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Fiber-optic Singlet Oxygen [$^1O_2$ ($^1\Delta_g$)] Generator Device Serving as a Point Selective Sterilizer

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ABSTRACT

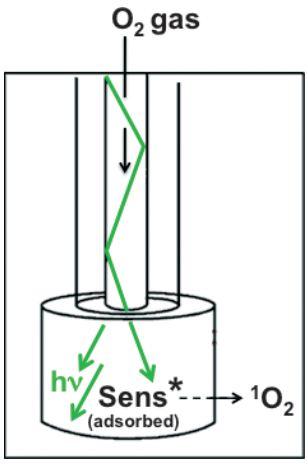
Traditionally, Type II heterogeneous photo-oxidations produce singlet oxygen *via* external irradiation of a sensitizer and external supply of ground-state oxygen. A potential improvement is reported here. A hollow-core fiber-optic device was developed with an "internal" supply of light and flowing oxygen, and a porous photosensitizer-end capped configuration. Singlet oxygen was delivered through the fiber tip. The singlet oxygen steady-state concentration in the immediate vicinity of the probe tip was *ca* 20 fM by *N*-benzoyl-DL-methionine trapping. The device is portable and the singlet oxygen-generating tip is maneuverable, which opened the door to simple disinfectant studies. Complete *Escherichia coli* inactivation was observed in 2 h when the singlet oxygen sensitizing probe tip was immersed in 0.1 mL aqueous samples of 0.1–4.4 $\times$ 10^7 cells. Photobleaching of the probe tip occurred after *ca* 12 h of use, requiring baking and sensitizer reloading steps for reuse.

INTRODUCTION

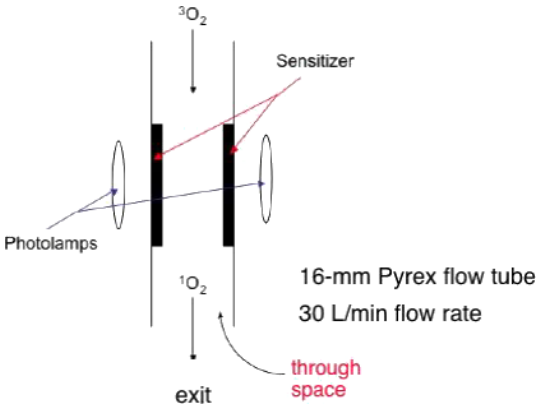
Only recently was a fiber-optic singlet oxygen (FOSG) device developed for the localized "delivery" of singlet oxygen (Scheme 1) (1). The device consisted of a porous Vycor glass (PVG) cap coated with *meso*-tetra(*N*-methyl-4-pyridyl)porphine (**1**) fixed to the end of a hollow fiber-optic (1). The hollow fiber flowed O_2 gas and guided 532 nm light from a continuous-wave or pulsed laser. The lifetime of photoexcited triplet-state adsorbed **1** was 57 μs and O_2 quenching at the water–PVG interface resulted in the formation of $^1O_2$ in aqueous solution (2).

To date, there are few devices specifically designed to deliver singlet oxygen through space (3–5). Perhaps the best documented example is that of Eisenberg *et al*. (5) who 24 years ago reported a Pyrex tube-bound rose bengal photosensitizer flowing singlet oxygen upon irradiation (Scheme 2). Singlet oxygen exited the distal end of the Pyrex tube and was transported through space ~1.5 mm due to a 54 ms lifetime. Despite the novelty of the Eisenberg Pyrex tube method (5) and other gas–solid methods to generate external $^1O_2$, they are not compatible in aqueous solution because of high oxygen gas flow rates, which would lead to water cooling and evaporation. Thus, interesting questions remain about devices whose distal ends generate singlet oxygen in aqueous solution.

We wondered whether the FOSG device could be exploited to inactivate *Escherichia coli* or whether the short diffusion distance of $^1O_2$ in water (~160 nm) (6–8) would prohibit it. Our interest in a disinfection application was due to interest in the



Scheme 1. Distal end of fiber-optic device where singlet oxygen exits.



Scheme 2. Device of Eisenberg *et al*. where singlet oxygen exited the distal end of the flow tube (5).

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fundamental factors of singlet oxygen generation water–solid interfaces, and because of the great potential that singlet oxygen photocatalysts possess for *E. coli* killing under visible light conditions (9). Fiber optics have been coupled to heterogeneous photosensitizers for the generation of $^1O_2$ (10–14), and used in the sensing of $^3O_2$ or $^1O_2$ in biological media (15,16), but no such device has been developed for *E. coli* inactivation.

