Visible Light Sensitized Inactivation of MS-2 Bacteriophage by a Cationic Amine-Functionalized C_{60} Derivative

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Recently, we reported the successful synthesis of various hexakis C_{60} derivatives (i.e., C_{60} with six functional groups containing NH_3^+-, CO_2H-, or OH-terminals) with enhanced stability in water for aqueous phase application (Lee et al., *Environ. Sci. Technol.* 2009, *43*, pp 6604−6610). Among these newly synthesized C_{60} derivatives, the cationic hexakis C_{60} derivative with amine functionality, $C_{60}(CR_2)_6$ ($R = CO_2(CH_2)_2NH_3^+CF_3CO_2^-$), was found to exhibit remarkable efficiency to inactivate *Escherichia coli* and MS-2 bacteriophage under UVA irradiation. Herein, we report that this amine-functionalized C_{60} derivative is also photoactive in response to visible light from both commercial fluorescence lamps and sunlight. Efficient production of $^1O_2$, facile reaction of $^1O_2$ with proteins in MS-2 phage capsid and electrostatic attraction between positively charged C_{60} derivative and negatively charged MS-2 phage collectively contributed to high efficiency of MS-2 phage inactivation in this photocatalytic disinfection system. The rate of $^1O_2$ production was evaluated using a probe compound, furfuryl alcohol, and $^1O_2$ *CT* (the product of $^1O_2$ concentration and exposure time) required to achieve a target level of virus inactivation was quantitatively analyzed. The unique visible-light sensitized virucidal property makes this C_{60} derivative highly desirable for the development of sustainable disinfection strategies that do not require continuous chemical addition nor an external energy source other than ambient light.

Introduction

The unique photochemical reactivity of C_{60}, through which reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) are readily generated in the presence of oxygen and irradiation of photon energy above 2.3 eV, has been recently explored to develop new, sustainable disinfection strategies. For instance, Käsermann and Kempf (*2*) showed that Semliki Forest virus and vesicular stomatitis virus could be inactivated by C_{60}, which was introduced into water via sonication (i.e. forming aggregates known as nC_{60}) under visible light irradiation. Another study also reported that influenza viruses could be inactivated by visible light photocatalysis mediated by similar nC_{60} aggregates in biological fluids (*3*). The mechanism of virus inactivation by C_{60} aggregates in water, however, has been controversial, as recent studies discovered that C_{60} loses most of its photoreactivity upon spontaneous aggregation in water (*4, 5*). Recently, with the goal of overcoming the difficulties of dispersing hydrophobic C_{60} in water and decrease in photocatalytic efficiency, C_{60} with hydrophilic functional moieties have been considered for applications in the aqueous phase. Hotze et al. (*6*) showed that fullerol ($C_{60}(OH)_{22-24}$), photosensitized by UVA light, produced $^1O_2$ which inactivated different types of viruses including single stranded RNA virus (MS-2 bacteriophage), double stranded DNA virus with (PRD1) and without (T7) internal lipid membrane. Tegos et al. (*7*) demonstrated that both gram negative and gram positive bacteria and fungi could be inactivated by cationic fullerenes (derivatized with pyrrolidinium groups) by white light irradiation. Cho et al. (*8*) reported that ozone-treated C_{60} (i.e., C_{60} with multiple hemiketal functional groups) readily penetrated into and inactivated *Escherichia coli* by photochemically producing hydroxyl radicals upon UVA irradiation.

Our past efforts also focused on developing various hexakis C_{60} derivatives (i.e., multifunctionalized C_{60} with NH_3^+-, CO_2H-, or OH-terminal groups) as new photocatalysts with enhanced aqueous applicability and disinfection efficiency (*1*). Among these newly synthesized C_{60} derivatives, we reported that cationic hexakis C_{60} derivative with amine functionality, $C_{60}(CR_2)_6$ ($R = CO_2(CH_2)_2NH_3^+CF_3CO_2^-$) showed a remarkable efficiency to inactivate *E. coli* and MS-2 bacteriophage under UVA irradiation (350−400 nm). We postulated that electrostatic attraction between negatively charged microorganisms and cationic C_{60} derivate (Figure 1, herein referred to as HC4, following the notation from our previous work (*1*)) contributed to rapid inactivation kinetics, in addition to very efficient photochemical production of $^1O_2$ by HC4. It was noteworthy that HC4 retained a majority of C_{60}'s intrinsic photochemical reactivity even though relatively large agglomerates were formed depending on solution compositions (e.g., over 1 μm in 10 mM phosphate

