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Chalcogenoxanthylium photosensitizers for the photodynamic purging of blood-borne viral and bacterial pathogens

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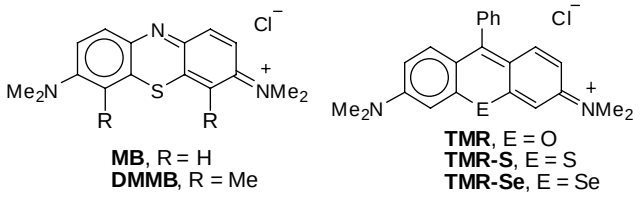
Abstract—Thio- and selenoxanthylium dyes were prepared by the addition of 2-lithiothiophene, 4-*N*,*N*-dimethylaminophenylmagnesium bromide, and 1-naphthylmagnesium bromide to the appropriate 2,7-bis-*N*,*N*-dimethylaminochalcogenoxanthen-9-one, followed by dehydration and ion exchange to the chloride salts. The corresponding chalcogenoxanthylium dyes were evaluated as photosensitizers for the inactivation of intracellular and extracellular virus in red blood cell suspensions and for the inactivation of selected strains of gram (+) and gram (−) bacteria in red blood cell suspensions. Selected combinations of photosensitizer and light gave >6 $\log_{10}$ inactivation of intracellular and extracellular virus, and >4 $\log_{10}$ inactivation of extracellular bacteria with varying levels of hemolyis, following a 42-day storage of red blood cell suspensions. Photocleavage experiments with plasmid DNA and the chalcogenoxanthylium dyes suggested the genomic material contained in the virus and in the bacteria as one possible target for the photodynamic action of some of these dyes.

1. Introduction

Blood transfusion exposes the recipient to some risk of becoming infected with a viral or bacterial pathogen from the donor. The safety of the blood supply has been drastically improved by the implementation of careful donor selection and extensive infectious disease testing. Despite these successful measures, a very small residual risk of pathogen transmission remains primarily due to collection of blood from infected individuals before they develop detectable levels of antigen, antibody, and/or nucleic acid. In the US, the residual risk of infection from a blood unit collected during this window period is 1 in 205,000 for HBV, 1 in 1,935,000 for HCV, 1 in 2,993,000 for HTLV-I and -2, and 1 in 2,135,000 for HIV.[1] Other known transmissible agents, such as *Babesia microti* and *Trypanosoma cruzi*, can also pose serious risks to recipients.[2–6] It is likely that unknown transfusion-transmissible agents will continue to emerge in the future. Pathogen reduction methods have been investigated in cellular blood components to minimize this risk.

One approach to the reduction of pathogens is photodynamic inactivation of pathogens using a photosensitizer and light.[7,8] The reduction of pathogens can be directed toward red blood cell (RBC) components and RBC-free components, such as plasma. Methylene blue (**MB**, Chart 1), is currently being used for the decontamination of freshly frozen plasma units.[7,8] Other phenothiazine photosensitizing dyes such as methylene violet and 1,9-dimethylmethylene blue (**DMMB**, Chart 1) have also been utilized in this approach.[9–14] In spite of the success of **MB** in the decontamination of plasma units,



MB, R = H
DMMB, R = Me

TMR, E = O
TMR-S, E = S
TMR-Se, E = Se

Chart 1. Structures of the phenothiazine dyes methylene blue (**MB**) and dimethylmethylene blue (**DMMB**), and thio- (**TMR-S**) and seleno-analogues (**TMR-Se**) of tetramethylrosamine (**TMR**).

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all earlier attempts to develop photosensitizers for pathogen inactivation of RBCs have failed, despite considerable efforts, either because of unacceptably low virucidal activity in blood or because of unacceptable hemolysis during storage.[15] These unsuccessful efforts reflect the difficulties associated with developing a method with sufficient specificity for $>6\log_{10}$ inactivation of extracellular pathogens, and, in addition to leukoreduction, $>4\log_{10}$ inactivation of intracellular pathogens, yet with <1% hemolysis (an FDA guideline) after a 42-day storage of red cells. The necessity for intracellular pathogen reduction is due to the need to inactivate infected white cells that may be present in red cell units. A typical leukoreplete red cell unit has approximately 2×10^9 white cells; a leukoreduced red cell unit typically contains fewer than 10^6 white cells.[16]

One disadvantage with **MB** is its inability to inactivate intracellular virus, presumably because the permanent positive charge of the hydrophilic dye prevents permeation through the plasma membrane of some cells.[17,18] The phenothiazine methylene violet, an uncharged dye, and **DMMB**, a more hydrophobic dye with permanent positive charge, overcome this deficiency and can inactivate intracellular virus.[11,14] However, these more hydrophobic dyes have a greater potential for hemolysis under more stringent virucidal conditions.[14,19] After a 42-day storage, hemolysis can be as extensive as 25% in traditional red cell storage media, but can be reduced to approximately 2% in an experimental medium that protects against colloidal osmotic hemolysis.[19,20] Red cell hemolysis from **DMMB** phototreatment can be reduced further to 1.2% by the addition of a molecule with a similar structure, quinacrine, which functions as a competitive inhibitor of dye binding to red cells.[20] The addition of red-cell-specific antioxidant ligands, such as dipyridamole, can also reduce the levels of hemolysis.[21,22] While these improvements are encouraging, levels of hemolysis have been unacceptable and new photosensitizers are a necessity for the robust inactivation of intracellular pathogens.

The synthesis of sulfur- and selenium-containing analogues (**TMR-S** and **TMR-Se**, respectively, Chart 1) of the xanthylium dye tetramethylrosamine (**TMR**, Chart 1) has recently been described.[23] The substitution of sulfur and, especially, selenium for oxygen in this series gave increased quantum yields for the generation of singlet oxygen. Both **TMR-S** and **TMR-Se** are photosensitizers in vitro against R3230AC[23] and AUXB1[24] cancer cells. In view of the similarity in the structures of **TMR-S** and **TMR-Se** with **MB** and related phenothiazine dyes, we examined **TMR-S**, **TMR-Se**, and a series of related structures for their ability to photoinactivate viral and bacterial pathogens in suspensions of RBCs.

