Pegylated and nanoparticle-conjugated sulfonium salt photo triggers necrotic cell death

Abstract: Photodynamic therapy (PDT) processes involving the production of singlet oxygen face the issue of oxygen concentration dependency. Despite high oxygen delivery, a variety of properties related to metabolism and vascular morphology in cancer cells result in hypoxic environments, resulting in limited effectiveness of such therapies. An alternative oxygen-independent agent whose cell cytotoxicity can be remotely controlled by light may allow access to treatment of hypoxic tumors. Toward that end, we developed and tested both polyethylene glycol (PEG)-functionalized and hydrophilic silica nanoparticle (SiNP)-enriched photoacid generator (PAG) as a nontraditional PDT agent to effectively induce necrotic cell death in HCT-116 cells. Already known for applications in lithography and cationic polymerization, our developed oxygen-independent PDT, whether free or highly monodispersed on SiNPs, generates acid when a one-photon (1P) or two-photon (2P) excitation source is used, thus potentially permitting deep tissue treatment. Our study shows that when conjugated to SiNPs with protruding amine functionalities (SiNP–PAG9), such atypical PDT agents can be effectively delivered into HCT-116 cells and compartmentalize exclusively in lysosomes and endosomes. Loss of cell adhesion and cell swelling are detected when an excitation source is applied, suggesting that SiNP–PAG9, when excited via near-infrared 2P absorption (a subject of future investigation), can be used as a delivery system to selectively induce cell death in oxygen-deprived optically thick tissue.

Keywords: oxygen-independent photodynamic therapy, photoacid generator, silica nanoparticles, stimuli-responsive, sulfonium salt

Introduction

The clinical usefulness of light was first recognized some 3,000 years ago when it was used to treat rickets, psoriasis, and other common ailments. More recently, the term photodynamic therapy (PDT) surfaced and has been used to describe the use of light to treat a variety of diseases, including cancer. Specifically, over the last 100 years, great strides have been made in developing photodynamic therapies for use in the clinical setting. PDT hinges on a few key components. The first component is the photosensitizer, the photosensitive material that targets diseased tissue, akin to chemotherapeutic agents. Biocompatibility and potential for targeting are important to the optimization of this component. The second component is the irradiation of the target area with light of a specific wavelength. Excitation of the photosensitive material to a triplet state in the presence of oxygen results in triplet–triplet annihilation, producing cytotoxic reactive oxygen species (ROS) that destroy cellular components, thus triggering cell death. Therefore, the success of photodynamic therapeutics depends largely on the photosensitizer concentration throughout the tissue, adequate intracellular
oxygen concentration, and proper source of excitation. The
discovery of this process was extremely promising in provid-
ing a more targeted, regulated method of cancer treatment.
However, one of the main drawbacks of this method of
PDT is that it is oxygen dependent. Although cancer cells
have abnormally high vascularization, intracellular oxygen
concentration is low due to poor morphology of the vessels
and abnormally high metabolic and oxygen consumption
rates, which are characteristics of cancerous tissues.² Without
oxygen available to interact with the photosensitizer, ROS
cannot be formed and the therapeutic method becomes rela-
tively ineffective or far less effective than intended.

Approach
In addressing the aforementioned issue, we recently took
an alternative approach in attempting to trigger cell death
by employing a photosensitive agent with low dark
cytotoxicity. Rather than producing ROS as the main
“damaging” component, we used a non-toxic sulfonium-
based photoacid generator (PAG) to cause intracellular pH
imbalance.² Upon proper light excitation, the PAG lowers
the pH in the moderately pH-sensitive intracellular envi-
rnonment; the acidic environment thus created leads to the
malfunction and destruction of many cellular components,
likely including key enzymes. This process is considered an
oxygen-independent PDT (OI-PDT) and, essentially, relies
on two main components, the sulfonium salt distribution in
the affected area and a proper source of excitation, rather
than the three components mentioned earlier. In essence,
reducing the number of variables in the therapeutic process
development improves the chances of success in material
design and subsequent treatment.

Given the hydrophobic nature of PAGs prepared in our
preliminary study,³ we opted to introduce a polyethylene
glycol (PEG) unit that can confer hydrophilicity to the PDT
agent while maintaining its photo-induced cytotoxicity.
Along the same line, we prepared highly monodispersed
silica nanoparticles (SiNPs) functionalized with amines
that served as handles to covalently bond PAG molecules
via an amide linkage. The incorporation of the PAG into
a nanomaterial delivery formulation is also intended to
improve delivery properties of the proposed PDT agent as
nanomaterials have emerged as a promising solution to the
issues of cellular specificity, time-controlled delivery, and
aqueous solubility.⁴ Herein, we report the design, synthesis
(Scheme 1 and Supplementary material), and investigation of
a new PAG and its incorporation on SiNPs (SiNP–PAG9),
which offers better solubility in aqueous media, greater
efficacy than water-soluble PEGylated PAG (PEG–PAG9),
and low dark cytotoxicity.

