Dense hydroxyl polyethylene glycol dendrimer targets activated glia in multiple CNS disorders

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Poor transport of neuropharmaceutics through central nervous system (CNS) barriers limits the development of effective treatments for CNS disorders. We present the facile synthesis of a novel neuroinflammation-targeting polyethylene glycol–based dendrimer (PEGOL-60) using an efficient click chemistry approach. PEGOL-60 reduces synthetic burden by achieving high hydroxyl surface density at low generation, which plays a key role in brain penetration and glia targeting of dendrimers in CNS disorders. Systemically administered PEGOL-60 crosses impaired CNS barriers and specifically targets activated microglia/macrophages at the injured site in diverse animal models for cerebral palsy, glioblastoma, and age-related macular degeneration, demonstrating its potential to overcome impaired blood-brain, blood-tumor-brain, and blood-retinal barriers and target key cells in the CNS. PEGOL-60 also exhibits powerful intrinsic anti-oxidant and anti-inflammatory effects in inflamed microglia in vitro. Therefore, PEGOL-60 is an effective vehicle to specifically deliver therapies to sites of CNS injury for enhanced therapeutic outcomes in a range of neuroinflammatory diseases.

INTRODUCTION

Diseases of the central nervous system (CNS) have some of the fastest-growing disparities between current clinical care and patient needs and are among leading causes of death in the elderly. The aging population in most countries results in a surge in the number of patients suffering from neurological diseases, leading to increased socioeconomic and health care burdens worldwide (1). Therefore, one should expect aggressive efforts from the pharmaceutical industry toward discovery, development, and translation of novel neurotherapeutics. On the contrary, many have either suspended or reduced their investments in CNS projects due to the high risk of failure in clinical trials (2). The primary clinical challenge in developing therapies for CNS diseases, such as autoimmune diseases, brain tumors, and ocular disorders, is achieving clinically relevant exposure of therapeutics to the site of injury, which is difficult to access due to CNS transport barriers. The blood-brain barrier (BBB), the primary impediment to neuro-drug development, is a dynamic and highly selective physical barrier that maintains brain tissue homeostasis by regulating the intake of chemicals and restricting the entry of the toxins and blood-borne pathogens (3). Drug delivery to other parts of the CNS faces similar challenges, such as the blood-retinal barrier (BRB) (4) for treating eye diseases, and pathology-dependent barriers, such as the traditional blood-brain-tumor barrier (5). The majority of treatments for neurological disorders require administration at high doses, resulting in systemic side effects and toxicities. Conventional methods to circumvent CNS barriers are highly invasive, thereby causing further collateral damage and limiting the number of doses possible in a repeat treatment scheme due to the high risk of complications. Therapies administered locally also often exhibit poor diffusion through the brain parenchyma, resulting in limited brain distribution and necessitating high dosages leading to toxicity. Recent strategies focusing on temporary disruption of the BBB using chemical or mechanical methods are often spatially nonspecific, allow undesired potentially detrimental molecules, and can induce deleterious immune responses (6). The clinically notable gaps in existing therapies for neurological disorders pose an urgent need to develop innovative, less-invasive, specialized drug delivery vehicles that can enhance the delivery of therapeutics across CNS barriers and target the key diseased cells at the site of injury.

Neuroinflammation mediated by activated microglia/macrophages (Mi/Ma) is a major hallmark of many neurological disorders (7). Proinflammatory Mi/Ma activation disrupts the BBB/BRB and can cause secondary damage through release of apoptotic signals to neurons and glia (8, 9). Anti-inflammatory activation promotes vascularization and cell growth while suppressing the immune response (10). Therefore, targeting both pro- and anti-inflammatory Mi/Ma phenotypes with immune-modulating agents is a potent therapeutic strategy to the disease pathology. Nanocarriers that can efficiently penetrate the CNS barriers upon systemic administration, diffuse freely through brain tissue, and localize to key pathological cells at the site of CNS injury are rare (11). A key goal in the development of nanomedicine-based therapeutics for CNS disorders is designing nanoconstructs that can be translated to the clinic in addition to having favorable brain transport properties. In addition to the ability to target key cells in the CNS, additional supplementary criteria include safety profile, water solubility, synthetic reproducibility, and feasibility for large-scale production toward successful commercialization. Dendrimers have shown potential in diverse biomedical applications including but not limited to targeted drug/gene delivery, diagnosis, and imaging (11–15). Dendrimers are three-dimensional, hyperbranched, monodisperse, globular nanoparticles whose surface groups, size, and molecular composition can be manipulated precisely
during their preparation for desired biomedical applications. Because of their unique layered branching structure, their nanoscale interactions with biological matrices can be finely tuned via careful selection of appropriate building blocks for the backbone and surface groups.

Our group has demonstrated that the systemically administered, hydroxyl-terminating, generation 4 poly(amideamine) dendrimers (PAMAM-G4-OH) with 64 terminal hydroxyl groups cross impaired CNS barriers and accumulate specifically in activated Mi/Ma at the site of brain injury while exhibiting minimal accrual in healthy brain tissue across multiple small- and large-animal models of neurodegenerative diseases (11, 16, 17). Similar neuroinflammation targeting was not observed with cationic and anionic dendrimers of equivalent size and similar backbone (18). This intrinsic targeting capability is theorized to arise from the high density of surface hydroxyl groups possible with dendrimers due to their unique layered branching structure (~1 OH terminal group/nm² at PAMAM-G4-OH) that is difficult to achieve with other polymeric nanoparticles. Motivated by these findings, we have designed and developed a high surface density hydroxyl-functionalized polyethylene glycol (PEG–based dendrimer nanocarrier for systemic targeting of activated Mi/Ma in CNS disorders. We designed this construct to exhibit greater hydroxyl surface density at lower generations than PAMAM dendrimers to have similar neuroinflammation targeting capabilities with lower synthetic burden (~5 OH terminal group/nm² at generation 2 compared to 1 for PAMAM–OH at generation 4). Keeping the requirements for clinical translation in mind, we developed this dendrimer consisting of water-soluble, inexpensive, and biocompatible building blocks in minimal reaction steps using an accelerated synthetic approach via highly efficient chemical transformations based on click chemistry. This dendrimer construct, referred to as PEGOL-60, is made up of low–molecular weight PEG-based building blocks and has 60 hydroxyl (neutral) surface groups at generation 2, which is produced in five reaction steps, compared to PAMAM-G4-OH, which has 64 hydroxyl surface groups at generation 4 achieved in more than eight synthesis steps. Recently, Ficker et al. (19) published an improved synthesis of PAMAM dendrimers, where they have used a huge excess (25 to 30 eq per site) of monomers and left the reactions on stirring for several days for its completion. The synthesis of PEGOL-60 is extremely facile and does not require the huge excess of reagents and monomers as in the case of PAMAM dendrimers. Moreover, there is no complication of defects in the synthesis of PEGOL-60, and the reactions are much faster due to the use of a highly efficient click chemistry approach. In addition, PEGOL-60 is designed to exhibit inherent neuroinflammation targeting, thereby streamlining the translation process by eliminating the requirement for postsynthetic modifications with targeting ligands. The short PEG chains are introduced in the dendrimer backbone because of several reasons: (i) to enhance the biocompatibility, (ii) to provide increased aqueous solubility, (iii) to improve the stability, and (iv) to decrease immunogenicity. Moreover, the PEGOL-60 dendrimer backbone is designed to consist predominantly of stable ether linkages to prevent enzymatic degradation or disintegration in the biological system, allowing it to be excreted intact through the kidneys. The ability for PEGOL-60 to target the relevant cells at the site of neuroinflammation was validated in vivo via fluorescence spectrometry–based quantification and confocal microscopy in three different models of CNS diseases to assess the ability of PEGOL-60 to cross both the BBB and the BRB, to penetrate solid tumor, and to target disease-associated Mi/Ma. To do this, we used a rabbit model of maternal uterine inflammation–induced cerebral palsy (CP), a murine orthotopic model of glioblastoma (GBM), and a rat model of subretinal lipid–induced age-related macular degeneration (AMD). This is perhaps the broadest demonstration of injured Mi/Ma targeting from systemic administration of a ligand-free delivery vehicle. We also explored the inherent therapeutic properties of PEGOL-60 in vitro based on previous findings that certain dendrimers exhibit anti–oxidant and anti–inflammatory effects without the addition of therapeutic payloads (20).

