

1 **An introduction to antibiotic-free techniques to eliminate**

2 ***Staphylococcus aureus* from blood**

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## Abstract

15           Here, we describe the implementation of three techniques for capturing and  
16 killing *Staphylococcus aureus* in blood *in vitro* inside a medical tube. The first technique  
17 involves capturing and removing pathogens using antibodies that are coated, via a  
18 simple chemical process, on the inner walls of a modified medical tube (tube capturing  
19 technique). In the second technique, a photosensitizer-antibody conjugate adheres to  
20 the pathogens while in circulation. When blood flows through the same kind of tube, the  
21 conjugate is activated by near-infrared (NIR) light to kill pathogens (photodynamic  
22 therapy technique). For the third technique, pathogens are exposed to light in the  
23 ultraviolet (UV) range while circulating through a similar tube (UV technique), which kills  
24 the pathogens.

25           We spiked blood with *S. aureus*, starting with about  $10^7$  CFU/mL and ending at  
26  $10^8$  CFU/mL after 5 hours. While the spiked bacteria rapidly grew in nutrition-rich whole  
27 blood, each of the three techniques were able to independently remove between 61%  
28 and 84% more *S. aureus* in the experimental blood sample compared to the controls  
29 groups. When combined, these techniques demonstrated a removal rate between 87%  
30 and 92%.

31

## 32 Introduction

33 Antimicrobial resistance is rapidly becoming a major health concern [1-3].  
34 Resistance to antibiotics used against common pathogens, such as *S. aureus*, poses  
35 significant medical risks [4-9]. For instance, Methicillin-resistant *S. aureus* (MRSA) killed  
36 approximately 11,000 Americans in 2012 and resulted in 278,203 hospitalizations [10,  
37 11]. The annual treatment cost associated with surgical site infections of MRSA is \$12.3  
38 billion in the USA [12]. It is evident that there is a dire need for improved treatments for  
39 patients with difficult-to-cure blood-borne infections such as MRSA.

40 Recently, a microfluidic device that relies on magnetic bead separation and a  
41 special bio-engineered molecule [13] was used to remove blood borne bacteria.  
42 Extracorporeal hemofiltration / hemoadsorption systems have been suggested to  
43 reduce cytokines [14] and endotoxins [15, 16] from septic blood.

44 Here, three antibiotic-free methodologies, which are activated while blood flows  
45 through a commercially available transparent tube, are introduced with a focus on *S.*  
46 *aureus* removal and killing. These techniques, shown in Fig. 1 are: (a) pathogen  
47 removal by a chemically modified medical tube coated with antibodies to capture the  
48 desired pathogens, (b) pathogen inactivation by photodynamic therapy (PDT) using  
49 photosensitizer-antibody conjugates that selectively bind to the pathogen while in  
50 circulation, allowing for the killing of these pathogens when exposed to NIR light as the  
51 blood flows through a transparent tube, and (c) UV (and more broadly near-UV)  
52 irradiation as the blood flows through the tube.

53 In previous efforts, we demonstrated the feasibility of the first two techniques for  
54 the removal and killing of human tumor cells [17, 18]. In this article, we describe the

55 extension of our effort to microbial organisms in constant flow and agitation to imitate *in*  
56 *vivo* conditions. We also report on our investigation into the effects of combining these  
57 techniques.

58

## 59 **Materials and Methods**

### 60 **Bacterial culture**

61 *S. aureus* were purchased from the American Type Culture Collection (ATCC  
62 12598). The bacteria were propagated in ATCC Medium 3 (nutrient broth or agar) at 37  
63 °C in a shaking incubator. Bacterial concentrations were determined by both OD-600  
64 value (optical density at 600 nm wavelength), which was measured with a UV-VIS  
65 spectrometer (Spectronic 20D+, Spectronic Instrument) in broth and its corresponding  
66 colony count from the agar plate. The initial OD-600 value in the range of 0.02-0.04  
67 (about  $1-2 \times 10^7$  CFU/mL, determined by calibration). Human whole blood with an  
68 anticoagulant (sodium citrate) was purchased from vendors including Innovative  
69 Research (Novi, MI).