MATERIALS AND METHODS

Device construction. A diagram of the fiber-optic apparatus is shown in Fig. 1. A similar fiber-optic geometry was chosen because it gave good results in our previous paper on singlet oxygen delivery (1). In the present study, a different fiber and different illumination source was used than in Ref. (1). The hollow core photonic bandgap fiber was Crystal Fibre's HC-440 having a 84 μm diameter, coated with a 136 μm layer of acrylate (attenuation of < 2 dB m^{-1}), which guided 473 nm light from a blue CW laser (20 mW, 2.0 mm beam diameter; Dragon Laser), which was within the wavelength range covered by the photonic bandgap in the cladding (415–485 nm). Unwanted melting of the acrylate coating by the incident 20 mW laser beam required the removal of a 0.5-cm segment of the acrylate, which was done by dipping the proximal end (beginning) of the fiber into dichloromethane while flowing oxygen (40 psi). This acrylate stripping method reduced the amount of light that could be transported through the fiber. The intensity of the illumination was 2.2 mW, incident *into* the photosensitizing cap hole during the experiment. The immobilization of **1** on PVG pieces was conducted as described previously for this heterogeneous reaction (1). PVG has pore sizes around 40 Å and increases in weight by 30% from its dry weight after soaking in water. Typically, 0.015 g PVG caps were loaded with 4×10^{-9} mol **1** producing ~0.5% sensitizer coverage. Caps were cut and polished into cylindrical shapes, ~2 mm o.d. × 3–4 mm. The sensitizer-impregnated PVG cap was then fixed to the distal end of the hollow fiber and glued into place at the hole entrance with ethyl-2-cyanoacrylate. The sensitizer-coated PVG cap absorbs 473 nm light at the tail of the Soret band (λ_{max} = 422 nm). Concentrations of oxygen delivered to 0.1 mL water samples *via* the FOSG generator system were determined with a pO_2 micro-oxygen electrode.

E. coli viability. Singlet oxygen toxicity was judged by *E. coli* K-12 cultures grown in tryptic soy broth to an early logarithmic phase (A_{590} = 0.2) and following the reduction in the number of colonies in treated *versus* control samples. Standard protocols were used in the growth and maintenance of the *E. coli* cultures and the experiments were carried out at ambient temperature (25°C). Two milliliters of the *E. coli* solutions was centrifuged, washed once with distilled water, re-suspended and diluted to 1.1×10^7 to 4.4×10^8 cells mL^{-1} in 100 μL reaction volumes. In a dark room, *E. coli* cells were exposed to singlet oxygen *via* the fiber-optic-based singlet oxygen generator with illumination with the 473 nm laser and introduction of the fiber tip into the 100 μL water solution. There was no headspace above the cell culture medium. The *E. coli* viability was evaluated at hourly intervals, in which 10 μL aliquots were removed and serial dilutions made ranging from 10^{-4} to 10^{-6}. Each 100 μL serial dilution was then added to 3 mL molten tryptic soy top agar (TSA), briefly vortexed and overlaid onto the TSA plates. Dilutions were made in duplicate. When the overlay solidified, the plates were inverted and incubated for 48 h. Temperatures were kept at 25°C throughout the course of the experiments. The number of colony-forming units was determined by direct count and the final concentration of *E. coli* is reported as the number of viable cells mL^{-1}.

