Macrophage-targeted nanoparticles

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A Macrophage-Targeted Theranostic Nanoparticle for Biomedical Applications**

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Sequela of atherosclerotic vascular disease (for example, acute myocardial infarction or stroke) are leading causes of death worldwide. Given that atherogenesis is an insidious process occurring over decades, there exists a need to first diagnose and then treat subclinical but high-risk (so-called vulnerable) atherosclerotic plaques prior to clinical manifestations.[1–5] In particular, the macrophage has emerged as a key biological, imaging, and therapeutic target for atherosclerosis.[6] Thus, antimacrophage therapeutic strategies are becoming more prevalent. These therapies include systemic modulation of macrophage activity and localized photodynamic therapy (PDT), a clinical approach that utilizes injectable photosensitizers and subsequent photoangioplasty to debulk atherosclerotic lesions.[6]

Commonly used photosensitizers are usually of porphyrinic origin and, once excited by light of the appropriate wavelength, are capable of producing cytotoxic singlet oxygen through interactions between the excited porphyrinic molecule and molecular oxygen. One of the main advantages of PDT is its ability to spare healthy tissues surrounding the site of interest, due to the fact that treatment only takes place upon illumination, which can be localized. PDT has recently found clinical utility in the treatment of cardiovascular diseases, such as atherosclerosis and in-stent restenosis.[7,8] A number of photosensitizers have shown preferential uptake in atherosclerotic plaques, probably due to the interactions between the relatively hydrophobic porphyrin derivatives and the lipid-rich core of the atheroma.[9–13] The most studied of these, motexafin lutetium (Antrin) is currently in clinical trials for the treatment of coronary artery disease.[7,8,14] Unfortunately, systemic delivery of most photosensitizing agents also leads to significant side effects, such as prolonged photosensitivity due to localization of the drug within the skin.

Numerous methodologies have been developed to increase the efficacy of PDT, such as conjugation of photosensitizers to peptides and antibodies, for the active targeting of cell-surface receptors or other biomolecules.[15–20] PDT agents have also been encapsulated within polymeric nanoparticles, in order to yield higher local concentrations at therapeutic sites.[22–24] We hypothesized that the efficacy of PDT in killing local macrophages could be augmented by conjugation of photosensitizers to macrophage-avid nanoparticles. Using a mouse model of atherosclerosis (apoE−/−), we have previously shown that a dextran-coated magnetofluorescent nanoparticle (MFNP)[22–24] had enhanced uptake in atherosclerotic plaques in vivo, as well as specificity for macrophages.[24,30] Importantly, these nanoparticles do not localize in the epidermis in significant amounts, a fact suggesting that a MFNP-based PDT approach could lessen the risk of skin toxicity as compared to that with available PDT agents.

Based on the previous results and biodistribution studies of magnetic nanoparticles (MNPs) in clinical use,[52] we reasoned that an atherosclerotic-plaque/macrophage-targeted delivery vehicle could be based upon dextran coatings. Indeed, macrophages contain a previously cloned dextran receptor, SIGNR1.[53] Other design criteria included the need for the nanoparticles to be detectable by magnetic resonance imaging (MRI; magnetic core) as well as by catheter-based fluorescence imaging. Since dextrans and other carbohydrates dissociate from iron oxide crystal surfaces, we crosslinked the coating through epichlorohydrin and functionalized the coating with primary amines. The monocrystalline nanoparticles had an overall diameter (volume weighted) in aqueous solution of 33 nm (polydispersity = 0.21), a superparamagnetic core of 5 nm containing 8000 Fe atoms per particle and magnetic relaxivities, R1 and R2, of 21 and 62 mms⁻¹ (37 °C, 0.5 T), respectively. Alexa Fluor 750 (AF750; Invitrogen) was conjugated to the particle in a ratio of 3 fluorophores per particle, through reaction of the amine groups with the corresponding succinimidyl ester of AF750.

In order to impart upon the nanoparticle the ability to generate singlet oxygen, a potent photosensitizer, 5-(4-carboxyphenyl)-10,15,20-triphenyl-2,3-dihydroxychlorin (TPC), was covalently conjugated to the primary amines of the MFNP. TPC has a singlet-oxygen quantum yield of 0.65 when excited at 646 nm. It has also proven more efficacious than the commonly used chlorin e₅.[54] This sensitizer is convenient to synthesize and utilize as it is generated directly from the corresponding 5,10,15,20-tetraphenylporphyrin derivative in relatively high yields and purity. The presence of one carboxylic acid moiety, which is easily activated, also simplifies conjugation to peptides or other amine-containing molecules.

