

H₂O₂- and Light-Triggered MnO₂-Based Nanoswitches for Microenvironment Reprogramming and Photodynamic Therapy of Hypoxic Atherosclerotic Plaques

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Abstract

Background

M1 macrophage-enriched hypoxic microenvironment within high-risk plaques accelerates atherosclerosis progression. Notably, intraplaque hypoxia severely limits the therapeutic efficacy of oxygen-dependent photodynamic therapy (PDT). In this study, we report a rationally engineered oxygen-generating manganese dioxide (MnO_2)-based nanoswitch system that integrates plaque microenvironmental reprogramming with enhanced, activatable PDT for the precise treatment of hypoxic atherosclerotic plaques.

Results

The HA-TEPA/ MnO_2 /Ce6 nanoswitches are readily synthesized via sequential reactions of tetraethylenepentamine-modified hyaluronic acid (HA-TEPA), potassium permanganate, and chlorine e6 (Ce6)- NH_2 . Under physiological conditions, the MnO_2 effectively quenches both fluorescence and singlet oxygen generation of Ce6, maintaining the nanoswitch in a photodynamically “off” state to minimize off-target phototoxicity and skin photosensitization. Following CD44-mediated selective uptake by pro-inflammatory M1 macrophages, endogenous hydrogen peroxide (H_2O_2)-triggered MnO_2 degradation induces H_2O_2 -to- O_2 transition. This trigger-responsive transition alleviates local hypoxia to enhance the PDT efficacy and reprogram macrophages toward a pro-resolving, anti-inflammatory M2 phenotype. Concurrently, H_2O_2 -triggered MnO_2 degradation restores the intrinsic Ce6 fluorescence and photodynamic activity, enabling activatable PDT in plaques under laser irradiation. In vivo evaluation using a hypoxic atherosclerotic plaque model demonstrates that photoactivated HA-TEPA/ MnO_2 /Ce6 nanoswitches significantly promote pro-inflammatory macrophage depletion, reduce lipid core burden, and enrich collagen content, thereby driving robust plaque stabilization and regression without inducing systemic toxicity.

Conclusions

Collectively, these findings establish this state-switchable therapeutic platform as a highly promising, stimulus-responsive nanomedicine strategy for environmentally activated atherosclerosis therapy.

Introduction

Atherosclerosis is a chronic, progressive vascular disease and a leading cause of fatal cardiovascular events [1, 2]. High-risk plaques are characterized by dysregulated lipoprotein metabolism and the development of lipid- and inflammatory cell-rich microenvironments beneath the arterial intima [3]. The atherosclerotic plaque microenvironment comprises diverse cellular and molecular components, including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), monocytes/macrophages, foam cells, oxidized low-density lipoprotein (ox-LDL), inflammatory cytokines, and chemokines [4]. This milieu is characterized by mild acidity (pH 6.0–6.8), elevated levels of reactive oxygen species (ROS) and

lipids, and localized hypoxia [4–6]. Macrophages play a central role in plaque progression and stability [7]. Circulating monocytes infiltrate plaques and differentiate into classically activated, pro-inflammatory M1 macrophages that promote atherogenesis by secreting cytokines and producing ROS [8]. M1 macrophages also amplify inflammatory cell recruitment and activate neighboring cells [9, 10]. The recruited inflammatory cells, including macrophages and foam cells, increase local metabolic activity and contribute to severe intraplaque hypoxia ($< 1\% \text{ O}_2$) [6]. The hypoxic microenvironment induces hypoxia-inducible factor (HIF)-1 α expression, thereby accelerating atherosclerotic plaque progression by enhancing inflammation and vascular cell proliferation [11]. Together, M1 macrophages and a hypoxic microenvironment promote plaque progression and sustain inflammation, making them attractive therapeutic targets in atherosclerosis.

Photodynamic therapy (PDT) is a minimally invasive therapeutic strategy for treating atherosclerosis and multiple cancers [12, 13]. PDT relies on photosensitizers (PSs) that generate cytotoxic singlet oxygen (SO) upon light irradiation in oxygenated environments [12, 14]. Chlorin e6 (Ce6), a second-generation PS, offers a high singlet oxygen yield, favorable biocompatibility, and relatively deep tissue penetration [15]. However, the intrinsic hydrophobicity of Ce6 leads to poor aqueous solubility and limited tissue accumulation owing to self-aggregation under physiological conditions [14, 16]. To address these limitations and improve macrophage-targeted delivery to atherosclerotic plaques, Ce6 is frequently incorporated into nanoparticle systems functionalized with ligands such as dextran sulfate, D-mannosamine, laminarin, and β -glucan [17–20]. Despite the enhanced plaque selectivity, hypoxic microenvironments remain a major barrier that limits the efficacy of oxygen-dependent PDT [21, 22]. In addition, clinical trials have reported substantial skin photosensitization associated with Ce6 [13, 15, 23]. This adverse effect is largely attributable to the constitutive (“always on”) photoactivity of many PSs, which persists in normal tissues [24, 25]. Although nanocarrier-based delivery can enhance the bioavailability and tissue accumulation of Ce6, incomplete quenching of fluorescence and photoreactivity may leave residual photoactivity, increasing the risk of off-target phototoxicity in healthy tissues [26].

To address both the reduced efficacy of PDT in hypoxic microenvironments and the constitutive (“always on”) photodynamic activity of PSs in normal tissues, we focused on nanoswitch systems that can be selectively activated by specific environmental triggers. Nanoswitches have emerged as promising platforms for stimuli-responsive biomedical applications, although studies remain limited. A nanoswitch is generally defined as a material system that undergoes a functional state transition in response to specific triggers (e.g., enzymes, pH, ROS, light, or temperature), enabling controlled activation under pathological conditions [27–29]. For example, Xu et al. [27] reported a near-infrared (NIR)-operated chitosan-based nanoswitch that converts from an inactive carrier to an activated therapeutic state upon NIR irradiation, enabling controlled photothermal activation, drug release, and immunogenic ferroptosis induction. Similarly, Chen et al. [28] developed a triplex DNA-based nanoswitch that undergoes a pH-triggered conformational transition from a linear, drug-retaining state under neutral conditions to a folded triplex structure in acidic environments, enabling intracellular release of multiple therapeutics in cancer cells. Consistent with these concepts, activatable PDT strategies have been developed to keep

PSs in a photodynamically “off” state in normal skin while selectively switching them “on” in pathological environments in response to local triggers [15, 24, 30, 31]. In this context, Zhang et al. [15] reported a switchable PDT platform in which Ce6 photoactivity was suppressed by fluorescence and singlet oxygen quenching and subsequently restored through NIR irradiation-induced nanoparticle disassembly, reactivating Ce6 fluorescence and PDT efficacy while reducing skin photosensitization.

Building on these nanoswitch concepts, we propose a manganese dioxide (MnO_2)-based nanoswitch that couples oxygen generation with fluorescence and singlet oxygen quenching. In recent years, oxygen-generating MnO_2 nanomaterials have attracted considerable attention owing to their ability to enhance therapeutic responses through microenvironment reprogramming [22, 32, 33]. MnO_2 catalyzes endogenous hydrogen peroxide (H_2O_2) to molecular oxygen, thereby alleviating hypoxia in solid tumors or atherosclerotic plaques [22, 33–35]. Sustained MnO_2 -mediated oxygen production can further increase singlet oxygen generation during PDT [22, 36]. More recently, MnO_2 has also been reported to promote macrophage polarization from the pro-inflammatory M1 phenotype toward the anti-inflammatory M2 phenotype via HIF-1 α downregulation within atherosclerotic plaques [34, 35]. In parallel, MnO_2 nanomaterials have been widely used as efficient energy acceptors and fluorescence-quenching agents in Förster resonance energy transfer (FRET)-based sensing platforms [37–39]. Following H_2O_2 -triggered MnO_2 degradation, the released PSs recover their intrinsic fluorescence, enabling H_2O_2 -responsive “turn on” fluorescence systems [38, 40, 41]. Moreover, FRET-based quenching systems can effectively suppress singlet oxygen generation, thereby maintaining PSs in a photodynamically “off” state until nanoparticle degradation occurs [42, 43]. Collectively, these features make MnO_2 -based FRET nanosystems attractive for activatable PS design.

In this study, we designed novel MnO_2 -based nanoswitches to achieve synergistic microenvironment reprogramming and enhanced activatable PDT in hypoxic atherosclerotic plaques (Scheme 1). To construct the MnO_2 -based nanoswitches, tetraethylenepentamine (TEPA) ligands (as a metal-ion chelator) were first conjugated to hyaluronic acid (HA; an M1 macrophage-targeting ligand), which lacks metal-binding moieties. The resulting TEPA-modified HA (HA-TEPA) was then sequentially reacted with potassium permanganate (KMnO_4) and Ce6- NH_2 to yield HA-TEPA/ MnO_2 /Ce6 nanoswitches. These nanoswitches are rationally engineered to undergo multiple functional transitions—including H_2O_2 -to- O_2 conversion, M1-to-M2 macrophage polarization, fluorescence off-to-on switching, and photodynamic off-to-on activation—in response to two triggers: H_2O_2 (the primary environmental stimulus) and light (an additional therapeutic trigger). After systemic administration, the nanoswitches preferentially accumulate in hypoxic atherosclerotic plaques via CD44-mediated interactions with injured endothelium [44]. The accumulated nanoswitches subsequently target CD44-positive M1 macrophages via receptor-mediated endocytosis. Exposure to endogenous H_2O_2 induces MnO_2 degradation, leading to in situ oxygen generation, hypoxia alleviation, and HIF-1 α downregulation; together, these effects promote macrophage reprogramming from the pro-inflammatory M1 state to the anti-inflammatory M2 state. Concurrently, MnO_2 -mediated fluorescence and singlet oxygen quenching maintain Ce6 in a fluorescence

“off” (FL “off”) and a photodynamically “off” (PDT “off”) state under physiological conditions, thereby reducing off-target phototoxicity. Upon H_2O_2 -triggered MnO_2 degradation followed by 670 nm light irradiation, the nanoswitch transitions to restore Ce6 fluorescence (FL “on”) and activate PDT (PDT “on”). Furthermore, the in-situ oxygenation generated by H_2O_2 -triggered MnO_2 degradation can further improve PDT by increasing oxygen availability during irradiation. As shown in Scheme 1, this H_2O_2 - and light-responsive MnO_2 -based nanoswitch integrates microenvironment reprogramming with enhanced activatable PDT, yielding synergistic therapeutic outcomes, including plaque modulation, stabilization, and regression in hypoxic atherosclerotic plaques.

Results and discussion

Synthesis and characterization of HA-TEPA

HA-TEPA was synthesized via N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) chemistry between the carboxyl groups of HA (a natural ligand for CD44) and the primary amine of TEPA (a metal-chelating agent) (Figure S1A). In the proton nuclear magnetic resonance (^1H NMR; VNS-600, Varian Inc., Palo Alto, CA, USA) spectrum, characteristic HA backbone signals were observed between 3.0 and 4.0 ppm, corresponding to glucosidic ring protons, together with a singlet at 1.95 ppm assigned to acetamide protons (Figure S1B). Additional peaks at 2.65–2.90 ppm assigned to the ethylene protons of TEPA were also evident [36]. The presence of these TEPA-specific signals alongside the HA signals confirms successful TEPA conjugation to HA.

Comparison of reducing reactivity of HA and HA-TEPA towards KMnO_4

TEPA exhibits strong chelation with metal ions (e.g., Mn^{2+} , Cu^{2+} , Cr^{3+} , Co^{2+} , and Ni^{2+}) [36, 45]. It was therefore hypothesized that HA-TEPA, which incorporates TEPA moieties, would more effectively reduce KMnO_4 , consistent with prior reports that metal-chelating molecules and polymers can promote MnO_2 formation via KMnO_4 reduction [36, 46]. To compare the reducing reactivity of HA and HA-TEPA, we evaluated their ability to convert KMnO_4 to MnO_2 , a key component of the nanoswitch system, by monitoring visual color changes and UV/Vis spectra over time (Figure S2). As shown in Figure S2A, the HA/ KMnO_4 mixture retained the characteristic purple color of KMnO_4 after 24 h, and the absorbance peaks at 508, 526, and 544 nm (unreacted KMnO_4) remained essentially unchanged. These findings suggest that HA exhibits insufficient reducing capacity to convert KMnO_4 to MnO_2 under these conditions. By contrast, when HA-TEPA was reacted with KMnO_4 (Figure S2B), the solution gradually changed from purple to reddish-brown over 24 h, consistent with progressive reduction. In parallel, the KMnO_4 absorbance features at 508–544 nm decreased over time, while a broad band below 360 nm increased, consistent with the formation of colloidal MnO_2 [33, 47]. Following purification via dialysis, the reaction mixture became yellowish, and the characteristic KMnO_4 peaks disappeared. Collectively, these

results demonstrate that TEPA modification imparts strong reducing ability to HA, enabling efficient and rapid MnO₂ nanoparticle formation.

Synthesis and characterization of HA-TEPA/MnO/Ce6 Nanoswitches

As shown in Fig. 1A, HA-TEPA/MnO₂/Ce6 nanoswitches were synthesized via a two-step sequence involving KMnO₄ reduction and subsequent Ce6-NH₂ conjugation. First, HA-TEPA was reacted with KMnO₄; TEPA moieties reduced KMnO₄ to MnO₂, forming HA-TEPA/MnO₂ via TEPA-Mn²⁺ chelation, consistent with the reduction behavior shown in Figure S2. After purification by dialysis, the primary amine of Ce6-NH₂ was conjugated to carboxyl groups on the HA-TEPA/MnO₂ backbone via EDC/NHS coupling, yielding the final HA-TEPA/MnO₂/Ce6 nanoswitches.

To verify the stepwise formation of HA-TEPA/MnO₂/Ce6 nanoswitches, the zeta potential (ζ) of HA, HA-TEPA, HA-TEPA/MnO₂, and HA-TEPA/MnO₂/Ce6 was measured using a Zetasizer (Malvern Panalytical Ltd., Malvern, UK) (Fig. 1B). The zeta potential of HA was -55.43 ± 3.85 mV, while that of HA-TEPA increased to -41.93 ± 0.50 mV, indicating successful conjugation of positively charged TEPA molecules to the HA backbone. After reaction with KMnO₄, the surface charge of HA-TEPA/MnO₂ became markedly more negative (-84.7 ± 0.85 mV), likely reflecting incorporation of MnO₂, which has a low point of zero charge (below pH 3) [48]. Subsequent conjugation of Ce6-NH₂ increased the zeta potential to -52.7 ± 0.91 mV, likely owing to Ce6 conjugation and adsorption on the HA-TEPA/MnO₂ surface. These stepwise shifts in zeta potential across the synthetic sequence confirm the successful formation of HA-TEPA/MnO₂/Ce6 nanoswitches. Notably, the highly negative surface charge ($|\zeta| > 30$ mV) also suggests good colloidal stability of the final nanoswitches in aqueous environments [33, 49].

The particle size and morphology of HA-TEPA/MnO₂/Ce6 nanoswitches were evaluated by dynamic light scattering (DLS; SZ-100, Horiba, Kyoto, Japan) and a field-emission transmission electron microscope (FE-TEM; JEM-F200, Tokyo, Japan) (Fig. 1C and Figure S3). DLS indicated a hydrodynamic mean diameter of 143.6 ± 34.2 nm and a Z-average diameter of 141.9 nm, with a moderate polydispersity index (PDI) of 0.326, suggesting a relatively narrow size distribution. TEM images revealed small and spherical particles (30–40 nm in diameter) clustered into larger agglomerates, with dimensions consistent with the hydrodynamic size measured by DLS. Collectively, these results indicate that the synthesized HA-TEPA/MnO₂/Ce6 nanoswitches possess a uniform and well-dispersed morphology.