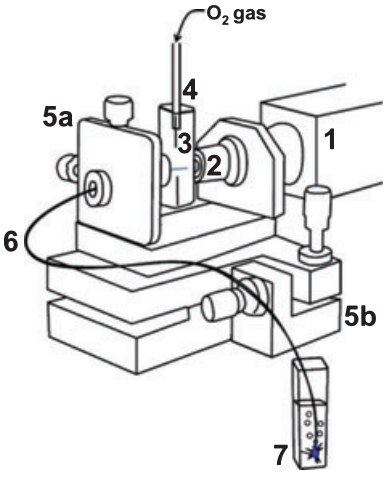


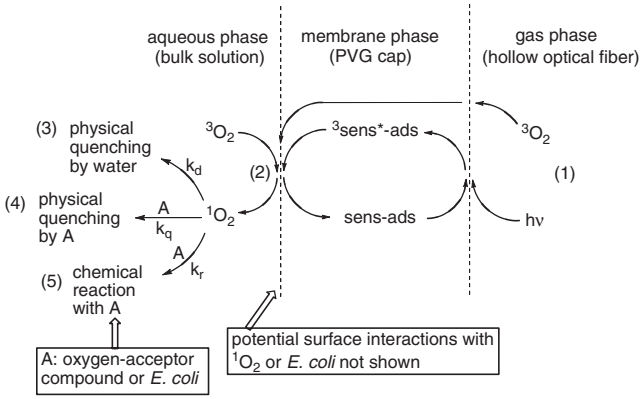
Figure 1. Schematic of the fiber-optic-based singlet oxygen generator (FOSG): (1) continuous-wave laser; (2) microscope objective; (3) cuvette; (4) cuvette top with tube coupled to compressed oxygen tank; (5) laser-to-fiber coupler with micrometer *x–y* (a) and *y–z* (b) translation resolution; (6) hollow core fiber-optic transporting oxygen gas and 473 nm light; (7) aqueous reaction solution containing the distal end of the fiber capped with the singlet oxygen sensitizing porous Vycor glass tip.

Photo-oxidation. Reports of *N*-benzoyl-DL-methionine anion (**2**), 9,10-anthracenedipropionic acid (**4**) and *trans*-2-methyl-2-pentenoate anion (**6**) as chemical probes for the evaluation of singlet oxygen in aqueous solution are available in the literature (1,17,18). The experiments in this manuscript were conducted at room temperature with the FOSG device with 40 psi oxygen gas and blue CW laser light passing through a hollow core bandgap fiber. Steady-state concentrations of singlet oxygen were determined as described previously (19, 20), but with the use of methionine anion **2** rather than furfuryl alcohol due to adsorption of the latter onto the PVG surface.

Chemicals. Deionized H_2O was obtained from a U.S. Filter Corporation deionization system. PVG (Corning 7930) was purchased from Advanced Glass and Ceramics. *N*-benzoyl-DL-methionine sodium salt (Aldrich), 9,10-anthracenedipropionic acid (Aldrich), sodium azide (Aldrich), *meso*-tetra(*N*-methyl-4-pyridyl)porphine tetratosylate (Frontier Scientific) and adipic acid (Monsanto Chemical Co.) were used as received. Deuterium oxide-d_2 (Aldrich), chloroform-d_1 (Aldrich) and acetonitrile-d_3 (Isotec, Inc.) were of spectrophotometric grade.

RESULTS AND DISCUSSION

A mechanism that involves the generation of singlet oxygen as outlined in Scheme 3 is consistent with our results. As can be seen, the FOSG generator is essentially a three-phase system: the gas phase is the hollow core of the fiber, the solid phase is the fiber cap and the aqueous phase is the bulk solution. Blue light and ground-state triplet O_2 were delivered through a hollow fiber to the porous cap (Eq. [1]) and sensitized to $^1O_2$ at the water–solid interface (Eq. [2]). The heterogeneous sensitizer was the probe tip coated with porphyrin **1** (2.5×10^{-7} mol **1** g^{-1} PVG). Subsequent physical and chemical quenching reactions of singlet oxygen can take place by processes involving solvent, substrates or biological materials (Eqs. [3–5]).



Scheme 3. Three-phase system.

Flowing oxygen through the probe tip

Gas diffusion through hollow core fibers has been studied before (21), but not with the aim of generating singlet oxygen. The FOSG generator was operated at 40 psi O_2 pressure as the pieces glued at the cuvette/fiber and fiber/cap junctions otherwise come apart. The transmission rate of O_2 through the porous tip was $\sim$1.0 μL min^{-1} ($\sim$0.1 ppm h^{-1}). After 4 h at 40 psi, an increase in oxygen concentration of 4–12% ($\sim 2 \times 10^{-5}$ M) can be obtained beyond the starting air-saturated concentration (2.6×10^{-4} M; 4.68 ppm). The variation in the percent of oxygen transported across the PVG tip can be explained by the differences in the cap shape, its length and outer diameter, and the hole inner diameter (Fig. 2). When illuminated, oxygen rapidly quenches the triplet PVG adsorbed **1**; its lifetime in N_2-purged water ($\tau_0 = 57 \pm 1$ μs) was reduced by oxygen in air-saturated solution ($\tau = 7 \pm 1$ μs) (2).