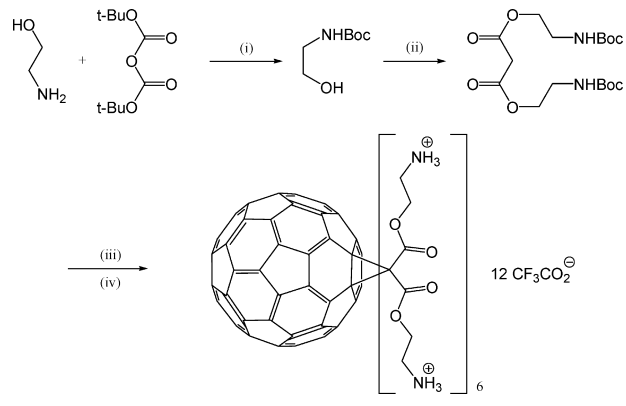

FIGURE 1. Synthesis of cationic hexakis C_{60} derivative (herein called HC4) (*1*). (i) room temperature, overnight; (ii) malonyl chloride, Hünig's base, in CH_2Cl_2, 2 h, −20 °C → room temperature; (iii) CBr_4, toluene: CH_2Cl_2 = 3: 1, DBU, 5 h; (iv) TFA: dioxane = 1: 9, 2 h, room temperature.

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buffer at pH 7.0) (*1*), which was in marked contrast to aggregates of pristine C_{60} (i.e., nC_{60}) (*9*).

Herein, we report that this particular amine-functionalized C_{60} derivative, HC4, also efficiently produces $^1O_2$ and inactivates MS-2 bacteriophage in response to visible light irradiation, suggesting a novel way of developing sustainable disinfection technology which requires no energy supply other than ambient visible light. Experiments were performed under both commercial fluorescence lamp light and sunlight irradiation. $^1O_2$ production was measured using an indicator chemical, furfuryl alcohol, and $^1O_2$ exposure required to achieve a target level of virus inactivation was quantitatively analyzed in terms of *CT* (the product of steady-state $^1O_2$ concentration and exposure time), the parameter frequently used for the disinfection process design and assessment.

Experimental Section

Preparation of C_{60} Derivative. HC4 was prepared as shown in Figure 1, based on the method recently developed by Lee et al. (*1*) with minor modifications. First, β-aminoethanol and di*tert*-butyl pyrocarbonate (1:1) were reacted at room temperature overnight. The resulting (2-hydroxyethyl)-carbamic acid *tert*-butyl ester was isolated in vacuo (yield 99%), combined with *N*,*N*-diisopropylethylamine in methylene chloride, and mixed with malonyl chloride (0.49 equivalents) under cooling. The malonic acid bis-(2-*tert*-butoxycarbonylamino-ethyl) ester, obtained as yellow oil according to a slightly modified procedure (*10*), was purified by liquid chromatography on silica gel using methylene chloride, giving white solid (mp = 92 °C), recrystallized from CH_2Cl_2-hexanes (yield 86%). The Bingel-Hirsch reaction of C_{60} (99+%, MER Corporation, Tucson, AZ) with malonate (20 equivalents) was then performed in toluene-methylene chloride mixture with CBr_4 using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a base. Resulting hexakis-adduct was isolated by liquid chromatography on silica gel using chloroform-ethanol mixture (yield 30%). The hexakis adduct was characterized by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS; calculated mass = 3052.1 and observed mass = $[M+H^+]$ 3052.4). NMR data was also obtained: ^1H NMR (400 MHz, $CDCl_3$, TMS reference) δ (ppm) = 5.19 (br, 12H, NH), 4.38 (t, ^3J = 14 Hz, 24H, OCH_2), 3.48 (m, 24H, CH_2NH), and 1.44 (s, 108H, $C(CH_3)_3$). Deprotection of $-NH_2$ groups was achieved by treatment with trifluoroacetic acid (TFA) in dioxane, resulting in HC4 (with quantitative yield) which was further purified by dialysis using a cellulose membrane (molecular weight cut-off = 2000) and vacuum drying. Properties of HC4 including hydrodynamic radius in the aqueous phase measured by dynamic light scattering are available in our previous report (*1*).

Preparation of MS-2 Bacteriophage Stock and Viability Assay. MS-2 bacteriophage (ATCC 15597) and *E. coli* (C3000) were obtained from American Type Culture Collection (ATCC). The stock of MS-2 phage was prepared via soft agar overlay (double-agar layer) of confluent lysis and the number of viable MS-2 phage was quantified by the soft agar overlay method described previously (*11*). In brief, top and bottom agars were prepared by adding 7 and 15 g/L of agar, respectively, in tryptone broth containing 10 g/L tryptone, 1 g/L glucose, 1 g/L yeast extract, 8 g/L NaCl, and 0.22 g/L $CaCl_2$. The host *E. coli* was grown in tryptone broth for 18 h and recultured in a new broth for 4 h. An aliquot (0.3 mL) of diluted sample and 0.1 mL of cultured host were spiked onto 5 mL of soft agar. These mixed soft agars were poured on top of the bottom agar. After incubation for 24 h at 37 °C, the cultured MS-2 phage in soft agar was harvested and purified via centrifugation twice (3000×*g*) and subsequent membrane filtrations (i.e., by collecting filtrate after 0.1 μm microfiltration and retentate after subsequent 20 000 MWCO ultrafiltration). The viable MS-2 phage was assayed by counting the number of plaque forming unit (pfu) after incubation. For viable assay, each sample was 10-fold diluted up to 1/100 and 0.3 mL aliquot of the undiluted and diluted solutions was inoculated onto three replicate plates.