2. Results

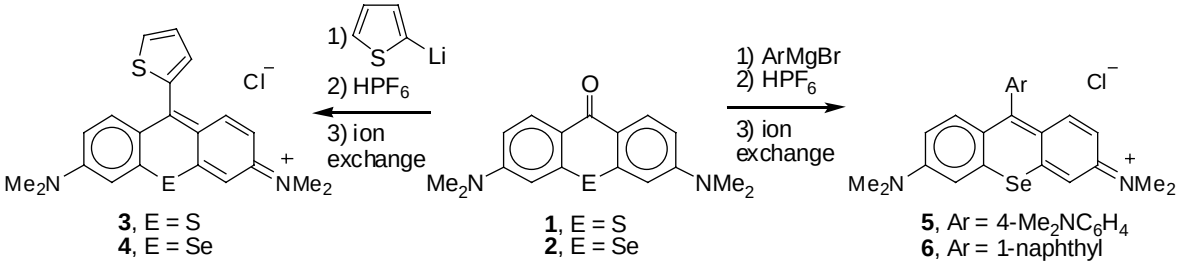
2.1. Synthesis of dyes

TMR-S and **TMR-Se** were prepared via the addition of phenylmagnesium bromide to chalcogenoxanthen-9-ones **1** and **2**, respectively, followed by acid-induced dehydration and ion exchange.[23] A similar approach was used to prepare 9-2-thienyl-substituted derivatives **3** and **4** (Scheme 1). The addition of 2-lithiothiophene to **1** and **2**, followed by dehydration with HPF_6, gave the PF_6^- salts of dyes **3** and **4**, respectively, in 80% isolated yield for each. Ion exchange with Amberlite IRA-400 chloride exchange resin gave the chloride salts **3** in 95% isolated yield and **4** in 92% isolated yield. The addition of 4-*N*,*N*-dimethylaminophenylmagnesium bromide to **2**, followed by dehydration with HPF_6, gave the PF_6^- salt of **5** in 93% isolated yield. Similarly, the addition of 1-naphthylmagnesium bromide to **2** followed by dehydration with HPF_6 gave the PF_6^- salt of **6** in 45% isolated yield. Ion exchange with Amberlite IRA-400 chloride exchange resin gave the chloride salts **5** and **6** in 69% and 90% isolated yields, respectively.

2.2. Chemical and photophysical properties

Absorption maxima (λ_{max}), quantum yields for the generation of singlet oxygen [$\phi(^1O_2)$], and values of the *n*-octanol/water (pH 6 phosphate buffer) partition coefficient ($\log P$) for **TMR-S**, **TMR-Se**, and **3**–**6** are given in Table 1. Substituent changes at the 9-position have significant impact on the values of λ_{max}, $\phi(^1O_2)$, and $\log P$.

Values of λ_{max} are sensitive to the nature of the 9-substituent on the chalcogenoxanthylium nucleus (Table 1). Dyes based on the thioxanthylium nucleus have values of λ_{max} approximately 10 nm shorter than the values of λ_{max} for the corresponding selenoxanthylium dye (**TMR-S** vs **TMR-Se**; **3** vs **4**). The 9-2-thienyl substituent gives a 20-nm bathochromic shift, relative to the 9-phenyl substituent. Thus, **3** has λ_{max} of 590 nm and **TMR-S** has λ_{max} of 571 nm. The 4-*N*,*N*-dimethylaminophenyl



Scheme 1. Synthesis of chalcogenoxanthylium dyes **3**–**6** from chalcogenoxanthen-9-ones **1** and **2**.

Table 1. Absorption maxima (λ_{max}) in CH_2Cl_2, quantum yields for the generation of singlet oxygen [$\phi(^1O_2)$] in MeOH, and *n*-octanol/water partition coefficients (log *P*) for **TMR-S**, **TMR-Se**, and **3**–**6**

Compound	λ_{max} (nm) (log ε)	$\phi(^1O_2) \pm SD$[a]	log *P*[b]
TMR	552 (4.92)[c]	0.06 ± 0.03	—
TMR-S	571 (4.70)[c]	0.21 ± 0.02[c]	0.07 ± 0.02
TMR-Se	582 (4.84)[c]	0.87 ± 0.01[c]	0.09 ± 0.07
3	590 (4.57)	0.08 ± 0.02	−0.49 ± 0.03
4	601 (4.78)	0.43 ± 0.02	−0.31 ± 0.04
5	576 (4.75)	0.02 ± 0.01	0.49 ± 0.03
6	587 (4.60)	0.71 ± 0.02	1.80 ± 0.05

[a] Direct detection of singlet-oxygen luminescence using rose Bengal as a standard.
[b] pH 6 phosphate buffer as the aqueous phase.
[c] Ref. 23.

substituent imparts a small hypsochromic shift (6 nm), relative to the phenyl substituent, while the 9-(1-naphthyl) substituent imparts a small (5 nm) bathochromic shift, relative to the phenyl substituent.

Values of $\phi(^1O_2)$ in MeOH were quite sensitive to the 9-substituent in these molecules (Table 1). While **TMR-S** with a 9-phenyl substituent has $\phi(^1O_2)$ of 0.21, compound **3** with a 9-2-thienyl substituent has a $\phi(^1O_2)$ of 0.08. A similar halving of $\phi(^1O_2)$ was observed when the 9-phenyl substituent of **TMR-Se** [$\phi(^1O_2)$ 0.87] was replaced with a 9-2-thienyl substituent in selenoxanthylium dye **4** [$\phi(^1O_2)$ 0.43]. The 9-phenyl and 9-1-naphthyl substituents have a similar impact on $\phi(^1O_2)$ with the values of $\phi(^1O_2)$ of 0.87 and 0.71 for **TMR-Se** and selenoxanthylium dye **6**, respectively. Selenoxanthylium dye **5** with a 9-4-*N*,*N*-dimethylaminophenyl substituent has a value of $\phi(^1O_2)$ of only 0.02, which is much smaller than that of $\phi(^1O_2)$ for any of the other molecules of this study. Internal conversion dominates the photophysics of the excited state of **5**.

Values of log *P* also cover a wide range in the molecules of this study (Table 1). In pairwise comparisons, the chalcogen atom has little impact on log *P*, relative to the impact of the 9-substituent. Thus, **TMR-S** and **TMR-Se** have nearly identical values of log *P* (0.07 and 0.09, respectively) as do compounds **3** and **4** (log *P* of −0.49 and −0.31, respectively). Values of log *P* indicate that compounds **3** and **4** are somewhat hydrophilic, that **TMR-S** and **TMR-Se** are equally hydrophilic and lipophilic, that compound **5** is somewhat lipophilic (log *P* 0.49), and that compound **6** (log *P* 1.80) is highly lipophilic.