Successful synthesis of HA-TEPA/MnO₂/Ce6 nanoswitches was further confirmed by UV/Vis spectroscopy (NEO-S490, NEOGEN Inc., Daejeon, Korea), inductively coupled plasma optical emission spectroscopy (ICP-OES; Optima 7300 DV, PerkinElmer, USA), Fourier transform infrared (FT-IR) spectroscopy (Shimadzu 8400S, Kyoto, Japan), and X-ray photoelectron spectroscopy (XPS; K-alpha+, Thermo Scientific, MA, USA). The UV/Vis spectra (Fig. 1D) of the HA-TEPA/MnO₂/Ce6 nanoswitches exhibited characteristic absorption features of Ce6, including the Soret band at 405 nm and Q bands at

500–700 nm. Relative to free Ce6, these bands were broadened, and the Q bands were slightly red-shifted (~ 10 nm), likely owing to nanoparticle-induced light scattering (turbidity) and changes in the local microenvironment upon Ce6 loading [50]. A broad absorption feature spanning 280–600 nm further indicated the presence of MnO_2 , overlapping with the Soret and Q bands of Ce6. Quantification showed that 1 mg of HA-TEPA/ MnO_2 /Ce6 nanoswitches contained approximately 0.125 mg of Ce6. ICP-OES further verified Mn incorporation, indicating that 1 mg of nanoswitches contained 0.105 mg of Mn^{2+} . Accordingly, quantitative analysis demonstrated that the Ce6 loading content (wt%) of HA-TEPA/ MnO_2 /Ce6 nanoswitches reached 12.5%, surpassing the typical loading efficiencies of conventional drug delivery systems (generally $< 10\%$) [51]. This high drug loading underscores the potential of HA-TEPA/ MnO_2 /Ce6 nanoswitches as an efficient and versatile nanomedicine platform.

In the FT-IR spectra (Figure S4), both HA-TEPA and HA-TEPA/ MnO_2 /Ce6 nanoswitches showed characteristic bands attributable to O–H (3430 cm^{-1}), C–H (2900 cm^{-1}), C = O (1650 cm^{-1}), and C–O (1090 cm^{-1}) vibrations. Notably, HA-TEPA/ MnO_2 /Ce6 nanoswitches exhibited a distinct peak at 730 cm^{-1} assigned to the chlorin ring of Ce6 [52], together with a strong band at 608 cm^{-1} corresponding to Mn–O–Mn vibrations of MnO_2 [53]. XPS analysis further confirmed the chemical composition and Mn oxidation state (Fig. 1E). The spectrum of HA-TEPA/ MnO_2 /Ce6 nanoswitches showed two distinct Mn 2p peaks at 642.2 eV (Mn 2P_{3/2}) and 654 eV (Mn 2P_{1/2}), with a separation of 11.8 eV, indicating the presence of Mn^{4+} in the form of MnO_2 [54]. In addition, the appearance of a characteristic N 1s peak at 399.5 eV confirmed the successful incorporation of TEPA [36]. Collectively, these results confirm the successful synthesis of HA-TEPA/ MnO_2 /Ce6 nanoswitches with appropriate incorporation of each component.

The colloidal stability of HA-TEPA/ MnO_2 /Ce6 nanoswitches was assessed by monitoring the visual appearance and Z-average size over time in various aqueous media. As shown in Figure S5A, no visible aggregation or precipitation was observed in deionized water (DW), 10% fetal bovine serum (FBS), 0.9% normal saline (NS), or phosphate-buffered saline (PBS) during 7 days at 25°C, indicating good stability. Consistently, the Z-average diameter remained nearly unchanged in all media throughout the observation period (Figure S5B), confirming their excellent colloidal stability. This stability is likely attributable to the relatively high negative zeta potential of HA-TEPA/ MnO_2 /Ce6 nanoswitches (-52.7 ± 0.91 mV; Fig. 1B), which provides sufficient electrostatic repulsion to prevent nanoparticle aggregation in aqueous environments [46, 55].

Degradation behavior, oxygen generation, and singlet oxygen production of HA-TEPA/ MnO /Ce6 nanoswitches under hypoxic conditions

MnO₂ readily decomposes in the presence of H₂O₂, generating additional molecular oxygen (O₂) [21, 36]. To evaluate this property, we monitored the degradation of HA-TEPA/MnO₂/Ce6 nanoswitches under physiologically relevant H₂O₂ concentrations (50, 100, and 200 μM) [21, 36] by measuring the absorbance at 320 nm, which corresponds to the characteristic MnO₂ peak [47]. As shown in Fig. 1F, the absorbance of the HA-TEPA/MnO₂/Ce6 nanoswitches decreased steadily in a time- and H₂O₂ concentration-dependent manner, indicating progressive MnO₂ degradation by H₂O₂. The oxygen-generating capability of the nanoswitches was then evaluated under hypoxia-mimicking conditions using N₂-purged PBS. As shown in Fig. 1G, the generated O₂ levels increased proportionally with H₂O₂ concentration, demonstrating efficient catalytic decomposition of H₂O₂ into O₂. Collectively, these results confirm that the HA-TEPA/MnO₂/Ce6 nanoswitches undergo H₂O₂-responsive degradation, consume H₂O₂, and generate oxygen under hypoxic conditions.

Hypoxia significantly reduces the efficacy of type-II PDT, which requires O₂ to generate cytotoxic SO [22, 36]. As shown in Fig. 1G, HA-TEPA/MnO₂/Ce6 nanoswitches generated O₂ upon exposure to endogenous levels of H₂O₂ under hypoxic conditions. To determine whether this oxygen generation could enhance PDT performance, we quantified SO production in N₂-purged PBS containing different H₂O₂ concentrations using the singlet oxygen sensor green (SOSG) probe. As shown in Fig. 1H, the fluorescence intensities (FIs) of SOSG increased with both laser irradiation time and H₂O₂ concentration, indicating enhanced SO generation under hypoxic conditions in the presence of H₂O₂. These results suggest that MnO₂-mediated oxygen release alleviates oxygen limitations and amplifies the photodynamic activity of Ce6 under hypoxic conditions.

HO-triggered fluorescence and PDT off-to-on switching effects of HA-TEPA/MnO/Ce6 nanoswitches

Before additional in vitro and in vivo studies, Ce6-conjugated HA (HA-Ce6) was synthesized as an MnO₂-free control. Briefly, HA carboxyl groups were covalently coupled to the primary amine of Ce6-NH₂ using EDC/NHS chemistry (Figure S6A). The resulting HA-Ce6 contained approximately 0.05 mg Ce6 per milligram of HA-Ce6. UV/Vis spectroscopy confirmed that Ce6 retained its characteristic absorption profile, including a strong Soret band at 405 nm and Q-bands spanning 480–700 nm (Figure S6B). Notably, the HA-Ce6 Q-bands were significantly broader than those of free Ce6, likely owing to aggregation-induced turbidity arising from the hydrophobic Ce6 nature.

MnO₂ nanomaterials exhibit broad absorption (280–600 nm), enabling efficient fluorescence quenching of various fluorophores, such as quantum dots, fluorescein isothiocyanate (FITC), and Ce6 [33, 38, 40]. In addition, MnO₂ can be rapidly reduced to Mn²⁺ in response to pathological stimuli, including elevated glutathione, low pH, and increased H₂O₂ levels. These features have been widely exploited to construct “off-to-on” fluorescence systems via FRET-based quenching between MnO₂ and fluorophores.

Particularly, the use of photosensitizers as fluorophores enables switchable PDT systems with minimized off-target photosensitization in normal tissues [15, 24, 30, 31]. On this basis, we hypothesized that HA-TEPA/MnO₂/Ce6 nanoswitches would remain in a fluorescence-quenched, PDT-inactive “off” state under normal tissue conditions owing to efficient MnO₂-mediated quenching of Ce6.

To validate the H₂O₂-triggered fluorescence off-to-on switching of the nanoswitches, we compared the fluorescence emission spectra of HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches under aqueous conditions in the presence or absence of H₂O₂ (Fig. 2A, B). In both groups, the Ce6 emission peak exhibited a slight blue shift from 670 to 660 nm, attributable to an aggregation-induced spectral shift [56]. HA-Ce6 exhibited relatively weak FI under both conditions compared with free Ce6 dissolved in dimethyl sulfoxide (DMSO), likely owing to partial self-quenching caused by the close packing of hydrophobic Ce6 moieties within the polymer architecture. This result indicates that HA-Ce6 forms mildly aggregated structures in aqueous media, leading to attenuated fluorescence emission. By contrast, HA-TEPA/MnO₂/Ce6 nanoswitches showed substantially lower FI than HA-Ce6 in the absence of H₂O₂, owing to additional MnO₂-mediated quenching on adsorbed Ce6. Notably, FI markedly increased upon H₂O₂ addition, suggesting that MnO₂ degradation releases weakly quenched HA-Ce6 and thereby partially restores fluorescence. Together, these findings support the HA-TEPA/MnO₂/Ce6 nanoswitches as H₂O₂-responsive “off-to-on” fluorescence systems.

To evaluate H₂O₂- and light-triggered PDT off-to-on switching, SO generation was assessed in aqueous solution using SOSG as a fluorescent probe (Fig. 2C, D). In the absence of 670 nm laser irradiation, both HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches produced negligible SOSG fluorescence. Upon 670 nm irradiation, HA-Ce6 generated a distinct SO signal owing to its partial fluorescence emission in water, whereas HA-TEPA/MnO₂/Ce6 nanoswitches did not yield detectable SO, consistent with the strong MnO₂-induced quenching observed in Fig. 2B. These results confirm that Ce6 remains effectively inactivated within HA-TEPA/MnO₂/Ce6 in the absence of stimuli, thereby reducing the potential for off-target phototoxicity. Notably, after H₂O₂ (200 μM) and light (670 nm) exposure, HA-TEPA/MnO₂/Ce6 nanoswitches produced higher FI than HA-Ce6 owing to additional O₂ generation accompanying MnO₂ degradation. Collectively, these findings demonstrate that HA-TEPA/MnO₂/Ce6 nanoswitches enable controlled and enhanced PDT applications through H₂O₂- and light-triggered off-to-on switching, coupled with oxygen-generating effects from MnO₂ degradation.

Comparison of intracellular H₂O₂ and CD44 Expression in macrophages and activated macrophages under normoxic and hypoxic conditions

Macrophages stimulated with lipopolysaccharide (LPS) and/or interferon-γ can polarize toward an M1 phenotype that plays key roles in pro-inflammatory responses and host defense [57]. M1 macrophages are known to overexpress ROS [58, 59], particularly owing to LPS-driven metabolic reprogramming toward glycolysis. This metabolic shift supports ROS generation and hypoxia adaptation via HIF

stabilization [60] while also enhancing pro-inflammatory cytokine expression through HIF-1 α signaling [61].

To evaluate ROS expression, we compared intracellular H₂O₂ levels in normal macrophages and LPS-activated macrophages (activated macrophages) under normoxic and hypoxic conditions using 2',7'-dichlorofluorescein diacetate (DCFDA), a widely used ROS probe that is oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) by reactive species (including H₂O₂, hydroxyl radicals, and peroxynitrite) [62]. As shown in Fig. 3A, hypoxic macrophages exhibited stronger DCF fluorescence than normoxic macrophages, indicating increased ROS under hypoxia. Notably, activated macrophages showed significantly enhanced fluorescence under both normoxic and hypoxic conditions relative to normal macrophages. These results suggest that H₂O₂ levels increase under hypoxia and are further elevated following LPS-induced macrophage activation.

Pro-inflammatory M1 macrophages are known to overexpress CD44 on their membranes, making CD44 a promising biomarker for selective targeting using HA-based nanosystems [44, 63, 64]. In addition, HIF-1 α has been reported to upregulate CD44 expression and increase the proportion of CD44-positive cells in several cancer cell types, including human gastric cancer, triple-negative breast cancer, and glioma stem-like cells [65–67].

To confirm CD44 upregulation in LPS-induced M1 macrophages under hypoxic conditions, we evaluated CD44 expression in both normal and activated macrophages using a fluorescence-labeled anti-CD44 antibody (Fig. 3B). Hypoxic macrophages exhibited higher CD44 FIs than normoxic macrophages, indicating increased CD44 expression under hypoxia. Activated macrophages showed significantly stronger CD44 fluorescence than resting macrophages under both normoxic and hypoxic conditions. Collectively, these findings demonstrate that CD44 expression increases in hypoxic environments and is further enhanced in pro-inflammatory activated macrophages, supporting CD44-targeted delivery under hypoxic conditions.

In vitro cytotoxicity and intracellular uptake of HA-TEPA/MnO₂/Ce6 nanoswitches

The cytotoxicity of the HA-TEPA/MnO₂/Ce6 nanoswitches was assessed in resting and activated macrophages using the cell counting kit (CCK)-8 assay (Figure S7). Under normoxic conditions (Figure S7A), cell viability remained above 98% across all tested nanoswitch concentrations, indicating negligible cytotoxicity. Under hypoxic conditions (Figure S7B), macrophages treated with HA-TEPA/MnO₂/Ce6 nanoswitches maintained viabilities above 80%, confirming general biocompatibility. However, in hypoxic activated macrophages, cell viability decreased slightly in a concentration-dependent manner, which could be attributed to intracellular Mn²⁺ generation following MnO₂ degradation at higher nanoswitch concentrations [68]. Overall, these results suggest that HA-TEPA/MnO₂/Ce6 nanoswitches are relatively non-toxic at Ce6 concentrations below 5 μ M.

To evaluate CD44-dependent cellular uptake under normoxia, HA-TEPA/MnO₂/Ce6 nanoswitches were incubated with macrophages and activated macrophages, which express high levels of CD44 [44, 63, 64]. Confocal laser scanning microscope (CLSM) imaging and quantitative analysis (Figure S8A) showed significantly higher FIs in activated macrophages than in resting macrophages, consistent with their higher CD44 expression (Fig. 3B). In both cell types, nanoswitch-associated FIs increased in a dose-dependent manner. To verify CD44-mediated endocytosis, cells were pretreated with free HA (MW: 215,000) to competitively block CD44 binding sites (Figure S8B). This pretreatment significantly reduced nanoswitch uptake in activated macrophages, indicating that internalization occurred predominantly via CD44-mediated endocytosis [44, 63, 64]. Collectively, these results suggest that HA-TEPA/MnO₂/Ce6 nanoswitches preferentially target CD44-overexpressing activated macrophages and that uptake efficiency correlates with CD44 expression.

To compare intracellular uptake of HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches in high CD44-expressing activated macrophages under normoxic and hypoxic conditions, CLSM imaging was conducted (Figure S9). Under normoxic conditions, HA-Ce6-treated cells exhibited relatively stronger red fluorescence than cells treated with HA-TEPA/MnO₂/Ce6 nanoswitches, likely owing to insufficient H₂O₂ available to degrade MnO₂, resulting in minimal release of weakly quenched Ce6 (Figure S9, 2A, 2B, and 3A). Consistent with the CD44 expression results (Fig. 3B), HA-Ce6-treated cells under hypoxia exhibited stronger fluorescence than their normoxic counterparts, likely reflecting increased CD44 expression under hypoxia. Notably, hypoxic cells treated with HA-TEPA/MnO₂/Ce6 nanoswitches showed similarly elevated FIs to hypoxic cells treated with HA-Ce6 (Figure S9). This fluorescence recovery is attributed to H₂O₂-triggered MnO₂ degradation and the resulting dequenching of Ce6 (Fig. 2A, B). Collectively, these results indicate that HA-TEPA/MnO₂/Ce6 nanoswitches enable robust intracellular fluorescence recovery in hypoxic activated macrophages, consistent with elevated intracellular H₂O₂ levels.

In vitro HO-to-O switching effects of HA-TEPA/MnO/Ce6 nanoswitches

MnO₂ can catalyze the decomposition of endogenous H₂O₂ to generate molecular O₂, potentially alleviating hypoxia (Fig. 3C) [22, 33, 69]. To evaluate whether HA-TEPA/MnO₂/Ce6 nanoswitches decompose intracellular H₂O₂ and thereby mitigate hypoxia-induced oxidative stress, intracellular ROS levels were measured using DCFDA fluorescence imaging (Figure S10). Compared with the normal macrophage control group, the activated macrophage control displayed markedly increased DCF fluorescence, indicating elevated intracellular ROS levels, consistent with earlier results (Fig. 3A). The HA-Ce6-treated group, which lacks MnO₂, showed no significant reduction in FI compared with the activated macrophage control, suggesting that HA-Ce6 alone did not modulate intracellular ROS. In contrast, cells treated with HA-TEPA/MnO₂/Ce6 nanoswitches showed markedly reduced DCF fluorescence, indicating efficient MnO₂-mediated catalytic decomposition of H₂O₂. These findings

indicate that HA-TEPA/MnO₂/Ce6 nanoswitches can effectively decompose intracellular H₂O₂, generate O₂, and thereby modulate oxidative stress in hypoxic activated macrophages.