Photoinduced Virus Inactivation Experiments. Reaction suspensions were prepared by adding HC4 (5−20 μM) and MS-2 phage ($4-6 \times 10^4$ pfu/mL) to sterile phosphate buffer (10 mM, pH 7.0). Selected experiments were performed using 10 μM Rose Bengal (Aldrich) as an alternative photocatalyst. Disinfection experiments to simulate indoor lighting condition were conducted using a collimated beam apparatus equipped with four commercial fluorescence lamps (4 W, Philips Co.). A 60 × 50 cm long collimating tube placed below the lamps produced a parallel irradiation of light. Prior to the experiment, the lamps were turned on for at least 10 min to obtain a constant light intensity output. A sterile Pyrex dish containing 20 mL reaction suspension was placed normal to the incident light for the initiation of the experiments. The experimental temperature was controlled at 22 °C by placing the reactor insider a thermostat chamber. Disinfection experiments under sunlight condition were conducted on sunny days (August 2009, between 3:00 to 4:30 pm) on Georgia Institute of Technology campus in Atlanta, GA (84° 39′W, 33° 77′N). Reaction solution was placed in a 20 mL cylindrical quartz reactor. The side of the reactor opposite to the solar irradiation was in contact with a water circulation tube for the experiments performed under constant temperature. Selected experiments were performed with a UV filter (which blocks lights < 400 nm) placed between the experimental suspension and light source. Another set of experiments was conducted using a commercial TiO_2 (P25, Degussa Co.) instead of HC4 under Black Light Blue (BLB, Philips Co.) irradiation in which photochemical produced •OH inactivated MS-2 phage. Decay of a probe compound, *p*CBA (*para*-chlorobenzoic acid), assayed using a HPLC to measure •OH radical concentration (*13*).

Five to seven samples were taken during each experiment and the viability of MS-2 phage was assessed following the method described above. Each experiment was triplicated. The light intensity of fluorescent lamps and sunlight at a representative wavelength of 365 nm was measured using a UVX radiometer with 365 longwave sensor (UVP Co., Upland, CA). Production of $^1O_2$ was monitored and quantified using a $^1O_2$ probe compound, furfuryl alcohol (FFA, 2 μM or 1 mM, k (FFA + $^1O_2$) = 1.2×10^8 $M^{-1}s^{-1}$ (*12*)). The concentration of FFA was measured using a Waters 2695 HPLC system equipped with a photodiode-array detector and a C-18 column. The surface charge of MS-2 phage was measured using a Zetasizer Nano ZS zetapotentiometer (Malvern Instruments Co.).

For selected experiments, concentrations of total proteins and protein carbonyls in the suspension were quantified using Bradford assay and OxyELISA oxidized protein quantitation kit (Millipore Co., Kankakee, IL) (*14*). Bradford assay (*15*) is a widely used method to quantify the total amount of proteins. Briefly, 0.06 mL of diluted and undiluted sample containing MS-2 stock or standard protein was added to 3 mL of $^1/_5$ diluted Bio-Rad protein assay dye reagent (BMS Co., U.S.). After stirring for 20 min, protein concentration was calculated by measuring the absorbance at 595 nm using a spectrophotometer. The amount of protein carbonyls was measured during the course of MS-2 phage inactivation. Experimental procedures were identical except that MS-2 phage was concentrated to approximately 3×10^{12} pfu/mL (equivalent to approximately 10 μg/mL) using a membrane filter (10 000 MWCO). The carbonyl groups in the protein side of treated MS-2 phages and standards are derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone), when they were reacted with DNPH (2,4-dinitrophenylhydrazine) in 96 wells. After incubation for 45 min in dark condition at 25 °C

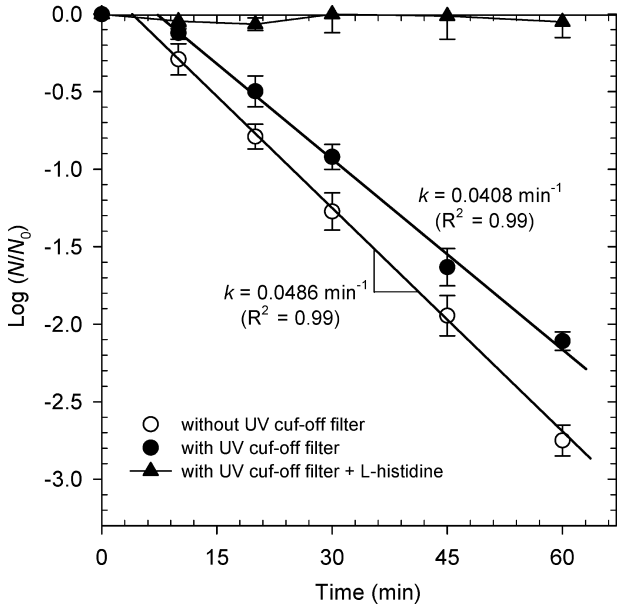

FIGURE 2. Inactivation of MS-2 phage by HC4 under fluorescence light irradiation (22 °C, pH 7.0, $[MS\text{-}2]_0 = 4-6 \times 10^4$ pfu/mL, $[HC4]_0 = 15\ \mu M$, light intensity at 365 nm = 165 $\mu W/cm^2$).

and washing steps, blocking buffer and anti-DNP working solution was consecutively added to stop the reaction. Subsequent incubation with the enzyme substrate 3,3′,5,5′-tetramethylbenzidine (TMB) showed colored products and the absorbance at 450 nm was assayed using a UV/vis spectrophotometer (Agilent 8453, Agilent Co., Germany). Detailed experimental procedure is available in the instruction manual (*14*).