### 70 **Technique (a): Tube capturing**

71 A polydimethylsiloxane (PDMS) tube (Dow Corning Silastic laboratory tubing with  
72 an internal diameter of 1.02 mm) was used for this study. The tube length was  
73 approximately 120 cm and was prepared in a manner similar to what has been  
74 described in our previous publication [18]. Specifically, the tube's internal surface was  
75 activated by treatment with an acidic hydrogen peroxide solution ( $H_2O:HCl:H_2O_2$  in 5:1:1  
76 volume ratio) for five minutes at room temperature [19]. The tube was rinsed with  
77 excess deionized (DI) water five times and dried in air. The tube was then filled with

78 aminopropyltrimethoxysilane (APTMS) for 10 minutes. The tube was rinsed with excess  
79 DI water at least five times and dried in air.

80 *S. aureus* polyclonal antibody (PA1-7246, Life Technologies) was treated for 1.5  
81 hour with Traut's reagent (2-iminothiolane HCl, 2-IT) to generate an available sulfhydryl  
82 group (-SH) (antibody:2-IT=1:10 in mole ratio) in PBS (pH 7.4). Then, unbound 2-IT was  
83 removed from the antibodies using a protein concentrator (MWCO 30 kDa, Corning  
84 Spin-X protein concentrator) at 5000 RCF for 30 minutes. The concentrated *S. aureus*  
85 pAb was re-suspended in PBS, and the volume was adjusted to 1.2 mL. During the  
86 antibody-2-IT reaction, the amine functionalized tube was filled with a hetero-  
87 bifunctional crosslinker, sulfo-SMCC (sulfosuccinimidyl 4-[N-  
88 maleimidomethyl]cyclohexane-1-carboxylate) in 2 mg/mL concentration in PBS (pH 7.4).  
89 After the 2-IT treated pAb was spun down, the sulfo-SMCC solution was removed, and  
90 the tube was rinsed in PBS and re-filled with 1.2 mL re-suspended *S. aureus* pAb  
91 solution. The reaction was run on a shaker for two hours at room temperature and  
92 continued overnight at 4 °C. The next day, after the unbound antibody solution was  
93 collected, the tube was gently rinsed with PBS and then refilled with 2 mg/mL L-cysteine  
94 for another two hours. The tube was rinsed and filled with PBS and stored at 4 °C until  
95 use.

96 0.5 mL of *S. aureus* (10 x concentrated) were added to 4.5 mL of whole blood,  
97 resulting in 5 mL of 1x bacterial solution in blood (estimated bacterial input was 1-2 x  
98 10<sup>7</sup> CFU/mL) in sterile 15 mL culture tube. The initial bacterial concentration was  
99 estimated by monitoring the OD-600 value of sample diluted in the same way in broth  
100 instead of blood. Blood-bacteria mixtures were stirred with a mini magnetic stirrer to

101 prevent blood from settling down by gravity over time. *S. aureus* pAb immobilized tubes  
102 were connected to the blood-bacteria mixture with both ends submerged into the  
103 solution.

#### 104 **Technique (b): PDT**

105 2 mg of a NIR photosensitizer, Chlorin E6 (Ce6, Frontier Scientific) were mixed  
106 with 6.5 mg of 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC,  
107 crosslinker) (Sigma-Aldrich) and 7.6 mg of sulfo-NHS (stabilizer for EDC) (Pierce) in 1  
108 mL 10% Dimethyl sulfoxide - PBS buffer (DMSO:PBS=10:90), (Ce6:EDC:sulfo-  
109 NHS=1:10:10 in mole ratio). The reaction was run at room temperature with agitation for  
110 2 hours. Then, 1 mL of 200 µg/mL *S. aureus* pAb in 10% DMSO-PBS mixture was  
111 mixed with 1 mL of Ce6 mixture. The conjugation reaction was run at room temperature  
112 with agitation for 3 hours. The reaction mixture was spin-filtered with a protein  
113 concentrator to remove the unbound Ce6 and other chemicals from the desired Ce6-  
114 antibody conjugates at 5000 RCF for 15 min, and the procedure was repeated 4 times  
115 with refilling excess 10% DMSO-PBS solution. The final product was re-suspended in  
116 PBS, adjusting the final volume of 1 mL. The produced Ce6-conjugated *S. aureus* pAb  
117 was stored at 4 °C. All conjugates were consumed within 1-2 weeks from their  
118 preparation.