Because HA-TEPA/MnO₂/Ce6 nanoswitches are intrinsically responsive to endogenous H₂O₂, their hypoxia-alleviating capability was evaluated in vitro using a commercially available hypoxia probe that fluoresces under oxygen-deficient conditions (Fig. 3D) [70]. As shown in the CLSM images, normoxic-activated macrophages exhibited negligible fluorescence across all treatment groups, confirming the absence of hypoxic conditions and verifying that neither HA nor Ce6 induced hypoxia alleviation under normoxia. Conversely, hypoxic activated macrophages treated with HA-TEPA/MnO₂/Ce6 nanoswitches exhibited a substantial decrease in FIs, by approximately 60% compared with the untreated hypoxic control, demonstrating a significant reduction in intracellular hypoxia. This marked fluorescence decrease can be attributed to MnO₂-mediated H₂O₂ consumption with concomitant oxygen generation, enabled by rapid MnO₂ degradation upon exposure to high levels of H₂O₂ in hypoxic cells. Collectively, these findings highlight efficient hypoxia alleviation by HA-TEPA/MnO₂/Ce6 nanoswitches and support their use as oxygen-generating materials to improve phototherapeutic performance under hypoxic conditions.

In vitro M1-to-M2 macrophage polarization effects of HA-TEPA/MnO/Ce6 nanoswitches

Under hypoxic conditions, LPS-stimulated macrophages tend to polarize toward the M1 phenotype, driven by elevated intracellular ROS and stabilization of HIF-1 α [60, 61]. Accumulated ROS activates inflammatory signaling cascades, thereby promoting the progression of atherosclerosis [71]. Accordingly, MnO₂-based nanodrugs with ROS-scavenging and hypoxia-alleviating capabilities have been proposed as tools for reprogramming macrophages from the M1 to the M2 phenotype [34, 35]. As shown in Fig. 3B, hypoxic, LPS-treated activated macrophages exhibited enhanced CD44 expression, which is associated with M1 polarization and metabolic reprogramming. To determine whether HA-TEPA/MnO₂/Ce6 nanoswitches can reprogram the M1-to-M2 polarization via hypoxia alleviation (Fig. 3C), we assessed CD44 expression by CLSM imaging after treatment with HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches (Fig. 3E). As shown in Fig. 3E, no significant changes were observed in the FIs of CD44 among normoxic normal or activated macrophages across treatment groups, indicating that HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches did not modulate CD44 expression under normoxia. Conversely, in hypoxic activated macrophages, HA-TEPA/MnO₂/Ce6 nanoswitches markedly reduced CD44 expression relative to the HA-Ce6-treated group. This downregulation is likely attributed to efficient intracellular H₂O₂ consumption (Figure S10) and hypoxia alleviation (Fig. 3D) mediated by MnO₂ degradation. These findings suggest that HA-TEPA/MnO₂/Ce6 nanoswitches possess potent M1-to-M2 macrophage reprogramming activity, as reflected by CD44 downregulation.

To further validate the M1-to-M2 macrophage reprogramming effects of HA-TEPA/MnO₂/Ce6 nanoswitches in hypoxic M1 macrophages, we performed additional CLSM analyses using CD86 (M1 marker) and CD206 antibodies (M2 marker) (Figure S11). Following LPS pre-treatment, CD86 expression increased significantly while CD206 remained unchanged, confirming successful polarization to the M1 phenotype. Treatment with HA-Ce6 did not significantly alter CD86 or CD206 fluorescence, suggesting that HA and Ce6 alone do not induce phenotypic transition. In contrast, cells treated with HA-TEPA/MnO₂/Ce6 nanoswitches showed significantly reduced CD86 expression together with a marked increase in CD206 expression. This shift is consistent with the catalase-like activity of MnO₂, which decomposes H₂O₂ and generates oxygen, thereby relieving hypoxia and downregulating HIF-1 α , a key regulator of M1 polarization [34, 35, 60]. Overall, these results demonstrate that the HA-TEPA/MnO₂/Ce6 nanoswitches can effectively drive M1-to-M2 reprogramming under hypoxic conditions, contributing to inflammation reduction and plaque stabilization.

In vitro photodynamic effects on normal macrophages and activated macrophages under normoxic and hypoxic environments

PDT requires molecular O₂ to generate cytotoxic SO and is therefore inherently less effective in hypoxic microenvironments with limited oxygen availability. Furthermore, PDT can further aggravate hypoxia owing to local oxygen consumption during treatment [21]. Because hypoxic regions occur throughout all stages of atherosclerotic plaque development [6, 72, 73], PDT efficacy within plaques is consequently constrained. Our findings demonstrate that HA-TEPA/MnO₂/Ce6 nanoswitches generate additional O₂ via H₂O₂-triggered MnO₂ degradation in hypoxic activated macrophages with elevated intracellular ROS levels (Fig. 3D), indicating that these nanoswitches can effectively alleviate hypoxia.

Before evaluating the phototherapeutic potential of HA-TEPA/MnO₂/Ce6 nanoswitches, cytotoxicity was initially assessed under dark conditions (without laser irradiation) in both macrophages and activated macrophages under normoxic and hypoxic conditions (Fig. 4A). Across all treatment groups, including the untreated control and HA-Ce6-treated cells, cytotoxicity was negligible under both oxygen conditions, confirming the biocompatibility of HA-Ce6. Consistent with Figure S7, HA-TEPA/MnO₂/Ce6 nanoswitches showed no apparent cytotoxicity under normoxic conditions, whereas hypoxia caused a slight reduction in cell viability. Nevertheless, cell viability remained above 80%, indicating acceptable biocompatibility of HA-TEPA/MnO₂/Ce6 nanoswitches.

Subsequently, the phototherapeutic efficacy of HA-TEPA/MnO₂/Ce6 nanoswitches was investigated under 670 nm laser irradiation (50 mW/cm², 2 min) in normal and activated macrophages under normoxic and hypoxic conditions (Fig. 4B). In all treatment groups, activated macrophages exhibited higher phototoxicity than normal macrophages under both oxygen conditions, consistent with increased nanoparticle uptake via CD44-mediated endocytosis. Under normoxia, activated macrophages displayed

significantly higher PDT-induced cytotoxicity than under hypoxia, reflecting the oxygen dependence of PDT. In normoxic activated macrophages, HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches reduced cell viability by 73.6% and 88.1%, respectively. The superior efficacy of HA-TEPA/MnO₂/Ce6 nanoswitches is attributed to their ability to continuously supply O₂ during PDT via MnO₂-mediated catalytic degradation. Under hypoxia, HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches decreased cell viability by 40.7% and 73.9%, respectively, indicating that they can effectively alleviate hypoxia and sustain SO production even when oxygen is limited. Collectively, these results indicate that HA-TEPA/MnO₂/Ce6 nanoswitches enable in situ oxygen generation and thereby enhance PDT efficacy in hypoxic regions of atherosclerotic plaques.

HA-TEPA/MnO₂/Ce6 nanoswitches undergo H₂O₂-triggered degradation to generate O₂, which is subsequently converted to cytotoxic SO upon laser irradiation and induces SO-mediated apoptosis (Fig. 4C) [12, 74]. To validate this mechanism, we evaluated intracellular SO production and apoptosis using SOSG staining and an Annexin V-PI assay, respectively. SO generation was further evaluated using SOSG staining in both normal and activated macrophages under normoxic and hypoxic conditions (Fig. 4D). CLSM images and the corresponding quantification showed negligible SOSG fluorescence in all untreated groups, irrespective of oxygen conditions. Under laser irradiation, all treated groups exhibited higher SO-associated fluorescence in activated macrophages than in normal macrophages, consistent with enhanced intracellular uptake mediated by CD44 overexpression. Notably, activated macrophages under normoxic conditions also exhibited significantly higher SO levels than those under hypoxia, likely owing to greater dissolved O₂ availability for Ce6 activation. Importantly, HA-TEPA/MnO₂/Ce6 nanoswitch-treated activated macrophages showed markedly stronger SOSG fluorescence than HA-Ce6-treated cells under both normoxia and hypoxia. This enhancement is attributed to the catalytic decomposition of endogenous H₂O₂ by MnO₂, generating O₂ and thereby alleviating hypoxia, supporting sustained SO production even in oxygen-deficient environments. These findings are consistent with the cell viability results in Fig. 4B, confirming that HA-TEPA/MnO₂/Ce6 nanoswitches effectively enhance PDT efficacy by enabling sufficient SO generation under both normoxic and hypoxic conditions.

To further confirm that the elevated SO production translates into increased PDT-induced apoptosis, we conducted Annexin V-PI staining (Fig. 4E). Untreated controls displayed negligible fluorescence, indicating minimal baseline apoptosis. After laser irradiation, Annexin V and PI signals were substantially higher in activated macrophages than in normal macrophages, consistent with the higher SO levels observed in SOSG staining. Furthermore, apoptosis-associated markers (Annexin V and PI) were notably stronger under normoxia than under hypoxia, reflecting the oxygen dependence of PDT. Most importantly, HA-TEPA/MnO₂/Ce6 nanoswitch-treated activated macrophages exhibited markedly higher Annexin V and PI fluorescence than HA-Ce6-treated cells under both oxygen conditions. These results support the conclusion that MnO₂-mediated oxygen generation relieves intracellular hypoxia and enhances SO-driven apoptotic cell death even under limited oxygen availability. The observed trends align with the phototoxicity and SOSG results (Fig. 4B, D), supporting the premise that HA-

TEPA/MnO₂/Ce6 nanoswitches potentiate PDT-induced apoptosis by providing a continuous oxygen supply in hypoxic activated macrophages.

In vivo biodistribution and skin phototoxicity of HA-TEPA/MnO₂/Ce6 nanoswitches

Prior to the in vivo experiments, the physical appearance of the HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches was assessed at the injection dose (equivalent to 3 mg/kg Ce6) (Figure S12). The HA-Ce6 solution exhibited the characteristic green color of Ce6, whereas the HA-TEPA/MnO₂/Ce6 nanoswitches appeared dark brown because of MnO₂. When the samples were inverted (180°), the HA-Ce6 solution exhibited high viscosity at this concentration, likely owing to the high molecular weight and high concentration of HA [75, 76]. Conversely, the HA-TEPA/MnO₂/Ce6 nanoswitches maintained a substantially lower viscosity at the same Ce6 concentration, consistent with their high colloidal stability in aqueous media (Figure S5A). This reduced viscosity improves formulation fluidity and facilitates injection relative to HA-Ce6. Collectively, these findings suggest that HA-TEPA/MnO₂/Ce6 nanoswitches offer superior injectability at clinically relevant doses, which is advantageous for in vivo administration.

To investigate the in vivo biodistribution, whole-body fluorescence imaging was conducted at predetermined time points after intravenous injection of HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 3 mg/kg Ce6) (Fig. 5A). HA-Ce6 exhibited a time-dependent increase in fluorescence in the liver and lungs up to 3 h, followed by gradual systemic clearance over 72 h. In contrast, HA-TEPA/MnO₂/Ce6 nanoswitches showed transient accumulation primarily in the liver at 3 h with negligible lung signals, and were rapidly cleared by 72 h. Across all time points, HA-Ce6 demonstrated stronger and more sustained whole-body fluorescence than HA-TEPA/MnO₂/Ce6 nanoswitches. This difference is consistent with the quenching assay (Fig. 2A, B), which showed that HA-TEPA/MnO₂/Ce6 nanoswitches exhibit pronounced fluorescence quenching owing to Ce6 adsorption on the MnO₂ surface.

Ex vivo near-infrared fluorescence (NIRF) imaging (Figure S13) revealed that HA-Ce6 accumulated predominantly in the liver and lungs, which are enriched in immune cells expressing CD44 [63, 77]. In contrast, fluorescence from HA-TEPA/MnO₂/Ce6 nanoswitches was largely restricted to the liver, with markedly reduced signals in the lungs and spleen. These findings suggest that HA-TEPA/MnO₂/Ce6 nanoswitches undergo faster systemic clearance and limit off-target, prolonged accumulation, thereby reducing potential toxicity to normal tissues.

To assess cutaneous photosensitivity, mice were intravenously injected with saline, HA-Ce6, or HA-TEPA/MnO₂/Ce6 nanoswitches, followed by 670 nm laser irradiation (50 mW/cm², 3 min) 1 h after injection (Fig. 5B). At this time point, whole-body fluorescence imaging revealed substantially stronger systemic fluorescence for HA-Ce6 than for HA-TEPA/MnO₂/Ce6 nanoswitches (Fig. 5A), suggesting that HA-Ce6 existed in a more photoactive state. After 48 h of irradiation, mice treated with HA-Ce6 exhibited pronounced erythema and clear epidermal injury on hematoxylin and eosin (H&E)-stained skin sections,

indicating phototoxic responses. Conversely, the HA-TEPA/MnO₂/Ce6 nanoswitches showed no significant histological changes compared with the control group, confirming their low cutaneous photosensitivity. This difference is attributed to the strong SO generation by HA-Ce6 upon laser irradiation, whereas the HA-TEPA/MnO₂/Ce6 nanoswitches remain largely quenched and therefore produce minimal SO, as shown in Fig. 2C and D. Collectively, these results support the biocompatibility of HA-TEPA/MnO₂/Ce6 nanoswitches and their superior safety profile for in vivo applications.

In vivo accumulation of HA-TEPA/MnO₂/Ce6 nanoswitches in hypoxic atherosclerotic plaques

To evaluate the developed nanoswitches, a hypoxia-enhanced atherosclerotic plaque model was established by comparing mice fed a high-cholesterol diet (HCD; 40 kcal% fat, 1.25% cholesterol; Research Diets, Inc., New Brunswick, NJ, USA) for 12 weeks (general atherogenic diet) with those fed HCD for 8 weeks followed by a warfarin-containing diet (WD; 1.25% cholesterol, 0.3% warfarin, 0.15% vitamin K1; Doo Yeol Biotech, Seoul, Korea) for an additional 4 weeks (hypoxia-enhanced atherogenic diet). Warfarin, a vitamin K antagonist, has been reported to accelerate atherosclerotic calcification and promote a vulnerable plaque phenotype [78]. In addition, atherosclerotic calcification induces an immunomodulatory microenvironment that enhances recruitment of pro-inflammatory macrophages [79]. As inflammatory cells, particularly macrophages and foam cells, accumulate within atherosclerotic plaques, their elevated metabolic activity increases oxygen consumption, thereby forming hypoxic regions (< 1% O₂) [6]. Notably, these hypoxic areas are strongly associated with CD68⁺ macrophage clusters in atherosclerotic lesions [6, 72]. Collectively, these observations suggest that warfarin-supplemented HCD promotes immune cell infiltration and oxygen depletion, thereby establishing a hypoxic plaque microenvironment.

To confirm local hypoxia induction, a commercial hypoxia-sensitive fluorescent reagent was administered to mice in both the general atherogenic diet and hypoxia-enhanced atherogenic diet groups (Figure S14). Mice subjected to the hypoxia-enhanced atherogenic diet exhibited significantly higher FIs and more fluorescent foci within atherosclerotic plaques, indicating more pronounced hypoxic regions than in the general atherogenic diet group. These findings confirm that the hypoxia-enhanced atherogenic diet effectively induces a more hypoxic plaque microenvironment and is therefore an appropriate in vivo model for evaluating the oxygen-generating capacity and therapeutic efficacy of the HA-TEPA/MnO₂/Ce6 nanoswitches.

HA has been widely used as a targeting ligand for CD44, which is expressed on injured endothelium, enabling selective delivery to atherosclerotic plaques [44, 63, 64]. The in vivo plaque-targeting ability of HA-TEPA/MnO₂/Ce6 nanoswitches was evaluated using CLSM in apolipoprotein E-deficient (ApoE^{-/-}) mice fed a hypoxia-enhanced atherogenic diet (Fig. 5C). Atherosclerotic plaques were readily identified in the distal common carotid artery and its bifurcation as filling defects on the FITC angiogram. In these angiograms, healthy arteries showed strong green fluorescence reflecting normal blood flow, whereas

plaques exhibited disrupted or attenuated signals indicative of restricted blood flow. Both HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches produced comparable red fluorescence signals co-localized with these filling-defect regions, suggesting successful targeting to atherosclerotic plaques. Consistently, quantitative analysis of plaque-to-background ratio (pTBR) (Fig. 5E) revealed no significant difference between the two groups, confirming that the HA-TEPA/MnO₂/Ce6 nanoswitches retained CD44-mediated targeting efficiency comparable to HA-Ce6 ($P = 0.69$).