MS-2 phage inactivation experiment by UVC irradiation was performed using a collimated beam apparatus (*16*). This equipment produced a parallel irradiation of UV light by means of a 60 × 50 cm long collimating tube placed below the UV lamps. Prior to the experiment, the UV lamps were turned on for at least 30 min to obtain a constant UV intensity output of 100 $\mu W/cm^2$ at 254 nm. A 60 × 15 mm sterile Petri dish containing 40 mL experimental suspension was immediately placed normal to the incident light for the initiation of the experiment.

Results and Discussion

MS-2 Phage Inactivation under Fluorescence Lamp Irradiation. Figure 2 shows the kinetics of MS-2 phage inactivation by 15 μM HC4, in which more than 1 log inactivation (90%) was achieved within approximately 24 min of ambient white light irradiation. Control experiments confirmed that MS-2 phage was not inactivated without light irradiation in the presence of HC4 or without HC4 in the presence of light. The kinetics shown in Figure 2 did not change after repeating inactivation experiments seven times by adding MS-2 phages to the same HC4 suspension after each experiment (data not shown). The presence of 1 mg/L Suwannee River natural organic matter (purchased from International Humic Substance Society) also did not affect the kinetics (Supporting Information (SI) Figure S3). The inactivation curves were characterized by the presence of an initial time lag during which inactivation was relatively slow followed by a first-order decrease in viability with respect to time. These data are quantitatively analyzed in the subsequent section.

The observed inactivation resulted mostly from visible light photocatalysis. When a UV filter was used to remove a small amount of the UVA below 400 nm which is emitted

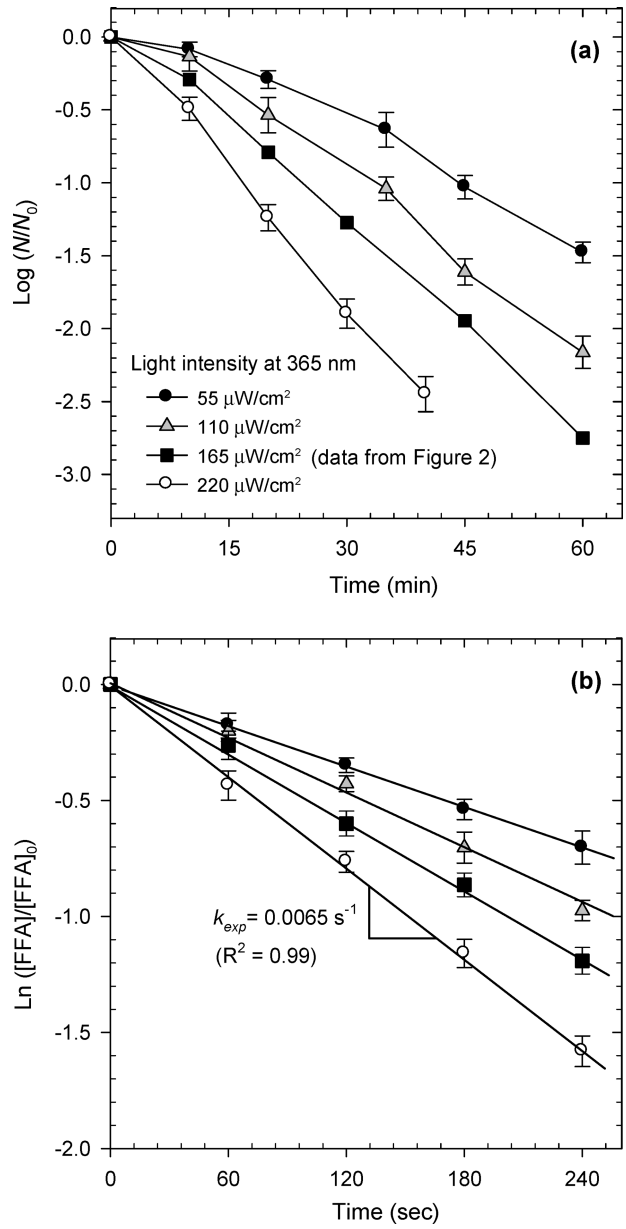

FIGURE 3. Effect of fluorescence lamp light intensity on (a) MS-2 phage inactivation and (b) FFA degradation by HC4 ($[FFA]_0 = 2\ \mu M$, $[HC4]_0 = 15\ \mu M$).

