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Intrinsic targeting of inflammatory cells in the brain by polyamidoamine dendrimers upon subarachnoid administration

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Abstract

Aim—Understanding the interactions between nanomaterials and disease processes is crucial for designing effective therapeutic approaches. This article explores the unusual neuroinflammation targeting of dendrimers (with no targeting ligands) in the brain, with significant consequences for nanoscale materials in medicine.

Method—The *in vivo* biodistribution of fluorescent-labeled neutral generation-4-polyamidoamine dendrimers (~4 nm) in a rabbit model of cerebral palsy was explored following subarachnoid administration.

Results—These dendrimers, with no targeting ligands, were localizing in activated microglia and astrocytes (cells responsible for neuroinflammation), even in regions far moved from the site of injection, in newborn rabbits with maternal inflammation-induced cerebral palsy.

Conclusion—This intrinsic ability of dendrimers to localize inactivated microglia and astrocytes can enable targeted delivery of therapeutics in disorders such as cerebral palsy, Alzheimer's and multiple sclerosis.

Keywords

cerebral palsy; microglia-targeted drug delivery to the brain; neuroinflammation; PAMAM dendrimers

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Nanoscale materials are emerging as potentially powerful tools in medicine. Polyamidoamine (PAMAM) dendrimers have been increasingly employed in drug delivery, gene therapy and imaging. Dendrimers offer a new length scale (~5–10 nm) in nanomedicine, with significant versatility to incorporate multiple active molecules, such as targeting moieties and imaging agents [1–6]. The unique properties of dendrimers, such as their high degree of branching, multivalency, globular architecture and well-defined molecular weight, make them promising scaffolds for drug delivery, with improved solubility, pharmacokinetics and biodistribution compared with small molecule drugs. PAMAM dendrimers have been reported recently as carriers for anti-cancer drugs (e.g., 5-fluorouracil) [1], as solubility enhancers [7], intracellular delivery agents [2,3], imaging contrast agents [8,9], and as delivery vehicles for antisense and siRNA oligonucleotides [10]. *In vivo* biodistribution studies following intravenous administration in normal animals and animal with tumor xenografts have shown that dendrimers are cleared rapidly by the blood-stream, predominantly accumulating in the liver, kidney and the lung, with very low levels detected in the brain [8,11–14]. The relatively fast total body clearance of dendrimers in general and PAMAM dendrimers in particular largely prevents ‘long-term’ accumulation in nontargeted organs, such as kidney and liver, reducing the potential for side effects [12]. Physical factors, such as molecular size, chemical modifications and surface charge, can impact the pharmacokinetics and biodistribution of dendrimers [12–14]. Biological factors related to specific disease processes may also play a role in the biodistribution of dendrimers. Ligand-targeted dendritic polymers were found to accumulate in areas of angiogenesis in a mouse model of hindlimb ischemia, with a significant increase when targeted using peptides specific for integrin receptors [15]. Various tumor models have shown increased accumulation in the tumor xenografts with receptor targeting [2,13]. Increased accumulation of folate dendrimer conjugates at sites of inflammation has been demonstrated in animal models of arthritis [16].

Understanding and manipulating the tissue localization and targeting of nanomaterials to different disease processes are keys to improving their efficacy for specific applications. For example, therapy of several debilitating neurodegenerative and neuroinflammatory conditions of the CNS, such as hypoxic-ischemic injury, cerebral palsy (CP), Alzheimer’s, multiple sclerosis and Huntington’s disease, have not been feasible due to the inability to deliver adequate concentrations of the drug into the CNS. Although intraventricular delivery of drugs into the cerebrospinal fluid (CSF) is known to result in greater drug concentration with a longer half-life in the CSF, drug penetration in the parenchyma is limited, with most of the drug being taken up by the ependymal cells lining the ventricles rather than the target cells [17,18]. Implants or injections of drugs or convection-enhanced delivery into the brain interstitium are other methods that have been attempted in delivering drugs or nanoparticles/microsomes loaded with drugs into the brain [17]. These methods are useful for localized areas of injury or disease where diffusion of the drug occurs in the area surrounding the site of insertion or delivery. The drug concentration decreases with increase in the distance from the site of injection. Hence, these techniques of drug delivery may not be suitable for diffuse neuroinflammatory or neurodegenerative disorders where multiple regions in the brain may be affected. Drug-delivery vehicles that can target the inflammatory cells in different areas of the brain may provide new alternatives for sustained therapy.