2.3. Inactivation of viral pathogens with chalcogenoxanthylium dyes

Oxygenated leukodepleted 20% hematocrit RBC suspensions in a citrate-containing additive solution, Erythrosol, were deliberately inoculated with extracellular vesicular stomatitis virus (VSV), a negative single stranded RNA virus used as a model for HIV, or pseudorabies virus (PRV), a double stranded DNA virus used as a model for cytomegalovirus, or intracellular VSV, incubated with various concentrations of the dye and subsequently illuminated with 2.0–7.4 J cm^{-2} of 400–750-nm white light. Controls and treated samples were assayed for virus and results are given in Table 2.

The photosensitizer, **TMR-Se**, is extremely virucidal in red cell suspensions. Only 1 μM **TMR-Se** and 2.0 J cm^{-2} 400–750-nm light were required to inactivate 7.0 $\log_{10}$ of extracellular VSV in 20% hematocrit red cells (Table 2). Unfortunately, no significant inactivation of intracellular virus (≈0.2 $\log_{10}$) was observed under these conditions. Higher concentrations (5 or 10 μM) of **TMR-Se** resulted in the inactivation of extracellular PRV (>8 $\log_{10}$) and intracellular VSV (2.5 and 4.4 $\log_{10}$, respectively). The sulfur-containing analogue, **TMR-S**, required 50 μM concentrations with 2.5 J cm^{-2} of 400–750-nm light to give comparable effectiveness toward extracellular VSV (>8.0 $\log_{10}$ inactivation). However, under these conditions, **TMR-S** was also effective against intracellular VSV (5.5 $\log_{10}$ inactivation).

As shown in Figure 1 for **TMR-S**, the inactivation of virus is a function of both light dose and photosensitizer

Table 2. Inactivation of extracellular VSV and PRV, and intracellular VSV with chalcogenoxanthylium dyes **TMR-S**, **TMR-Se**, and **3**–**6** and 400–750-nm white light

Compound	Concentration (μM)	*hν* (J cm^{-2})	$\log_{10}$ extracellular VSV	Inactivation extracellular PRV	Intracellular VSV	% hemolysis (42 days)
TMR-S	20	7.4	>7.98 ± 0.02	>8.15 ± 0.08	5.46 ± 0.89	9.2
	50	2.5	>8.00 ± 0.14	>8.26 ± 0.06	5.20 ± 0.78	1.2
TMR-Se	1	2.0	6.95 ± 0.54	0.90 ± 0.19	0.21 ± 0.07	0.22
	5	4.9	>7.48 ± 0.15	>8.28 ± 0.51	2.51 ± 0.25	9.2[a]
	10	2.5	>7.62 ± 0.14	>8.41 ± 0.19	4.37 ± 0.37	10.0[a]
3	10	7.4	>7.87 ± 0.04	6.40 ± 0.90	1.8 ± 1.0	0.90
	25	4.9	>7.91 ± 0.03	>8.20 ± 0.09	5.51 ± 0.60	1.3
4	5	2.5	>7.39 ± 0.58	3.27 ± 0.82	—	—
	10	4.9	>7.89 ± 0.01	>8.17 ± 0.06	3.2 ± 1.3	8.5[a]
5	10	7.4	>7.85 ± 0.05	3.14 ± 2.86	0.47 ± 0.35	1.6
6	5	2.5	>7.83 ± 0.14	1.95 ± 0.07	1.21 ± 0.96	—
	5	4.4	<7.82 ± 0.10	3.21 ± 0.17	1.28 ± 0.57	47[a]

[a] After 7 days.

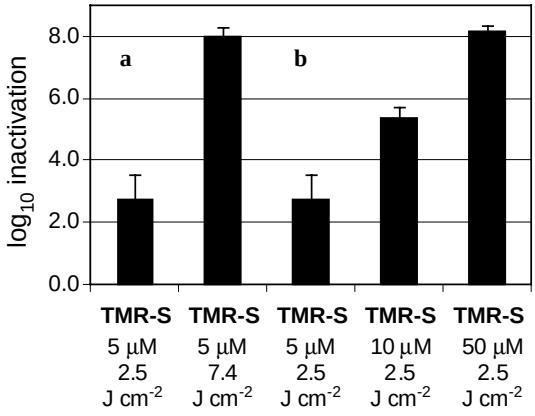


Figure 1. Inactivation of extracellular VSV as a function of: (a) light dose with 400–750-nm light and 5 μM **TMR-S** and (b) **TMR-S** concentration with 2.5 J cm^{-2} of 400–750-nm light.

concentration. Both increased light dose and increased concentration of photosensitizer give increased viral inactivation. Inactivation levels of approximately $8\log_{10}$ indicate the limit of detection of the VSV assay.

Selenoxanthylium dye **4** with a 2-thienyl substituent in the 9-position required a higher concentration of photosensitizer to achieve the same virucidal activity as **TMR-Se**. At 10 μM **4** and 4.9 J cm^{-2} of 400–750-nm light, $>7.9\log_{10}$ inactivation of extracellular VSV and $3.2\log_{10}$ inactivation of intracellular VSV were observed. As was observed in a comparison of **TMR-S** and **TMR-Se**, the thioxanthylium analogue **3** was less active than the selenium analogue **4** against extracellular VSV and PRV, and intracellular VSV. With 10 μM **3** and 7.4 J cm^{-2} of 400–750-nm white light, $6.4\log_{10}$ inactivation of extracellular PRV and $1.8\log_{10}$ inactivation of intracellular VSV were observed. With 10 μM **4** and only 4.9 J cm^{-2} of 400–750-nm light, $>8.1\log_{10}$ inactivation of extracellular PRV and $3.2\log_{10}$ inactivation of intracellular VSV were observed. Inactivation of intracellular VSV ($5.5\log_{10}$) with **3** could be achieved using greater concentrations of dye (25 μM) and 4.9 J cm^{-2} of 400–750-nm light.

Selenoxanthylium dye **5** with a 4-*N*,*N*-dimethylaminophenyl substituent at the 9-position was active against extracellular VSV and PRV, and intracellular VSV. At 10 μM with 7.4 J cm^{-2} of 400–750-nm light, $>7.9\log_{10}$ inactivation of extracellular VSV was observed under conditions that inactivated PRV and intracellular VSV by 3.1 and $0.4\log_{10}$, respectively. Selenoxanthylium dye **6** with a 1-naphthyl substituent at the 9-position gave $>7.8\log_{10}$ inactivation of extracellular VSV, with less inactivation of PRV ($3.2\log_{10}$) and intracellular VSV ($1.3\log_{10}$) using 5 μM **6** and 4.4 J cm^{-2} of 400–750-nm light.