from the fluorescence lamps (typically approximately 4% of total light energy), the inactivation kinetics was only slightly decreased. Figure S1 (SI) shows UV−vis spectrum of HC4 compared to the emission spectrum of a commercial fluorescence lamp, which suggests that HC4 absorbs lights in the visible range (and hence assume a dark red color). It is noteworthy that no measurable inactivation was observed in the presence of excess (20 mM) singlet oxygen scavenger, L-histidine (k(L-histidine + $^1O_2$) = $4.6 \times 10^7\ M^{-1}s^{-1}$ (*12*)). This indicates that MS-2 phage inactivation resulted mostly from $^1O_2$ that was photochemically produced by HC4, consistent with our previous findings with UVA irradiation (*1*). The occurrence of a lag phase during the initial phase of inactivation, therefore, might have resulted from the time required for $^1O_2$ to react with vital components of the MS-2 phage (*17*).

The MS-2 phage inactivation rate increased as light intensity was increased from 55 to 220 $\mu M/cm^2$ at a constant HC4 concentration of 15 μM (Figure 3a), and as the HC4 concentration was increased from 5 to 20 μM at a constant

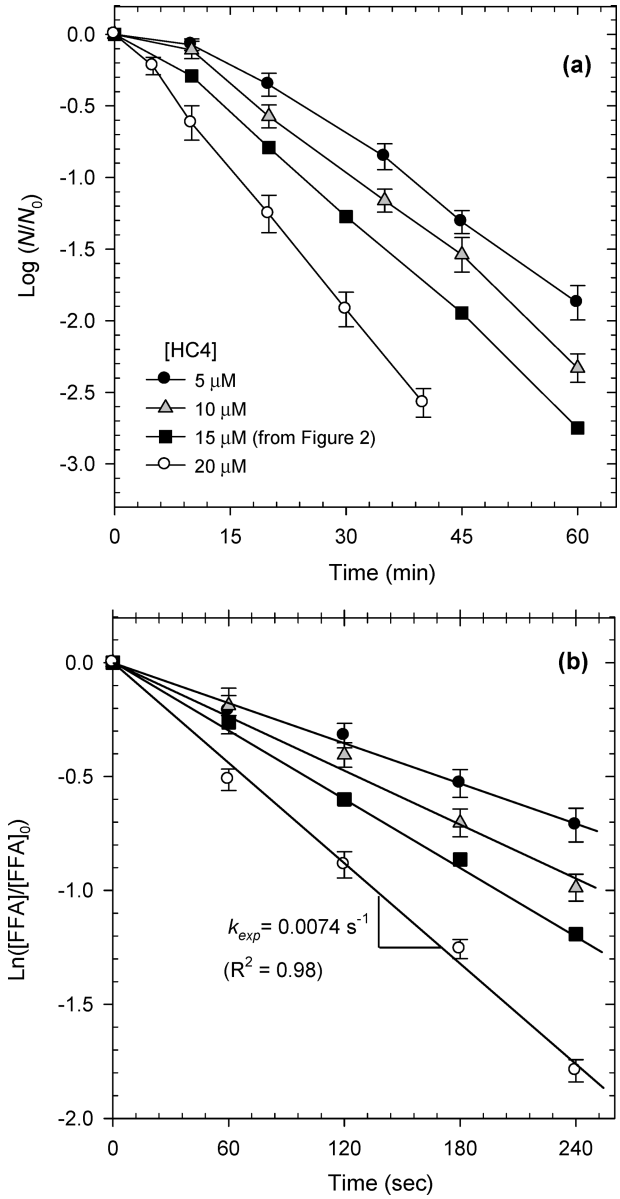

FIGURE 4. Effect of HC4 concentration on (a) MS-2 phage inactivation and (b) FFA degradation by HC4 under a constant fluorescent lamp light intensity of 165 μW/cm^2 at 365 nm ([FFA]$_0$ = 2 μM).

light intensity of 165 μW/cm^2 (Figure 4a). The increase in the inactivation kinetics correlated well with the increase in $^1O_2$ production, measured by the degradation of FFA (Figures 3b and 4b). Under the condition that led the fastest MS-2 phage inactivation within the range investigated, 1 log inactivation was achieved within approximately 17 min of white light irradiation (lag time = 1.7 min and rate constant = 0.0653 min^{-1} (R^2 = 0.996)).

Determination of $^1O_2$ CT. The same MS-2 phage inactivation kinetics was observed when 10 μM Rose Bengal dye instead of HC4 was used to photochemically generate $^1O_2$, reconfirming that MS-2 phage inactivation was solely due to $^1O_2$. Figure S2 (SI) shows MS-2 phage inactivation (Figures S2a and S2b) and FFA decomposition kinetics ([FFA]$_0$ = 2 μM; insets of Figures S2a and S2b) when $^1O_2$ was produced by different concentrations of Rose Bengal under varying fluorescence light intensities. Control tests confirmed that MS-2 phage was not inactivated without either light irradiation or dye within the experimental time scale. In addition, MS-2 phage inactivation was completely prohibited with the addition of excess (20 mM) L-histidine (data not shown) similar to the results presented in Figure 2.