To further verify in vivo plaque targeting, carotid arteries containing hypoxic atherosclerotic plaques were harvested from mice injected with either the HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches. Signal-rich regions were cryo-sectioned and analyzed via CLSM (Fig. 5D, F). Both groups exhibited strong NIRF signals within plaques that co-localized with CD68⁺ macrophages, indicating effective plaque targeting and macrophage internalization. NIRF signals were also observed in CD68⁺ (pan-macrophage) and CD44⁺ (M1 marker) regions, suggesting preferential accumulation in pro-inflammatory M1-type macrophages. Although the HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches showed comparable fluorescence intensity within the plaques, the CD44 signal (green) was significantly reduced in the HA-TEPA/MnO₂/Ce6 nanoswitch-treated group, consistent with the in vitro trend of CD44 downregulation (Fig. 3E). This decrease is likely attributable to MnO₂-mediated oxygen generation, which alleviates local hypoxia and subsequently suppresses CD44 expression in M1 macrophages. Importantly, expression of HIF-1 α (blue), a hypoxia marker, was also markedly reduced in plaques treated with HA-TEPA/MnO₂/Ce6 nanoswitches compared with HA-Ce6, further supporting the hypoxia-alleviating effect of the nanoswitches. Consistent with these findings, fluorescence imaging with a hypoxia-specific reagent confirmed significantly lower hypoxia signals in the HA-TEPA/MnO₂/Ce6 nanoswitch group than in the HA-Ce6 group (Figure S15). Taken together, although both nanoswitch types exhibited comparable plaque-targeting efficiency via CD44-mediated uptake, only the HA-TEPA/MnO₂/Ce6 nanoswitches effectively relieved hypoxia and reduced HIF-1 α expression, thereby inducing downregulation of CD44-positive M1 macrophages [6].

To further validate the M1-to-M2 reprogramming effect of HA-TEPA/MnO₂/Ce6 nanoswitches, we examined the polarization states of macrophages within atherosclerotic plaques (Figure S16). The HA-Ce6-treated group exhibited a dominant CD86-positive area (51%). Conversely, HA-TEPA/MnO₂/Ce6 nanoswitch treatment significantly reduced CD86 expression to 22.49% ($P = 0.0012$). At the same time, the CD206-positive area increased markedly from 17.76% in the HA-Ce6 group to 40.36% in the HA-TEPA/MnO₂/Ce6 nanoswitch group ($P = 0.0012$). Collectively, these results highlight the therapeutic advantage of the HA-TEPA/MnO₂/Ce6 nanoswitches in targeting and reprogramming the inflammatory microenvironment of hypoxic atherosclerotic plaques.

In vivo effects of HA-TEPA/MnO₂/Ce6 nanoswitches photoactivation on hypoxic atherosclerotic plaques

To evaluate the in vivo phototherapeutic efficacy of the HA-TEPA/MnO₂/Ce6 nanoswitches, serial in vivo CLSM imaging was performed at baseline and 1 week after laser irradiation in ApoE^{-/-} mice bearing hypoxic atherosclerotic plaques (Fig. 6A). Mice received intravenous free Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches 72 h before photoactivation and imaging. Photoactivation was applied to the carotid plaque region using a previously reported CLSM-guided laser system that enables focal irradiation with a homogeneous beam profile [17]. To distinguish laser-dependent effects from nanoswitch-associated effects, additional control groups without laser irradiation were included. As shown in Fig. 6B and C, mice treated with free Ce6 and laser irradiation did not show any notable change in plaque area, macrophage area (Ce6 fluorescence), or pTBR over 1 week, suggesting that free Ce6 does not effectively target plaques and therefore provides negligible therapeutic benefit under these conditions. Similarly, in mice administered HA-TEPA/MnO₂/Ce6 nanoswitches without laser exposure (Fig. 6D, E), plaque area, macrophage area, and pTBR remained unchanged, indicating that nanoswitch accumulation alone was insufficient to induce therapeutic effects in the absence of photoactivation. In contrast, mice treated with HA-TEPA/MnO₂/Ce6 nanoswitches followed by laser irradiation exhibited a pronounced therapeutic response (Fig. 6F, G). After photoactivation, the Ce6 fluorescence signal corresponding to macrophage accumulation within plaques was significantly reduced (Fig. 6F, G). Quantitative analysis revealed that both the macrophage signal and pTBR decreased by approximately 56.7% ($P = 0.0337$) and 37.6% ($P = 0.0352$), respectively, relative to the baseline. Plaque area was also significantly reduced by 38.4% ($P = 0.001$), indicating substantial plaque regression. Collectively, these findings demonstrate that only the photoactivated HA-TEPA/MnO₂/Ce6 nanoswitches induced macrophage depletion and plaque reduction, likely owing to the synergistic photodynamic effect enhanced by MnO₂-mediated hypoxia alleviation. Neither free Ce6 nor non-irradiated HA-TEPA/MnO₂/Ce6 nanoswitches produced comparable therapeutic outcomes. Overall, these results confirm the requirement of both targeted delivery and laser activation for successful phototherapy in hypoxic atherosclerotic plaques.

To further validate in vivo phototherapeutic efficacy, excised carotid plaques were analyzed by immunofluorescence (IF) and histological staining to assess inflammation, lipid content, and fibrotic remodeling (Fig. 7). CD44, an overexpressed marker on M1 macrophages, was significantly reduced in both HA-TEPA/MnO₂/Ce6 nanoswitch-treated groups, regardless of laser irradiation, relative to the free Ce6 control. Quantification showed a reduction in CD44-positive area by approximately 84.2% ($P < 0.0001$) in the non-irradiated group and by 72.1% ($P < 0.0001$) in the irradiated group. These findings suggest that MnO₂-mediated oxygen generation may promote a phenotypic shift from pro-inflammatory M1 to anti-inflammatory M2 macrophages during PDT, consistent with the in vivo observations in Fig. 5D. Notably, only the photoactivated HA-TEPA/MnO₂/Ce6 nanoswitch group demonstrated a significant reduction in CD68-positive macrophage area by 68.1% ($P < 0.0001$), compared with controls. Furthermore, the CD44/CD68-colocalized area, representing inflammatory M1 macrophages, was also significantly decreased in the photoactivated HA-TEPA/MnO₂/Ce6 nanoswitch group by 89.2% ($P < 0.0001$) relative to the control groups. Collectively, these results suggest that photoactivation of HA-TEPA/MnO₂/Ce6 nanoswitches selectively eliminates CD44-expressing M1 macrophages. Accordingly,

HA-TEPA/MnO₂/Ce6 nanoswitches appear to act through a synergistic mechanism in which MnO₂-driven in situ oxygenation promotes (i) hypoxia alleviation and M1-to-M2 macrophage polarization for plaque microenvironment reprogramming and (ii) improved PDT efficacy by sustaining oxygen availability during irradiation. The Oil Red O (ORO)-stained area (lipid accumulation) was also significantly reduced in the photoactivated HA-TEPA/MnO₂/Ce6 nanoswitch group by 46.26% ($P = 0.02902$) and 55.05% ($P = 0.00162$) compared with the free Ce6 control and the non-irradiated HA-TEPA/MnO₂/Ce6 nanoswitch group, respectively. These results indicate that photoactivated HA-TEPA/MnO₂/Ce6 nanoswitches effectively reduce lipid-rich cores, a hallmark of high-risk plaques. Meanwhile, the Picrosirius Red (PSR)-stained area (collagen content) increased by 174% ($P = 0.003354$) in the photoactivated nanoswitch group relative to the free Ce6 control group, suggesting the enhanced fibrotic stabilization of the plaques.

M1-to-M2 reprogramming induced by photoactivated HA-TEPA/MnO₂/Ce6 nanoswitches was also evaluated (Figure S17). Relative to free Ce6, the HA-TEPA/MnO₂/Ce6 nanoswitches significantly reduced the CD86-positive area (45.88% to 19.68%; $P = 0.0003$) and increased the CD206-positive area (13.79% to 38.30%; $P = 0.0018$). This therapeutic synergy was further enhanced by laser irradiation. While free Ce6 Laser (+) had minimal impact, likely owing to poor plaque targeting, the HA-TEPA/MnO₂/Ce6 Laser (+) group produced the most pronounced reprogramming, with reduced CD86 expression (50.46% to 15.04%; $P < 0.001$) and increased CD206 expression (11.58% to 45.84%; $P < 0.001$). These results demonstrate that integrating CD44-mediated targeting, MnO₂-mediated oxygenation, and photoactivation effectively shifts the plaque microenvironment from a pro-inflammatory state toward a pro-resolving, M2-dominant phenotype.

Taken together, these results indicate that photoactivated HA-TEPA/MnO₂/Ce6 nanoswitches exhibit superior anti-atherogenic activity by reducing pro-inflammatory M1 macrophages and lipid burden while promoting fibrotic plaque stabilization.

Biosafety of HA-TEPA/MnO₂/Ce6 nanoswitches

Ce6 has been approved by the United States Food and Drug Administration for photodynamic cancer therapy [80]. To evaluate the biosafety of the HA-TEPA/MnO₂/Ce6 nanoswitches, a comprehensive assessment was performed that included blood biochemistry and histological analysis of the major organs (Figure S18). Mice received a single injection of HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 3 mg/kg Ce6). Blood biochemistry analyses indicated no evidence of hepatic or renal dysfunction following nanoswitch administration (Figure S18A). Consistently, histological analyses of the kidney, liver, lung, spleen, and heart revealed no detectable tissue damage in the HA-TEPA/MnO₂/Ce6 nanoswitch groups compared with the control group (Figure S18B).

Materials and methods

Materials

Sodium hyaluronate (HA; Mw: 215 kDa) was purchased from Lifecore Biomedical (Chaska, MN, USA). TEPA and Ce6-NH₂ were obtained from Acros Organics (CA, USA) and Frontier Scientific (Logan, UT, USA), respectively. KMnO₄, EDC, NHS, potassium bromide (KBr), H₂O₂ solution (30% w/w), DMSO, deuterium oxide (D₂O), and DCFDA were purchased from Sigma–Aldrich (St. Louis, MO, USA). MnO₂ nanopowder (98%, 50 nm) was obtained from US Research Nanomaterials, Inc. (Houston, TX, USA). SOSG, Image-iT™ Green Hypoxia Reagent, and Image-iT™ Red Hypoxia Reagent were procured from Thermo Fisher Scientific (Waltham, MA, USA). Dialysis membranes (MWCO: 12–14 kDa) were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). PBS and 0.9% NS solution were obtained from Lonza (Walkersville, MD, USA) and Dai Han Pharm (Seoul, Korea), respectively. Dulbecco's Modified Eagle Medium (DMEM), FBS, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Welgene Inc. (Seoul, Korea). CCK-8 was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Furthermore, 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) was obtained from GBI Labs (Bothell, WA, USA). FluoreGuard Mounting Medium was procured from ScyTek Laboratories (Logan, Utah, USA).

Synthesis and characterization of HA-TEPA

HA (0.3 g) was dissolved in 30 mL of DW for 24 h. Next, 22 mL of DMSO was added to the HA solution. EDC (0.143 g, 0.746 mmol) and NHS (0.129 g, 1.119 mmol), each dissolved in 4 mL of DMSO, were added to the HA solution to activate HA carboxyl (COOH) groups for 30 min. TEPA (42.9 µL, 0.224 mmol) was then added, and the reaction was stirred for 24 h at room temperature. The reaction mixture was dialyzed against DW (MWCO, 3.5 kDa) for 3 days and lyophilized to obtain HA-TEPA. The synthesized HA-TEPA was characterized via ¹H NMR spectroscopy after dissolving in D₂O containing 1% tetramethylsilane (TMS) at a concentration of 5 mg/mL.

Synthesis and characterization of HA-TEPA/MnO₂/Ce6 nanoswitches

To compare the reducing reactivity of HA and HA-TEPA toward KMnO₄, each sample (30 mg) was reacted with KMnO₄ (60 mg) in DW (15 mL) for 24 h. Time-dependent changes in absorbance were monitored using a UV/Vis spectrophotometer at predetermined time points (1 min, 12 h, and 24 h), and photographs of the reaction mixture were also acquired. After 24 h, the resulting solutions were purified via dialysis (MWCO, 12–14 kDa) against DW for 1 day. The absorption spectra and photographs of the purified solutions were obtained using the same instrument, and the samples were subsequently lyophilized for 3 days for further use.

HA-TEPA/MnO₂/Ce6 nanoswitches were synthesized via sequential reactions among HA-TEPA, KMnO₄, and Ce6-NH₂. Briefly, HA-TEPA (50 mg) was reacted with KMnO₄ (50 mg) in DW (30 mL) for 4 h. The reaction product was purified by dialysis (MWCO, 12–14 kDa) to remove unreacted KMnO₄. The obtained

HA-TEPA/MnO₂ solution was then mixed with EDC (23.8 mg, 0.1244 mmol) and NHS (21.47 mg, 0.1866 mmol) dissolved in 2 mL of DMSO to activate carboxyl groups on the HA backbone. After 1 h, Ce6-NH₂ (22.27 mg, 0.03732 mmol) dissolved in 4 mL of DMSO was added and allowed to react for 12 h. HA-TEPA/MnO₂/Ce6 was purified by dialysis (MWCO, 12–14 kDa) against 30% DMSO in DW for 2 days, followed by dialysis against DW for an additional 3 days. The purified solution was lyophilized for 3 days.

The sequential formation of the HA-TEPA/MnO₂/Ce6 nanoswitches was confirmed by measuring the zeta potentials of HA, HA-TEPA, HA-TEPA/MnO₂, and HA-TEPA/MnO₂/Ce6 using a Zetasizer. Absorption spectra of free Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches were also recorded using a UV/Vis spectrophotometer (NEO-S490). The Ce6 content in the HA-TEPA/MnO₂/Ce6 nanoswitches was quantified using a Ce6 standard calibration curve ($Y = 173.81x + 0.024$; $R^2 = 1$). The manganese ion (Mn²⁺) content was determined via ICP-OES (Optima 7300 DV) after treatment with nitric acid and H₂O₂. The Ce6 loading content in the HA-TEPA/MnO₂/Ce6 nanoswitches was then calculated using the following equation:

$$\text{Ce6 loading content (wt\%)} = \frac{\text{Weight of loaded Ce6}}{\text{Weight of nanoswitches}} \times 100\%$$

To further confirm nanoswitch synthesis, the lyophilized HA-TEPA and HA-TEPA/MnO₂/Ce6 powders were analyzed by FT-IR spectroscopy (Shimadzu 8400S) at a resolution of 4 cm⁻¹ over the range of 4000–400 cm⁻¹ using the KBr pellet method. XPS (K-alpha+) equipped with a Cu-Kα radiation source was also performed.

For size and morphology characterization, HA-TEPA/MnO₂/Ce6 nanoswitches (0.5 mg/mL) were dispersed in DW and sonicated for 5 min using an Ultrasonic Processor (KFS-150N, Korea Process Technology Co., Ltd., Seoul, Korea) at 22.5 W. Size distribution, hydrodynamic diameter, Z-average size, and PDI were measured using a particle size analyzer (SZ-100). TEM images were acquired using a FE-TEM (JEM-F200) after depositing the nanoswitch dispersion onto a carbon-coated copper grid (Carbon Type-B, Ted Pella Inc., Redding, CA, USA) and drying under ambient conditions.

To assess nanoswitch stability, HA-TEPA/MnO₂/Ce6 nanoswitches (0.1 mg/mL) were dispersed in four solutions: DW, DMEM containing 10% FBS, 0.9% NS, and PBS 1X (pH 7.4). Samples were stored at 25 °C, and changes in Z-average size and visual appearance were monitored over 7 days at predetermined intervals (0, 1, 2, 3, 4, 5, 6, and 7 days).

Degradation behavior, oxygen generation, and singlet oxygen production of HA-TEPA/MnO₂/Ce6 nanoswitches

To evaluate the H₂O₂ responsiveness of the HA-TEPA/MnO₂/Ce6 nanoswitches, their degradation behavior was assessed by monitoring the absorbance at 320 nm using a UV/Vis spectrophotometer

(NEO-S490). The HA-TEPA/MnO₂/Ce6 nanoswitches (0.1 mg/mL) were incubated in PBS (pH 7.4) with different H₂O₂ final concentrations (0, 50, 100, and 200 μ M), and their absorbance was recorded over 5 min.