RESULTS

Synthesis, physicochemical characterization, and fluorescent labeling of PEGOL-60 dendrimer with inherent neuroinflammation-targeting potential from systemic administration

To overcome traditional challenges in scaling up dendrimer synthesis, novel synthetic strategies have been designed to achieve complex dendritic structures with high purity and efficiency. For the construction of PEGOL-60, we used a combination of hypercore-hypermonomer and orthogonal approaches and developed an accelerated scheme to achieve high surface hydroxyl densities (21, 22). Using this accelerated approach, we synthesized PEGOL-60 in two reaction steps starting from the core via highly efficient and orthogonal chemical transformations based on Cu(I)-catalyzed alkyn azide (CuAAC) (23) and thiol–ene click (24) chemistry. The key to produce defect-free dendrimers lies in the chemical transformations used to couple building blocks in a layer-by-layer fashion. Conventional chemical reactions that appear to be efficient at lower generations become sluggish at higher generations with a huge number of reactive terminals due to steric crowding, which leads to structural defects and asymmetry. The click chemistry concept has become a valuable synthetic tool that comprises a pool of reactions that are easy to execute, highly robust, high yielding, atom economical, and modular in nature. Among the list of click transformations, CuAAC and thiol–ene click are the two most powerful and widely applied because they are highly facile, orthogonal, and stereoselective. These click transformations have been successfully used in polymer chemistry, bioconjugation reactions, dendrimer synthesis, and the generation of huge libraries of novel chemical entities (25).

The synthesis of PEGOL-60 began with the construction of hypercore (2) by performing propargylation on dipentaerythritol (1) in the presence of NaH and propargyl bromide to achieve the hexa-propargylated product (2) in 60% yield (Fig. 1A). The 1H NMR spectrum showed the presence of six propargyl protons at 2.4 ppm (spectrum for compound 2; Fig. 1B). We then constructed the hypermonomer (5) in two synthetic steps. In the first step, the allylation reaction was carried out on compound 1 to obtain AB₃ monomer (3) with five allyl groups and one unreacted hydroxyl group. The pure product (3) was isolated by performing column chromatography. Compound (3) was then reacted with mono tosylated triethylene glycol azide (4) to achieve AB₃ orthogonal hypermonomer (5) with one azide functional group and five allyl arms. The purpose of the azide group is to participate in the CuAAC click reaction on the core (2), while the alkene groups can be exploited for photo–catalyzed thiol–ene click reaction with thiolated monomers. Compounds (2) and (5) were then subjected to a CuAAC click reaction to yield generation 1 dendrimer (D1–allyl-30, 6) with 30 terminal alkene functions. It is worth mentioning here that the azide monomer (5) was used only 1.2 eq per site as compared to a huge excess (25 to 20 eq) of reagents.
Fig. 1. Synthesis and characterization of PEGOL-60 and PEGOL-60-Cy5. (A) Key steps in synthetic pathway of PEGOL-60 (8). Reagents and conditions: (i) NaH, propargyl bromide, 0°C to room temperature (RT), 6 hours; (ii) NaH, allyl bromide, 0°C to RT, 2 hours; (iii) NaH, 2-(2-(2-azidoethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (4), 0°C to RT, 3 to 4 hours; (iv) CuSO₄·5H₂O, sodium ascorbate, 11,11,15,15-tetraakis(allyloxy)methyl)-1-azido-3,6,9,13,17-pentaoxaicos-19-ene (5), N,N′-dimethylformamide (DMF), tetrahydrofuran (THF), H₂O, microwave, 50°C, 6 hours, 76%; (v) 2,2-dimethoxy-2-phenylacetophenone, 1-thioglycerol (7), DMF, 12 hours. (B) ¹H NMR comparison of hexa-propargylated core (2), D1-allyl-30 (6), PEGOL-60 (8), and fluorescently labeled dendrimer PEGOL-60-Cy5 (11) (from bottom to top) showing the appearance/disappearance of characteristic proton signals. TEG, triethylene glycol. (C) Comparative HPLC chromatogram of D1-allyl-30 (6), PEGOL-60 (8), D-GABA-NHBOC (9), and PEGOL-60-Cy5 (11) showing evident shifts in retention times at each stage (absorption at 210 nm for 6, 8, and 9 and at 650 nm for 11). (D) MALDI-TOF spectrum of PEGOL-60 (8) showing a peak at mass of 7.4 kDa. (E) Synthesis of fluorescently labeled PEGOL-60-Cy5 (11): (i) N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl), 4-(dimethylamino)pyridine (DMAP), DMF, γ-(boc-amino)butyric acid, 12 hours; (ii) dichloromethane (DCM)/trifluoroacetic acid (3:1), 8 hours; (iii) N,N′-diisopropylethylamine (DIPEA), Cy5 N-hydroxysuccinimide (NHS) ester, pH 7 to 7.4, DMF, 12 hours. (F) Stability analysis of fluorescently labeled PEGOL-60-Cy5 (11) by HPLC: HPLC chromatogram of PEGOL-60-Cy5 in water (left), extracted from human plasma in vitro after incubation for 24 hours at 37°C (middle), and urine obtained from rabbit bladder in vivo 24 hours after injection of PEGOL-60-Cy5 (right).
in the synthesis of PAMAM dendrimers (19). The $^1$H NMR revealed the appearance of allyl protons at δ 5.8, 5.2, and 3.9 ppm while showing the evident disappearance of propargyl protons at δ 2.4 ppm (spectrum for 6; Fig. 1B). In addition, a distinct sharp peak for triazole protons appeared in $^1$H NMR at δ 7.69 ppm. The theoretical molecular weight of compound (6) is 4153.24 Da, and matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) analysis revealed a peak at 4156.49 Da, confirming the formation of the product. Compound (6) was reacted with 1-thio glycerol (7) via thiol–ene click reaction to yield the generation 2 dendrimer with 60 hydroxyl groups at the surface (PEGOL-60, 8). $^1$H NMR showed the complete disappearance of allyl end groups and the appearance of protons from thio-glycerol groups (δ 2.8 to 2.5 ppm), along with the characteristic appearance of allyl end groups and the appearance of protons from \( \gamma \)-O-ester (Fig. 2A). D1-allyl 30 (6) has a retention time of 12.8 min, and the final dendrimer PEGOL-60 (8), which is more polar, has a retention time of 7.1 min at 210 nm. MALDI-TOF spectrum shows a peak at 7343 Da (Fig. 1D), in close agreement with the theoretical molecular weight of PEGOL-60 of 7398 Da. PEGOL-60 has a size of 1.9 ± 0.2 nm and a near-neutral \( \zeta \) potential (−1.9 ± 0.7 mV) (Fig. S1). All other intermediates and final compounds were characterized using $^1$H NMR, MALDI-TOF, high resolution mass spectroscopy (HRMS), and HPLC (figs. S2 to S6).