The unique interactions between dendrimers (with no targeting moieties) and *in vivo* neuroinflammatory processes are investigated in this study. Inflammatory responses in the brain are associated with the activation of microglial cells, the resident macrophages of the CNS that serve the role of immune surveillance and host defense under normal conditions. Microglial cells are known to be activated by stimuli such as trauma, infection, inflammation and ischemia, resulting in the secretion of proinflammatory mediators, generation of reactive oxygen species and peroxynitrites that may lead to further neuronal

damage. The distribution of dendrimers in the presence and absence of neuroinflammation was studied using our previously established newborn rabbit model of maternal inflammation-induced CP. We have demonstrated that intrauterine injection of endotoxin near-term in pregnant rabbits leads to neuroinflammation as indicated by robust microglial activation in the periventricular regions of the brain [19]. This was associated with a phenotype of CP in the newborn rabbits [20]. Consequently, delivering anti-inflammatory agents in a targeted manner to activated microglial cells in the CNS may result in attenuation of the motor deficits and brain injury seen in CP. This strategy will also have broad applications in decreasing microglial activation in other neuroinflammatory disorders, such as Alzheimer's disease, multiple sclerosis and Parkinson's disease. Although *in vivo* studies have shown that there is very low accumulation of dendrimers in the brain, most of these studies have been conducted in healthy animals, or in animals with tumor xenografts [3,5,13]. The biodistribution of dendrimers appears to be closely related not only to its surface moieties, which would dictate the interactions of the dendrimers with various cells, but also the disease state and *in vivo* conditions that may influence the extent of uptake of the dendrimers by various cells.

In the present study, we imaged the cellular uptake and distribution of fluorescein-labeled neutral PAMAM dendrimers (FITC-G4-OH), following subarachnoid injection, in the neonatal rabbit brain with and without neuroinflammation. Neutral dendrimers were chosen owing to their improved biocompatibility, reduced cytotoxicity and reduced protein interactions [4,11]. Moreover, neutral and cationic nanoparticles have been shown to have the greatest diffusivity in the brain parenchyma when administered by convection-enhanced delivery [21–23]. In newborns, the CSF is easily accessible by injection into the subdural/subarachnoid space through the bregma, which corresponds to the anterior fontanelle or the 'soft spot' in humans, since the cranial sutures are not completely fused. Hence, local delivery of drugs into the CSF in newborns can be achieved without the more invasive mechanism of injection into the intraventricular space.

Materials & methods

Ethylenediamine-core PAMAM dendrimers (diagnostic grade, generation 4 with 64 OH end groups) was purchased from Dendritech (Midland, MI, USA). Other reagents were obtained from assorted vendors in the highest quality available. Fluorescein isothiocyanate (FITC, Alfa Aesar, MA, USA), ethyl(dimethylaminopropyl) carbodiimide (EDC), 6-diamidino-2-phenylindole (DAPI) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (MO, USA). Dialysis membrane (molecular weight cut-off: 1000 Da) was obtained from Spectrapor (CA, USA), dimethyl sulphoxide (DMSO), dimethyl formamide, methanol and diethyl ether were purchased from Fischer scientific (NJ, USA).

¹H-NMR spectra were recorded on Varian Mercury (400 MHz) spectrometer. Chemical shifts are reported in ppm and tetramethylsilane was used as the internal standard. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) spectra were recorded on a Bruker Ultraflex system equipped with a pulsed nitrogen laser (337 nm), operating in positive ion reflector mode, using 19 kV acceleration voltage and a matrix of 2,5-dihydroxybenzoic acid.