Photo-oxidation and kinetics

Ene and [4+2]-cycloaddition reactions are often diagnostic and verify the presence of singlet oxygen (22–24) in solution. Here, we found the formation of 9,10-anthracene-9,10-endoperoxide dipropionate dianion (**5**) took place *via* a [4+2] cycloaddition of singlet oxygen with 9,10-anthracene dipropionate dianion (0.1–0.2 M, pH = 10, **4**) (Scheme 4). Control reactions demonstrated that >99% of **4** was photo-oxidized by the device tip, and was not self-sensitized under the reaction conditions. *N*-benzoyl-DL-methionine **2** was also photo-oxidized by the FOSG giving predominantly *N*-benzoyl-DL-methionine S-oxide (**3**). Products **3** and **5** were stable in aqueous solution at room temperature and identified by ^{1}H NMR and by LCMS. We next wanted to learn if the FOSG production could be quantified with the chemical trap **2**.

Control experiments showed that anions, such as methionine anion **2**, do not penetrate through nor associate with PVG (Scheme 5), suggesting **2** could be used as a trapping agent for the FOSG and its concentration followed in the surrounding aqueous solution. The chemical quenching (k_r), and solvent (k_d) and substrate (k_q)-induced physical quenching reactions of singlet oxygen are shown in Eqs. (3–5) (Scheme 3). The quantum yield of singlet oxygen production is Φ, and the rate of absorption of 473 nm light by the PVG-sensitizer **1** is I_a (Eq. [6]). As a 2 mW output of the CW laser through the fiber was constant to $\pm$0.1 mW over the course of the experiments, we applied a steady-state approximation for singlet oxygen (Eqs. [7] and [8]) in a similar fashion as Haag and Hoigné (19). Protic solvents suppress the physical quenching (k_q) of singlet oxygen by organic sulfides (typically 0% physical quenching) (25) thus permitting the rate of formation of singlet oxygen in the solution around the probe tip to be estimated by the reduction in the concentration of **2**. The rate constant of the reaction between $^1O_2$ and DL-methionine (k_T) has been examined previously ($\sim 3 \times 10^7$ M^{-1} s^{-1} in water at pH 6–11) (26) and we assumed a similar or identical rate constant between $^1O_2$ and *N*-benzoyl-DL-methionine **2**. The initial concentration of **2** was 0.1 mM so that k_d was seven times greater than k_T[**2**] and the loss of **2** was first-order in **2**. The k_d of singlet oxygen in water is 2.5×10^5 s^{-1} (27). Under constant 2-mW light intensity, pseudo-first order plots with the FOSG generator were obtained by Eq. (8), which suggested a $[^1O_2]_{ss}$ of ~ 1–4×10^{-14} M in the immediate vicinity of the probe tip.

$$-\frac{d[\mathbf{2}]}{dt} = \left(\frac{I_a \Phi}{k_d}\right) k_r[\mathbf{2}] \quad (6)$$

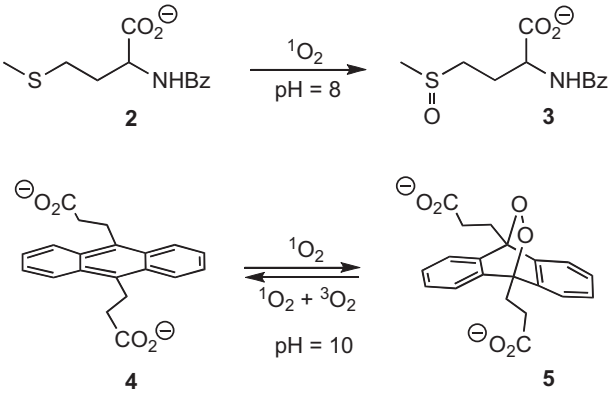
$$-\frac{d[\mathbf{2}]}{dt} = k_r[^1O_2]_{ss}[\mathbf{2}] = k_{obs}[\mathbf{2}] \quad (7)$$

(ss = steady state)

$$\frac{k_{obs}}{k_r} = [^1O_2]_{ss} \quad (8)$$

Inactivation of *E. coli*

Figure 3 shows the results of *E. coli* inactivation through singlet oxygen sensitized damage together with control data. The reduced number of cells was compared against blanks



Scheme 4. Photo-oxidation of *N*-benzoyl-DL-methionine anion and 9,10-anthracene dipropionate dianion.