We further attached a near-infrared fluorescent tag, cyanine 5 (Cy5), to PEGOL-60 (Fig. 1E) to study the in vivo biodistribution using fluorescence spectroscopy and confocal microscopy. The fluorophore was attached only on 2 of the 60 arms to maintain the intrinsic pharmacokinetic properties of the dendrimer and to avoid any effects on biodistribution. PEGOL-60 (8) was coupled with \( \gamma \)-(boc-amino)butyric acid using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and 4-(dimethylamino)pyridine (DMAP) to obtain dendrimer (9, D-GABA-NHBOC), after which the BOC group was depleted under mild acidic conditions [trifluoroacetic acid (TFA)/dichloromethane (DCM), 1/5] to get dendrimer (10). Dendrimer (10), having two amine groups as TFA salts, was lastly reacted with Cy5 mono N-hydroxysuccinimide (NHS) ester at pH 7.0 to 7.5 to achieve Cy5-labeled dendrimer (PEGOL-60-Cy5, 11). $^1$H NMR revealed the presence of Cy5 protons in the aromatic region upon conjugation (spectrum for 11; Fig. 1B), and HPLC chromatogram showed a clear shift in retention time from 8.4 min for D-GABA-NHBOC (9, 210 nm) to 7.6 min for PEGOL-60-Cy5 (11, 210 and 650 nm; Fig. 1C). We further evaluated the stability of PEGOL-60-Cy5 in human plasma (Fig. 1F). The fluorescently labeled dendrimer was incubated in human plasma at 37°C for 24 hours. The plasma proteins were precipitated, and dendrimer was extracted in methanol. The HPLC chromatogram of the methanolic extract containing dendrimer was analyzed and compared to that of pure PEGOL-60-Cy5 in water. The plasma extract showed the peak at the same retention time (7.6 min) as the retention time of PEGOL-60-Cy5 in water at 650 nm (Cy5 wavelength). No additional peaks corresponding to free Cy5 or hydrolyzed Cy5 products were found, confirming the stability of PEGOL-60-Cy5 in plasma at physiological temperature. Moreover, the concentration of PEGOL-60-Cy5 obtained for the T0 and T24 samples was the same, further confirming its stability in plasma (fig. S7). We further analyzed the urine samples obtained from the bladders of CP rabbit kits 24 hours after PEGOL-60-Cy5 injection. The urine HPLC chromatogram showed peak at the same retention time as PEGOL-60-Cy5 in water at 650 nm, further confirming the stability of Cy5-labeled dendrimer and suggesting that the dendrimer is clearing intact from the kidneys. We further evaluated the CNS barrier penetration and inflammation-targeting ability of PEGOL-60 from systemic administration in three different animal models.

**Qualitative and quantitative brain and organ distribution of systemically administered PEGOL-60 in a neonatal rabbit model of CP**

First, we investigated the in vivo BBB penetration and microglial targeting capabilities of PEGOL-60-Cy5 in a neonatal rabbit model of CP both qualitatively and quantitatively using confocal microscopy and fluorescent spectroscopy, respectively. CP is caused by an injury/insult to the developing brain, including maternal infection/inflammation, and results in offspring with motor, sensory, and cognitive impairment (26). Periventricular leukomalacia, characterized by diffuse microglial and astrocyte activation in the immature white matter, is one of the pathophysiological hallmarks of CP in humans (27). Apart from white matter injury, CP also involves neuronal injury in gray matter areas, including the cerebral cortex and hippocampus in CP patients (28). Here, we investigated the uptake of PEGOL-60-Cy5 in the corpus callosum (white matter), hippocampus, and cortex at 1, 4, and 24 hours after a single systemic dose in a maternal intrauterine inflammation–induced fetal neuroinflammation model of CP in rabbits. This model recapitulates the hallmark microglial and astrocytic proinflammatory activation seen in human patients, as well as signature behavioral markers such as hind limb rigidity and spasticity. CP kits (\( n = 3 \)) received an intravenous administration of PEGOL-60-Cy5 (55 mg/kg) on postnatal day 1 (PND1); euthanized at 1, 4, and 24 hours after injection; and were compared to healthy controls (\( n = 3 \)) euthanized at 24 hours after intravenous administration of equivalent dose. The colocalization of PEGOL-60-Cy5 with activated Mi/Ma, indicated by Iba1-positive cells with amoeboid soma with shortened processes, at the corpus callosum hippocampus and cortex in CP kits strongly suggests dendrimer accumulation in the activated microglia (Fig. 2, A to C) at these injured sites in the brain (29). PEGOL-60-Cy5 is mainly distributed in the perinuclear cytoplasm of these activated Mi/Ma. We show that PEGOL-60-Cy5 was able to extravasate from the leaky blood vessels and rapidly localize in activated Mi/Ma within 1 hour in the injured brain region (Fig. 2A). PEGOL-60-Cy5 signal strength increased in activated Mi/Ma by 4 hours and was present at similar levels 24 hours after injection (Fig. 2, B and C), demonstrating dendrimer accumulation at the site of injury with potential for local sustained release. In contrast, no colocalization with Mi/Ma cells in healthy controls was observed (Fig. 2D).

Next, we studied the quantitative brain and organ biodistribution of PEGOL-60-Cy5 at three different time points (1, 4, and 24 hours) in CP kits (\( n = 6 \)) and compared it to the age-matched healthy controls (\( n = 5 \)). Instead of measuring whole brain dendrimer levels as is conventionally done, we microdissected the perfused brains to separate the periventricular region (PVR), hippocampus, and cortex to measure the local uptake in these regions where activated microglia are present in this model (15). We have shown high engagement of activated Mi/Ma in the PVR, potentially due to the role of the ventricles.
as pathways for macrophage recruitment into the brain (30). The hippocampus and cortex are the regions implicated in the pathology of CP due to their roles in learning, memory, and motor function (29). This microdissection enables the evaluation of local dendrimer uptake in these clinically relevant subregions of the brain as opposed to overall brain quantities. To avoid the interference of blood and dendrimer stuck in the blood vessels, the kits were perfused with phosphate-buffered saline (PBS). A significant increase (~10-fold)
in the dendrimer uptake was detected in the brain of CP animals as compared to healthy controls (\(P < 0.01\), Student’s \(t\) test compared to healthy controls) (Fig. 2E). The selective uptake of PEGOL-60 in the injured brain regions of CP animals could be explained because of its ability (i) to cross the impaired BBB, (ii) to diffuse efficiently within the brain parenchyma due to its neutral charge, and (iii) to be picked up by phagocytic activated Mi/Ma. On the basis of our previous experience with PAMAM dendrimer–drug conjugates (12, 13), we envision that the drug loading capacity of PEGOL-60 should be \(\approx 20\) weight % (wt %) for the dendrimer targeting properties to be conserved.

A major concern for the clinical translation of nanomedicine-based therapeutics is their unwanted accumulation in organs other than the area of disease. We further assessed the biodistribution of PEGOL-60-Cy5 in all major organs (heart, lungs, liver, spleen, and kidneys) and plasma. Our results suggest a rapid clearance of the dendrimer from the body with an accumulation of less than 1% of the injected dose in any major organ at all time points (Fig. 2F). A similar trend was observed for all organs with a peak accumulation at 4 hours and then clearance at 24 hours. The presence of less than 4% dendrimer of injected dose in serum at 4 hours after injection shows that the dendrimer clears rapidly from circulation. Moreover, the clearance of dendrimer from control animals followed the same pattern with less than 0.2% dendrimer of injected dose in the serum at 24 hours for both CP and healthy animals (Fig. S8). There was no significant difference in PEGOL-60-Cy5 levels in plasma in both CP and healthy kits at 24 hours (not significant: \(P > 0.05\), Student’s \(t\) test). This is in agreement with our previous work on PAMAM dendrimer nanoparticles in this size range (sub–5 nm), which did not exhibit differences in clearance from plasma in healthy versus CP kits (31). This dendrimer shows similar levels of brain uptake as we have previously observed in this CP model with PAMAM-G4-OH, nanoparticles of similar size, shape, and number of surface hydroxyl groups, but exhibits much faster clearance rate from circulation and other vital organs (31). This rapid clearance rate from the body within 4 hours, along with the sustained cellular accumulation in the injured regions of the brains of neonatal CP rabbits, makes this dendrimer a promising candidate to design therapies for CNS disorders, where organ side effects from systemic therapies are critical.