One of the limits for the use of photosensitizers and light for pathogen inactivation in RBC suspensions is the extent of hemolysis observed in the red blood cells following treatment. Identically prepared, but uncontaminated, RBC suspensions with selected concentrations of **TMR-S**, **TMR-Se**, and **3**–**6** were phototreated under identical conditions, and subsequently stored under standard blood bank conditions for 42 days to determine the levels of hemolysis, which are also given in Table 2. With 1 μM **TMR-Se** and 2.0 J cm^{-2} of 400–750-nm light, only 0.22% hemolysis was observed during a 42-day storage, well under the FDA guideline of <1.0%. However, under conditions that inactivated $4.4\log_{10}$ intracellular VSV, 10% hemolysis was observed in **TMR-Se** samples after 1 week of 1–6 °C storage. In contrast, 50 μM **TMR-S** and 2.5 J cm^{-2} of 400–750-nm light gave 1.2% hemolysis at 42 days and inactivated $5.2\log_{10}$ of intracellular VSV (Table 2). While it is encouraging, this level of hemolysis is somewhat higher than the FDA guideline. Similar results were obtained with 25 μM of the thioxanthylium dye **3** and 4.9 J cm^{-2} of light where conditions that gave acceptable levels of intracellular and extracellular inactivation of virus produced levels of hemolysis that were only slightly above the FDA guideline at day 42 of storage (1.3%, Table 2). In contrast, the selenoxanthylium dye **4** at 10 μM with 4.9 J cm^{-2} of 400–750-nm light gave 8.5% hemolysis after only 7 days of storage and did not adequately inactivate intracellular VSV.

Selenoxanthylium dyes **5** and **6** represent an interesting contrast. Under conditions where both dyes give $7.8\log_{10}$ inactivation of extracellular VSV, dye **5** displays only 1.6% hemolysis after 42 days of storage, while dye **6** displays 47% hemolysis after only 7 days.

2.4. Inactivation of bacterial pathogens

Oxygenated leukodepleted 20% hematocrit RBC suspensions in a citrate-containing additive solution, Erythrosol, were deliberately inoculated with both gram (+) (*Staphylococcus aureus*, *Staphylococcus epidermidis*) and gram (−) (*Pseudomonas fluorescens*, *Yersinia enterocolitica*) extracellular bacteria, incubated with 10 μM **TMR-Se** or 10 μM selenoxanthylium dye **4**, and subsequently illuminated with 2.5 or 4.9 J cm^{-2} of 400–750-nm light, respectively. Controls and treated samples were assayed for bacteria and results are given in Table 3. Both **TMR-Se** and **4** were effective against gram (+) bacteria with $\geqslant 2.5\log_{10}$ inactivation of *S. aureus* and $>8\log_{10}$ inactivation of *S. epidermidis*. Among the gram (−) bacteria, *P. fluorescens* and *Y. enterocolica* are responsible for up to 75% of clinical sepsis from red cells.[25] **TMR-Se** was effective against *Y. enterocolitica* with $4.0\log_{10}$ inactivation, but was much less effective against *P. fluorescens* with only $0.4\log_{10}$ inactivation. Selenoxanthylium dye **4** also did not inactivate *P. fluorescens* yet showed $4.5\log_{10}$ inactivation of *Y. enterocolitica*.

2.5. DNA as a target for photodynamic action

Both **TMR-Se** and **4** damage plasmid DNA in solution upon irradiation. Solutions of plasmid DNA (7.5 μM in base pairs) and photosensitizer (1 μM) were irradiated for various times with 500–800-nm light delivered at 50 mW cm^{-2}. As shown in Figure 2 for **TMR-Se**, irradiation for 60 min (180 J cm^{-2}) gave essentially complete

Table 3. Inactivation of gram (+) and gram (−) bacterial pathogens with chalcogenoxanthylium dyes **TMR-Se** and **4** and 400–750-nm light

Compound	Concentration (μM)	*hv* (J cm^{-2})	$\log_{10}$ inactivation			
			S. aureus	*S. epidermidis*	*P. fluorescens*	*Y. entercolitica*
TMR-Se	10	2.5	2.5 ± 1.2	>8.45 ± 0.08	0.37 ± 0.15	3.97 ± 0.34
4	10	4.9	>6.7 ± 1.7	>8.46 ± 0.03	0.33 ± 0.26	4.5 ± 1.6

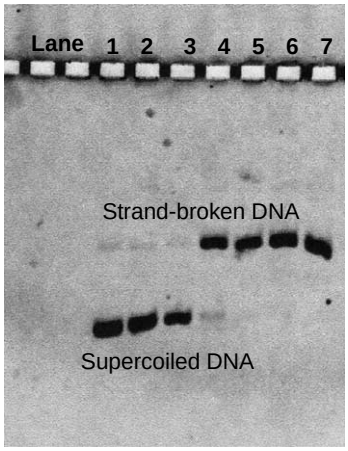


Figure 2. Photochemical damage to supercoiled plasma DNA with **TMR-Se** and 0–180 J cm^{-2} of 500–800-nm light (50 mW cm^{-2} for 0–60 min). Lane 1, plasmid DNA (no dye, no light); Lane 2, plasmid DNA + 60 min light; Lane 3, plasmid DNA + 1 μM **TMR-Se** (no light); Lanes 4–7, plasmid DNA + 1 μM **TMR-Se**; Lane 4, 15 min light; Lane 5, 30 min light; Lane 6, 45 min light; Lane 7, 60 min light.

'nicking' of supercoiled plasmid DNA to give strand-broken DNA with both **TMR-Se** and **4**.[26] The plasmid DNA was stable to 180 J cm^{-2} of 500–800-nm light and was stable in the dark to 1 μM solutions of photosensitizer (shown in Figure 1 for **TMR-Se**). As illustrated in Figure 3 for **4**, the 'nicking' of supercoiled DNA was directly proportional to the light dose with both dyes. It is not clear whether the DNA damage is a result of intercalation or less specific association with the anionic deoxyriboses of the DNA. The addition of plasmid DNA to either **TMR-Se** or **4** gives a small bathochromic shift (3 nm) in λ_{max}.