To investigate the oxygen-generating capability of the HA-TEPA/MnO₂/Ce6 nanoswitches under hypoxic conditions, 10 mL of the HA-TEPA/MnO₂/Ce6 nanoswitch solution (0.2 mg/mL) and 10 mL of H₂O₂ solutions (0, 100, 200, and 400 μ M in PBS, pH 7.4) were each purged with N₂ for 10 min. Equal volumes of the N₂-purged solutions were then mixed to yield final H₂O₂ concentrations of 0, 50, 100, and 200 μ M. The reaction proceeded for 5 min, during which dissolved oxygen was recorded using an Oakton® DO 6 + dissolved oxygen meter (EW-35643-15, Cole-Parmer®, Vernon Hills, IL, USA).

To evaluate SO generation under hypoxic conditions, the HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μ M Ce6) were prepared in N₂-purged PBS (pH 7.4) containing various concentrations of H₂O₂ (0, 50, 100, or 200 μ M). Each solution was supplemented with SOSG (1 μ M) and then irradiated with a 670-nm laser (50 mW/cm²) for 0–4 min. SOSG fluorescence (Ex/Em: 504/525 nm) was measured using a fluorescence spectrophotometer (FS-2, Scinco, Korea) to quantify SO generation as a function of irradiation time and H₂O₂ concentration.

H₂O₂-triggered FL and PDT off-to-on switching effects of nanoswitches

For comparative studies, HA-Ce6 was synthesized as a MnO₂-free control. Briefly, HA (30 mg) was dissolved in DW (15 mL), and EDC (14.3 mg, 74.6 μ mol) and NHS (12.9 mg, 111.9 μ mol) were dissolved in DMSO (2 mL). After mixing, the HA solution was activated by reaction with EDC/NHS solution for 1 h to activate the COOH groups. Subsequently, Ce6-NH₂ (4.5 mg, 7.46 μ mol) dissolved in 3 mL of DMSO was then added, and the reaction mixture was stirred for 24 h. Excess EDC/NHS and unreacted Ce6-NH₂ were removed by dialysis (MWCO: 12–14 kDa) against DW containing 30% DMSO for 2 days, followed by further dialysis in DW alone for 3 days. The resulting HA-Ce6 was lyophilized for 3 days. Absorption spectra of free Ce6 and HA-Ce6 were recorded using a UV/Vis spectrophotometer (NEO-S490), and the Ce6 content was quantified using a Ce6 standard curve ($Y = 42.8x + 0.0075$; $R^2 = 0.9995$).

To confirm the H₂O₂-triggered FL off-to-on switching of nanoswitches, the Ce6 fluorescence spectra of HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 0.25 μ M Ce6) were measured in the absence or presence of H₂O₂ (200 μ M) using a fluorescence spectrophotometer (FS-2). In addition, the fluorescence spectrum of free Ce6 (0.25 μ M) in DMSO was recorded using the same instrument.

To further verify PDT off-to-on switching, the SO-generating capability of HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches (each equivalent to 5 μ M Ce6) was evaluated via the SOSG assay. The fluorescence spectra of SOSG were recorded with and without laser irradiation (670 nm, 50 mW/cm², 2 min exposure) using the same spectrophotometer (FS-2). To further assess H₂O₂-triggered activation,

HA-TEPA/MnO₂/Ce6 nanoswitches were incubated in the presence of H₂O₂ (200 μM) and then irradiated under the same conditions (670 nm, 50 mW/cm², 2 min exposure); SOSG fluorescence spectra were subsequently recorded.

In vitro cellular uptake in macrophages or activated macrophages

RAW 264.7 murine macrophages were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

To evaluate cellular uptake, RAW 264.7 cells (1×10^5 cells/well) were seeded on 4-well chamber slides and cultured for 24 h. For macrophage activation, cells were treated with LPS (200 ng/mL) for an additional 24 h. After washing with DPBS, untreated (macrophages) or LPS-stimulated (activated macrophages) cells were incubated with HA-TEPA/MnO₂/Ce6 nanoswitches at concentrations equivalent to 5, 10, or 20 μM Ce6 for 1 h. For CD44-specific uptake analysis, a receptor-blocking assay was performed by preincubating macrophages or activated macrophages with free HA (20 mg/mL) for 2 h, followed by treatment with HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μM Ce6) for 1 h. After incubation, the cells were washed with DPBS and fixed in 3.7% paraformaldehyde for 30 min, followed by three DPBS washes. Cell nuclei were counterstained with a fluoromount solution containing DAPI (SouthernBiotech, AL, USA).

Cellular uptake and receptor-blocking results were assessed using a customized CLSM (Ex/Em: 633 nm/650–710 nm). Quantitative fluorescence analysis was performed by calculating FI per cell from randomly selected regions of interest in the CLSM images using ImageJ software (ver. 1.53a, NIH, MD, USA).

Intracellular ROS detection in macrophages and activated macrophages under normoxic or hypoxic conditions

To compare intracellular ROS levels, RAW 264.7 cells (1×10^5 cells/well) were divided into two groups (macrophages or activated macrophages) as described above. Cells were then incubated for 4 h under either normoxic conditions in a humidified 5% CO₂ incubator or hypoxic conditions in an N₂-purged, laboratory-built hypoxic chamber, following a previously reported protocol [21]. After incubation, the cells were treated with 5 μM DCFDA and incubated at 37 °C for 30 min to detect intracellular ROS. The cells were subsequently washed with DPBS, fixed with 3.7% formaldehyde for 30 min, and counterstained with DAPI (SouthernBiotech). Fluorescence from oxidized DCF was imaged by CLSM, and intracellular ROS levels were quantified by calculating FI from CLSM images using ImageJ. This approach enabled comparison of oxidative status between macrophages and activated macrophages under normoxic and hypoxic conditions.

In vitro expression of CD44 receptors on macrophages and activated macrophages under normoxic or hypoxic conditions

RAW 264.7 cells (1×10^5 cells/well) were prepared as macrophages or activated macrophages. Cells were then incubated for 4 h under either normoxic or hypoxic conditions. After incubation, the cells were washed with DPBS and fixed with 3.7% formaldehyde for 30 min. Following fixation, permeabilization was conducted with 0.2% Triton X-100 in DPBS for 10 min. Cells were then stained with Alexa Fluor 594® anti-mouse/human CD44 antibody (1:100 dilution, 100 μ L/well) for 1 h, followed by DAPI nuclear counterstaining. Fluorescence imaging was performed by CLSM, and the FIs of CD44 expression were quantified using ImageJ.

In vitro cytotoxicity of HA-TEPA/MnO₂/Ce6 nanoswitches under normoxic or hypoxic conditions

To evaluate the in vitro cytotoxicity of the HA-TEPA/MnO₂/Ce6 nanoswitches, RAW 264.7 cells were seeded in 96-well plates at 3×10^4 cells/well and incubated for 24 h. Cells were then stimulated with or without LPS (200 ng/mL) for an additional 24 h and incubated under normoxic or hypoxic conditions. HA-TEPA/MnO₂/Ce6 nanoswitches were then added at varying concentrations (0–20 μ M Ce6 equivalent) and incubated for an additional 24 h. After treatment, cells were washed with fresh medium and incubated with CCK-8 solution for 4 h. Cell viability was determined by measuring absorbance at 450 nm using a microplate reader (Multiskan Go, Thermo Fisher Scientific).

Comparison of intracellular uptake of HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches under normoxic or hypoxic conditions

For cellular uptake analysis, RAW 264.7 cells (1×10^5 cells/well) were seeded and activated with LPS (200 ng/mL) and then incubated under normoxic or hypoxic conditions. Cells were subsequently treated with HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches (Ce6-equivalent concentration, 5 μ M) for 1 h. After washing with DPBS, the cells were fixed with 3.7% formaldehyde for 30 min and counterstained with DAPI. Ce6 fluorescence was imaged using a customized CLSM (Ex/Em: 633 nm/650–710 nm) and quantified using ImageJ.

In vitro H₂O₂-to-O₂ switching effects of HA-TEPA/MnO₂/Ce6 nanoswitches

MnO₂ can generate molecular oxygen (O₂) by decomposing endogenous H₂O₂, particularly in activated macrophages under hypoxic conditions, where H₂O₂ levels are elevated. To assess the H₂O₂-consuming capacity of HA-TEPA/MnO₂/Ce6 nanoswitches, RAW 264.7 cells (1×10^5 cells/well) were seeded and treated with or without LPS for 24 h. Cells were then incubated under hypoxic conditions and treated

with HA-Ce6 (5 μ M Ce6 equivalent) or HA-TEPA/MnO₂/Ce6 nanoswitches (5 μ M Ce6 equivalent) for 1 h. Following treatment, cells were washed with DPBS, fixed with 3.7% formaldehyde for 30 min, and stained with DAPI. Intracellular ROS levels were evaluated by measuring oxidized DCF fluorescence (Ex/Em: 488 nm/510–535 nm) using CLSM. FIs were quantified using ImageJ to compare ROS levels among groups.

To assess the intracellular oxygen-generating capacity of the nanoswitches via H₂O₂ consumption, RAW 264.7 cells (1 $\times$ 10⁵ cells/well) were cultured with or without LPS and maintained under either normoxic or hypoxic conditions. The cells were treated with HA-Ce6 (equivalent to 5 μ M Ce6) or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μ M Ce6) for 1 h. After washing with DPBS, the cells were incubated with Image-iT™ Green Hypoxia Reagent (5 μ M) for 30 min according to the manufacturer's instructions. After replacing the medium with fresh medium, normoxic groups were incubated for 4 h in a humidified 5% CO₂ incubator. Hypoxic groups were initially placed in an N₂-purged, laboratory-made hypoxia chamber for 20 min, followed by an additional 4 h incubation in a humidified CO₂ incubator. The cells were subsequently fixed with 3.7% formaldehyde, counterstained with DAPI, and imaged by CLSM (Ex/Em: 488 nm/510–535 nm). Their FIs were quantified from randomly selected images using ImageJ.

In vitro M1-to-M2 macrophages polarization effects of HA-TEPA/MnO₂/Ce6 nanoswitches

To evaluate the potential of HA-TEPA/MnO₂/Ce6 nanoswitches to downregulate CD44 expression, RAW 264.7 cells (3 $\times$ 10⁴ cells/well) were seeded in 8-well cell culture plates and incubated with or without LPS for 24 h to generate macrophages or activated macrophages, respectively. The cells were then subjected to either normoxic or hypoxic incubation, followed by treatment with HA-Ce6 (equivalent to 5 μ M Ce6) or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μ M Ce6) for 1 h. To minimize interference from dissolved O₂ in the culture medium, after replacing the medium with fresh medium, normoxic groups were incubated for an additional 1 h in a humidified CO₂ incubator, whereas hypoxic groups were placed in a N₂-purged laboratory-made hypoxia chamber for 10 min and subsequently incubated in a humidified CO₂ incubator for 1 h. After incubation, the cells were washed with DPBS, fixed with 3.7% formaldehyde at 25 °C for 30 min, and permeabilized with 0.2% Triton X-100 in DPBS for 10 min. CD44 expression was evaluated by IF staining using Alexa Fluor 594® anti-mouse/human CD44 antibody (1:100 dilution, 100 μ L/well) for 1 h, followed by DAPI nuclear counterstaining. Fluorescence images were acquired by CLSM, and the FIs of CD44 were quantified using ImageJ software.

To investigate macrophage phenotype switching, RAW 264.7 cells (3 $\times$ 10⁴ cells/well) were seeded and activated as described above. Activated macrophages were incubated under hypoxic conditions and treated with either HA-Ce6 (equivalent to 5 μ M Ce6) or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μ M Ce6) for 1 h. Following medium replacement, the cells were placed in an N₂-purged hypoxia chamber for 10 min and then incubated for 1 h in a humidified 5% CO₂ incubator. Cells were washed, fixed, and permeabilized (0.2% Triton X-100, 10 min). A 100 μ L/well antibody mixture containing Alexa Fluor 405® Mouse B7-2/CD86 antibody (M1 marker; 1:50 dilution) and FITC anti-mouse CD206 antibody

(M2 marker; 1:100 dilution) was then applied for 1 h, followed by mounting with FluoreGuard Mounting Medium (ScyTek Laboratories). CLSM was used to visualize CD86 and CD206 expression on activated macrophages, and FIs were quantified using ImageJ.

In vitro phototherapeutic effects of HA-TEPA/MnO₂/Ce6 nanoswitches under normoxic or hypoxic conditions

RAW 264.7 cells (3×10^4 cells/well) were seeded in 96-well black plates and prepared as macrophages or activated macrophages under normoxic or hypoxic conditions. The cells were treated with either HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μ M Ce6) for 1 h. After treatment, the cells were washed three times with DPBS and replenished with fresh culture medium (normoxia) or N₂-purged culture medium (hypoxia). Subsequently, the cells were irradiated with or without a 670 nm laser (50 mW/cm², 2 min), followed by incubation for 24 h. Cell viability was assessed using CCK-8. After 4 h of incubation with CCK-8, absorbance at 450 nm was measured using a microplate reader (Multiskan Go), and cell viability was calculated relative to untreated controls.

To compare the intracellular generation of SO, RAW 264.7 cells (3×10^4 cells/well) were seeded in 8-well chamber slides and differentiated into macrophages or activated macrophages under normoxic or hypoxic conditions. The cells were treated with HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μ M Ce6) for 1 h, washed with DPBS, and incubated with SOSG reagent (5 μ M) in fresh culture medium (normoxic groups) or N₂-purged medium (hypoxic groups) for 30 min. Subsequently, the cells were irradiated with a 670 nm laser (50 mW/cm², 2 min). Immediately after irradiation, the cells were washed with DPBS, fixed with 3.7% formaldehyde for 30 min, and counterstained with DAPI. SOSG fluorescence (Ex/Em: 488 nm/510–535 nm) was imaged by CLSM, and FIs were quantified using ImageJ.

For apoptosis analysis, RAW 264.7 cells (3×10^4 cells/well) were seeded in 8-well chamber slides and differentiated into macrophages or activated macrophages as described above. After the cells were treated with HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μ M Ce6) for 1 h under normoxic or hypoxic conditions, washed, and replaced with fresh medium (normoxia) or N₂-purged medium (hypoxia), followed by laser irradiation (670 nm, 50 mW/cm², 2 min). After an additional 24 h incubation, cells were stained with CF® 488 Annexin V and PI at 25 °C for 30 min according to the manufacturer's instructions. Cells were then washed with DPBS, fixed with 3.7% formaldehyde (30 min, 25 °C), and nuclei were counterstained with DAPI. CLSM was used to visualize early apoptotic (Annexin V, Ex/Em: 488 nm/510–535 nm) and late apoptotic (PI, Ex/Em: 488 nm/617 nm) cells. FIs were also quantified using ImageJ.

In vivo biodistribution and skin phototoxicity of HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches

All animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85 – 23, revised 1996) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Chung-Ang University (A2022001) and Korea University College of Medicine (KOREA-2021-0084).

Before the in vivo experiments, injectability at the intended injection concentration was verified by photographing solutions of HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches. To evaluate in vivo biodistribution, seven-week-old BALB/c nude mice (Doo Yeol Biotech, Seoul, Korea) were intravenously injected with HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches at an equivalent Ce6 dose of 3 mg/kg. Whole-body fluorescence imaging was performed in the lateral position at predetermined time points (0, 0.25, 0.5, 1, 3, 6, 12, 24, 48, and 72 h post-injection) using a small-animal imaging system (InVivo Smart-LF, VIEWWORKS, Anyang, Republic of Korea). At 72 h post-injection, the mice were sacrificed, and major organs, including the liver, lung, spleen, kidney, and heart, were harvested. Ex vivo fluorescence images of the excised organs were acquired using the same imaging system (InVivo Smart-LF) to assess organ-specific accumulation.

To assess cutaneous phototoxicity, Balb/c mice (7-week-old, male; Doo Yeol Biotech, Seoul, Korea) were randomly assigned to three groups and intravenously injected with 100 µL saline, HA-Ce6, or HA-TEPA/MnO₂/Ce6 nanoswitches (each equivalent to 3 mg/kg Ce6). One hour after injection, the shaved dorsal skin was irradiated with a 670 nm laser (50 mW/cm²) for 3 min. Two days after irradiation, the treated skin areas were photographed at a controlled distance and under controlled lighting conditions. The irradiated skin tissues were then harvested, fixed in 3.7% paraformaldehyde, paraffin-embedded, and sectioned for histological analysis. H&E staining was performed to assess epidermal damage and inflammatory cell infiltration. Stained tissues were imaged using an automated slide scanner (Axio Scan Z1, Zeiss, Oberkochen, Germany).