Tumor penetration and tumor-associated macrophage targeting via systemic administration of PEGOL-60 in a mouse model of GBM

PEGOL-60 was further evaluated in a murine model of GBM to assess whether this specific colocalization with activated Mi/Ma in pro-inflammatory diseases extends to targeting anti-inflammatory M2 phenotype Mi/Ma as well and whether it could uniformly distribute throughout the solid tumor. These tumor-associated macrophages (TAMs) are host macrophages that have an induced anti-inflammatory phenotype to promote tumor growth and suppress the tumor killing immune response by secretory signals from cancer cells, making them ideal therapeutic targets for immune-modulating agents to repolarize into cancer-fighting cells (32). Despite the discovery of many powerful new anticancer therapies, clinical outcomes have not translated to GBM because these treatments fail to penetrate through the BBB and into the solid brain tumor in clinically relevant quantities, thereby necessitating high doses that lead to systemic toxicity. We explored the tumor-targeting ability of PEGOL-60-Cy5 in the syngeneic GL261 intracranial injection mouse model of GBM, which has been extensively characterized and is known to closely recapitulate the immune profile of humans (33). GL261 murine GBM cells were inoculated into the striatum of mice, and PEGOL-60-Cy5 was systemically administered 15 days after inoculation. Here, we demonstrate that PEGOL-60-Cy5 can specifically target TAMs within the solid tumor upon systemic administration while exhibiting minimal signal in the contralateral hemisphere (Fig. 3A). The dendrimer is able to fully penetrate and distribute throughout the solid tumor, overcoming both the BBB and traditional barriers to solid tumor delivery such as poorly developed vasculature and high interstitial fluid pressure (Fig. 3B) (34). The signal of PEGOL-60-Cy5 delineates the tumor region, demonstrating its specificity for TAMs over Mi/Ma in healthy parts of the brain, even in the peritumor area. On the basis of the similar accumulation of PEGOL-60 and PAMAM-G4-OH at the site of injury in CP, we expect PEGOL-60 to perform similarly in tumor targeting compared to PAMAM-G4-OH in GBM as well. We have previously shown that PAMAM-G4-OH accumulates in the brain tumor to tissue (~2.5 \(\mu\)g/g) after 24 hours, while free chemotherapies are rapidly cleared from the brain within 2 hours (35, 36). Therefore, we expect PEGOL-60 to exhibit similar tumor targeting while experiencing faster systemic clearance, which is highly beneficial for anticancer therapies. These findings suggest that PEGOL-60 may be a promising nanoplatform
with which to specifically and systemically deliver immunotherapies to the solid tumor in GBM without damaging healthy brain tissue.

**Intravenously administered PEGOL-60 dendrimer demonstrates pathology-dependent ocular biodistribution in a rat model of AMD**

In addition to brain penetration, we also looked at PEGOL-60-Cy5 colocalization with activated Mi/Ma in a subretinal lipid injection–induced model of AMD to demonstrate its ability to cross the BRB for applications in diseases of the back of the eye. AMD is a multifactorial ocular degenerative disease that involves multiple activated Mi/Ma-mediated pathologies including oxidative stress, inflammation, and neovascularization (37). Pathological buildup of toxic lipids leads to vision loss in patients, and currently, there are no viable therapies available for dry/early AMD. We tested the targeting capabilities of PEGOL-60-Cy5 in a rat model of AMD consisting of a subretinal injection of lipid to induce cell damage, resulting in a region of neovascularization referred to as a bleb. Confocal imaging of choroidal flat-mounts shows PEGOL-60-Cy5 signal localized with activated Mi/Ma specifically in the bleb area after systemic administration, with minimal signal in healthy tissues (Fig. 4A). In retinal tissue, PEGOL-60-Cy5 was found localized in Mi/Ma cells that are accumulated in the near vicinity of the radial blood vessels and the capillaries at the bleb borders (Fig. 4B). Cross sections corroborate these findings, showing that PEGOL-60 localized within activated Mi/Ma in the bleb area and not in healthy regions of the eye (Fig. 4C). These results suggest that PEGOL-60 may be a promising drug carrier for systemic targeted therapies in AMD where there are few viable therapeutic interventions available, as well as other posterior segment ocular diseases such as diabetic retinopathy, retinopathy of prematurity, retinitis pigmentosa, and uveitis (38).

**Intrinsic anti-oxidant and anti-inflammatory properties of PEGOL-60 in LPS-stimulated microglia**

On the basis of reports showing inherent anti-oxidant effects of some dendrimers, we next explored the inherent anti-oxidant and anti-inflammatory efficacy of PEGOL-60 (39). First, PEGOL-60 was not found to be cytotoxic up to at least 1000 μg/ml after 24 hours of exposure via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in BV2 murine microglia (n = 3, P < 0.05; fig. S9). Then, to assess the therapeutic efficacy of PEGOL-60, BV2 murine microglia were challenged with lipopolysaccharide (LPS) to induce a proinflammatory state, then cotreated with LPS and PEGOL-60, and assessed for markers of inflammation and oxidative stress. Treatment with PEGOL-60 without the addition of any anti-oxidant or anti-inflammatory agents resulted in significantly decreased expression of tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), IL-10, and inducible nitric oxide synthase (iNOS) (Fig. 5A), as well as up-regulation of anti-inflammatory markers CD206, Arg1, and IL-4 (Fig. 5B), in generally dose-dependent manners. CD206 also exhibits up-regulation with PEGOL-60 treatment, although lower doses were more efficacious than higher doses. Both pro- and anti-inflammatory markers were restored to near healthy levels with high PEGOL-60 treatment (500 μg/ml). At the protein level, this dose resulted in significant reduction in extracellular secreted TNFα (P < 0.001; Fig. 5C) and nitrite ions (P < 0.001; Fig. 5D). Because of this powerful anti-oxidant effect, we also tested the efficacy of PEGOL-60 following oxidative stress insult. Pretreatment with PEGOL-60 resulted in significant improvement to cell viability after H₂O₂ challenge (P < 0.001; Fig. 5E). This anti-oxidant effect is likely due to the free electrons in the dendrimer backbone acting as scavengers to neutralize reactive oxygen species (ROS). Access of these scavengers in the backbone to ROS may be sterically inhibited by the branching arms, with each successive generation layer further shielding the interior in case of high-generation dendrimers. While comparative studies are lacking, we theorize that PEGOL-60 may be a superior nanocarrier because it achieves the high density of hydroxyl groups at relatively lower generation, allowing for accessibility of the backbone for ROS scavenging.

**DISCUSSION**

Systemically administered nanoparticles that exhibit cell type–specific targeting in CNS diseases and high specificity for regions of CNS injury compared to healthy CNS tissue and peripheral organs, and meet synthetic criterion for simple scalable clinical translation are highly desirable. We have developed a PEG-based dendrimer nanoparticle, PEGOL-60, with a high density of surface hydroxyl groups at low generation via highly efficient click chemistry transformations with minimal reaction steps, mild reaction conditions, scalable synthesis route, and reduced synthetic burden. PEGOL-60 is noncytotoxic in target cells in vitro up to 1 mg/ml and is excreted intact through the kidney, showing rapid systemic clearance in vivo, still showing significant accumulation in the CNS target cells involved in neuro-inflammation after systemic administration in vivo in three different CNS disease models: (i) CP, (ii) GBM, and (iii) AMD. In CP, PEGOL-60 crosses the BBB and selectively localizes to proinflammatory, activated Mi/Ma in regions of brain injury while clearing rapidly from healthy brain tissue, peripheral organs, and circulation. In GBM, systemically administered PEGOL-60 overcomes traditional solid tumor barriers to uniformly distribute throughout the brain tumor and specifically target TAMs. In AMD, PEGOL-60 crosses the BRB to target activated Mi/Ma in the injured choroid and retina and is retained at least 7 days after administration. Last, we showed that PEGOL-60 exhibits inherent anti-oxidant and anti-inflammatory therapeutic effects in activated microglia in vitro. Together, these results indicate that this dendrimer construct is a promising and scalable platform with significant potential for clinical translation in targeted systemic drug delivery for inflammatory diseases of the CNS.