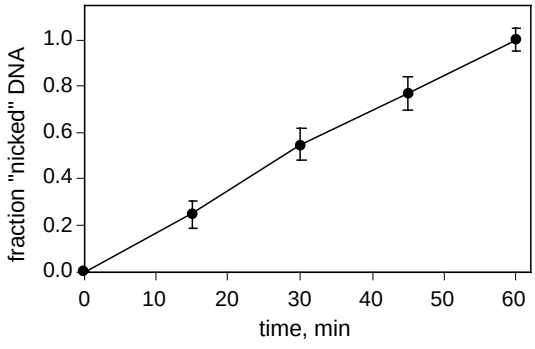


Figure 3. Light-dose-dependent damage to plasmid DNA with 1 μM **4** and 500–800-nm light delivered at 50 mW cm^{-2} for indicated time periods. The ratio of supercoiled DNA and 'nicked' DNA was determined via densities using a phosphoimager, following gel electrophoresis. The line between points shows only their connectivity and has no significance. Bars are SEM.

3. Discussion

The selective inactivation of viral and bacterial pathogens in RBC suspensions via treatment with a photosensitizer and light places several requirements on the photosensitizer. The combination of photosensitizer and light should give >6 $\log_{10}$ inactivation of extracellular virus or bacteria and, in addition to leukodepletion, >4 $\log_{10}$ inactivation of intracellular virus. Ideal for the purging of blood-borne pathogens, the photosensitizer will absorb strongly with λ_{max} > 580 nm to avoid hemoglobin bands at 541 and 575 nm, and the photosensitizer should generate singlet oxygen or other reactive oxygen species upon irradiation. The photosensitizer should also target a site in the virus or bacterium that is not targeted in RBCs. Red blood cells lack RNA, DNA, and mitochondria.[27] Photosensitizers that target RNA or DNA should selectively bind to the virus or bacterium. Although dyes, such as **MB**, **TMR-S**, and **TMR-Se**, target mitochondria in cells,[22,15] the absence of mitochondria in mature RBCs eliminates these sites as targets for photosensitizers in the RBCs. Finally, the combination of photosensitizer and light should not be hemolytic toward RBCs.

Evaluation of the series of chalcogenoxanthylium dyes **TMR-S**, **TMR-Se**, and **3-6** indicates that the chalcogenoxanthylium dyes may be a promising new class of compounds for inactivation of blood-borne pathogens. With the exception of **TMR-S** with λ_{max} of 571 nm, the remaining five dyes all absorb with values of λ_{max} > 580, which place them safely above the absorption bands of hemoglobin.

What is most interesting about the correlation of photophysical properties with virucidal activity in these molecules is that there is no correlation of $\phi(^1O_2)$ with virucidal activity. All six dyes at some combination of 1–20 μM dye and 2.0–7.4 J cm^{-2} of 400–750-nm light gave >6 $\log_{10}$ inactivation of extracellular VSV, while **TMR-S**, **TMR-Se**, **3**, and **6** also gave >5 $\log_{10}$ inactivation of extracellular PRV (Table 2). Thioxanthylium dyes **TMR-S** and **3** were most effective at inactivating intracellular virus with >6 $\log_{10}$ inactivation of intracellular VSV with 50 μM **TMR-S** and 2.5 J cm^{-2} of 400–750-nm light, and 25 μM **3** and 4.9 J cm^{-2} of 400–750-nm light. Although these concentrations are higher than those used for inactivation of extracellular virus, hemolysis levels under these more stringent conditions were only 1.2% and 1.3%, respectively. The selenoxanthylium dyes **TMR-Se** and **4-6** gave >6 $\log_{10}$ inactivation of extracellular virus at concentrations lower than those of the thioxanthylium dyes, but had slightly less to much less virucidal activity against intracellular virus compared to **TMR-S** and compound **3**. The

selenoxanthylium dyes also displayed a greater level of hemolytic activity toward RBCs than the thioxanthylium dyes under conditions that inactivated >7 $\log_{10}$ extracellular virus and only moderate levels of intracellular virus. Thus, **TMR-Se**, or compound **4** or **6** produced >8% hemolysis after only 1 week of storage under conditions that inactivated >7 $\log_{10}$ of extracellular and 1.3–4.4 $\log_{10}$ of intracellular virus.

The two thioxanthylium dyes of this study produced the least hemolysis in RBCs after 42 days of storage. **TMR-S** gave 1.2% hemolysis, while compound **3** gave 1.3% hemolysis. These two molecules also had two of the smaller values of $\phi(^1O_2)$ with $\phi(^1O_2)$ of 0.20 for **TMR-S** and $\phi(^1O_2)$ of 0.08 for **3**. One can speculate that the presence of the sulfur atom in the ring rather than a selenium atom may be responsible for the reduced hemolysis or that the slower production of singlet oxygen with smaller values of $\phi(^1O_2)$ may lead to reduced oxidative damage in RBCs. An argument for the latter point is found in the behavior of selenoxanthylium dye **5** where $\phi(^1O_2)$ is only 0.02 and the 42-day hemolysis is only 1.6%. Hemolysis may be a result of the red blood cell's inability to fight oxidative damage from the photosensitizers with high values of $\phi(^1O_2)$.

The 1.2% hemolysis observed with **TMR-S**, the 1.3% hemolysis observed with thioxanthylium dye **3**, and the 1.6% hemolysis observed with selenoxanthylium dye **5** are without the benefit of added competitive inhibitors of dye binding, such as quinacrine,[20] or without the benefit of red-cell-specific antioxidant ligands, such as dipyridamole.[21,22] Red cell hemolysis from **DMMB** phototreatment was reduced from 25% to 1.2% through the use of a storage medium that protects against colloidal osmotic hemolysis and by the addition of quinacrine.[20] It should be possible to reduce further the already low levels of hemolysis observed with **TMR-S**, **3**, and **5**.

Two chalcogenoxanthylium dyes were investigated for inactivation of bacterial pathogens. Both **TMR-Se** and **4** were effective photosensitizers for >5 $\log_{10}$ inactivation of the gram (+) bacteria *S. aureus* and *S. epidermidis*, but were less effective photosensitizers for the inactivation of the gram (−) bacteria *P. fluorescens* and *Y. enterocolitica* (Table 3). Only a combination of 10 μM **TMR-Se** and 2.5 J cm^{-2} of 400–750-nm light gave ≈4 $\log_{10}$ inactivation of *Y. enterocolitica*. These conditions yielded only 0.4 $\log_{10}$ inactivation of *P. fluorescens*. The combination of 10 μM **4** and 4.9 J cm^{-2} of 400–750-nm light gave no inactivation of *P. fluorescens* and 4.5 $\log_{10}$ inactivation of *Y. enterocolitica*.

Irradiation of plasmid DNA treated with chalcogenoxanthylium dyes **TMR-Se** and **4** produced strand cleavage of the supercoiled DNA (Figs. 2 and 3). The DNA damage was directly proportional to the light dose, as shown in Figure 3. The virucidal activity of the chalcogenoxanthylium dyes was also directly related to light dose and to photosensitizer concentration, as shown in Figure 1. These data suggest that genomic material (DNA, RNA) could be one target for the photodynamic action of these dyes.