#### 119 **Technique (c): UV (or near UV) exposure of extracorporeal tube**

120 Its germicidal effects have made UV light a valuable tool for killing bacteria and  
121 viruses. UV irradiation has been used in surgical wound disinfection with high success  
122 rates for eliminating bacteria [20, 21]. Here, *S. aureus* spiked blood samples were  
123 circulated through an unmodified PDMS tube, which was inserted into an illumination

124 chamber. UV light (ODYSSEA UVC-18W, centered at 254 nm) was used to illuminate  
125 the tube inside the illumination chamber.

## 126 **Combination of multiple techniques**

127 Three individual techniques were combined in different configurations of two and  
128 three. The combinations used were: PDT-tube capturing (using 1 capturing tube and 1  
129 unmodified tube), UV-tube capturing (using 1 capturing tube and 1 unmodified tube),  
130 and PDT-UV-tube capturing (using 1 capturing tube and 1 unmodified tube for both PDT  
131 and UV). In all combinations, each tube was directly connected to one blood sample in  
132 a parallel connection. Tubes for PDT, UV, or both were inserted into illumination  
133 chambers with NIR LED lamps and UV lamps installed on top.

## 134 **Experimental setup**

135 The experimental setup included three major components: a temperature-  
136 controlled bath, a peristaltic pump, and an illumination chamber. *S. aureus* spiked blood  
137 samples were placed in 15 mL sterile culture tubes and in a water bath heated to 37° C  
138 on a small heating stirrer plate. In PDT, the Ce6-pAb conjugate (200 µL) was added to  
139 the blood. The blood sample was agitated with a mini magnetic stirrer (7 mm x 2 mm)  
140 inside the solution. A 120 cm tube for techniques (a), (b), and (c) was connected to the  
141 blood sample in the water bath. The tube was installed through a peristaltic pump (P-3,  
142 Pharmacia) to maintain a constant flow of blood. Part of the tube was inserted into a  
143 cube-shape illumination chamber (12 x 12 x 12 inch) with mirror walls to reflect the light.  
144 The temperature inside the illumination chamber was monitored and tested for several  
145 hours to ensure that heat generated by the 660 nm LED lamp and the UV lamp did not  
146 reach temperatures that could cause thermal damage to cells. The chamber

147 temperature was equilibrated in the range of 29 - 31° C. The tubes were connected to  
148 the blood samples in a water bath to complete the circulation system. The entire set up  
149 (shown in Fig 1 (c)) was installed inside a biosafety cabinet to prevent contamination.  
150 The *S. aureus* containing blood mixture was circulated through the tube with peristaltic  
151 pumping at 0.5 mL/min for 5 hours. In the PDT case, in order to allow sufficient time for  
152 the antibody conjugates to bind with *S. aureus*, the blood mixture was circulated through  
153 the tube without illumination for the first 2 hours. Illumination by NIR light was then  
154 performed for 3 hours.

### 155 **Processing samples and colony counting**

156 *S. aureus* was pipetted from a culture tube and diluted until the value fell  
157 between  $1 \times 10^7$  and  $2 \times 10^7$  CFU/mL (initial OD-600 0.02-0.04). We included a control  
158 each time the experiment was performed to account for the variation in bacteria  
159 concentration and growth patterns between stocks (deviation from stock to stock).

160 During the procedure, 50  $\mu$ L blood samples were extracted at the following time  
161 intervals: 0, 1, 3, and 5 hours. The sample was diluted in 450  $\mu$ L of nutrient broth (10 x  
162 dilution) and diluted 3 more times in same manner (100 x, 1000 x, 10000 x dilution). 10  
163  $\mu$ L of each of the diluted samples were streaked on a 5% sheep blood agar plate  
164 (Fisher Scientific) that was divided into quadrants. The bacteria colonies were allowed  
165 to grow overnight. The colonies were then counted using the particle analysis function in  
166 ImageJ (National Institutes of Health). Each experiment and control was repeated 5  
167 times.