**MATERIALS AND METHODS**

**Synthesis and characterization of PEGOL-60 and PEGOL-60-Cy5**

**Materials and reagents for the synthesis and characterization of intermediates, dendrimers, and fluorescently labeled conjugates**

All the reagents were used as received unless otherwise stated. Propargyl bromide solution (80 wt % in toluene), allyl bromide, sodium hydride (60% dispersion in mineral oil), 2,2-dimethoxy-2-phenylacetophenone, 1-thioglycerol, EDC·HCl, DMAP, N,N'-diisopropylcarbodiimide (DIEA), p-toluenesulfonyl chloride, tetraethyleneglycol, TFA, γ-(Boc-amino)butyric acid (BOC-GABA-OH), copper sulfate pentahydrate, sodium ascorbate, anhydrous DCM, anhydrous tetrahydrofuran (THF), and anhydrous N,N'-dimethylformamide (DMF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cy5-mono-NHS ester and fluorescein isothiocyanate (FITC) were purchased from Amersham Biosciences–GE Healthcare. All other American Chemical Society (ACS) grade solvents were from Fisher Scientific. Deuterated solvents dimethylsulfoxide (DMSO-d₆), water (D₂O), methanol (CD₃OD), and chloroform (CDCl₃) were purchased.
Fig. 4. PEGOL-60 demonstrates pathology-dependent biodistribution in the retina and choroid in rat AMD model upon systemic administration. Mi/Ma were stained using Iba-1 (green), blood vessels were stained with lectin (blue) and PEGOL-60-Cy5 (red), and nucleus was stained using DAPI (orange). (A) Confocal image of choroidal flat-mount demonstrating the bleb area (white dashed lines) with choroidal neovascularization (CNV) formation, macrophage accumulation, and PEGOL-60-Cy5 found only in the bleb area. Higher magnification (×20 and ×40) demonstrates cellular colocalization of PEGOL-60 in choroidal macrophages in the bleb area (white arrows). (B) Retinal flat-mounts also demonstrated PEGOL-60-Cy5 signals only in the injured area corresponding to that of the bleb area in the choroid. High-magnification images demonstrate PEGOL-60-Cy5 colocalization in the retinal microglia pertaining to the injured area. (C) Cross-sectional analysis of the posterior segment (retina + choroid complex) demonstrates the bleb area with PEGOL-60-Cy5 localization. Further, high magnification (×20) of the healthy area (a) shows no dendrimer signal, whereas in the bleb area (b), PEGOL-60-Cy5 was found localized in activated Mi/Ma (white arrows) and CNV blood vessels (orange arrows) in retina and choroid. Scale bars for choroidal flat-mounts: 500 μm (×5), 200 μm (×20), and 50 μm (×40). Scale bars for retinal flat-mounts: 200 μm (×5) and 50 μm (×20 and ×40). Scale bars for cross sections: 200 μm (×5) and 50 μm (×20).
from Cambridge Isotope Laboratories Inc. (Andover, MA). Dialysis membrane (molecular weight cutoff of 1000 Da) was obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA).

Characterization of intermediates, dendrimers, and fluorescently labeled conjugates

Nuclear magnetic resonance (¹H and ¹³C{¹H} NMR). ¹H and ¹³C{¹H} NMR spectra were recorded on a Bruker 500-MHz spectrometer at ambient temperatures. The chemical shifts are reported in parts per million relative to tetramethylsilane as an internal standard. The residual protic solvent of CDCl₃ [¹H, δ 7.27 ppm; ¹³C, δ 77.0 ppm (central resonance of the triplet)], D₂O [¹H, δ 4.79 ppm], and CD₃OD [¹H, δ 3.31 ppm; ¹³C, δ 49.0 ppm] were used for chemical shift calibration. The resonance multiplicity in the ¹H NMR spectra are indicated as “s” (singlet), “d” (doublet), “t” (triplet), and “m” (multiplet). The broad resonances are expressed by “b.”

High-performance liquid chromatography. The purities of compounds were analyzed using HPLC (Waters Corporation, Milford, MA).

The HPLC is equipped with a 1525 binary pump, an In-Line degasser AF, a 717 plus autosampler, a 2998 photodiode array detector, and a 2475 multi-λ fluorescence detector interfaced with Waters Empower software. A Symmetry C18 reversed-phase column (Waters) was used, having a particle size of 5 μm, a length of 25 cm, and an internal diameter of 4.6 mm. The HPLC chromatograms were monitored at 210 nm using a photodiode array (PDA) detector, and the fluorescently labeled conjugate was monitored at both 650 and 210 nm using both PDA and fluorescence detectors. The injection was run using a gradient flow starting with 90:10 (H₂O/ACN), gradually increasing to 10:90 (H₂O/ACN) in 20 min and returning to 90:10 (H₂O/ACN) in 25 min maintaining a flow rate of 1 ml/min.

Mass spectroscopy. Accurate mass measurements (HRMS) were performed on a Bruker microTOF-II mass spectrometer using electrospray ionization (ESI) in positive mode and direct flow sample introduction in the CH₃CN:H₂O (9:1) solvent system. Either protonated molecular ions [M + nH]⁺ or adducts [M + nX]⁺

Fig. 5. PEGOL-60 alone (no drug) displays anti-inflammatory and anti-oxidant properties in vitro. BV2 murine microglia were stimulated with LPS (100 ng/ml) for 3 hours, followed by cotreatment with dendrimer for 24 hours. Cells were lysed, extracted for RNA, and analyzed with real-time quantitative polymerase chain reaction (RT-qPCR) (A and B). Media were collected and analyzed for extracellular TNFα with ELISA (C) and nitrite production with a Griess reaction (D). (A) PEGOL-60 treatment significantly reduces the expression of proinflammatory cytokines TNFα, IL-6, and IL-10 and marker iNOS. (B) PEGOL-60 treatment also significantly elevates anti-inflammatory cytokines. (C) Secreted TNFα into the media is reduced with PEGOL-60 treatment. (D) PEGOL-60 significantly reduces production of reactive species nitrite in a dose-dependent manner. (E) Separately, PEGOL-60 was tested for protective properties against oxidative stress–induced cell death. BV2s were treated with PEGOL-60 for 24 hours, followed by insult with 500 μM H₂O₂ for 3 hours. Cell viability was assessed via trypan blue exclusion. PEGOL-60 pretreatment confers significant protection against oxidative stress–induced cell death in a dose-dependent manner. ***P < 0.001, *P < 0.05 in Student’s t test compared to LPS and H₂O₂ groups from n = 3 independent trials with three internal replicates each.
(X = Na, K, NH₄) were used for empirical formula confirmation. MALDI-TOF experiments were performed on a Bruker Autoflex MALDI-TOF instrument using linear positive mode and a laser power of 55 to 100%. Sinapinic acid was used as the matrix.

Dynamic light scattering and ζ potential. The size and ζ-potential distribution of PEGOL-60 was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instrument Ltd., Worcestershire, UK) equipped with a 50-mW He-Ne laser (633 nm). The dendrimer was dissolved in deionized water (18.2 ohms) to make a concentration of 0.5 mg/ml. The solution was filtered through a cellulose acetate membrane (0.2 μm; Pall Life Science), and DLS measurements were performed in triplicate, at 25°C with a scattering angle of 173°. For ζ-potential measurement, the dendrimer was dissolved in 10 mM sodium chloride solution to obtain a concentration of 0.1 mg/ml. The readings were performed in triplicate, and an average value was recorded.