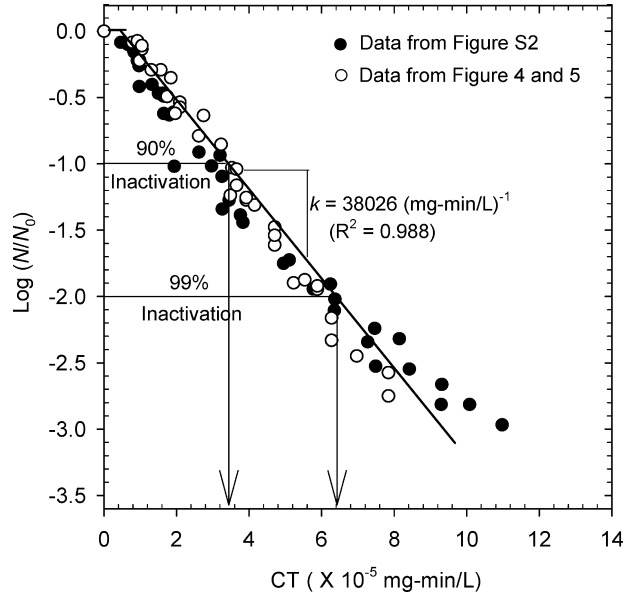

FIGURE 5. MS-2 phage inactivation versus *CT* at 22 °C (replotted data from Figure 3, Figure 4, and SI Figure S2).

From the results shown in Figure 3, Figure 4 and SI Figure S2, *CT* (i.e., the product of $^1O_2$ concentration and contact time) required to achieve a target MS-2 phage inactivation was analyzed. The *CT* is a commonly used engineering design parameter to evaluate the level of microorganism inactivation from the overall exposure to disinfectant. For the determination of *CT*, the kinetics of FFA degradation by $^1O_2$ was first expressed as follow (*18*):

$$\frac{d[FFA]}{dt} = -k_{^1O_2,FFA}[^1O_2]_{ss}[FFA] \quad (1)$$

where $[^1O_2]_{ss}$ represents the steady-state $^1O_2$ concentration and $k_{^1O_2,FFA} = 1.2 \times 10^8$ M^{-1}s^{-1} (*12*). Integrating eq 1 over time and solving for *CT* (where *C* represents $[^1O_2]_{ss}$) yields:

$$CT = [^1O_2]_{ss} \times t = \frac{-\ln([FFA] / [FFA]_0)}{k_{^1O_2,FFA}} \quad (2)$$

Figure 5 shows that all MS-2 phage survival ratio data plotted versus *CT* merge into a single line, suggesting that the same level of MS-2 phage inactivation would be achieved as long as the same *CT* is targeted. Similar to the inactivation kinetics observed with various microorganisms with chemical disinfectants (*13*), the *CT* concept is thus valid for $^1O_2$ inactivation of MS-2 phage. This trend could be represented using the following delayed Chick−Watson kinetics (*13, 19*):

$$\frac{N}{N_0} = \begin{cases} 0 & if\ CT \le CT_{lag} \\ \exp\{-k(CT - CT_{lag})\} & if\ CT > CT_{lag} \end{cases} \quad (3)$$

where N_0 = initial viable MS-2 phage concentration (pfu/mL); N = concentration of viable MS-2 phage concentration at time t (pfu/mL); k = post lag phase inactivation rate constant (L/(mg-min)). CT_{lag} in eq 3 represents *CT* required to overcome the initial lag phase.

From Figure 5, k and CT_{lag} were determined as 38 026 L/mg-min and 0.54 × 10^{-5} mg-min/L at 22 °C, respectively, for MS-2 phage inactivation by $^1O_2$. For example, for 1 log (90%) and 2 log (99%) inactivation of MS-2 phage, *CT* values of 3.6 × 10^{-5} and 6.4 × 10^{-5} mg-min/L would be required, regardless of light intensity, irradiation time and concentra-

tion of HC4 (hence the concentration of $^1O_2$). The *CT* value for 2 log inactivation of MS-2 phage measured herein was 33 times lager than the *CT* value of 1.9×10^{-6} mg-min/L that we estimated based on experimental data by Kohn and Nelson (*17*) in which 2 log MS-2 phage inactivation was achieved by 10 h exposure to $^1O_2$ at a steady-state concentration of 10^{-13} M produced by visible-light photosensitization of natural organic matter. The *CT* value for $^1O_2$ produced by HC4 was also slightly larger than the *CT* value of 1.1×10^{-5} mg-min/L measured for 2 log inactivation of MS-2 phage by •OH (i.e., generated by UVA photosensitization of a commercial TiO_2).