In vivo accumulation of HA-Ce6 and HA-TEPA/MnO/Ce6 nanoswitches in hypoxic atherosclerotic plaques

ApoE^{-/-} mice with a genetic background of Balb/c (Balb/c.KOR/StmSlc-Apoeshl) were obtained from Japan SLC (Shizuoka, Japan). All experiments used male mice to minimize hormonal effects on atherosclerosis progression [81]. To establish a hypoxic atherogenic mouse model, mice were divided into two groups (n = 3 per group). The first group (general atherogenic diet) was fed HCD for 12 weeks. The second group (hypoxia-enhanced atherogenic diet) received the same HCD for 8 weeks, followed by a WD for an additional 4 weeks.

To confirm plaque hypoxia, mice were intravenously injected with Image-iT™ Red Hypoxia Reagent (200 nmol/mouse) [70]. After 2 h, the mice were euthanized, and the carotid arteries were harvested, cryosectioned (10 µm), and imaged using a custom-built CLSM to visualize hypoxic regions. FIs were

quantified in ImageJ. Based on these quantitative data, the group fed HCD for 8 weeks followed by WD for 4 weeks was selected as the hypoxic atherogenic mouse model for subsequent experiments.

To evaluate plaque-targeting efficiency, hypoxic atherogenic mice (n = 6) were intravenously injected with either HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 3 mg/kg Ce6; n = 3 per group). Mice were anesthetized with 1.5% isoflurane (JW-Pharma, Republic of Korea), and the carotid artery was surgically exposed. A 0.5 mm fiber was placed beneath the vessel to minimize motion artifacts [17]. Immediately before imaging, FITC-dextran was intravenously injected for carotid angiography. Fluorescence images were acquired using a customized CLSM equipped with two laser lines (488 nm for FITC and 642 nm for Ce6). The mean Ce6 FI within the plaque region was quantified, and the pTBR was calculated as follows: [17]

$$pTBR = \frac{FI_{plaque}}{FI_{adjacent\ normal\ artery}}$$

For IF analysis, mice were euthanized, and the imaged carotid arteries were harvested, embedded, and serially cryosectioned (10 µm). To assess plaque accumulation of the HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches, sections were imaged using a custom-built CLSM. For co-localization analysis with CLSM and macrophages, CD44, and HIF-1α, the cryosections were incubated with a CD68 antibody (1:100; BIO-RAD) at 4 °C overnight, washed with Tris-buffered saline with Tween 20 (TBST) for 3 min, then incubated with Alexa Fluor 594® anti-rat IgG (1:100) at 37 °C for 2 h in the dark, followed by three washes with TBST. The CD68-stained sections were then incubated with a CD44 antibody (1:100; Novus Biologicals) at 4 °C overnight, followed by Alexa Fluor 488® anti-rabbit IgG (1:100) at 37 °C for 2 h in the dark. After additional washing, HIF-1α staining was performed by incubation with anti-HIF-1α (1:100; Santa Cruz) at 4 °C overnight, followed by Alexa Fluor 350® anti-mouse IgG (1:100) at 37 °C for 2 h in the dark. Finally, the stained slides were mounted with FluoreGuard Mounting Medium (ScyTek Laboratories, Logan, Utah, USA), and fluorescence images were acquired using a Zeiss LSM 900 confocal microscope (Carl Zeiss).

To assess the hypoxia-alleviating capability of HA-TEPA/MnO₂/Ce6 nanoswitches, hypoxic atherogenic mice were intravenously administered PBS (n = 3), HA-Ce6, or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 3 mg/kg Ce6; n = 3 per group). After 22 h, all mice received an additional intravenous injection of Image-iT™ Red Hypoxia Reagent (200 nmol/mouse).⁷⁰ After 2 h, the mice were euthanized, and the carotid arteries were harvested and cryosectioned (10 µm). The frozen sections were imaged with CLSM to visualize hypoxic regions, and FI was quantified using ImageJ.

In vivo imaging-guided photoactivation and histopathological analysis

Fifteen atherogenic mice (7-week-old, male) were fed a hypoxia-enhanced atherogenic diet for 12 weeks (HCD for 8 weeks followed by WD for 4 weeks). To investigate the therapeutic potential of photoactivation, the mice were intravenously administered either free Ce6 (3 mg/kg) or HA-

TEPA/MnO₂/Ce6 nanoswitches (equivalent to 3 mg/kg Ce6). Baseline intravital imaging of the carotid artery was performed 72 h post-injection using a custom-built CLSM system. Following baseline imaging, the mice were randomly assigned to three groups: (i) free Ce6 with laser irradiation (photoactivation, n = 5), (ii) HA-TEPA/MnO₂/Ce6 nanoswitches with laser irradiation (photoactivation, n = 5), and (iii) HA-TEPA/MnO₂/Ce6 nanoswitches without laser irradiation (non-irradiated control, n = 5). For photoactivation, the imaged carotid plaque region was externally illuminated with a 670 nm diode laser at a fluence rate of 1 W/cm² for 300 s. After irradiation, the incision site was closed surgically, and all mice were maintained on normal chow. After 1 week, the same dose of free Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches was administered intravenously, and follow-up imaging of the same carotid plaque region was performed under identical CLSM conditions. Serial intravital imaging was used to evaluate therapeutic responses by quantifying changes in three atherosclerosis-related parameters: (i) plaque burden, determined from the FITC angiogram; (ii) macrophage infiltration, indicating inflammatory status; and (iii) pTBR, calculated from Ce6 fluorescence. After follow-up CLSM imaging, the mice were euthanized by CO₂ inhalation and perfused with PBS. The imaged carotid arteries were dissected and frozen-embedded for histopathological analysis.

For histopathological evaluation of atherosclerotic plaque composition, the dissected carotid arteries were embedded, and serial cryosections were obtained at a thickness of 10 µm. To assess the effects of photoactivation of HA-TEPA/MnO₂/Ce6 nanoswitches on plaque composition, serial cryosections were stained with ORO (ScyTek) to quantify lipid accumulation and with PSR (ScyTek) to evaluate collagen distribution. The stained slides were scanned using an automated slide scanner (Axio Scan Z1). For IF staining, adjacent cryosections were incubated with anti-CD68 (1:100) to label macrophages and anti-CD44 (1:100) to identify M1-type macrophages. After overnight incubation at 4 °C, the slides were washed with TBST and then stained with Alexa Fluor 594® anti-rat IgG (1:100) and Alexa Fluor 488® anti-rabbit IgG (1:100) for 2 h in the dark. The stained sections were then mounted and imaged using a Zeiss LSM 900 confocal microscope (Carl Zeiss).

To validate macrophage phenotype within plaques, sister frozen sections were incubated with anti-CD86 (M1 marker; 1:100 dilution) overnight at 4 °C. After washing, the slides were incubated with Alexa Fluor 488® anti-rat IgG for 2 h in the dark. The CD86-stained slides were then incubated with anti-CD206 (M2 marker; 1:100), followed by Alexa Fluor 594® anti-mouse IgG. The stained tissues were mounted and imaged using an LSM 900 (Carl Zeiss).

In vivo biosafety of HA-TEPA/MnO₂/Ce6 nanoswitches

To evaluate the in vivo biosafety of the HA-TEPA/MnO₂/Ce6 nanoswitches, hypoxic atherogenic mice were intravenously injected with either PBS (n = 3) or HA-TEPA/MnO₂/Ce6 nanoswitches (n = 3, equivalent to 3 mg/kg Ce6). At 72 h post-injection, blood samples were collected from the inferior vena cava under isoflurane anesthesia (1.5% in 2 L/min oxygen). Immediately after blood collection, the mice were sacrificed, and major organs (liver, lung, spleen, and kidney) were harvested for histological analysis. Serum was isolated by centrifuging whole blood (without anticoagulant) at 1500 ×g for 15 min

at 4 °C. For serum biochemistry analysis, liver function was evaluated by quantifying aspartate aminotransferase, alanine transaminase, and alkaline phosphatase, whereas kidney function was assessed by quantifying serum uric acid. All biochemical parameters were measured using an automatic analyzer (DRI-CHEM NX500i, FUJIFILM, Japan). For histology, harvested organs were cryosectioned (10 μ m) and stained with H&E; the stained tissues were imaged using an automated slide scanner (Axio Scan Z1).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The data are presented as mean $\pm$ standard deviation (SD). Paired t-tests were used to compare plaque area, macrophage area, and pTBR between baseline and follow-up imaging. Comparison of normally distributed data was performed using an unpaired t-test (two groups) or one-way analysis of variance (ANOVA; three or more groups) with an appropriate post hoc test. $P < 0.05$ was considered statistically significant; $P > 0.05$ was considered not significant (n.s.).

Conclusion

In this study, we developed HA-TEPA/MnO₂/Ce6 nanoswitches, a MnO₂-based nanoswitch platform that integrates microenvironment reprogramming with enhanced activatable photodynamic therapy for the treatment of hypoxic atherosclerotic plaques. The HA-TEPA/MnO₂/Ce6 nanoswitches were readily synthesized via sequential reactions of HA-TEPA, KMnO₄, and Ce6-NH₂. The nanoswitches were designed to remain in a photodynamically “off state” under physiological conditions, while being selectively activated by pathological H₂O₂ and light irradiation. Upon accumulation in CD44-positive M1 macrophages, the nanoswitches undergo H₂O₂-triggered MnO₂ degradation, resulting in in-situ oxygen generation, hypoxia alleviation, and subsequent macrophage reprogramming from the pro-inflammatory M1 phenotype toward the anti-inflammatory M2 phenotype. In parallel, MnO₂-mediated fluorescence and singlet oxygen quenching effectively minimize off-target phototoxicity under normal physiological conditions. Following H₂O₂-triggered MnO₂ degradation and laser irradiation, the photoactivity of the nanoswitches is restored and further enhanced in hypoxic microenvironments. Consequently, these H₂O₂- and light-triggered functional transitions produce synergistic therapeutic effects, including macrophage depletion, lipid-core reduction, collagen enrichment, and overall plaque stabilization and regression in vivo, without systemic toxicity or skin photosensitization. Collectively, this study establishes MnO₂-based nanoswitches as a trigger-responsive therapeutic platform that integrates microenvironment reprogramming with activatable photodynamic therapy. This state-switchable, stimulus-responsive behavior supports both biosafety and robust efficacy and provides a broadly applicable design strategy for environmentally activated treatment of inflammatory diseases.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Supplementary Information

The online version contains supplementary material available.

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Author Contribution

Y.G.L. and J.H.K. contributed equally as co-first authors. Y.G.L., J.H.K., J.W.K., and K.P. conceived and designed the experiments. Y.G.L., J.H.K., and R.H.K. were responsible for the methodology and conducted the investigations. Y.G.L. and J.H.K. conducted validations, performed the formal analysis and data curation, and wrote the original manuscript draft. H.Y., J.W.K., and K.P. were responsible for project administration, reviewing, and editing the manuscript. J.W.K. and K.P. handled funding acquisition.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Scheme

Scheme 1 is available in the Supplementary Files section.

Figures

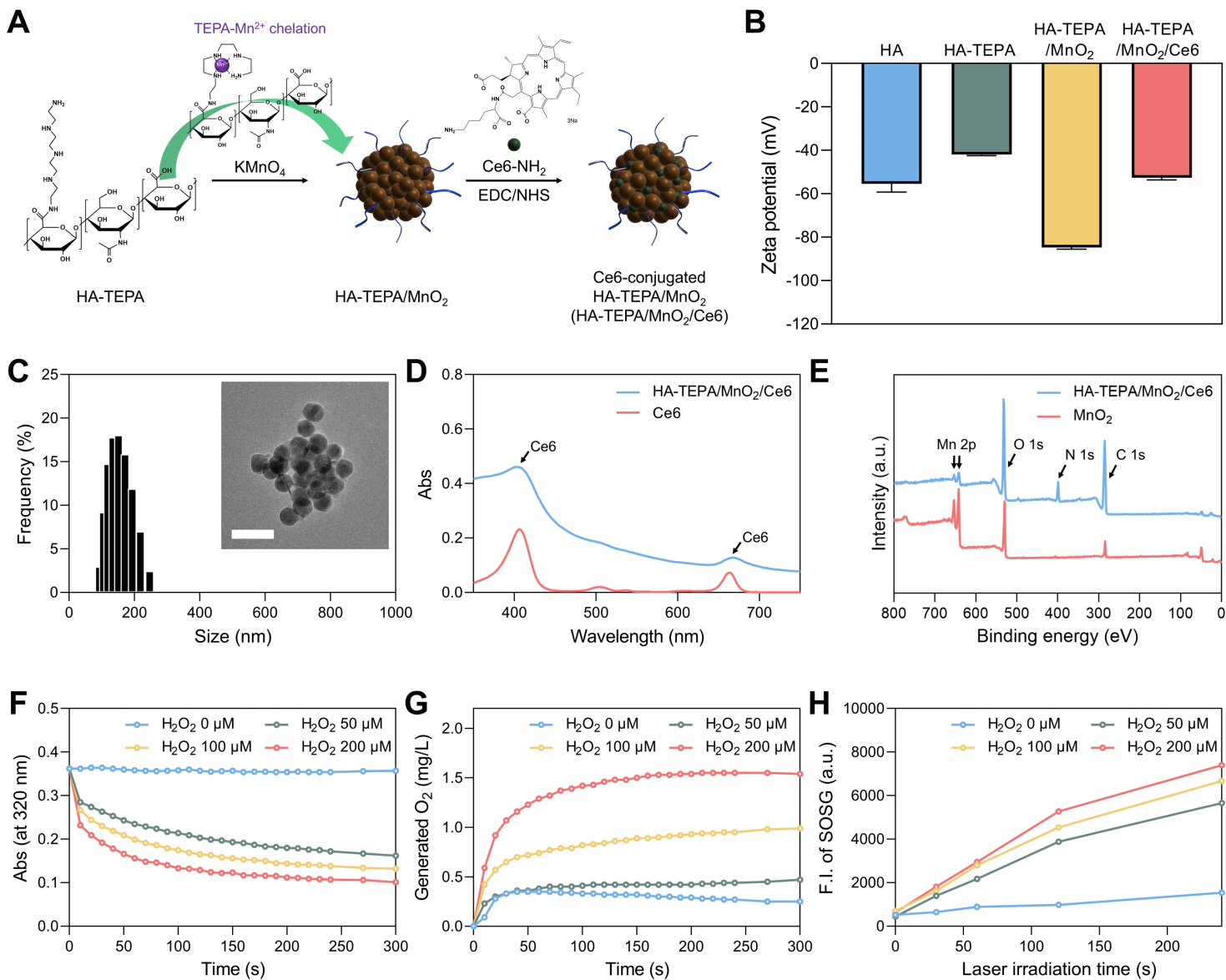


Figure 1

Synthesis and characterization of HA-TEPA/MnO₂/Ce6 nanoswitches. (A) Schematic of HA-TEPA/MnO₂/Ce6 nanoswitch synthesis. HA-TEPA/MnO₂/Ce6 nanoswitches were prepared through sequential reactions of HA-TEPA with KMnO₄, followed by conjugation with Ce6-NH₂. HA-TEPA/MnO₂ was first synthesized by mixing HA-TEPA and KMnO₄, and the resulting HA-TEPA/MnO₂ was then conjugated with Ce6-NH₂ to produce HA-TEPA/MnO₂/Ce6 nanoswitches. (B) Zeta potential values of HA, HA-TEPA, HA-TEPA/MnO₂, and HA-TEPA/MnO₂/Ce6 nanoswitches. (C) Size distribution of HA-TEPA/MnO₂/Ce6 nanoswitches determined by DLS. Inset: TEM image of HA-TEPA/MnO₂/Ce6 nanoswitches. Scale bar: 100 nm. (D) UV/Vis spectra of free Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches. (E) Wide-scan XPS spectra of MnO₂ and HA-TEPA/MnO₂/Ce6 nanoswitches. (F) In vitro degradation of HA-TEPA/MnO₂/Ce6 nanoswitches at different H₂O₂ concentrations (0–200 μM). (G) Oxygen generation by HA-TEPA/MnO₂/Ce6 nanoswitches in N₂-purged PBS (hypoxia) at different H₂O₂

concentrations (0, 50, 100, and 200 μM). (H) Singlet oxygen generation by HA-TEPA/ MnO_2 /Ce6 nanoswitches as a function of irradiation time and H_2O_2 concentration under hypoxic conditions.

