Despite the biofilm heterogeneity, statistical analyses (ANOVA and PCA, supplementary material – Figure S5) have not revealed a statistical difference between both extremities and among the same positions on different training days. Equally, the ORO and LG contamination have been followed throughout seven days of operation (Fig. 5B). The system ability to transport infection through the ET is proved by the obtained results (Fig. 5) and the variations observed along the ET are a demonstration of a uniform contamination in fewer days. Nevertheless, in the group circulated for seven days, positions from 16 to 20 cm, positions closer to ORO box, revealed a tendency to increase bacterial load. This is in agreement with observations in the literature showing a more efficient adhesion of bacterial cells in the proximal part [14]. Furthermore, proximal extremity showed a more significant variability when compared to the distal part and it can be due to the proximity to ORObox, where the contaminant solution circulated always, as microorganisms enter there. The variability in this part of ET was expected, as the mouth is easily accessible for the microorganism to enter, and there are already many commensal strains on it. Although the ORObox seems to always have slightly higher values of contaminants, it remains in the same order of colonies concentration as in the lungs.

The second analyze was based on studying parameters for optimizing photodynamic inactivation. ET-curc applying 4R condition showed more heterogeneous inactivation along the tube than 5R concentration, with less significant biofilm inhibition. Figure 6C and D confirmed the direct relationship between the light doses number and inactivation action along the tube. A more effective S. aureus photodynamic inactivation is achieved when the light dose increases. One light dose decreased 0.2 logs of CFU/mL and two light doses decreased 0.5 log CFU/mL of 24 hours of biofilm formation. Three light doses increased biofilm inactivation by almost 2 logs of CFU/mL, and four light doses delivered in 24 hours achieved 7 logs of CFU/mL decrease along all tube. Equivalently, Fig. 6D shows the decrease in contamination of the left and right lungs occurred while the ORObox contaminations was kept at a high level. Applying 3 or 4 doses of daily illumination resulted in no contamination of the lungs. The prevention of the contamination through the ET to the lungs was achieved. This is an important result and a proof of principle that our method for photodynamic inactivation can prevent infections from lower respiratory tract during endotracheal intubation. According to these results, four light doses per day, each of 100 J/cm², were chosen to analyze the kinetics and dynamics of the system with the circulation of bacteria for seven days. Figures 6E and F describe the results for a seven-day period of experiments with 4 irradiations per day using an ET-curc 5R. Comparing the dark groups (control) with the illuminated ones, the difference is quite remarkable. The complete infection transmission blockage is observed and maintained over time without deactivation of the ET-curc. Although few colonies were found in the LGs (second and third day), they have not been statistically relevant according to the ANOVA test. ET-curc irradiated four times a day inactivated bacterial biofilms and prevented the colonization of the surface of the tube. The irradiation of ET-curc tubes may avoid bacteria cells to spread from ET to the lungs of patients and prevent VAP development.
When the mechanical respirator was introduced and coupled to the system, similar photodynamic inactivation results were obtained when it was compared to the previous one with natural transport. Therefore, all photodynamic inactivation groups are statistically different from their controls, demonstrating an effective inactivation, even at the highest contamination at the lowest exposure time with the mechanical ventilator associated. The results from controls indicated a change in the biofilm development dynamics along the ET when the ventilator was coupled. When the ventilator was not operating, the biofilm developed homogeneously along the tube, whereas with MV it shows an increase in the central region. A cuff at the distal end can also contribute to the adherent increase in cells on it. To understand the treatment dynamics, a 24-hour kinetic experiment was performed with and without the ventilator in PI groups. Both groups were irradiated four times (100 J/cm² each time) and resulted in a more variance in the *S. aureus* cells number in ORO box (Fig. 7B), probably due to the circulation of contaminated liquid over time. Both LGs have a similar behavior with or without MV. In addition to the reported results, equivalent experiments were carried out using MRSA. The contamination prevention with ET-curc and illumination was similar to that of non-resistant bacteria. Such findings imply that bacterial-resistant species are not an obstacle to our approach to photodynamic disinfection. Therefore, based on these studies, which the photodynamic therapy acted such as a photodynamic barriers to avoid the infection to spread, it was possible to find the best parameters to perform pre-clinical assays.