PAG–PAG9 and SiNP–PAG9 were designed having
similar A–π–A’ core structure to the one reported in our initial
work, where the two acceptor units (A), −NO₂ and −SPh₂⁺,
flank a fluorenyl-stilbene π-spacer. Using the C(9) of
fluorene 1 to attach a handle for further functionalization, a
propanoic acid unit was added as depicted in Scheme 1. Ini-
tially, treatment of 1 with one equivalent of n-BuLi at −78°C
generated a carbanion, stabilized by resonance through the
fluorene π-system; this was followed by careful addition of
ethyl bromide to afford the monoalkylated intermediate 2.
Bi-substituted fluorene 3 was obtained through a Triton
B-assisted Michael addition of 2 with acrylonitrile in satis-
factory yield (77%).

Materials and methods
Although later stages of the synthesis that use aqueous acids
have resulted in the hydrolysis of the nitrile moiety, such
acidic hydrolysis was incomplete and often resulted in low
yields and multiple side products. Therefore, we opted to
hydrolyze the nitrile group using aqueous NaOH to afford
the carboxylic acid derivative 4 in good yield. Subsequent
intermediates 5–9 were prepared following our reported
procedure to afford PAG 9 in ~61% yield over four steps.
This compound was conjugated to an amine-terminated PEG
moiety by preparing the benzotriazole intermediate in situ,
followed by the addition of the amine-terminated PEG to
afford PEG–PAG9 in 77% yield. On the other hand, SiNPs
having amine appendages SiNP(NH₂)₁ (preparation detailed
in the Supplementary material) were treated with 1-ethyl-
3-(3-dimethylaminopropyl)carbodiimide condensation
agent, followed by the addition of an excess of PAG 9, the
good solubility of PAG 9 in water allowed the dialysis of the
resulting SiNP–PAG9 to separate any unreacted PAGs.
Surface modification of SiNP(NH₂)₁ by addition of PAG 9
molecules was first assessed using a Zetasizer Nano system
(Malvern Instruments, Malvern, UK), where such addition
resulted in particle size increase from an average of 4 nm
to 9 nm (Supplementary material). In addition, the nuclear
magnetic resonance (NMR) spectrum of SiNP–PAG9 in
D₂O (Supplementary material) showed the presence of peaks
in the aromatic region that corresponded to PAG 9, hence
verifying the successful functionalizing of the SiNPs with
the PDT (PAG) molecules.

Next, the photophysical properties of PEG–PAG9 and
SiNP–PAG9 were examined to ensure that the magnitude of
photoacid generation is preserved after modification.

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Next, the photophysical properties of PEG–PAG9 and
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photoacid generation is preserved after modification.
Initially, the absorption profiles of PEG–PAG9 and SiNP–PAG9 were obtained in water, where both show a similar absorption band to previously reported PL-127®-encapsulated PAG, with an absorption \( \lambda_{\text{max}} \) of 384 nm. More importantly, the photoacid generation quantum yield (\( \Phi_{\text{H}^+} \)) of PEG–PAG9 was maintained at \(~0.4\) (Supplementary material), suggesting that the aqueous environment has little effect on the relevant photophysical properties of PEG–PAG9. Furthermore, the pH drop inside cell lysosomes was estimated by recording the changes in the absorption intensity of Rhodamine B (RhB) base. Conceptually, it is safe to assume that the number of protons generated by PEG–PAG9 upon exposure is the same as the number of RhB base molecules converted to RhB+, which is visualized as an increase in the absorption peak at ca. 555 nm (Figure 1A). Extrapolation of the calibration curve (Figure 1B) suggests that 50 \( \mu \)M of PEG–PAG9 would result in an increase of \([\text{H}^+]_{\text{lysosomal}}\) by \(9.1 \times 10^{-6} \) M after a radiation dose of 10 min; consequently, the lysosomal pH would be

**Scheme 1** Synthetic route for the preparation of PEG–PAG9 and SiNP–PAG9.

**Note:** “7” is phenyl 4-vinylphenyl sulfide.

**Abbreviations:** PEG, polyethylene glycol; PAG, photoacid generator; SiNP, silica nanoparticle; THF, tetrahydrofuran; MEG, monoethylene glycol; DMF, dimethylformamide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DPIHFP, diphenyliodonium hexafluorphosphate.
reduced by at least 0.3 pH units to \( \approx 4.4 \) (Supplementary material).9