If viral and/or bacterial DNA/RNA are targets for the photosensitizer, one can reasonably ask whether the relative values of $\phi(^1O_2)$ are higher in the photosensitizer-DNA/RNA complex, relative to solution values. In the case of **TMR-S**, **3**, and **5**, if internal conversion is responsible for the low values of $\phi(^1O_2)$ in solution, then complexation may give a significant increase in singlet-oxygen generation in the pathogen, relative to solution, by limiting internal conversion. In the selenoxanthylium dyes **TMR-Se**, **4**, and **6**, values of $\phi(^1O_2)$ are large and the photophysics of these molecules is dominated by intersystem crossing to the triplet. Internal conversion is less important and singlet-oxygen generation in the photosensitizer-DNA/RNA complex may be comparable to that observed in solution.

In summary, the chalcogenoxanthylium dyes represent a new class of photosensitizers that may be useful in the inactivation of blood-borne pathogens. Selected members of the series show adequate inactivation of extracellular and intracellular virus, as well as inactivation of gram (+) and gram (−) bacteria. The levels of hemolysis of RBCs ranged from an acceptable 0.22% with **TMR-Se** after a 42-day storage to an unacceptable 47% with **5** after only a 7-day storage. We are preparing new derivatives of this class that hopefully will provide >6 $\log_{10}$ inactivation of extracellular, >4 $\log_{10}$ intracellular virus, and >4 $\log_{10}$ of extracellular bacteria with <1% hemolysis after a 42-day storage of treated, leukodepleted RBC suspensions.

4. Experimental

4.1. General methods

Solvents and reagents were used as received from Sigma–Aldrich Chemical Co. (St. Louis, MO) unless otherwise noted. Chalcogenoxanthones **1** and **2** were prepared according to Refs. 23,28, respectively. **TMR-S** and **TMR-Se** were prepared according to Ref. 23. Concentration in vacuo was performed on a Büchi rotary evaporator. NMR spectra were recorded on a Varian Inova 500 instrument with residual solvent signal as internal standard. UV–Vis-near-IR spectra were recorded on a Perkin-Elmer Lambda 12 spectrophotometer equipped with a circulating constant temperature bath for the sample chambers. Elemental analyses were conducted by Atlantic Microlabs, Inc. (Norcross, GA).

4.1.1. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(2-thienyl)selenoxanthylium chloride (4). *n*-Butyllithium (1.6 M, 1.5 mL, 2.4 mmol) was added dropwise to a solution of *N*,*N*,*N*,*N*-tetramethylethylenediamine (**TMEDA**, 0.40 mL, 2.4 mmol) and thiophene (0.19 mL, 2.3 mmol) in 12 mL of anhydrous THF at −78 °C. The resulting mixture was stirred for 1 h at −78 °C and a solution of 2,7-bis-*N*,*N*-dimethylamino-9*H*-selenoxanthen-9-one (**2**, 0.20 g, 0.58 mmol) in anhydrous THF (4 mL) was added dropwise. The resulting mixture was stirred for 1 h at −78 °C and was then heated at reflux for 0.5 h, allowed to cool to ambient temperature, and poured into acetic acid (3.0 mL).

Hexafluorophosphoric acid (60%-by-weight solution in water) was added dropwise until a color change was observed. Water (50 mL) was added and the solution was cooled to −10 °C. The resulting precipitate was collected by filtration and the solid was washed with water (10 mL) and diethyl ether (10 mL), followed by recrystallization from acetonitrile and a small amount of diethyl ether to give 0.27 g (80%) of a dark green solid. The hexafluorophosphate salt (0.26 g) was dissolved in 20 mL acetonitrile and 1.00 g of Amberlite IRA-400 Chloride ion exchange resin was added. The resulting mixture was stirred for 0.5 h, followed by removal of the exchange resin via filtration. The process was repeated with two additional 0.5 g aliquots of the ion exchange resin. Following the final ion exchange, the filtrate was concentrated and the crude product was recrystallized from acetonitrile and a small amount of diethyl ether to give 0.24 g (92%) of the product as a dark green solid,. mp 265–266 °C: ^{1}H NMR [500 MHz, CD_3OD] δ 7.86 (d × d, 1H, J = 1.0, 5.2 Hz), 7.64 (d, 2H, J = 9.8 Hz), 7.52 (d, 2H, J = 2.7 Hz), 7.34 (d × d, 1H, J = 3.5, 5.2 Hz), 7.26 (d × d, 1H, J = 1.2, 3.5 Hz), 7.05 (d × d, 2H, J = 2.7, 9.8 Hz) 3.28 (s, 12H); ^{13}C NMR [300 MHz, CD_3OD] δ 152.3, 144.6, 136.9, 135.7, 130.0, 127.7, 126.6, 119.6, 114.3, 108.1, 99.4, 38.9; λ_{max} (CH_2Cl_2) 601 nm ($\varepsilon = 6.0 \times 10^4$ M^{-1} cm^{-1}); HRMS (ESI) m/z 413.0584 (Calcd $C_{21}H_{21}N_2S^{80}Se$: 413.0585). Anal. Calcd for $C_{21}H_{21}N_2SSePF_6$: C, 45.25; H, 3.80; N, 5.03. Found: C, 45.43; H, 3.80; N, 5.01. Calcd for $C_{21}H_{21}N_2SSePCl{\cdot}0.25\ H_2O$: C, 55.75; H, 4.79; N, 6.19. Found: C, 55.88; H, 4.95; N, 5.94.

The chloride salt of **4** is hygroscopic and elemental analysis reflects the incorporation of 0.25 water of crystallization. The water of crystallization was observed in ^{1}H NMR spectra where the water/chloroform ratio increases upon the addition of the chloride salt. Compounds **3**, **5**, and **6** incorporated 0.5–2.5 waters of crystallization.