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169



## 170 **Results**

171           Technique (a), tube capturing, yielded bacterial growth  $11.4 \pm 4.3 \times 10^7$  CFU/mL  
172 (mean  $\pm$  standard error of mean (SEM), n=5), while the control growth (from same  
173 stock) was  $60.5 \pm 16.3 \times 10^7$  CFU/mL at 5 hour as shown in Fig. 2 (a). Using only PDT,  
174 there was a reduction of  $10.9 \pm 3.0 \times 10^7$  CFU/mL, whereas the control group produced  
175 a reduction of  $40.9 \pm 10.1 \times 10^7$  CFU/mL (Fig. 2 (b)). UV tube exposure was the least  
176 effective of the techniques when applied alone with  $32.8 \pm 4.2 \times 10^7$  CFU/mL vs.  
177  $83.8 \pm 2.5 \times 10^7$  CFU/mL for the control (Fig. 2 (c)). When PDT and tube capturing were  
178 combined, there was a reduction in bacteria of  $9.2 \pm 3.5 \times 10^7$  CFU/mL vs.  $69.4 \pm 8.31 \times$   
179  $10^7$  CFU/mL for the control (Fig. 2 (d)). When combining UV and tube capturing the  
180 CFUs were  $9.0 \pm 2.7 \times 10^7$  CFU/mL vs.  $80.4 \pm 4.3 \times 10^7$  CFU/mL for the control (Fig. 2  
181 (e)). Finally, when all three techniques were combined, the experimental group's  
182 reduction in bacteria was  $4.2 \pm 1.0 \times 10^7$  CFU/mL, compared to  $59.7 \pm 2.5 \times 10^7$   
183 CFU/mL for the control group (Fig. 2 (f)).

184

## 185 **Discussion**

186           Given that the initial bacteria count was not exactly the same for the control and  
187 the experiment groups, the values in Fig. 3 were normalized before comparison with  
188 controls. Using tube capturing, there were 83.4% fewer bacteria, while using PDT there  
189 were 71.4% fewer bacteria. UV light yielded a 61.6% bacteria reduction. Among the  
190 three individual techniques, capturing appeared to be the most effective for removing  
191 bacteria. Combining these techniques yielded improved suppression of *S. aureus*  
192 growth. When PDT and tube capturing were combined, there was a reduction in

193 bacteria CFU by 87.1%. When UV and tube capturing were combined, the percent  
194 reduction of the bacteria CFU was 89%. Finally, when all three techniques were  
195 combined, the experimental group's reduction in bacteria was 92%.

196 When combined, these techniques reduce bacterial growth at the given bacteria  
197 concentrations *in vitro* with higher efficiency than individual techniques. These  
198 conclusions are dependent on the initial bacteria concentration, type of bacteria, and  
199 organism. The utilization of a thin plastic transparent tube provides the foundation of  
200 these three techniques. Tube capturing does not kill bacteria, so using this technique  
201 alone will likely lead to bacteria repopulation. Thus, the two additional methods were  
202 employed to further inactivate the pathogens.

203 The utilization of a thin tube was essential for PDT to function in blood. PDT is  
204 based on the activation of photosensitizers by light. The presence of hemoglobin in  
205 blood (a strong light absorber) blocks the majority of light to achieve effective PDT. PDT  
206 has been used in applications where deep tissue penetration by light is not required (e.g.  
207 skin cancer [22, 23], lung [24, 25], head and neck cancer [26, 27], and some dental  
208 conditions [28, 29]). Our set-up allowed PDT to function successfully in blood by  
209 utilizing: (a) a thin transparent tube that allows the entire blood sample volume flowing  
210 through the tube to be exposed to NIR light, and (b) a NIR photosensitizer with an  
211 excitation wavelength of 660 nm where the light absorption by hemoglobin is minimal.

212 PDT's efficacy is based on oxidative damage by locally induced reactive oxygen  
213 species. PDT efficacy depends on oxidative stress, which is non-specifically effective  
214 within narrow vicinities. Thus, PDT can kill antibiotic-resistant microorganisms, such as  
215 MRSA [30, 31]. The photosensitizer must be selectively delivered by conjugating with

216 an adhesion molecule to target organisms to prevent collateral damage to other blood  
217 components.