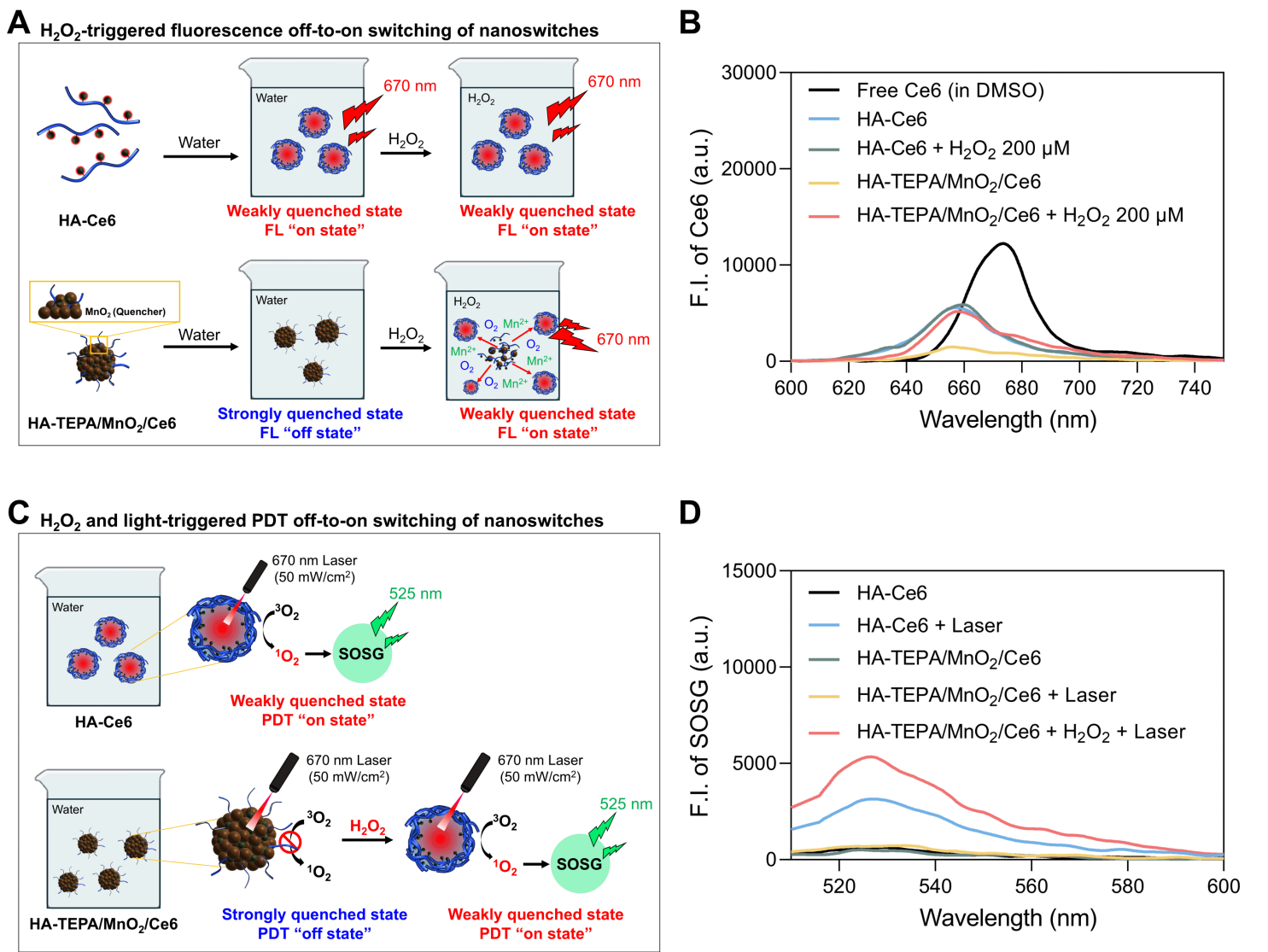


Figure 2

H_2O_2 -triggered fluorescence (FL) and photodynamic therapy (PDT) off-to-on switching behavior of HA-TEPA/ MnO_2 /Ce6 nanoswitches. (A) Schematic of the fluorescence quenching and recovery behaviors of HA-Ce6 and HA-TEPA/ MnO_2 /Ce6 nanoswitches under aqueous conditions in the absence and presence of H_2O_2 . Free Ce6 exhibits strong fluorescence emission ($\lambda_{\text{Em}} = 670 \text{ nm}$) in DMSO, whereas HA-Ce6 shows weakly quenched fluorescence in aqueous solution irrespective of H_2O_2 treatment. Conversely, HA-TEPA/ MnO_2 /Ce6 nanoswitches remain in a strongly quenched fluorescence state owing to MnO_2 -mediated quenching, followed by fluorescence recovery upon H_2O_2 -triggered MnO_2 degradation. (B) Fluorescence emission spectra of free Ce6 in DMSO and of HA-Ce6 and HA-TEPA/ MnO_2 /Ce6 nanoswitches in aqueous solution, recorded in the absence and presence of H_2O_2 . (C) Schematic of singlet oxygen (SO ; $^1\text{O}_2$) generation and PDT activation for HA-Ce6 and HA-TEPA/ MnO_2 /Ce6

nanoswitches under different conditions. Under 670 nm laser irradiation (2 min), weakly quenched HA-Ce6 produces detectable SO, leading to an increased SOSG fluorescence signal. In contrast, HA-TEPA/MnO₂/Ce6 nanoswitches in a strongly quenched state exhibit negligible SO generation both in the absence and presence of laser irradiation. Following exposure to H₂O₂, SOSG fluorescence is restored for HA-TEPA/MnO₂/Ce6 nanoswitches, consistent with H₂O₂-triggered MnO₂ degradation. (D) SOSG fluorescence intensity profiles of the HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches under 670 nm laser irradiation (2 min), measured in the absence and presence of H₂O₂.

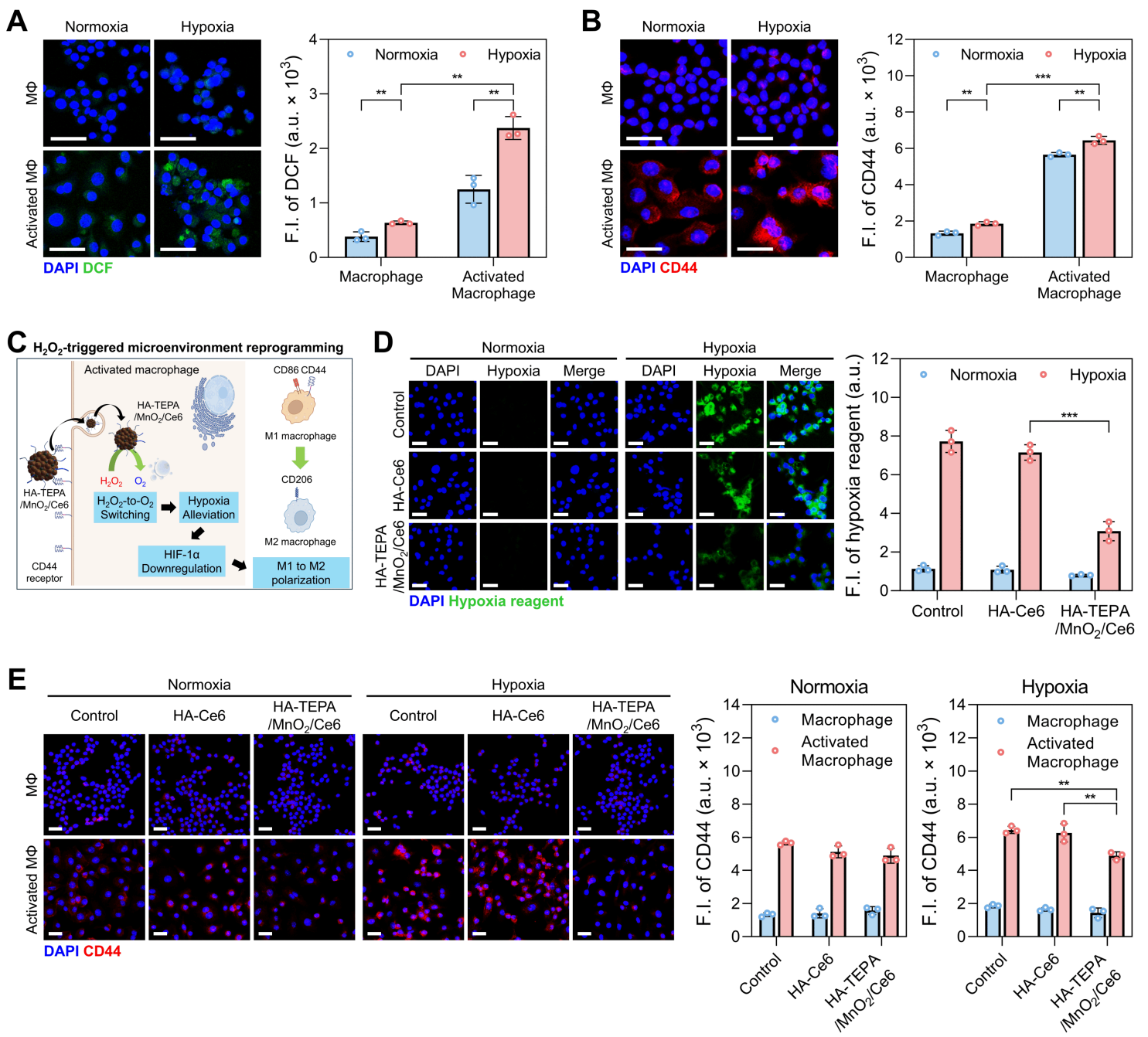


Figure 3

(A) Intracellular H_2O_2 levels in macrophages and activated macrophages under normoxic or hypoxic conditions. Representative confocal laser scanning microscope (CLSM) images and quantitative analysis of DCFDA-stained cells. Scale bar: 30 μm . $**P < 0.01$. (B) In vitro CD44 expression evaluation. Representative immunofluorescence CLSM images and quantitative analysis of CD44 expression in macrophages and activated macrophages under normoxic and hypoxic conditions. Scale bar: 30 μm . $**P < 0.01$, $***P < 0.001$. (C) Schematic of the proposed mechanism underlying H_2O_2 -triggered microenvironment reprogramming mediated by HA-TEPA/ MnO_2 /Ce6 nanoswitches internalized into CD44-positive activated macrophages via CD44-mediated endocytosis. Upon exposure to elevated intracellular H_2O_2 , the nanoswitches undergo MnO_2 degradation, resulting in in situ oxygen generation and hypoxia alleviation. The relieved hypoxic microenvironment subsequently downregulates HIF-1 α expression, which promotes anti-inflammatory responses and drives macrophage polarization from the pro-inflammatory M1 phenotype toward the anti-inflammatory M2 phenotype. This figure, created in BioRender.com, is licensed under CC BY 4.0. (D) Comparison of hypoxia alleviation by HA-Ce6 and HA-TEPA/ MnO_2 /Ce6 nanoswitches in normoxic- and hypoxic-activated macrophages. Representative CLSM images and quantitative analysis of normoxic- and hypoxic-activated macrophages treated with HA-Ce6 (equivalent to 5 μM Ce6) or HA-TEPA/ MnO_2 /Ce6 nanoswitches (equivalent to 5 μM Ce6). Scale bar: 30 μm . $***P < 0.001$. (E) Comparison of CD44 modulation by HA-Ce6 and HA-TEPA/ MnO_2 /Ce6 nanoswitches in macrophages and activated macrophages under normoxic or hypoxic conditions. Representative CLSM images and quantitative analysis of CD44 expression after treatment with HA-Ce6 (equivalent to 5 μM Ce6) or HA-TEPA/ MnO_2 /Ce6 nanoswitches (equivalent to 5 μM Ce6). Scale bar: 30 μm . $**P < 0.01$.

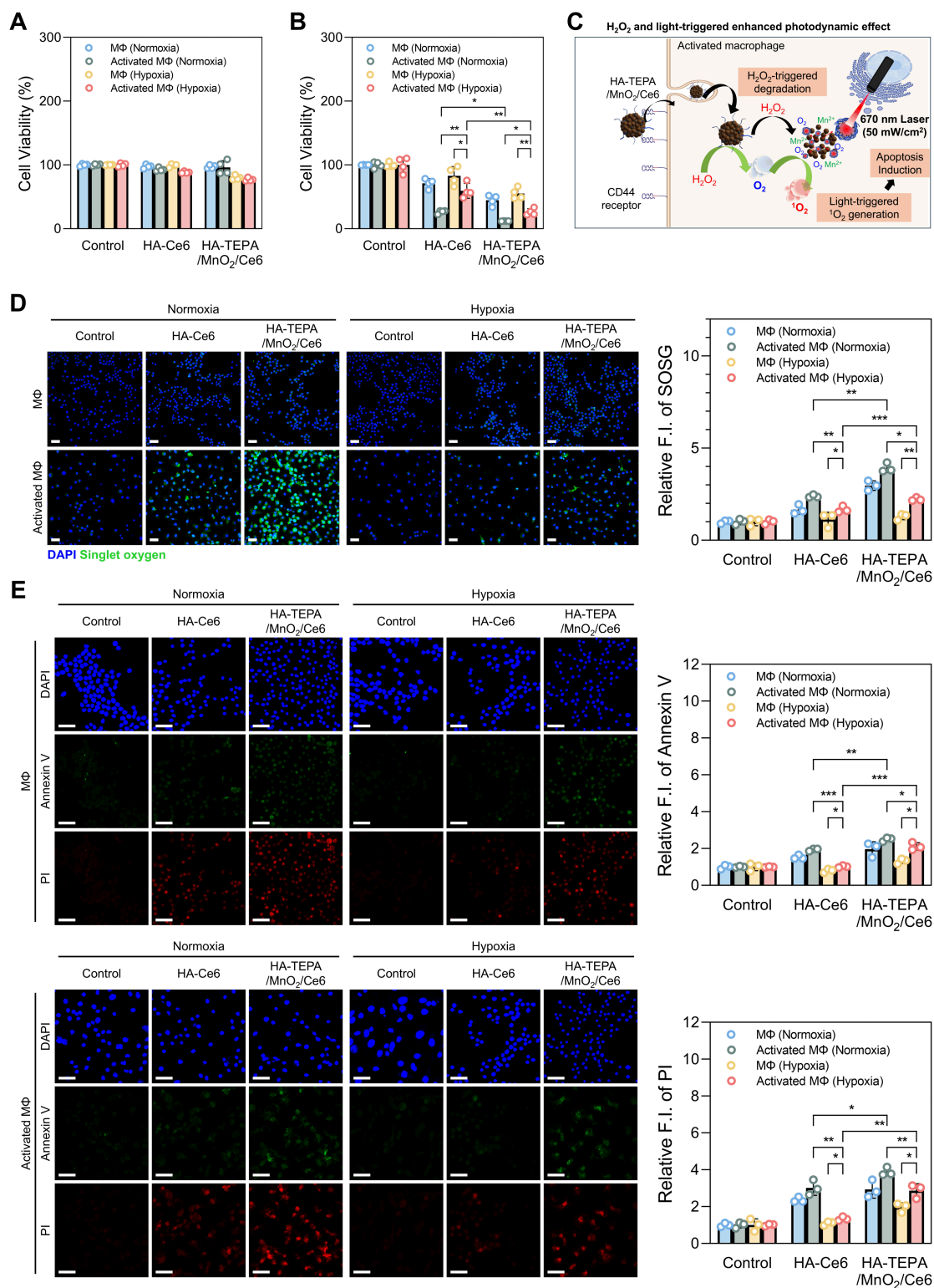


Figure 4

(A) In vitro dark toxicity of cells treated with HA-Ce6 (equivalent to 5 μ M Ce6) or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μ M Ce6) without laser irradiation. (B) In vitro phototoxicity of cells treated with HA-Ce6 (equivalent to 5 μ M Ce6) or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μ M Ce6) under 670 nm laser irradiation. * P < 0.05, ** P < 0.01. (C) Schematic of the H₂O₂- and light-triggered

phototherapeutic mechanism of HA-TEPA/MnO₂/Ce6 nanoswitches. Following CD44-mediated endocytosis, the nanoswitches undergo H₂O₂-triggered MnO₂ degradation, leading to in situ oxygenation and Ce6 release. Upon 670 nm laser irradiation, reactivated Ce6 converts the generated O₂ into cytotoxic singlet oxygen (SO; ¹O₂), thereby inducing apoptosis even under hypoxic conditions. This figure, created in BioRender.com, is licensed under CC BY 4.0. (D) In vitro intracellular SO detection. Representative CLSM images and quantitative analysis of SOSG fluorescence in macrophages and activated macrophages treated with HA-Ce6 (equivalent to 5 μM Ce6) or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μM Ce6) under normoxic or hypoxic conditions following laser irradiation (670 nm). Scale bar: 30 μm. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (E) In vitro apoptosis induction by HA-TEPA/MnO₂/Ce6 nanoswitches in macrophages and activated macrophages under normoxic or hypoxic conditions. Representative CLSM images and quantitative analysis of cells stained with DAPI (blue), Annexin V (green), and propidium iodide (PI, red) after treatment with HA-Ce6 (equivalent to 5 μM Ce6) or HA-TEPA/MnO₂/Ce6 nanoswitches (equivalent to 5 μM Ce6). Scale bar: 30 μm. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