TPC (2) was synthesized from the corresponding meso-tetraphenylporphyrin analogue as shown in Scheme 1. OsO₄-mediated dihydroxylation of porphyrin 1 yields the chlorin 2,[55] while activation of 2 with DSC in DMF gives 3. In order to conjugate the activated chlorin to the AF750–MFNP, the phosphate-buffered saline (PBS) in which the particles were originally synthesized and suspended was exchanged for dimethylsulfoxide (DMSO) by filtration through Sephadex G-25 with DMSO as the eluent. Conjugations of cell-surface receptors or other biomolecules, for the active targeting of cell-surface receptors or other biomolecules. Thus, antimacrophage therapeutic strategies are key biological, imaging, and therapeutic target for atherosclerosis. This work was supported by a National Institutes of Health (NIH) T32 Fellowship and by NIH grants CA79443, R01 HL080731, and P50 CA86355 (all to R.W.).

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tion was accomplished by stirring of 3 with the particle suspension for 16 h, followed by filtration through Sephadex G-25 with PBS as the eluent to yield the theranostic (diagnostic and therapeutic) nanoparticle (TNP).

TNP s were stable in aqueous solutions in physiological buffer at pH 7.4 for months without precipitation and were detectable by MRI and fluorescence imaging (Figure 1, 0.01 mg Fe per mL). The UV/Vis absorption spectrum of the TNP shows characteristic features of all three components (λ_max(iron oxide) < 300 nm, λ_max(TPC) = 648 nm, λ_max(AF750) = 755 nm; Figure 2A). This spectrum was also used to estimate the number of TPCs per particle (n = 30). It is notable that the roughly 100-nm difference between the longest wavelength absorption for TPC and that for AF750 minimizes energy transfer when the particles are excited at 650 nm (the therapeutic wavelength). This feature should avoid cell killing while imaging the spatial distribution of the agent within cells (750 nm excitation). As evident in the fluorescence emission spectrum (Figure 2B), there is minimal intermolecular energy transfer between the chlorin and AF750 (that is, there is minimal fluorescence emission from AF750 when the particle is excited at 630 nm), even though there is slight overlap between the fluorescence emission of the chlorin and the absorption of the AF750.

Cellular uptake and light-induced phototoxicity of the TNP were subsequently investigated in the RAW 264.7 murine macrophage cell line. Flow cytometry showed a time-dependent increase in TNP cell-associated fluorescence, as is expected for dextranated nanoparticles (Figure 3A). Uptake and localization of the particles within the cells was also observed by fluorescence microscopy, as shown in Figure 3B. At saturation, we calculated an average of 10^6–10^7 nanoparticles per macrophage. Importantly, the TNP s were not any more toxic (dark toxicity) to macrophages than the control nanoparticles lacking TPC (which are similar to clinically used preparations). However, when the same experiments were performed with the cells being irradiated with light at 650 nm (1 h incubation, 42 mW cm⁻², 7.5 J), a dose-dependent phototoxicity was observed. By a cell-proliferation (MTS) assay, only 35% of the cells re-

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**Scheme 1.** Synthesis and activation of 5-(4-carboxyphenyl)-10,15,20-triphenyl-2,3-dihydroxychlorin. i) OsO₄, CH₂Cl₂:pyridine (2:1); ii) disuccinimidyl carbonate (DSC), N,N-dimethylformamide (DMF).
mained viable after treatment at a concentration of 0.1 mg Fe per mL for 1 h (Figure 3D). Longer incubation times and higher doses resulted in complete cell killing. Importantly, when the cells were irradiated at 750 nm, under conditions comparable to intravital fluorescence imaging of atherosclerosis (0.8 mW total power, scanning laser, 512×8J92M in DMF. The reaction was stirred for 16 h, at which time it was evaporated to dryness. The crude product was used in the conjugation reaction (below).