Results
In order to evaluate the intrinsic toxicity of PEG–PAG9 and SiNP–PAG9, cell viability assays with CellTiter 96® AQueos One Solution Reagent (Promega Corporation, Fitchburg, WI, USA) were performed in the dark (dark viability) to prevent the production of acid and determine suitable concentration for effective PDT (Supplementary material). In this regard, HCT-116 cells (human colorectal carcinoma) were incubated with various concentrations of PEG–PAG9 and SiNP–PAG9 for 24 h at 37°C. The results showed that PEG–PAG9 has very low dark cytotoxicity, even at 100 \( \mu \)M dose concentration (Figure 2A). On the other hand, cell viability decreased from 99% to 75% when SiNP–PAG9 loading was doubled from 10 \( \mu \)M to 20 \( \mu \)M (Figure 2B). In order to further assess the cytotoxicity of PDT agents, post-exposure viability assays were performed using 50 \( \mu \)M and 10 \( \mu \)M of PEG–PAG9 and SiNP–PAG9, respectively. As can be seen in Figure 3, exposure of HCT-116 cultures incubated with PEG–PAG9 prompted a drop from 97% viability to 64% viability within 10 min. Within the same exposure timeframe, cultures incubated with SiNP–PAG9 experienced a reduction in viability from 98% to 42%. Such a drastic decrease in cell viability can be the result of PAG distribution within the lysosomes

Figure 1 (A) Absorption spectra of PEG–PAG9 (10^{-4} M) and RhB base (10^{-5} M) (indicator) in CH_2Cl_2 irradiated at 366 nm at different time intervals. (B) Dose-dependent calibration curve using 5.4 mW/cm² irradiation.

Abbreviations: PEG, polyethylene glycol; PAG, photoacid generator; RhB, Rhodamine B; au, arbitrary units; OD, optical density.

Figure 2 Dark cytotoxicity of HCT-116 cells incubated with (A) PEG–PAG9 and (B) SiNP–PAG9.

Abbreviations: PEG, polyethylene glycol; PAG, photoacid generator; SiNP, silica nanoparticle.
and endosomes. Mechanistically, photoexcitation of PAG 9 molecules that are densely grafted on SiNP(NH$_2$)$_n$ generates a highly localized dose of protonated species, and, thus, a greater localized pH drop. Consequently, a localized surge in the protonated species concentration creates an acidic microenvironment that could override mechanisms by which cells tend to regulate intracellular [H]$^+$, resulting in severe cytoplasmic acidification and inducing cell death.

Although this report is largely qualitative, the error bars of the cell viability in Figures 2 and 3 provide some insight into the statistical significance of the results. Thus, SiNP–PAG9 exhibits high cytotoxicity at very low concentration and short exposure time.

In order to determine the localization of PEG–PAG9 in HCT 116 cells, LysoTracker Green (Thermo Fisher Scientific, Waltham, MA, USA), a commercial dye that localizes in lysosomes after cell uptake, was used along with PEG–PAG9. Fluorescence of PEG–PAG9 was collected inside cells (Figure 4A–C), showing a good uptake efficiency of PEG–PAG9. Overlay image (Figure 4D) exhibited good colocalization between PEG–PAG9 and LysoTracker Green, which indicated that PEG–PAG9 mainly built up in lysosomes and endosomes. Next, we verified the reduced lysosomal pH by employing LysoSensor Green as a suitable intracellular pH sensor. Since the fluorescence quantum yield of LysoSensor Green increases in increasingly acidic environments,
fluorescence intensity as a function of exposure time was studied, which showed a drop in intralysosomal pH following irradiation in HCT-116 cells incubated with PEG–PAG9 (Figure 5 and Supplementary material).

As demonstrated in our previous study, time-lapsed micrographs (Figure 6) of HCT-116 cells incubated with PEG–PAG9 and SiNP–PAG9 (Figures 6 and 7, respectively) show significant cell swelling, loss of cell adhesion that is followed by a “blebbing”-like activity, which is a characteristic of necrotic cell death. Figure 8 shows a simplified Jablonski diagram summarizing the process of photoacid generation by either one-photon or two-photon (2P) excitation.

**Conclusion**

We demonstrated that sulfonium salts can be used to selectively induce cell death by photoexcitation. PAGs induced necrotic cell death by creating a pH imbalance in the HCT-116 cells via generation of photoacid within the lysosomes. OI-PDT was shown to be effective using water-soluble PAG molecules. Higher efficacy was achieved when these PAG molecules were conjugated to silica-based nanoparticles,
Figure 6 Time-lapse images of HCT-116 cells incubated with PEG–Pag9 show the process of cell death by light irradiation (5.4 mW/cm²).

Notes: Orange arrows show loss of cell adhesion, blue arrows show blebbing-like activity, and red arrows show cell swelling.

Abbreviations: PEG, polyethylene glycol; PAG, photoacid generator.

Figure 7 Time-lapse images of HCT-116 cells incubated with SiNP–Pag9 show loss of cell adhesion (yellow arrows), blebbing-like activity (blue arrows), and cell swelling (red arrows).

Abbreviations: SiNP, silica nanoparticle; PAG, photoacid generator.
resulting in a higher cytotoxic effect. Ultimately, near-infrared (IR) 2P excitation of the water-soluble PAG should afford deep tissue excitation and PDT. Thus, light-guided acid generation could be considered an attractive tool for treatment of hypoxic tumors and other diseases. Currently, our efforts are focused on evaluating the prepared PAGs in vivo and for 2P excitation in the near-IR spectral range.

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**Disclosure**

The authors report no conflicts of interest in this work.

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