High-performance liquid chromatography analysis

High-performance liquid chromatography (HPLC) characterization of the conjugate was carried out with Waters HPLC instrument equipped with two pumps, an auto sampler and dual UV and florescent detector interfaced to millennium software. The mobile phase used was acetonitrile/water (acetonitrile and water containing 0.14% [v/v] of trifluoroacetic acid),

and the water phase had a pH of 2.25. Mobile phases were freshly prepared, filtered and degassed prior to use. Supelco discovery BIO wide pore C5 HPLC column (5 μ m particle size, 25 cm $\times$ 4.6 mm length $\times$ internal diameter) equipped with two C5 supelguard cartridges (5 μ m particle size, 2 cm $\times$ 4.0 mm length $\times$ internal diameter) was used for characterization of the conjugates. The gradient method used for analysis was 100:0 water:acetonitrile to 60:40 water:acetonitrile in 25 min, followed by returning to initial conditions in 15 min. The flow rate was 1 ml/min. The dual florescent detector was used at λ_{ex} = 495 nm, λ_{em} = 521 nm. Calibration curves were prepared for conjugate using the florescence detector, with λ_{ex} = 495 nm and λ_{em} = 521 nm. These fluorescence calibrations were used for quantification of biological samples. The extracted cytosolic samples were injected (20 μ l) in triplicates and quantified with calibration curve.

Preparation of FITC-G4-OH dendrimers

To a solution of FITC (80 mg) in anhydrous DMSO, EDC (60 mg) and catalytic amount of *N*-dimethyl amino pyridine were added [24]. The reaction was stirred for 20 min and G4-OH (250 mg) was added to it; the reaction was allowed to proceed further for 18 h at room temperature in the dark. To remove unreacted FITC and EDC, the reaction mixture was dialyzed (molecular weight cut-off of membrane: 1000 Da) in DMSO for 24 h (by changing the DMSO every 8 h). The DMSO was lyophilized to get FITC-G4-OH (see FIGURE 1C) conjugate as an orange-colored solid (270 mg). The FITC-G4-OH compound was reconstituted into methanol and precipitated in acetone. Absence of free FITC in the conjugate was verified by thin-layer chromatography using chloroform and methanol (ratio 1:1) as the mobile phase, and further by ¹H-NMR and HPLC analysis. ¹H-NMR (DMSO-*d*₆, 400 MHz) δ ppm 2.18 (m, G4-OH protons), 2.39–2.70 (m, G4-OH protons), 3.00–3.16 (m, G4-OH protons), 3.22–3.41 (m, G4-OH protons), 4.65–4.78 (bs, OH protons of G4-OH), 6.47–6.59 (d, 6H, aromatic protons of FITC), 6.61–6.72 (m, 3H, aromatic protons of FITC) and 7.63–7.79 (br. d, NH, interior amide protons of G4-OH).

Animal model of CP

All the animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Wayne State University, MI, USA. The details of the surgical procedures were described previously by our group [19,20]. Briefly, New Zealand White rabbits (Covance Research Products Inc., Kalamazoo, MI, USA) with timed pregnancies that were confirmed breeders with a history of delivering 7–11 kits per litter, underwent laparotomy under general anesthesia (2–3% isoflurane by mask) on gestational day 28 (E28, term pregnancy is 31–32 days). A total of 1 ml of saline for the control group (*n* = 3) or 1 ml of saline containing 20 μ g/kg of LPS (*Escherichia coli* serotype O127:B8; Sigma-Aldrich) for the endotoxin group (*n* = 3), was equally divided and injected into the uterine wall between the fetuses using a 27-gauge needle, taking care not to enter the amniotic sac. This ensured that all the kits were exposed to the same amount of endotoxin. Normothermia was maintained using a water circulating blanket, and heart rate, oxygen saturations and arterial blood pressure measured through a 20G arterial catheter placed in the marginal ear artery were monitored continuously during the procedure. The dams were monitored daily for changes in activity, feeding and fever. A surveillance camera was placed in the rabbit room and the dams monitored remotely to determine the time of delivery. The kits were born spontaneously at 31 days gestational age. Rabbit kits that were exposed to maternal endotoxin *in utero* were born with a phenotype of CP, while those exposed to maternal saline injection had a normal phenotype, as previously described by our group [19].