Nanoparticle (MFNP) synthesis: The magnetic nanoparticle (MNP) used in this study consisted of a monocrystalline iron oxide core (5 nm diameter, 8000 Fe per particle) with a shell of 10-kD dextran. MNPs were synthesized by crosslinking the dextran coating with epichlorohydrin and treating it with ammonia to provide primary amine groups. The number of amines per nanoparticle was determined by using the N-succinimidyl 3-[2-pyridyldithio]-propionate (SPDP) method described previously.[34] Alexa Fluor 750 (AF750; 380 m

Synthesis of the succinimidy ester of 2: Disuccinimidyl carbonate (43 mg, 4 equiv) and diisopropylethylamine (7.5 mL, 4 equiv) were added to a solution of 2 (30 mg, 4.3×10⁻³ mmol) in DMF. The reaction was stirred for 16 h, at which time it was evaporated to dryness. The crude product was used in the conjugation reaction (below).

Photosensitizer–MFNP conjugate (TNP) synthesis: In order to conjugate TPC (2) to the nanoparticle, the liquid of the particle suspension (16.26 mg Fe per mL, 0.125 mL) was first switched using a B.W. Tek Inc. 650-nm laser.

5-(4-Carboxyphenyl)-10,15,20-triphenyl-2,3-dihydroxychlorin (TPC, 2) was synthesized as previously reported.[34]
from PBS to DMSO by filtration through Sephadex G-25 with DMSO as the eluent. The concentration of the suspension and the free amines were calculated from the absorbance of AF750 (3.2 AF750 per particle, 60 free amines per particle). The activated chlorin (2.1 × 10^{-2} M in DMSO, 7.68 μL) was added to the resulting DMSO suspension (750 μL), along with water (55 μL). The reaction was allowed to proceed for 16 h, after which time it was filtered through Sephadex G-25, with PBS as the eluent, to yield the final nanoparticle solution (0.2 mg Fe per mL, 30 TPCs per particle). The number of TPCs per particle was calculated from the extinction coefficients of the respective dyes (ε_{250} = 1.5 × 10^{4} M^{-1} cm^{-1}, ε_{AF750} = 2.4 × 10^{4} M^{-1} cm^{-1}, 3 molecules of AF750 per particle) by using the relative absorption of each molecule in solution.

**Cellular uptake:** In a typical experiment, 4 × 10^6 cells were seeded in 96-well cell-culture plates and incubated in a cell-culture incubator (37 °C, 5% CO₂) overnight before experiments. Cells were then treated with the appropriate nanoparticle suspension at a concentration of 0.1 mg Fe per mL and incubated for the appropriate time. Fluorescence uptake was quantified by flow cytometry. Briefly, treated cells were washed twice with PBS and treated with trypsin (200 μg/mL for 30 min). The trypsin reaction was stopped by adding ice-cold Dulbecco’s modified Eagle’s medium (DMEM; 1 mL) with fetal bovine serum, and the cells were spun down, washed twice, and resuspended in cold PBS (1 mL). The mean fluorescence intensity of a population of 10,000 cells was determined on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). A helium–neon laser at 633 nm was used for excitation and fluorescence emission was measured at 670 nm.

**Phototoxicity in cultured cells:** For these studies, 4 × 10^6 cells per well for RAW 264.7 cells or 2 × 10^6 cells per well for U937 cells were plated in a 96-well plate. Plates were then placed in an incubator (37 °C, 5% CO₂) overnight, washed with fresh media, and treated with the appropriate nanoparticle preparation. Samples were incubated for 1 h, washed with fresh media, and exposed to a 650-nm laser light source for 3 min each (42 mW cm⁻²). Plates were returned to the incubator overnight and cell viability was estimated colorimetrically by using the MTS assay. Cells incubated with PBS and no nanoparticles or with PDT treatment were used as controls.

**In vitro fluorescence microscopy:** RAW 264.7 cells were plated in a 96-well plate (4 × 10^6 cells per well) and placed in an incubator overnight (37 °C, 5% CO₂). The medium was then removed and replaced with the particle suspension (0.1 mg Fe per mL, 50 μL). The cells were incubated with the particles for 3 h, after which time they were washed with fresh media. Fluorescence microscopy was undertaken on a Nikon Eclipse TE-2000 microscope. The cells were observed in the Rhodamine channel, as well as by phase contrast (Phase L, white light images only).

**Keywords:**
drug design • iron • macrophages • nanoparticles • photosensitizers


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