218         The presence of excess and unbound photosensitizers (Ce6-pAb in this case) in  
219 the blood may cause non-specific damage to blood cells. Preliminary studies by other  
220 groups indicate that PDT in blood "is safe *in vivo*" [32, 33]. Potential toxicity will be  
221 investigated in the future given that toxicity investigation is more relevant when this  
222 technology is advanced enough for animal studies in which the bone marrow and the  
223 body's filtering organs create a more realistic scenario. Current clinical practices of PDT  
224 require a waiting period to minimize undesired collateral damage [34]. This period  
225 occurs between the application of the photosensitizer and the light illumination to allow  
226 for the photosensitizer to accumulate in target cells/tissues and for the excess  
227 photosensitizer to be cleared by the body through its natural filtering mechanisms. Such  
228 clearance cannot be emulated in *in vitro* conditions. The dosage of the photosensitizer  
229 and appropriate waiting time will have to be carefully determined by further studies to  
230 maximize the PDT's efficacy on targeted pathogens and at the same time to minimize  
231 adverse effects from unbound photosensitizers in circulation.

232         Another concern regarding toxicity is collateral damage to distant cells by  
233 reactive oxygen species' (ROS) diffusion/convection from targeted pathogens, which is  
234 highly unlikely. The predominant ROS in photosensitization is the molecular singlet  
235 oxygen ( $^1\text{O}_2$ ), which is extremely short lived and has very limited free diffusion distance  
236 (reportedly, 0.01-0.02  $\mu\text{m}$ ) in biological media [35, 36]. Also, prior research has  
237 demonstrated that PDT's efficacy is strictly confined to targeted cells in the mixture of  
238 different cells [37]. Future toxicity studies will analyze targeted versus non-targeted cell

239 death with such studies as complete blood count (CBC) differential test, applying  
240 separate fluorescent tags, or radiolabels, in addition to a cell viability assay and  
241 analyzing cell death with a cell sorting technology.

242 Utilizing thin tubes enables the use of UV in blood. The germicidal effect of UV  
243 light is well established and has been widely used in sterilization, including sterilization  
244 of wounds. Health risks by UV, including carcinogenesis, have restricted the use of UV  
245 light in humans. Here, UV irradiation was performed only on tubes residing in a mirrored  
246 chamber. Hemoglobin in blood significantly blocks light in the UV range, thus the  
247 resulting penetration depth of UV would be even shorter than that of PDT [38, 39].  
248 Therefore, it seems that bacterial killing by UV likely occurs very close to the tube's  
249 inner surface, which may explain the lower efficiency of this technique compared to  
250 capturing and PDT. The appeal of using germicidal light lays in the non-specific damage  
251 mechanism that allows for effectively eliminating most microorganisms. Potential toxicity  
252 by UV irradiation on normal blood components will be investigated in future work.  
253 However, it is worth noting that recent findings have shown that wavelengths in the low  
254 400 nm are also capable of disinfecting pathogens, including *S. aureus* and MRSA [40-  
255 43] and that short wavelength UV rays (UVC, 207 nm) can selectively kill bacteria with  
256 negligible damage to mammalian cells [20]. Further investigation will be conducted  
257 exploring various wavelengths to effectively inactivate pathogens without health risk.

258 There are a number of ways that these techniques can be further improved. For  
259 instance, introducing an additional antibody or binding molecule may further enhance  
260 efficiency. In this work, capturing and PDT both used the same antibody to bind to *S.*  
261 *aureus*. Introducing an additional binding molecule could reduce a potential competition

262 for binding sites. The technology presented here is currently limited in its capacity to  
263 handle high volumes of blood efficiently. Various engineering optimizations are  
264 underway to address conditions for larger animals and humans.

265 The capturing tube and the photosensitizer-antibody conjugates can be easily  
266 prepared with a specific antibody. Adhesion molecules that target a large group of  
267 pathogens will be included in future experiments, eliminating the need to first identify the  
268 pathogen. These general-purpose molecules can be used to coat the tube and create  
269 the conjugate. For instance, antibodies or molecules adhering to galactose-alpha-1,3-  
270 galactose (alpha gal), a carbohydrate found in the cell membrane of most organisms,  
271 but not in human cells, can be used as a target.