Subdural administration of FITC-G4-OH

The full-term kits born with CP ($n = 3$) or those born to healthy pregnant rabbits that were administered saline (referred to as 'healthy kits'; controls, $n = 3$) were injected with 2.5 μg of dendrimer-FITC (FITC-G4-OH) in 5 μl phosphate-buffered saline (PBS) into the CSF in the subarachnoid space through the skin and dura at the bregma and sacrificed 24 h later. Brains were fixed with paraformaldehyde, frozen and sectioned into 20 μm sections, and all sections were examined under fluorescence microscopy for the presence of FITC-G4-OH. Alternating sections from the para formaldehyde-fixed and frozen brains were stained for microglia (Texas-red tagged lectin) and astrocytes (rhodamine-labeled glial fibrillary acidic protein [GFAP]) to determine specific cellular colocalization of dendrimer-FITC.

Tissue processing

Animals in each group were euthanized 24 h after subarachnoid administration of FITC-G4-OH with an overdose of pentobarbital (120 mg/kg administered intraperitoneal). Following administration of the drug, animals were secured to a stainless steel surgical apparatus, the heart was exposed rapidly and a butterfly needle was inserted and secured in the left apex of the heart, the vena cava was incised and perfusion was initiated. Animals were perfused under pressure with 30 ml chilled physiological saline (0.9%) and 120 ml of 4% paraformaldehyde in PBS (0.1 M, pH 7.4) at a constant rate of 5 ml/min using a constant pressure pump, brains were removed and immersed in the same fixative for 48 h, cryoprotected using graded solutions of sucrose and frozen at -80°C until they were sectioned. Brains were embedded in 100% optimal cutting temperature media (Tissue-Tek[®]) and 20-micron thick coronal brain sections were cut using a cryostat (Leica Microsystems; Nuchloss, Germany) and mounted on poly-L-lysine coated slides (Sigma-Aldrich).

Lectin, GFAP & MBP fluorescence-histochemistry staining

Brain sections were washed with PBS followed by incubation in 1% hydrogen peroxide for 30 min in order to inactivate the endogenous peroxidase, and then in PBS solution containing 0.3% triton X-100 and 0.5% bovine serum albumin for 1 h following which slides were washed thrice with PBS for 5 min each. For microglial staining, the slides were covered with Texas-red labeled tomato lectin (1:100; Vector Laboratories, Burlingame, CA, USA) overnight. For GFAP immunolabeling, brain sections were incubated with mouse polyclonal GFAP to detect astrocytes (diluted 1/500; Dako Cytomation, Glostrup, Denmark). For myelin basic protein (MBP) immunolabeling, brain sections were incubated with rat monoclonal MBP to detect oligodendrocyte (diluted 1/100; Abcam, Cambridge, UK). After overnight incubation, slices were washed with PBS thrice for 5 min each and then incubated with the secondary antibody, which was rhodamine-conjugated goat antimouse for GFAP immunostaining (diluted 1/500; Abcam) or rhodamine-conjugated goat antirat for MBP immunostaining (diluted 1/200; Abcam) for 2 h. All slides were stained for nuclei using DAPI stain at a concentration of 1 $\mu\text{g}/\text{ml}$ for 10 min at room temperature. Slides were then rinsed in PBS, dehydrated in graded ethanol and cleared in xylene. The slides were then mounted with mounting medium (Sigma-Aldrich) and images obtained using a Leica DM2500 microscope (Leica Microsystems; Nuchloss, Germany) equipped with a camera or a confocal microscope (Zeiss LSM 310). For FITC, λ_{ex} of 495 nm and λ_{em} of 521 nm was used. Injection of equivalent amount of free FITC served as control.