Synthesis of intermediates and dendrimers
Preparation of compound 2. Dipentaerythritol (1) (5 g, 19.66 mmol) was dissolved in anhydrous DMF (30 ml) and stirred at 0°C. Sodium hydride (5.66 g, 235.83 mmol) was slowly added in portions to the stirring solution and was stirred for 15 min. It was followed by the addition of propargyl bromide [24.23 ml, 163.10 mmol, of an 80% (w/w) solution in toluene] at 0°C, and stirring was continued at room temperature (RT) for another 6 hours. The reaction mixture was cooled and partitioned between water (40 ml) and ethyl acetate (50 ml). The organic layer was washed with water (3 × 50 ml) and brine (2 × 50 ml), then dried (Na₂SO₄), filtered, and evaporated in vacuo. The crude product was purified by silica flash column chromatography [ethyl acetate/hexane, 25:75 (v/v)] to afford compound 2 in 60% yield.

Preparation of compound 3. Dipentaerythritol (1) (6 g, 23.59 mmol) was dissolved in anhydrous DMF (20 ml) and THF (50 ml), and the solution was stirred at 0°C. Sodium hydride (6.23 g, 259.55 mmol) was slowly added in portions to the stirring solution and was stirred for 15 min. It was followed by the slow addition of allyl bromide (11.2 ml, 129.77 mmol) diluted with anhydrous THF (20 ml) at 0°C, and the stirring was continued at 0°C for another 30 min followed by stirring at RT for 90 min. The reaction was constantly monitored with the help of thin-layer chromatography (TLC). Reaction was quenched with ice once the maximum product formation was observed on TLC. TLC was stained with KMnO₄ dip. The crude product was purified by silica flash column chromatography [ethyl acetate/hexane, 25:75 (v/v)] to afford compound 3 in 40% yield.

1H NMR (500 MHz, CDCl₃) δ 5.87 (dd, J = 22.5, 10.6, and 5.4 Hz, 5H), 5.24 (dd, J = 17.2 and 1.5 Hz, 5H), 5.21 to 5.10 (m, 5H), 4.06 to 3.90 (m, 10H), 3.82 to 3.55 (m, 10H), and 3.55 to 3.34 (m, 18H). HRMS (ESI-TOF) m/z: calculated for C₃₅H₆₅N₃O₉ [M + H]^+: 597.4917; found: 597.4771. HPLC purity, 97.3%; retention time, 12.8 min.

Preparation of compound 6. Hexapropargylylated compound 2 (1 eq) and an azido derivative (1.2 eq per acetylene) were suspended in a 1:1 mixture of DMF and water in a 5-ml microwave vial equipped with a magnetic stir bar. To this were added CuSO₄·5H₂O (0.5 eq per acetylene) and sodium ascorbate (0.5 eq per acetylene) dissolved in the minimum amount of water. The vial was tightly capped, and reaction was irradiated in a microwave at 50°C for 6 hours. Reaction completion was monitored with the help of TLC, and on completion, the reaction mixture was diluted with ethyl acetate (60 ml). The organic layer was washed three to four times with a saturated solution of EDTA and dried with anhydrous sodium sulfate, followed by concentration in vacuo. This procedure has been extensively demonstrated to remove trace amount of copper salts. Desired compound was purified using column chromatography using 3% methanol in DCM as eluent to afford transparent oil-like compound in 65% yield.

1H NMR (500 MHz, CDCl₃) δ 7.69 (s, 6H), 5.94 to 5.77 (m, 30H), 5.18 (dd, J = 57.0 and 13.8 Hz, 60H), 4.51 (s, 24H), 3.96 to 3.84 (m, 72H), 3.68 to 3.49 (m, 52H), and 3.51 to 3.27 (m, 108H). (MALDI-TOF) m/z: calculated for C₁₅₈H₂₃₀N₁₈O₁₀8 [M + Na]^+: 2626.1450; found: 2626.1444. HPLC purity, 99.9%; retention time, 12.8 min.

Preparation of compound 7. A 10-ml glass vial was charged with alkene-terminated dendrimer 6 (1.11 g, 0.24 mmol) and 1-thioglycerol 7 (2.31 ml, 26.7 mmol) in 12 ml DMF. 2,2-Dimethoxy-2-phenylacetophenone (420 mg, 1.59 mmol) was added, and the reaction mixture was stirred under ultraviolet light (365 nm) for 12 hours. After 12 hours, the reaction was stopped, and reaction mixture was precipitated using diethyl ether. The precipitates formed were washed several times with diethyl ether to remove excess 1-thioglycerol. The residue was dissolved in DMF and dialyzed against DMF for 6 hours, followed by water dialysis for 8 hours using dialysis membrane corresponding to a molecular weight cutoff of 1000 Da. The purified product was then lyophilized to achieve transparent oil in 70% yield.

1H NMR (500 MHz, MeOD) δ 7.99 (s, 6H), 4.57 (d, J = 29.3 Hz, 28H), 3.98 to 3.83 (m, 18H), 3.80 to 3.70 (m, 34H), 3.65 to 3.35 (m, 300H), 2.80 to 2.49 (m, 120H), 1.84 (s, 60H). ¹³C NMR (126 MHz, MeOD) δ 144.7, 124.3, 73.1, 71.4, 70.5, 70.1, 69.4, 64.6, 63.9, 50.0, 47.1, 45.6, 42.1, 35.0, 29.6, 29.1, and 26.8. (MALDI-TOF) m/z: calculated for C₃₀₂H₄₉₂N₁₈O₁₂₂S₃₀: 7397.8850; found: 7433.688. HPLC purity, 97.3%; retention time, 7.1 min.
Preparation of compound 9. BOC-GABA-OH (85 mg, 0.41 mmol) was added to a stirring solution of dendrimer 8 (620 mg, 0.08 mmol) in DMF (10 ml), followed by the addition of EDC (160 mg, 0.83 mmol) and DMAP (103 mg, 0.83 mmol). The reaction mixture was then stirred at RT for 24 hours. Upon completion, the reaction mixture was dialyzed against DMF for 6 hours, followed by water dialysis for 12 hours, changing water after every 4 hours. The aqueous solution was then lyophilized to yield compound 9.

1H NMR (500 MHz, DMSO) δ 7.94 (s, 7H), 4.54 to 4.48 (m, 24H), 3.88 (s, 16H), 3.75 to 3.63 (m, 29H), 3.60 to 3.53 (m, 66H), 3.53 to 3.47 (m, 44H), 3.47 to 3.42 (m, 56H), 3.41 to 3.36 (m, 24H), 3.33 (s, 55H), 3.26 (s, 38H), 2.71 to 2.45 (m, 122H), 1.78 (dd, J = 20.4 and 14.4 Hz, 68H), and 1.39 (s, 45H). HPLC purity, 93.3%; retention time, 8.4 min.

Preparation of compound 10. TFA (0.6 ml) was added dropwise to a stirring solution of compound 9 (620 mg, 0.08 mmol) in dry DCM (3 ml). The reaction mixture was stirred overnight at RT. The solvent was then evaporated, and the reaction mixture was diluted with methanol followed by evaporation on rotary evaporator. This process was repeated several times to remove traces of TFA. The solvent was evaporated to afford compound 10 as an off-white hygroscopic solid in quantitative yield.

Preparation of compound 11. DIPEA (0.2 ml to adjust pH of the solution to 7.4) was added to a stirring solution of compound 10 (600 mg, 0.07 mmol) in DMF (5 ml), followed by the addition of Cy5 NHS ester (55 mg, 0.15 mmol) dissolved in 1 ml of DMF. The stirring was continued at RT for 12 hours. The reaction mixture was dialyzed against DMF for 12 hours, changing DMF every 4 hours, followed by water dialysis for 6 hours. The aqueous solution was then lyophilized to afford compound 11 as a blue solid in percent yield.

1H NMR (500 MHz, DMSO) δ 8.37 (t, J = 12.9 Hz, Cy5 H), 8.00 (s, 6H), 7.82 (s, Cy5 H), 7.82 (s, Cy5 H), 7.64 (t, J = 6.9 Hz, Cy5 H), 7.33 (d, J = 8.3 Hz, Cy5 H), 6.59 (t, J = 12.3 Hz, Cy5 H), 6.31 (d, J = 7.6 Hz, Cy5 H), 6.0 to 5.93 (m, Cy5 H), 4.78 to 4.40 (m, 50H), 3.58 to 3.19 (m, 400H), 2.64 to 2.41 (m, 120H), 1.89 to 1.62 (m, 60H), 1.10 (t, J = 7.0 Hz, Cy5 H), and 0.99 (t, J = 7.2 Hz, Cy5 H). HPLC purity, 99.4%; retention time, 7.4 min.