Although $^1O_2$ is a much weaker and more selective oxidant than •OH (oxidation potentials of •OH = 2.80 V and $^1O_2$ = 0.65 V), the *CT* values were somewhat comparable. This might be related to the fact that the concentration of $^1O_2$ at the bulk phase would be much smaller than that near the site of production, since the lifetime of $^1O_2$ is extremely short in water (approximately 3 μs (*21*)). Underestimation of the amount of $^1O_2$ that participate in actual MS-2 phage inactivation would lead to significant underestimation of *CT* (i.e., overestimation of $^1O_2$ inactivation efficiency). Consistently, Latch and McNeill reported that the concentration of $^1O_2$ (produced by photosensitization of natural organic matter) measured using a hydrophobic dioxetane precursor was orders of magnitude higher than that measured using hydrophilic FFA (*20*). This *CT* underestimation was perhaps further augmented by electrostatic interaction between negatively charged MS-2 phage (zeta potential = -55.4 ± 5.75 mV in 10 mM phosphate buffer) and positively charged HC4 terminal groups, such that MS-2 phage was exposed to elevated $^1O_2$ concentrated near the HC4 photocatalyst. The extent of *CT* underestimation needs further in-depth study with efforts on quantifying the $^1O_2$ concentration distribution around the HC4 photocatalyst and physical interaction of the phage with photocatalyst, which was beyond the scope of this study.

Mechanism of MS-2 Phage Inactivation by $^1O_2$. Another reason for efficient MS-2 phage inactivation by photochemically produced $^1O_2$ might be related to the fact that $^1O_2$ readily reacts with proteins and protein side chains with bimolecular rate constants ranging from 10^5 to 10^9 $M^{-1}s^{-1}$ (*12*). These rate constants are much higher than those for other biomolecules including RNA, which typically range from 10^4 to 10^6 $M^{-1}s^{-1}$ (*22, 23*). The two major components of MS-2 phage are the outer protein coat and the encapsulated RNA (*24*). Figure 6 shows that the concentration of protein carbonyls, the most commonly used marker of protein oxidation (*25, 26*), gradually increased as MS-2 phage was inactivated by $^1O_2$. For 2 log inactivation of MS-2 phage, approximately 67% of total proteins (measured using Bradford assay with MS-2 phage stock prior to disinfection experiment) were oxidized. When MS-2 phage was inactivated by UVC irradiation, protein oxidation was not observed. These results suggest that MS-2 phage inactivation was primarily related to relatively efficient $^1O_2$ oxidation of virus capsid proteins.

Photocatalytic Inactivation by Sunlight. Figure 7 shows the results of MS-2 phage inactivation by HC4 under sunlight irradiation. Experiments were performed only when the sunlight intensity at 365 nm was constant at approximately 190 μW/cm^2 for the duration of the experiments. Note that the temperature of the experimental suspension would increase from 22 to 32 °C without temperature control under this irradiation and weather condition. Results from a control experiment performed at 32 °C under dark (Experiment 1 in Figure 7) suggested that MS-2 phage inactivation by heat alone was negligible. Since the sunlight spectrum contains UV light, a slight level of MS-2 phage inactivation (47% (0.27 log) over 60 min sunlight exposure) was also observed even when HC4 was not added (Experiment 2) (*17, 27*). When the suspension contained 10 μM of HC4, a rapid inactivation of

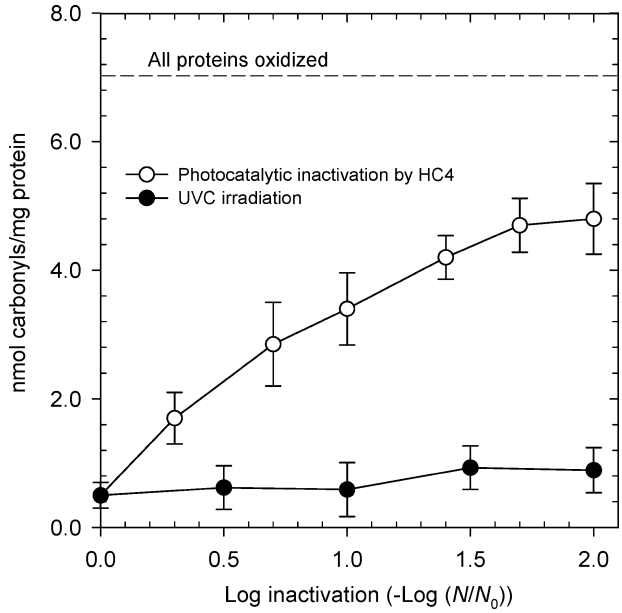

FIGURE 6. The amount of oxidized proteins (measured by protein carbonyls) during MS-2 phage photochemical inactivation by HC4 ($[HC4]_0$ = 15 μM, fluorescence lamp light intensity at 365 nm = 165 μW/cm^2) compared to inactivation by UVC irradiation (light intensity at 254 nm for UVC disinfection = 100 μW/cm^2).

MS-2 phage (faster than that observed under fluorescence lamp irradiation) was observed (experiment 3). When the temperature of the suspension was kept constant at 22 °C via water cooling as a control test (experiment 4), the kinetics was slightly slower. Finally, when UV light below 400 nm was cutoff using a UV filter (experiment 5), the inactivation kinetics also changed, but only slightly, suggesting that most inactivation resulted from visible-light photocatalysis.