272 In addition to removing *S. aureus*, these techniques can be applied to remove  
273 Gram-positive and Gram-negative bacteria, fungi, viruses, parasites and other types of  
274 microorganisms using appropriate adhesion molecules. These techniques can be used  
275 to reduce infectious particle load to minimal levels or at levels where conventional  
276 medication and the body's own immune system can fight the infection. Thus, they may  
277 be particularly useful for individuals experiencing immunosuppression or young children  
278 for whom antibiotics and antifungal medication can be highly toxic [44, 45]. Extending  
279 these concepts further, future work will include tubes coated with pathogen-killing  
280 agents. It will also be interesting to use the antibody capturing technique as an enrichment  
281 device. By circulating blood through a series of capturing tubes, each with a specific  
282 antibody (or other targeting molecules), microorganisms can be concentrated in the  
283 tubes without requiring further isolation steps. This approach could reduce the time  
284 required to identify a pathogen.

285

## 286 **Conclusion**

287         Three antibiotic-free techniques that can be adapted to reduce or eliminate  
288 bacterial infections from blood have been presented. In this manuscript, we used *S.*  
289 *aureus* and a combination of techniques to reduce bacteria load by about 90% *in vitro* in  
290 spiked blood. These techniques could be adaptable for use against other  
291 microorganisms and possibly against antimicrobial resistant strains. Several procedures  
292 should be implemented to improve these techniques, such as testing additional  
293 microorganisms, answering engineering challenges like throughput, and studying the  
294 toxicity of PDT and germicidal light.

295

## **Funding Information**

296 This research was funded by the National Institutes of Health grant number GM084520  
297 (PI: A.G.).

298

## **Acknowledgments**

299 We would like to thank Drs. Aileen Marty, Yogesh Gianchandani, Roma Gianchandani,  
300 Shekhar Bhansali, Horatiu Vinerean, and Paula Flicker for their feedback, comments,  
301 and support.

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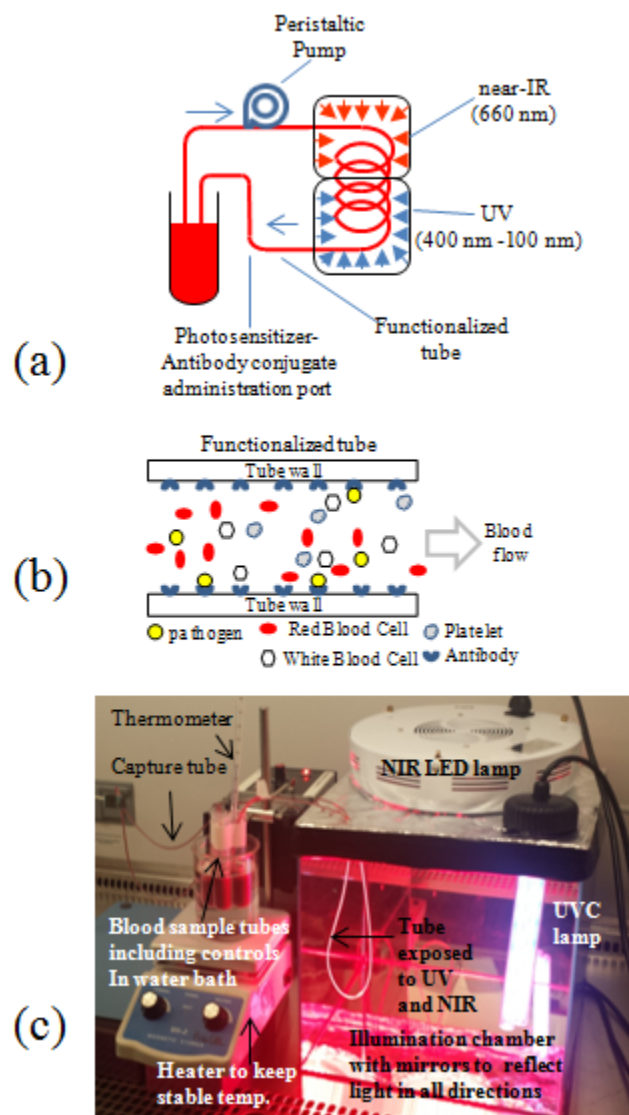
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430 Fig. 1. (a) Schematic of the set-up. (b) Capturing by antibody immobilization on tube. (c)  
431 Experimental setting of the system. The tubes that are exposed to UV and NIR along  
432 with the light sources are placed inside a mirror chamber. One side of the chamber was  
433 opened for this picture. The entire set-up resided inside a biosafety cabinet.