Qualitative and quantitative analyses of PEGOL-60-Cy5 biodistribution in a neonatal rabbit model of CP

Rabbit model of CP and administration of PEGOL-60-Cy5

New Zealand white rabbits were purchased from Robinson Services Inc. (NC, USA) and arrived at the facility 2 weeks before breeding. All animals were housed under ambient conditions (22°C, 50% relative humidity, and a 12-hour light/dark cycle), and necessary precautions were undertaken throughout the study to minimize pain and stress associated with the experimental treatments. Experimental procedures were approved by the Johns Hopkins University Animal Care and Use Committee (IACUC). Timed-pregnant rabbits underwent laparotomy on gestational day 28 (G28) and received a total of 3200 EU (endotoxin units) of LPS (Escherichia coli serotype O127:B8, Sigma-Aldrich, St. Louis, MO) injection along the wall of the uterus as previously described (40). Rabbits were induced on G30 with intravenous injection of Pitocin (0.5 U/kg) (JHP Pharmaceuticals, Rochester, MI). After delivery, rabbit kits were kept in incubators with a temperature of ~32°C to 35°C and a relative humidity of ~50 to 60% and fed three times per day with rabbit milk replacer (Wombaroo, South Australia, Australia). On PND1, healthy controls and CP kits received a single dose of PEGOL-60-Cy5 (55 mg/kg, 200 μl) or vehicle (0.9% sterile saline, 200 μl) intravenous injection. CP kits were euthanized at 1, 3, and 24 hours after injection. Healthy controls were euthanized at 24 hours after injection. All solutions used for administration were sterilized using 0.2-μm Acrodisc syringe filters (Pall Corporation, Port Washington, NY) before injection.

Immunohistochemistry for CP model

Animals received intravenous administration of PEGOL-Cy5 (55 mg/kg, 200 μl) on PND1 and euthanized at 24 hours after injection. The rabbits were anesthetized and transcardially perfused with PBS. All major organs (kidneys, lungs, liver, and heart) and plasma were isolated and flash-frozen. The brains were removed and divided into two halves. One-half was flash-frozen for fluorescence quantification, and the other half was postfixed in 10% formalin for 48 hours and cryoprotected in graded sucrose solutions. Coronal sections (30 μm, 1:6 series) were blocked by 3% normal donkey serum in 0.1 M PBS. For PEGOL-60-Cy5 and microglia colocalization, sections were incubated with goat anti-Iba1 (1:250; Abcam, MA, USA) overnight at 4°C. Sections were subsequently washed and incubated with donkey anti-goat Alexa Fluor 488 secondary antibodies (1:250; Life Technologies, MA, USA) for 2 hours at RT. Next, the sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000, Invitrogen) for 15 min. After washing, the slides were dried and cover-slipped with mounting medium (Dako, Carpinteria, CA, USA). Confocal images were acquired with Zeiss ZEN LSM 710 (Zeiss, CA, USA) and processed with ZEN software.

CP kits brain microdissection procedure

Following sacrifice and perfusion, one hemisphere of each rabbit kit brain was flash-frozen and saved at ~80°C until microdissection was performed following the procedure outline in our recently published manuscript (15). Briefly, the brains were warmed in disposable petri dishes on a bed of dry ice and then cut into five sections of equal thickness with a clean razor blade. The brainstem and the front section with the olfactory bulb were discarded, after which the cortex was removed from the remaining sections with a fresh scalpel blade and placed in premassed 1.5-ml Eppendorf tubes. The hippocampus and PVR were then isolated from the remaining tissue under a magnifying lens and placed in separate premassed 1.5-ml Eppendorf tubes. Each Eppendorf tube was massed again to determine the sample mass, and then all samples were stored at ~80°C until downstream processing by homogenization and extraction as previously described (15). Three samples of cortex, two samples of PVR, and one sample of hippocampus were obtained for each brain, which were each averaged together to provide one dendrimer uptake value for each brain subunit for each brain. Three cortex samples and two PVR samples were collected and analyzed. Then, they were averaged to create the PEGOL-60 concentration for that animal. The average concentrations for each animal were then averaged to obtain the overall average PEGOL-60 concentration. Blood samples were collected from cardiac puncture with EDTA-treated (lavender tops) tubes. The blood samples were centrifuged for 30 min at 2000g using a refrigerated centrifuge. The plasma was collected and stored at ~80°C until use.

Dendrimer extraction from tissue samples

Briefly, the organs (heart, lungs, liver, kidneys, spleen, and brain) were taken out at 80°C, slowly thawed on ice, and weighed. The organs were dissected to take a known amount of tissue samples (three from liver, lungs, and kidneys and two from heart and spleen). The brain hemispheres were further microdissected to separate cortex, hippocampus, and PVR using our recently published protocol (15). A known amount of tissue samples was taken from these subregions of brain (three from cortex, two from PVR, and one from hippocampus). The tissue samples were homogenized with 0.9- to 2.0-mm stainless
steel homogenization beads in methanol in a 1 ml:100 mg tissue ratio on a Bullet Blender Storm 24 tissue homogenizer (Next Advantage Inc., Averill Park, NY) for 10 min at a power level of 6 for brain and a power level of 12 for all other major organs at 4°C. The homogenized samples were then centrifuged at 20,000 g for 10 min at 4°C. The clear supernatant was transferred to protein LoBind Eppendorf tubes and stored at −80°C.

**Fluorescence quantification**

The supernatants were thawed and centrifuged again, and 130 μl of the supernatant was transferred to the micro cuvette (Starna Cell Inc., Atascadero, CA) for measurement. The fluorescence intensity in each sample was determined for Cy5 (λex = 645 nm, λem = 662 nm) using an RF5301PC spectrofluorophotometer running Panorama 3 software (Shimadzu Scientific Instruments, Columbia, MD). The background fluorescence was adjusted from the fluorescence values of healthy control tissue. The fluorescence intensity values were then converted to dendrimer concentrations using calibration curves of PEGOL-60-Cy5 for appropriate slit widths. Plasma was diluted 10-fold in Dulbecco’s PBS (Corning Inc.), then passed through a 0.2-μm pore polyethersulfone (PES) filter, and measured as described above for organ samples. Tissue concentrations are presented as %ID/g tissue; which were calculated by dividing the converted PEGOL-60 mass from the calibration curves by the organ mass and initial dose and multiplying by 100 to convert to percentages.

**PEGOL-60-Cy5 targeting of TAMs in a mouse model of GBM Mouse model of GBM and administration of PEGOL-60-Cy5**

Male and female C57BL/6 mice (6 to 8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), and experimental procedures were under Johns Hopkins IACUC–approved protocol. GL261 murine GBM tumor cells were grown in low glutamine RPMI 1640 (Gibco Laboratories, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco Laboratories), 1% penicillin/streptomycin antibiotic (Gibco Laboratories), and 1% l-glutamine (Gibco Laboratories) in an incubator at 37°C and 5% CO2. Cells were collected via trypsin detachment (Corning Inc., Corning, NY) for inoculation. Mice were anesthetized with intraperitoneal injection of a cocktail of ketamine (100 mg/kg; Henry Schein, Melville, NY) and xylazine (10 mg/kg; VetOne, Boise, Idaho) in normal saline (Quality Biological Inc., Gaithersburg, MD). An incision was made along the midline of the skull, and a burr hole was drilled for Hamilton syringe (Hamilton Company, Reno, NV) insertion. GL261 cells (100,000) in 2 μl of media were injected into the right hemisphere striatum at a rate of 0.2 μl/min via a stereotaxic frame and automatic syringe pump (Stoelting Co., Wood Dale, IL). Mice were then sutured (Ethicon Inc., Somerville, NJ) and monitored for surgical recovery. For administration of PEGOL-60-Cy5, mice were intravenously injected with PEGOL-60-Cy5 (55 mg/kg) 15 days after inoculation. Mice were perfused, and brains were collected 24 hours after administration. Immunohistochemistry and confocal imaging for GBM model