4.1.2. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(2-thienyl)thioxanthylium chloride (3). A solution of 2,7-bis-*N*,*N*-dimethylamino-9*H*-thioxanthen-9-one (**1**, 0.20 g, 0.67 mmol) in anhydrous THF (4 mL) was treated with 1.6 M *n*-butyllithium (1.7 mL, 2.7 mmol), **TMEDA** (0.41 mL, 2.8 mmol), and thiophene (0.21 mL, 2.7 mmol) in 12 mL of anhydrous THF, as described for the preparation of **4**. The reaction mixture was then treated with acetic acid (3.0 mL) and HPF_6 (60%-by-weight solution in water) as described. The crude product was recrystallized from acetonitrile and a small amount of diethyl ether to give 0.27 g (80%) of a dark green solid. The hexafluorophosphate salt (0.25 g) was dissolved in 20 mL acetonitrile and 1.00 g of Amberlite IRA-400 Chloride ion exchange resin was added. The resulting mixture was stirred for 0.5 h, followed by removal of the exchange resin via filtration. The process was repeated with two additional 0.5 g aliquots of the ion exchange resin. Following the final ion exchange, the filtrate was concentrated and the crude product was recrystallized from acetonitrile and a small amount of diethyl ether to give 0.24 g (95%) of the product as a dark green solid, mp 255–256 °C: ^{1}H NMR [500 MHz, CD_3OD] δ 7.91 (d × d, 1H, J = 1.2, 5.0 Hz), 7.65 (d, 2H, J = 9.8 Hz), 7.38 (d × d, 1H, J = 3.7, 5.0 Hz), 7.32 (d × d, 1H, J = 1.2, 3.7 Hz), 7.30 (d, 2H, J = 2.1 Hz), 7.15 (d × d, 2H, J = 2.7, 9.8 Hz), 3.21 (s, 12H); ^{13}C NMR [300 MHz, CD_3OD] δ 152.9, 151.8, 143.1, 135.1, 134.0, 130.4, 128.2, 126.7, 118.2, 114.8, 104.7, 38.8; λ_{max} (CH_2Cl_2) 590 nm ($\varepsilon = 3.7 \times 10^4$ M^{-1} cm^{-1}), HRMS (ESI) m/z 365.1131 (Calcd for $C_{21}H_{21}N_2S_2$: 365.1141). Anal. Calcd for $C_{21}H_{21}N_2S_2Cl{\cdot}2\ H_2O$: C, 57.71; H, 5.77; N, 6.41. Found: C, 57.60; H, 5.61; N, 6.32.

4.1.3. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(4-*N*,*N*-dimethylaminophenyl)selenoxanthylium chloride (5). A mixture of *N*,*N*-dimethyl-4-bromoaniline (0.23 g, 1.2 mmol) and ground magnesium turnings (0.030 g, 1.2 mmol) in 10 mL of anhydrous THF was heated at reflux for 2 h and then cooled to ambient temperature. The resulting solution was then added via cannula to a solution of 2,7-bis-*N*,*N*-dimethylamino-9*H*-selenoxanthen-9-one (0.10 g, 0.29 mmol) in anhydrous THF (3 mL). The reaction mixture was heated at reflux for 0.5 h, cooled to ambient temperature, and poured into acetic acid (3.0 mL). Hexafluorophosphoric acid (60 %-by-weight solution in water) was added dropwise until a color change was observed. Water (50 mL) was added and the solution was cooled to −10 °C. The resulting precipitate was collected by filtration. The solid was washed with water (10 mL), diethyl ether (10 mL), and was recrystallized from acetonitrile and a small amount of ether to give 0.16 g (93%) of the product as a dark green solid. The hexafluorophosphate salt (0.160 g) was dissolved in 20 mL acetonitrile and 1.00 g of Amberlite IRA-400 Chloride ion exchange resin was added. The resulting mixture was stirred for 0.5 h, followed by removal of the exchange resin via filtration. The process was repeated with two additional 0.5 g aliquots of the ion exchange resin. Following the final ion exchange, the filtrate was concentrated and the crude chloride salt was recrystallized from acetonitrile and a small amount of ether to give 0.089 g (69%) of the product as a dark green solid, mp 251–252 °C: ^{1}H NMR [500 MHz, CD_2Cl_2] δ 7.68 (d, 2H, J = 9.8 Hz), 7.24 (d, 2H, J = 2.4 Hz), 7.14 (d, 2H, J = 8.5 Hz), 6.87 (d, 2H, J = 8.5 Hz), 6.84 (d × d, 2H, J = 2.4, 9.8 Hz), 3.24 (s, 12H), 3.09 (s, 6H); ^{13}C NMR [500 MHz, CD_2Cl_2] δ 153.5, 144.4, 136.5, 135.4, 129.5, 129.2, 128.7, 119.2, 115.1, 114.2, 105.4, 40.5; λ_{max} (CH_2Cl_2) 576 nm ($\varepsilon = 5.6 \times 10^4$ M^{-1} cm^{-1}), 580 nm (sh, $\varepsilon = 5.7 \times 10^3$ M^{-1} cm^{-1}); HRMS (ESI) m/z 450.1440 (Calcd for $C_{25}H_{28}N_3{}^{80}Se$: 450.1443). Anal. Calcd for $C_{25}H_{28}N_3SeCl{\cdot}0.5\ H_2O$: C, 60.79; H, 5.92; N, 8.51. Found: C, 61.01; H, 5.98; N, 8.25.

4.1.4. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(1-naphthyl)selenoxanthylium chloride (6). A solution of 2,7-bis-*N*,*N*-dimethylamino-9*H*-selenoxanthen-9-one (0.15 g, 0.43 mmol) in anhydrous THF (3 mL) was treated with 1-bromonaphthalene (0.35 g, 1.7 mmol) and ground magnesium turnings (0.050 g, 1.7 mmol) in anhydrous THF (15 mL) as described for the preparation of **5**. The reaction mixture was then treated with acetic acid (3.0 mL) and hexafluorophosphoric acid (60%-by-weight solution in water) as described. The resulting precipitate was collected by filtration. The solid