Estimation of FITC-G4-OH in rabbit brain using HPLC

In order to get a semiquantitative measurement of the amount of dendrimer-FITC in the different regions of the neonatal rabbit brain, the hippocampus (which would be expected to localize dendrimer-FITC in the neuroinflammatory cells) and part of the frontal cortex

(which would lack dendrimer-FITC due to relative lack of neuroinflammatory cells in this region) were dissected from five adjacent 20- μ m sections of the brain starting from the level of the bregma in both healthy and CP kits. The tissue sections were homogenized in 1 $\times$ ice cold cytoplasmic lysis buffer with manual agitation and repeated five times. The samples were centrifuged at 8000 $\times$ g for 20 min and the supernatants containing the cytosolic portion of the cell lysate were obtained. The samples were analyzed by HPLC and the amount of dendrimer-FITC quantified using the standard calibration curve for FITC-G4-OH. All measurements were performed in triplicate for statistical analysis.

Results & discussion

Preparation of FITC-G4-OH dendrimers

We have covalently conjugated the dendrimer (FIGURE 1A) to FITC (FIGURE 1B) by one-step reaction through the formation of an ester bond [24]. We used G4-OH dendrimer, which contains 64 hydroxyl groups and is noncytotoxic well beyond concentration range used in the present study. The carboxylic acid group of FITC was conjugated with OH groups of G4-OH dendrimer by using EDC as a coupling agent (FIGURE 1). Since FITC was used just as an imaging agent, a payload of 2–3 per dendrimer was desired, so that most of the end groups are unaffected. The FITC-labeled compounds (FITC-G4-OH, FIGURE 1C) were purified by dialysis (membrane molecular weight cut off: 1000 Da) against DMSO for 24 h in dark to remove unreacted compounds. Purity of FITC-G4-OH conjugate was confirmed by HPLC (FIGURE 2) using fluorescence HPLC (λ_{ex} = 495 nm, λ_{em} = 521 nm). The FITC-G4-OH conjugate showed a single peak at 17.5 min in the reverse phase HPLC chromatogram, whereas free FITC eluted at 25 min, indicating absence of free FITC. ^1H -NMR was used to characterize the conjugate based on the appearance of dendrimer protons at 2.18 (m, G4-OH protons), 2.39–2.70 (m, G4-OH protons), 3.0–3.16 (m, G4-OH protons), 3.22–3.41 (m, G4-OH) and 4.65–4.78 (bs, OH protons of G4-OH), and aromatic protons at 6.47–6.59 (br.d, 6H, Ar) and 6.61–6.72 (s, 3H Ar), corresponding to the FITC protons and interior dendrimer amide protons at 7.793–7.63 (br.d, 1H, NH amide), respectively. The FITC payload on the surface of G4-OH was calculated by ^1H -NMR analysis comparing amidic protons of dendrimer and aromatic protons of FITC, which suggested that there were two molecules of FITC in the FITC-G4-OH conjugate. The MALDI-TOF MS of the G4-OH dendrimer (13,660 Da) and the FITC-G4-OH conjugate (14,540 Da) also suggests that approximately two molecules of FITC (389 Da) were conjugated to the dendrimer.