Consistent with the results obtained with fluorescence lamp irradiation, the inactivation was prohibited when excess L-histidine was added (results not shown). Figure 7(b) shows the degradation of FFA, confirming that $^1O_2$ was produced and acted as a virucidal agent in this system. Note that there were no significant differences in FFA degradation even when the temperature increased from 22 to 32 °C during the experiment, while inactivation was slightly faster. Independent experiments performed at constant temperature using Rose Bengal as a photocatalyst under sunlight suggested that the time required to achieve 2 log MS-2 phage inactivation at 28 °C and 32 °C, were 39 and 20 min, respectively (data not shown). The increase in inactivation kinetics is therefore the result of enhanced susceptibility of MS-2 phage toward $^1O_2$ attack, consistent with enhanced inactivation kinetics with chemical disinfectants (*28*).

Environmental Implication and Application. Efficient production of $^1O_2$, facile reaction of $^1O_2$ with proteins in MS-2 phage capsid and electrostatic attraction between the positively charged HC4 and negatively charged MS-2 phage collectively make HC4 an especially promising agent for disinfection applications. More importantly, HC4 exhibits this virucidal property in response to visible light. This is in marked contrast to other commonly used photocatalysts such as TiO_2 which, without addition of other elements (e.g., doping agent), is photoactive only in response to UV irradiation. This unique property of HC4 might prove to be highly useful for the development of novel disinfecting approaches, which could function without an external energy source other than visible light and without the continuous addition of chemicals. It should be noted that HC4 contains amine functional groups that might be used to attach the HC4 fullerene molecule to solid media such as a particle or

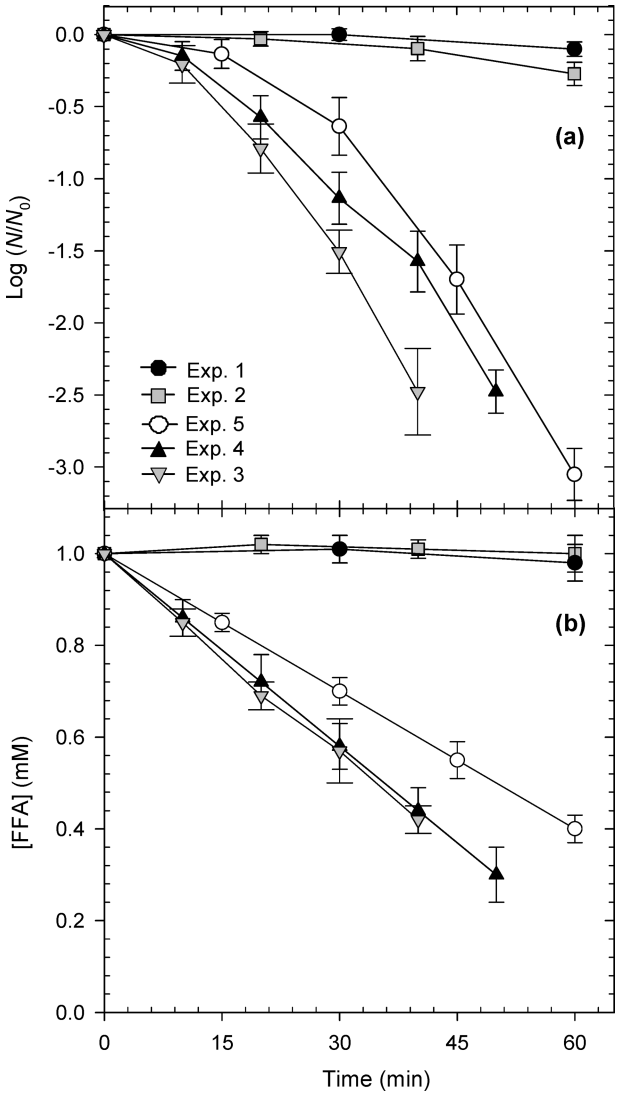

FIGURE 7. Inactivation of MS-2 phage by HC4 under sunlight irradiation (experiment 1: control experiment without HC4 at 32 °C under dark; experiment 2. control experiment without HC4 under sunlight; experiment 3: with 10 μM HC4 under sunlight; experiment 4.: with 10 μM HC4 under sunlight and at constant temperature of 22 °C; experiment 5: with 10 μM HC4 under sunlight after passing through a 400 nm UV filter).

a surface via covalent bonding. A gradual loss of physisorbed catalysts from such surfaces has been one of the key challenges for many inorganic catalysts, including TiO_2. Immobilization of HC4 onto surfaces of substances that can be easily recovered by common separation processes such as membrane filtration is therefore desirable for recycling and currently under investigation.

Acknowledgments

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Supporting Information Available

Additional figures showing the UV−visible spectra of the aqueous suspensions of C_{60} fullerene derivatives used in this work (Figure S1), MS-2 phage inactivation and $^1O_2$ production in Rose Bengal system (Figure S2), and the effect of natural organic matter on the inactivation efficiency of C_{60} fullerene derivative (Figure S3). This information is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

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