was washed with water (10 mL), ether (10 mL), and was then recrystallized from acetonitrile and a small amount of ether to give 0.100 g (45%) of the product as a dark purple solid. The hexafluorophosphate salt (0.100 g) was dissolved in 20 mL acetonitrile and 1.00 g of Amberlite IRA-400 Chloride ion exchange resin was added. The resulting mixture was stirred for 0.5 h, followed by removal of the exchange resin via filtration. The process was repeated with two additional 0.5 g aliquots of the ion exchange resin. Following the final ion exchange, the filtrate was concentrated and the crude chloride salt was recrystallized from acetonitrile and a small amount of ether to give 0.90 g (90%) of the product as a dark purple solid, mp 261–262 °C: ^{1}H NMR [500 MHz, CD_2Cl_2] δ 8.15 (d, 1H, $J = 8.2$ Hz), 8.06 (d, 1H, $J = 8.2$ Hz), 7.73 (d, 2H, $J = 9.8$ Hz), 7.73 (m, 1H), 7.58 (d × t, 1H, $J = 1.2$, 7.0 Hz), 7.46 (d × d, 1H, J = 1.2, 7.0 Hz), 7.40 (d × t, 1H, $J = 1.2$, 6.7 Hz), 7.25 (d, 2H, $J = 2.4$ Hz), 7.25 (m, 1H), 6.72 (d × d, 2H, $J = 2.7$, 9.8 Hz), 3.27 (s, 12H); ^{13}C NMR [500 MHz, CD_2Cl_2] δ 160.8, 153.5, 146.8, 138.5, 135.0, 133.8, 132.5, 129.9, 129.0, 128.9, 127.7, 127.1, 125.9, 125.6, 120.8, 119.0, 115.3, 41.1; λ_{max} (CH_2Cl_2) 587 nm ($\varepsilon = 4.0 \times 10^4\ M^{-1}\ cm^{-1}$); HRMS (ESI) *m/z* 457.1174 (Calcd for $C_{27}H_{25}N_2{}^{80}Se$: 457.1177). Anal. Calcd for $C_{27}H_{25}N_2SeCl{\cdot}2.5\ H_2O$: C, 60.39; H, 5.62; N, 5.22. Found: C, 60.77; H, 5.63; N, 5.18.

4.2. Quantum yield determination for the generation of singlet oxygen

The quantum yields for singlet-oxygen generation with chalcogenoxanthylium dyes **3**–**6** were measured by direct methods in MeOH, as described in Ref. 23.

4.3. Determination of partition coefficients

The octanol/water partition coefficients were all measured at pH 6 (phosphate-buffered) using UV–Vis spectrophotometry. The measurements were done using a 'shake flask' direct measurement.[29] Mixing for 3–5 min was followed by 1 h of settling time. Equilibration and measurements were made at 23 °C using a Perkin-Elmer Lambda 12 spectrophotometer. HPLC-grade 1-octanol was obtained from Sigma–Aldrich.

4.4. Viral assays for VSV and PRV

VERO cells (isolated from African green monkey kidney, CCL81, ATCC) were propagated in medium (RPMI-1640 supplemented with glutamine, Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum. Cells were seeded into six-well culture plates and allowed to grow to confluency. Control and phototreated samples were then serially diluted 10-fold, plated onto confluent monolayers, incubated for 30–60 min, depending on the virus, and gently rocked at 37 °C for virus adsorption to cells. The inoculum was then removed by aspiration and washed with PBS. A semi-liquid agar layer (0.2%) was added to each well and infected monolayers were incubated at 37 °C in air containing 5% CO_2. Incubation periods at 37 °C are 1 day for VSV and 4 days for PRV. After incubation, the agar layer was removed by aspiration and the monolayer was stained with 0.1% crystal violet in ethanol for at least 15 min. The stain was removed by aspiration, the plates were washed with water, and the plaques were then enumerated.

4.5. Bacterial assays

Overnight broth cultures were prepared from single colonies of *S. epidermidis*, *S. aureus*, *Y. enterocolitica*, or *P. fluorescens*, isolated from plate cultures. Broth cultures were incubated under aerobic conditions at 37 °C. Following inoculation into RBC suspensions, bacterial counts were determined in phototreated and unilluminated control samples containing the dye and maintained in the dark by 1-in-100 serial dilution of fully mixed samples in unbuffered saline, adding either 0.1 or 1.0 mL of the diluted or neat suspension, respectively, to 3 mL of 0.8% molten agar (39–40 °C) and pouring the molten agar over Luria broth agar plates. Up to 750 colonies per plate were counted after incubation for 24–72 h at 30 or 37 ° C, with time and incubation temperature depending on the strain.

4.6. Calculation of pathogen inactivation

The extent of viral or bacterial inactivation was determined by subtracting the $\log_{10}$ viral titer or bacterial count of phototreated samples from the $\log_{10}$ of their respective unilluminated controls containing dye. If no plaques or colonies were observed by the assay of neat phototreated samples, one plaque or colony was assumed for calculation purposes and the extent of inactivation was reported with a greater than value.

4.7. Red cell preparation and illumination

Extracellular and intracellular viruses were added to oxygenated, white cell reduced, RBCs at a final concentration of 20% hematocrit. The volume of the pathogen spike represents <10% of the volume of the total cell suspension. The chalcogenoxanthylium photosensitizer was then added to the virus inoculated RBC suspension. After mixing and incubating the suspension for 15 min at room temperature in the dark, the samples were divided into 2 mL aliquots in polystyrene culture dishes (50 mm bottom diameter) to produce 1 mm blood films. Control samples included unilluminated RBC suspensions containing sensitizer that were maintained in the dark.

For RBC storage studies, 25 petri dishes containing 2 mL of 20% hct RBCs with photosensitizer were illuminated with agitation on a reciprocal shaker, their contents pooled, resuspended in red cell additive solution, and transferred to a transfer container to provide sufficient volume (40–50 mL) for RBC storage studies. Control samples did not contain any sensitizer and were not illuminated.

Illumination was carried out using a bank of cool white fluorescent lamps with a Plexiglas stage, which limited wavelengths to the 400–750-nm range. Samples on the

stage, which was connected to a reciprocal shaker, were agitated during illumination.

4.8. Hemolysis assay

Total hemoglobin was measured using a hematology analyzer (Cell Dyne 3700, Abbott Laboratories, Abbott Park, IL). Supernatant hemoglobin was determined using the tetramethylbenzidine method.[30]

4.9. Photocleavage of DNA

pUC19 is a plasmid DNA vector, which is highly supercoiled, with over 90% of the plasmid being in the supercoiled form.[31] Ten microliters of 10 μM solutions of **TMR-Se** or **4** in, pH 7.4, sodium phosphate buffer was added to 10 μL of purified pUC19 plasmid DNA (75 μM in base pairs, roughly 2700 bp per strand) and diluted with 80 μL of pH 7.4 sodium phosphate buffer in an 8-mm-high Beckman microcell cuvette and irradiated with 25 mW of filtered 500–800-nm light for varying time periods. Ten microliter aliquots were taken at various time points, diluted with 10 μL of loading buffer [at pH 7.5] and mixed via centrifugation. Gel electrophoresis was run on each time point on an agarose gel. The gel was transferred to a phosphoimager slide and imaged using a Bio Rad Fx phosphoimager. As shown in Figure 2 above, the supercoiled and ''nicked'' (or damaged) plasmid DNA move at different rates on the gel. The phosphoimager was used to give quantitative densities to allow the determination of a time-course for DNA damage, as shown in Figure 3 above.

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