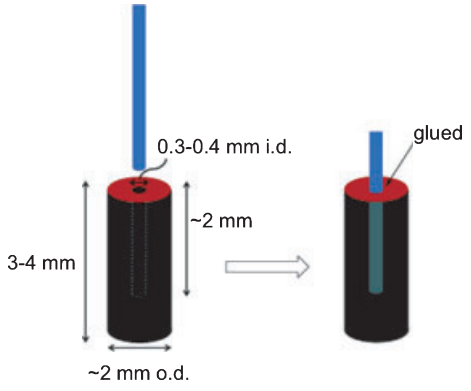
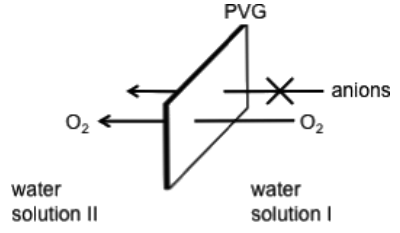


Figure 2. Schematic of the singlet oxygen sensitizing porous Vycor glass tip.



Scheme 5. Inability of anions to penetrate through or adsorb onto PVG.

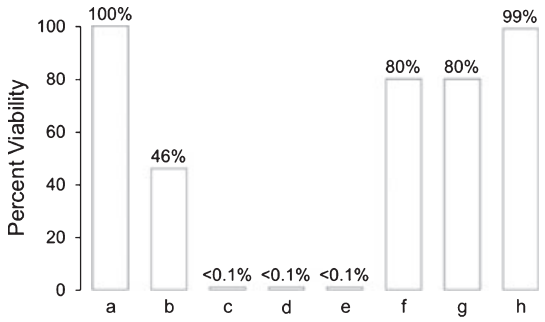


Figure 3. Percent survival of *Escherichia coli* cells (initial concentration = 1.1×10^7 to 4.4×10^8 cells mL^{-1}, 0.1 mL, pH = 7) in aqueous media. Each bar represents an average of two to three runs. (a) The values are shown as the increase in cell numbers relative to blanks containing the same initial *E. coli* cell concentration without introduction of the singlet oxygen generator tip. *Escherichia coli* inactivation in the presence of (b) the FOSG delivering 473 nm light and 40 psi O_2 over a 1 h period; (c) the FOSG delivering 473 nm light and 40 psi O_2 over a 2 h period; (d) the heterogeneous PVG sensitizer irradiated externally with 473 nm light over a 1 h period; (e) the homogeneous sensitizer **1** in the presence of oxygen and 473 nm light over a 2 h period; (f) sparging 20 μL min^{-1} oxygen in the dark over a 2 h period; (g) sparging 20 μL min^{-1} oxygen with 473 nm laser light over a 2 h period; and (h) the heterogeneous PVG sensitizer in the dark over a 2 h period.

containing from 1.1×10^7 to 4.4×10^8 cells mL^{-1} (entry a). A decrease in the number of viable cells was observed over a 1 h period with the FOSG (cf. control [2.1×10^8 cells mL^{-1}] with photo-oxidation sample [9.7×10^7 cells mL^{-1}]) (entry b). *Escherichia coli* was completely killed after 2 h (entry c). Additional data with higher oxygen concentrations (O_2-saturated solution [1.4 mM] *versus* air-saturated solution [0.26 mM]) revealed enhanced *E. coli* killing by a factor of ~2. Higher oxygen concentrations are known to enhance photodynamic bacterial inactivation (28). After a 4 h irradiation period the *E. coli* samples turned yellow, which we attribute to photochemical oxygen uptake. *Escherichia coli* was completely killed after 1 h when a 0.02 g piece of heterogeneous PVG sensitizer was irradiated externally with 473 nm light (entry d). A homogeneous solution of 5.0×10^{-8} M **1** in the presence of 473 nm light was also lethal concentration to *E. coli* growth after 2 h (entry e), which was expected due to previous reports of the phototoxicity of **1** toward *E. coli* in homogeneous solution (29). Singlet oxygen inactivation of *E. coli* with a number of homogeneous photosensitizers has been established (30,31). The viable number of *E. coli* significantly increased in the presence of blue light and/or oxygen without sensitizer. Twenty percent *E. coli* inactivation was observed in the presence of sparging oxygen in the dark (entry f), and in the presence of 473 nm light and sparging oxygen without sensitizer (entry g). The sensitizer **1**-coated PVG showed negligible dark toxicity toward *E. coli* (entry h), which was similar to the low dark toxicity previously reported for homogeneous **1** (32).