**Conclusion**

Our work using an artificial trachea model shows that photodynamic inactivation associated with curcumin-functionalized ET (ET-curc) can minimize the problem of biofilm formation and lung contamination in patients submitted to mechanically ventilated respiratory support. The functionalization of PVC tubes with curcumin was optimized to work under mild conditions, with much lower concentrations of base, and offer a homogenous functionalization. The reduction of bacterial load most successful conjugating curcumin functionalized ET with sequential delivery of light doses. These results demonstrate the potential of this technique to real-world ventilation-associated pneumonia prevention. The optimization of the photodisinfection conditions in a respiratory system model, such as done here, paves the way to evaluate our approach in intubated animals and minimizes the use of animals in such experiments. Preliminary investigations with animals are already in progress.

**Declarations**

**Acknowledgments**

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Ethical approval

No ethical approval was necessary for this paper.

Competing interest

The authors declare no competing interest.

Authors’ contributions

A.C.Z.: wrote the manuscript, performed the system experiments and data analysis, prepared the figures 1-2, 5-7, and the discussion.

F.M.R.: performed the synthesis tests, wrote this part of the manuscript and also prepared figures 3 and 4.

L.P: Built the circulated system and performed the system experiments.

M.M.P.: reviewed the manuscript and worked in discussion part;

L.A.: reviewed the manuscript and worked in discussion part;

V.S.B.: helped with the experimental ideas, discussion and reviewed the manuscript.

K.C.B.: helped with the experimental ideas, discussions and reviewed the manuscript.

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Availability of data and materials

All data acquired during the work are described in this paper and in supplementary material.

References


Figures
Figure 1

A) Circulating system image describing components and their positions in the system; from left to right: LGs, temperature control, trachea, inoculum solution, ET, peristaltic pumps, Orobox and mechanical ventilator; B) A representative figure describing the system.
Figure 2

Covalent linkage of curcumin photosensitizer onto PVC-based ET producing the ET-curc device.

Figure 3
Solid-state UV-Vis's spectrum of ET-curc 1R (black), UV-Vis spectrum of ET-curc 1R solution (120 mg in 10 mL THF) (red), UV-Vis spectrum of curcumin in THF solution, [Curcumin] = 1.2 × 10^{-5} M (blue) and UV-Vis spectrum of a THF solution of non-functionalized ET (100 mg in 10 mL THF) (green).

Figure 4

Selected regions (A, B, C) of ET-curc 5R prepared according to the procedure described in Table 2.

Figure 5

A) *S. aureus* CFU/mL biofilm development along the surface of the ET measured in linear position in the circulating system with four periods tested: one (black square line), two (red circle lines), three (blue
triangle line) and seven days (green triangle line); B) CFU/mL quantification in LGs and ORO box at first, second, third and seventh days of circulation system. * Statistical difference between the groups according to the Tukey ANOVA test one-way with p-value < 0.05. (0 days imply the starting point with zero CFU, this point is not shown on the plot).

Figure 6
A) PDT of biofilms developed for 24h in different functionalized tubes 4R and 5R and compared to control (ET) using only one light dose of 100 J/cm². B) *S. aureus* number cells from LGs and Oro box: control, ET-curc 4R and 5R; C and D) PDT of biofilms developed with 24 h exposure of ET-curc 5R for different light doses. C) 1D – one light dose, 2D – two light doses, 3D – three-light doses and 4D – four-light doses. D) *S. aureus* biofilm developed in the control groups (ET- light gray bar) and photodisinfection groups after 24 hours; each light dose corresponded to 100 J/cm²; bacterial cells were counted along each tube. E) Optimized results of 7 days of inhibition of PS-ET biofilm irradiated four times a day with 100 J/cm² compared to the biofilm formed by seven days on the ET-curc surface; F) Number of bacterial cells in the LGs and ORO box in the experiment with four dose lights per day (100 J/cm² each) during seven days of the circulation system. *Statistical difference between groups according to one-way ANOVA, Tukey's test (p<0.05).

**Figure 7**

A) CFU/mL of biofilm developed along ET and ET-curc 5R when the system was coupled with MV and *S. aureus*. The control groups were: ET-MV (blue triangle line), and ET (black square line); photodynamic inactivation groups: ET-curc-MV (green triangle line) and ET-curc (red circle line). B) *S. aureus* CFU/mL in the ORO box (light gray bar), in the Left Lung (medium gray bar) and Right Lung (dark gray bar) in ET and ET-MV (controls) and ET-curc and ET-curc-MV (photodynamic inactivation). * Statistical difference between groups according to the ANOVA one test way Tukey (p<0.05).

**Supplementary Files**

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