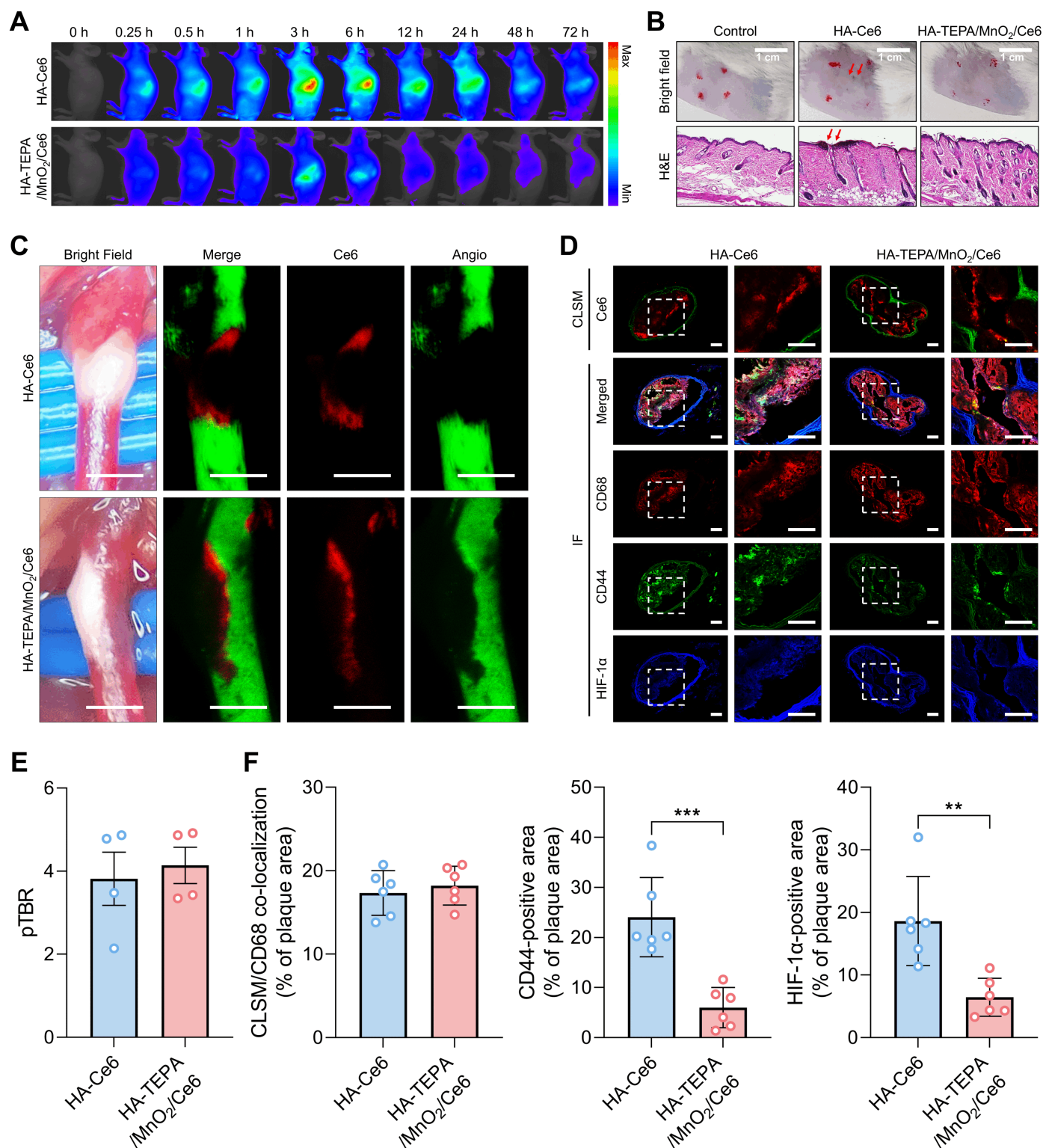
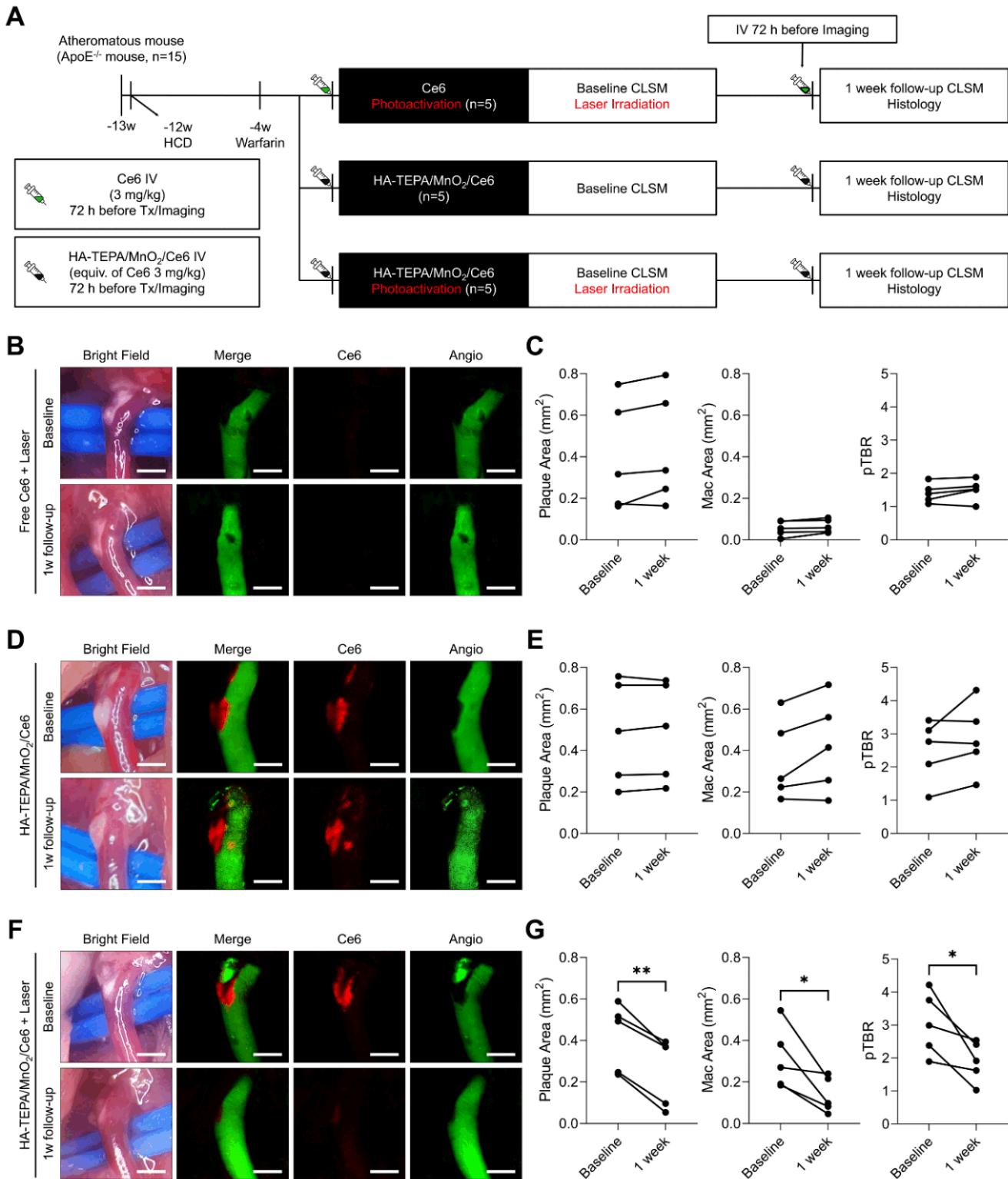


Figure 5

(A) In vivo biodistribution profile of HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches. Representative whole-body fluorescence images of BALB/c nude mice acquired at different time points (0, 0.25, 0.5, 1, 3, 6, 12, 24, 48, and 72 h) following intravenous injection. (B) Cutaneous phototoxicity evaluation of HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches. Mice treated with HA-Ce6 exhibited apparent erythema and epidermal damage, as indicated by the red arrows in the gross images and corresponding H&E-stained

skin sections. In contrast, mice treated with HA-TEPA/MnO₂/Ce6 nanoswitches showed no evident skin injury or histopathological abnormalities. Scale bar: 1 cm. (C) In vivo intravital imaging of carotid atherosclerotic plaques. Representative images show Ce6 fluorescence (red), indicating nanoswitch accumulation within carotid plaques, and FITC angiography (green), outlining the vasculature. Scale bar: 500 μm. (D) Confocal laser scanning microscopy (CLSM) and immunofluorescence analysis of sectioned carotid atheroma. CLSM images show the deposition of the HA-Ce6 or HA-TEPA/MnO₂/Ce6 nanoswitches (red) within plaques, with elastin shown in green. Immunofluorescence staining further reveals CD68 (red), CD44 (green), and HIF-1α (blue) expression in plaque regions. Scale bar: 200 μm. (E) Quantitative pTBR analysis shows no significant difference between HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches, indicating comparable plaque-targeting efficiency. (F) Quantitative immunofluorescence analysis of sectioned carotid atheroma. Consistent with the in vivo imaging of carotid plaques, the HA-Ce6 and HA-TEPA/MnO₂/Ce6 nanoswitches exhibited comparable plaque-targeting capability. However, only the HA-TEPA/MnO₂/Ce6 treatment group showed downregulation of CD44 and HIF-1α via hypoxia alleviation. ***P* < 0.01, ****P* < 0.001.



(CLSM) imaging was performed after 72 h, followed by laser irradiation (670 nm) and serial imaging to assess therapeutic response. (B-C) Representative serial CLSM images and quantification of plaque area, macrophage area, and pTBR at baseline and 1 week after laser irradiation in the free Ce6 photoactivation group (n = 5). No significant changes were observed after irradiation, indicating the limited therapeutic efficacy of free Ce6. Scale bar: 500 μ m. (D-E) Representative serial CLSM images and quantification of plaque area, macrophage area, and pTBR at baseline and 1 week in the HA-TEPA/MnO₂/Ce6 nanoswitch group without laser irradiation (n = 5). No significant differences were detected, confirming that the nanoswitches remain in a photodynamically “off” state in the absence of light activation. Scale bar: 500 μ m. (F-G) Representative serial CLSM images and quantification of plaque area, macrophage area, and pTBR at baseline and 1 week after laser irradiation in the HA-TEPA/MnO₂/Ce6 nanoswitch photoactivation group (n = 5). After irradiation, all parameters were significantly reduced, demonstrating effective plaque regression and macrophage depletion mediated by H₂O₂- and light-triggered activation of the nanoswitches. Scale bar: 500 μ m. **P* < 0.05, ***P* < 0.01.

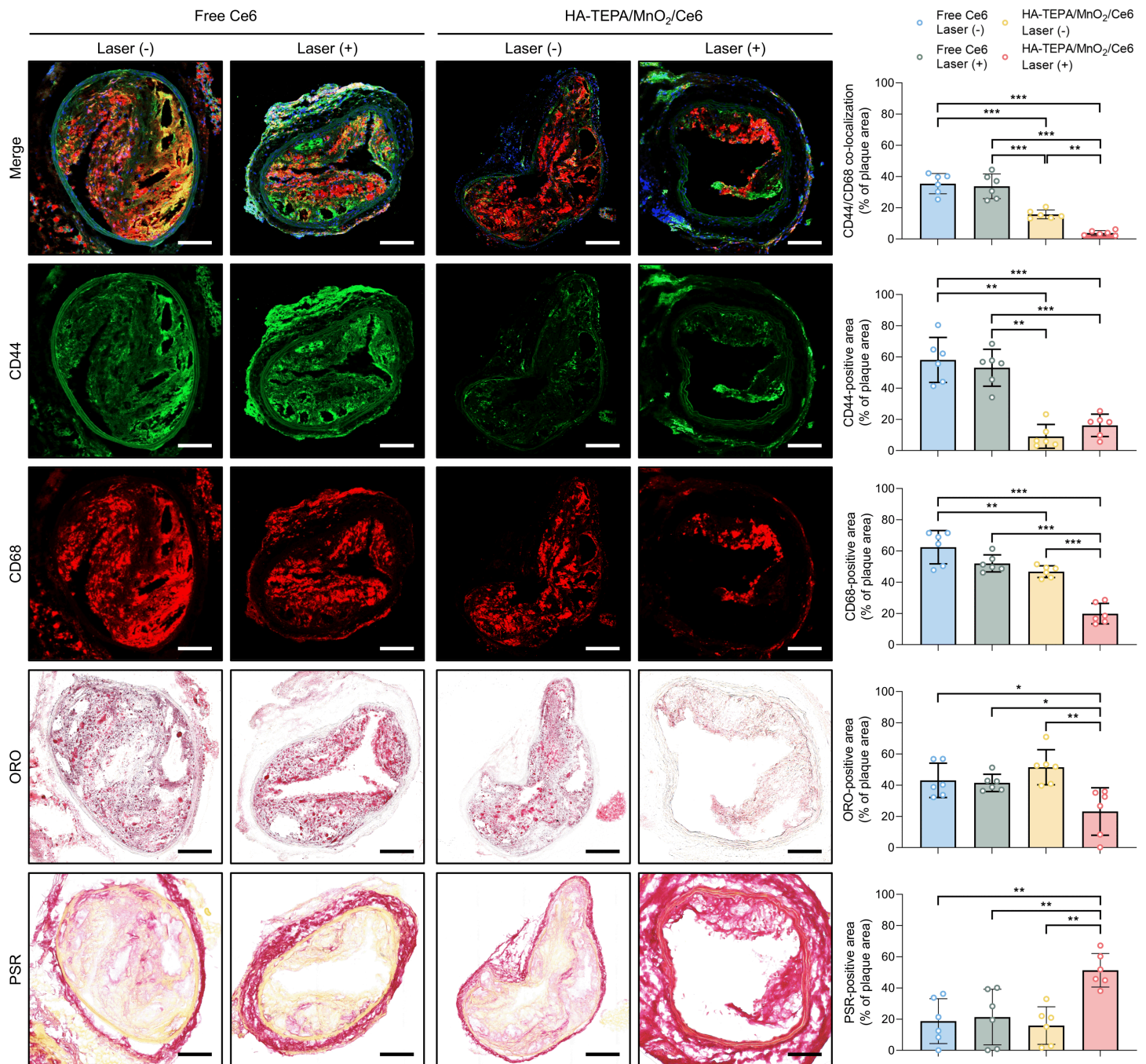


Figure 7

In vivo effects of photoactivated HA-TEPA/MnO₂/Ce6 nanoswitches on plaque composition and stabilization. Representative immunofluorescence (IF) images and histological staining of carotid atheroma sections from the different treatment groups. IF images show CD44 (green) and CD68 (red) staining to identify M1 macrophages and total macrophages, respectively. Lipid accumulation and collagen content within plaques were assessed by Oil Red O (ORO) and picrosirius red (PSR) staining, respectively. Scale bar: 100 μm. Quantification of CD44/CD68 co-localized M1 macrophages, CD44-positive area, CD68-positive area, ORO-positive lipid area, and PSR-positive collagen area per plaque. Relative to the control and free Ce6 groups, photoactivation of the HA-TEPA/MnO₂/Ce6 nanoswitches

markedly reduced CD44-positive M1 macrophages, decreased lipid content, and increased collagen deposition within plaques. These compositional changes indicate effective reprogramming of the plaque microenvironment and enhanced plaque stabilization mediated by H_2O_2^- and light-triggered nanoswitch activation. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1SupplementarymaterialHATEPAMnO2Ce6FINAL.pdf](#)
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