Animals were intravenously injected on day 14 after tumor inoculation with PEGOL-60-Cy5 (55 mg/kg), and brains were collected 24 hours later. Brains were fixed in 10% formalin (Sigma-Aldrich) for 24 hours, followed by sucrose gradient from 10 to 30% for 24 hours each. Brains were then frozen and sectioned coronally into 30-μm slices. Brain slices were blocked in 1× tris-buffered saline (Gibco Laboratories) supplemented with 0.1% Triton X-100 (Sigma-Aldrich), 1% bovine serum albumin (Sigma-Aldrich), and 5% normal goat serum (Sigma-Aldrich) for 4 hours at RT. Microglia were labeled with tomato lectin (1:1000; Vector Labs, Burlingame, CA), and cell nuclei were labeled with NucBlue DAPI cell stain (Invitrogen). Slides were then cover-slipped with mounting media (Dako). Confocal images were acquired on a Zeiss ZEN LSM710 (Zeiss) and processed with ZenLite software.

**Rat lipid–injected AMD model and administration of PEGOL-60-Cy5**

Male Sprague Dawley rats (6 to 8 weeks of age) were selected for this experimental AMD model. The studies were performed in accordance with ARVO (Association for Research in Vision and Ophthalmology) guidelines and Johns Hopkins IACUC–approved animal protocols. The rats were housed under ambient conditions (22°C, 50% relative humidity, and a 12-hour light/dark cycle). On day 0, 2 μl of HpODE (lipid) in 0.5 M borate buffer (20 μg/ml) was subretinally injected using a microinjector to form blebs underneath the retina. To evaluate the ocular biodistribution of PEGOL-60-Cy5, on day 3 after lipid injection, PEGOL-60-Cy5 was formulated in sterile saline (200 μl) and was administered intravenously at a concentration of 20 mg/kg. The rats were euthanized 7 days after dendrimer administration, and the eyeballs were enucleated and subjected for flat-mount and cross-sectional analysis.

**Tissue processing, immunohistochemistry, and confocal imaging of retinal and choroidal tissues**

Flat-mounts: The eyeballs were incubated in PBS in ice for 1 hour, and the anterior segment including lens were removed. Retina and choroids were separated and fixed in 2% paraformaldehyde (PFA) for 12 hours, followed by blocking with goat serum (Invitrogen, Rockford, IL, USA) for 6 hours. The Mi/Ma were stained using a rabbit anti-rat/mouse Iba-1 (FUJIFILM Wako Chemicals, Osaka, Japan) for 12 hours at 4°C and with Cy3-labeled goat anti-rabbit secondary antibody (Invitrogen, Rockland, IL, USA). FITC-labeled isoelectric (GS IIE) (Life Technologies, Eugene, OR, USA) was used to label and stain blood vessels and monocytes. The flat-mounts were prepared using four radial relaxation cuts, mounted on coverslip, and imaged under a confocal 710 microscope (Carl Zeiss, Oberkochen, Germany) using tile, Z-stack function. The images were processed using Zeiss software.

**Cross sections:** The eyeballs were fixed in 2% PFA with 5% sucrose for 3 hours, and the anterior segments including lens were removed and the posterior segments were subjected to sucrose gradient treatment from 5 to 20% to preserve the tissue structure during cryopreservation. The tissues were cryopreserved using optimal cutting temperature compound (OCT) and sectioned (10-μm sections) along the optic nerve. The sections were blocked with goat serum and stained using Iba-1 for Mi/Ma, lectin for blood vessels, and monocytes and DAPI for nucleus. Confocal images were acquired on a Zeiss ZEN LSM710 (Zeiss) and processed with ZenLite software.

**In vitro evaluation of anti-oxidant and anti-inflammatory activity of PEGOL-60**

**Cell culture**

BV2 murine microglia were obtained from the Children’s Hospital of Michigan Cell Culture Facility. Cells were cultured in an incubator at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Laboratories) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were maintained via trypsin detachment and passage every 3 days. At 80 to 90% confluence, cells were collected and seeded in 24-well plates for experiments.
MTT cell viability assay

BV-2 murine microglial cell lines were cultured as described in the cell culture section and seeded in alternating wells of 96-well plates (Sigma-Aldrich) at a concentration of 10,000 cells per well. Cells were then allowed to adhere and grow for 24 hours. A stock solution of PEGOL-60 (1000 µg/ml) was made in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin, vortexed, sonicated, and sterile-filtered. This solution was then diluted in medium to make stocks of PEGOL-60 (100, 10, 1, and 0.1 µg/ml). Medium was aspirated off all wells of the 96-well plate, and either medium containing PEGOL-60 or fresh medium was replaced on the cells, which were allowed to culture for an additional 24 hours. A 12 mM stock solution of MTT (Invitrogen, Carlsbad, CA) in sterile PBS was made and mixed by vortex and sonication. Medium was removed from all wells, and 100 µl of fresh medium was added to each well as in addition to 10 µl of MTT solution, which was mixed with the medium by pipetting. The cells were then allowed to incubate in the MTT for 4 hours, after which 85 µl of the MTT medium was removed and replaced with 150 µl of DMSO (Corning) as mixed thoroughly by pipetting. The DMSO incubated with the cells at 37°C for 10 min, after which the wells were mixed by pipetting again before the absorbance at $\lambda = 540$ nm of each well was read on a Synergy Mx Microplate Reader (BioTek, Winooski, VT) running Gen5 software (BioTek). The absorbances were all converted to ratios compared to untreated cells after subtracting off the background of media controls. Three plates were used, and treatments were done in triplicate on each plate and averaged to produce one data point.

LPS stimulation and evaluation

BV2 murine microglia were seeded in 24-well plates. Cells were stimulated with LPS (100 ng/ml; Sigma-Aldrich) for 3 hours in serum-free media, followed by coincubation with PEGOL-60 and LPS (100 ng/ml) for 24 hours. PEGOL-60 was solubilized in media and syringe-filtered through 0.2-µm pore filters. Media were collected. Isopropanol (500 µl; Thermo Fisher Scientific) was added to each sample, mixed, and incubated on ice for 15 min. Samples were then centrifuged for 15 min at 15,000 g, and aqueous fraction was collected. Isopropanol (500 µl; Thermo Fisher Scientific) was added to each sample, mixed, and incubated on ice for 10 min. Samples were again centrifuged for 15 min at 15,000 g and washed with 75% ethanol in diethyl pyrocarbonate water (Invitrogen). Samples were sonicated, and sterile-filtered. This solution was then diluted in medium and converted to complementary DNA (Applied Biosystems, Foster City, CA). Samples were measured on StepOne Plus real-time PCR system (Applied Biosystems) with SYBR Green reagent (Thermo Fisher Scientific). Samples were measured on StepOne Plus real-time PCR system (Applied Biosystems) with SYBR Green reagent (Thermo Fisher Scientific) on fast PCR plates (Thermo Fisher Scientific).

Oxidative stress–induced cell death

BV2 murine microglia were pretreated with PEGOL-60 for 24 hours, followed by oxidative stress insult with 500 µM H2O2 (Sigma-Aldrich) for 3 hours. Cells were collected via trypsinization, mixed 1:1 with trypsin blue (Corning), and counted for cell viability.

Statistical analyses

All data are presented as means ± SEM. The analyses were conducted in Excel 2013 and GraphPad Prism (version 6; La Jolla, CA). Student’s $t$ tests were performed to determine significant differences between different single groups: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

SUPPLEMENTARY MATERIALS

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/4/eaay8154/DC1

View/request a protocol for this paper from Bio-protocol.

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