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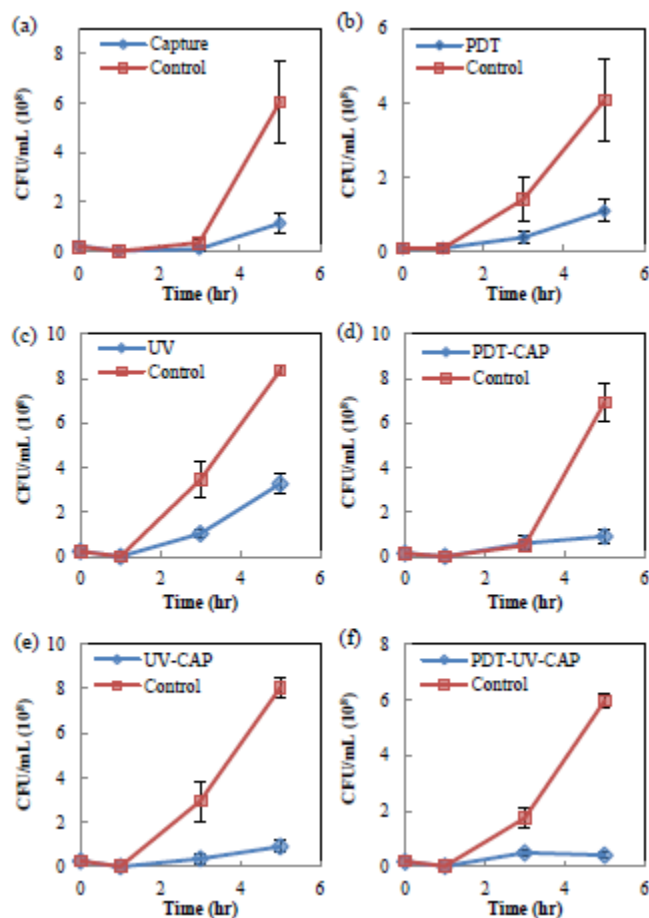
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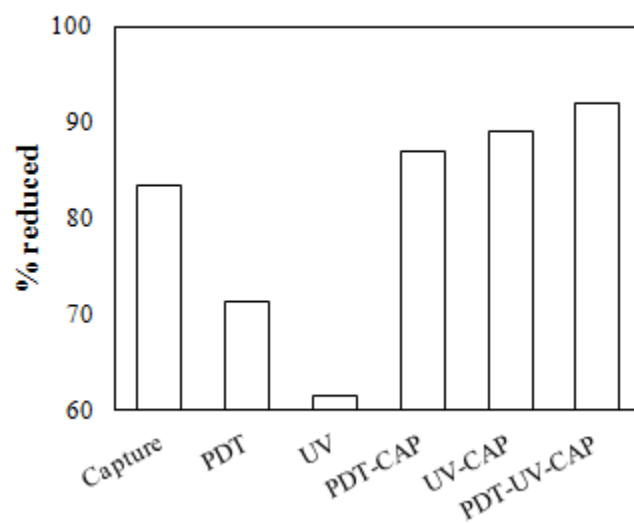
443 Fig. 2. Graph of bacterial growth using the techniques described vs. control after 5  
444 hours (n=5) (CFU/mL; error bars, SEM.), (a) Capturing (t-test  $P < 0.02$ ), (b) PDT (t-test  
445  $P < 0.03$ ), (c) UV (t-test  $P < 0.0001$ ), (d) PDT-Capturing combination (t-test  $P < 0.0002$ ),  
446 (e) UV-Capturing combination (t-test  $P < 0.0001$ ), and (f) PDT-UV-Capturing  
447 combination (t-test  $P < 0.0001$ ).

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453 Fig. 3. Summary of results of normalize values in % compared to control.