Sensitizer leaching and *E. coli* adhesion onto the PVG surface

Control experiments suggested that *E. coli* inactivation was not due to desorption of the sensitizer off the PVG and functioning as a homogeneous photosensitizer catalyst. Absorption spectra of *E. coli* cell solutions were identical in the 300–1100 nm range prior to and after removal of the PVG/adsorbed sensitizer **1**. Sensitizer **1** desorption was not detected when PVG (1.1×10^{-8} mol **1** adsorbed onto 0.04 g PVG) was placed in 2 mL of H_2O solution at pH = 7, and stirred for 8 h in the presence of *E. coli* in the dark. Figure 4 shows that even 1.6% desorption of **1** would be readily detected in the presence of *E. coli* M (Soret band, λ_{max} = 422 nm). Previous results also showed that porphyrin **1** remained immobilized on PVG when sensitizer-coated PVG was placed in H_2O solution at pH = 3, 7 and 10 (1), and that cations and metals tend to adsorb strongly onto PVG (2,33). The possible adhesion of *E. coli* to the PVG surface was then examined. A 0.3 g PVG sample coated with 8.0×10^{-8} mol **1** was submerged in 1.0 mL *E. coli* suspension (2.1×10^{-8} cells mL^{-1}) for 2 h. The PVG sample was washed with sterilized water and then plated onto TSA. An aliquot of the sterilized water from the last rinse was also plated onto TSA. The two plated samples showed no difference in colony growth, suggesting that *E. coli* adhesion did not take place onto the PVG photocatalyst surface. Electrostatic repulsion may be expected between *E. coli* and the negatively charged PVG surface, *E. coli* adhesiveness is known to correlate inversely with surface electro-negativity (34). *Escherichia coli* adhesion is generally favored on positively rather than negatively charged surfaces (35).

Photocatalyst bleaching and reuse

An important matter concerned the photostability of the probe tip. After 12 h of use, the probe tip began to photobleach and the rate of *E. coli* killing declined. We analyzed the photobleaching process with blue-light irradiation of a PVG sample (2.2×10^{-8} mol **1** adsorbed onto 0.084 g PVG) in D_2O. A dichloromethane solvent extract of the D_2O solution after removal of the PVG catalyst revealed organics presumably from fragmented/oxidized sensitizer molecules by GCMS in the *m/z* range 206.0–246.1. Baking the PVG catalyst at 500°C removed the residual bleached and unbleached material from the surface, and an active photocatalyst tip was regenerated by baking of the PVG and following the procedure of Ref. (1) to reload the tip with sensitizer **1**. Such baking and reloading steps could be done more than 10 times.

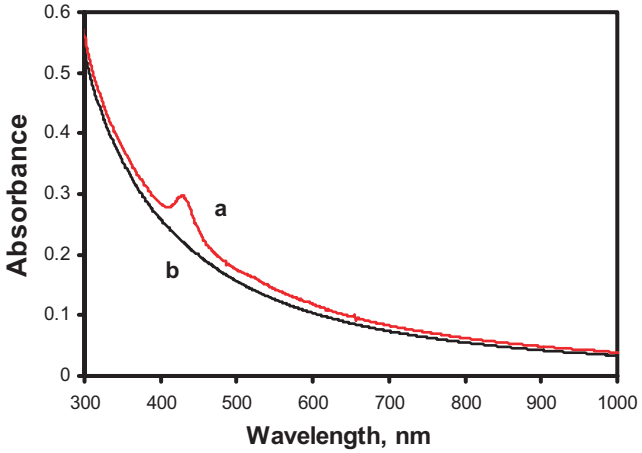


Figure 4. (a) 1.0×10^8 *Escherichia coli* cells mL^{-1} in aqueous solution with 6.6×10^{-7} M sensitizer **1**. (b) 1.0×10^8 *E. coli* cells mL^{-1} in aqueous solution.

CONCLUSION

Heterogeneous $^1O_2$-photosensitizers typically require externally supplied oxygen and light, and in some cases cannot be recovered for reuse. One way of improving heterogeneous photosensitizer performance would be if a hollow-core fiber-optic system flowing oxygen were coupled to a *porous* Type II heterogeneous photosensitizer tip, which is the subject of this paper. In this paper, it was shown that singlet oxygen can be delivered through the fiber tip into aqueous solution. *Escherichia coli* inactivation was analyzed to help establish conditions that can generate singlet oxygen, which extends our previous study (1) and provides a potential application in water disinfection. The porous fiber tip can be reused with baking and sensitizer reloading steps to enhance the photodynamic *E. coli* killing.

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