Subdural administration of FITC-G4-OH leads to localization in activated microglia & astrocytes in endotoxin kits with neuroinflammation

On examination of the tissue sections using confocal microscopy, very little FITC-G4-OH was noted in the brain parenchyma of the control kits (FIGURE 3, healthy kits). Surprisingly, in the kits with CP, the distribution of dendrimer-FITC in the brain parenchyma was found to be far-removed from the site of injection and localized to the periventricular white matter regions involving the corpus callosum, internal capsule, along the lateral ventricle and hippocampus, without any uptake noted in the cortex even near the site of injection (FIGURE 3, CP kits). The presence of FITC-labeled dendrimer in these regions was significantly greater in the CP kits with neuroinflammation than in the healthy kits. Based on our previous studies, these were the regions that were known to have an increased density of microglia and astrocytes in this rabbit model of CP [19]. In this study, we chose two time points (6 and 24 h) for detecting the biodistribution of the FITC-G4-OH in the brain (control and endotoxin) rabbit kits. We found the distribution patterns of FITC-G4-OH conjugate were similar at both time points (6 and 24 h). The latter time point has been shown to illustrate that the localization in the microglia and astrocytes are specific and persistent even at the longer time point.

When the microglia were stained with Texas-red tagged lectin, FITC-G4-OH was found to localize largely in the cytoplasm of activated microglial cells in both CP and healthy kits (FIGURE 4A & 4B). The activated microglia were recognized by their amoeboid cell body with short and thick processes. Since the CP kits had a significantly greater expression of activated microglial cells, there was increased dendrimer uptake noted in these animals. The colocalization of dendrimers in the astrocytes were investigated by labeling astrocytes with rhodamine-tagged GFAP. In CP kits, there is significant activation of astrocytes, indicated by an increase in number, along with the enlargement of the cell bodies and thickening of the processes. By contrast, in the healthy kits the astrocytes have thin processes and extensive branching with very small cell bodies. Dendrimer-FITC was found to colocalize significantly in activated astrocytes in CP kits (FIGURE 5A), with relatively no colocalization in astrocytes noted in the healthy kits (FIGURE 5B).

The increased uptake and specific distribution in the periventricular regions in the CP kits is related to the presence of activated microglia and astrocytes in these areas. This may be due to the increased endocytotic ability of activated microglial cells and astrocytes in CP kits with neuroinflammation. Interestingly, cells such as oligodendrocytes and neurons, which are typically not involved in causing inflammation, do not appear to take up the dendrimers to an appreciable extent (FIGURE 6). The dendrimer-FITC conjugate, where FITC is linked to the dendrimer through an ester linker, is stable under physiological conditions for several days, as we have demonstrated previously [25,26]. When equivalent amount of free FITC was injected into the CSF, both the CP and healthy kits showed nonspecific uptake in all layers of the cortex and ventricular region (FIGURE 7) with relatively minimal fluorescence seen in the regions associated with inflammatory activity where an increased density of activated microglial cells and astrocytes are noted. However, on conjugation of FITC with dendrimer, we did not observe any nonspecific uptake in the cortex or ventricular region at 24 h. This suggests that FITC-G4-OH conjugate was stable for 24 h in CSF. Therefore, the unique uptake profile described here is most likely related to the properties of the dendrimer, rather than FITC.

Activated microglia and astrocytes, which are the neuroinflammatory cells, are typically found in the periventricular white matter tracts and the hippocampus in the CP kits, with the cortex being relatively spared of these cells. Localization of dendrimer-FITC in the activated neuroinflammatory cells would be further confirmed by increased presence of the dendrimer-FITC in the periventricular regions and the hippocampus, with it being absent in the cortex in CP kits. Upon HPLC analysis of the tissue sections from both groups, there was no fluorescence detected in the cortex, indicating that there was no detectable uptake by cells in the cortex. In the hippocampus, there are normally a small amount of microglial cells in the control and a relatively large number of activated microglia and astrocytes in the CP kits. A 15-fold greater fluorescence was noted in the CP kits, indicating increased uptake by neuroinflammatory cells in this region, compared with control (0.03 $\mu\text{g}/\text{mg}$ FITC-G4-OH in CP kits vs 0.002 $\mu\text{g}/\text{mg}$ in healthy kits as detected by HPLC analysis) (TABLE 1). The data were reasonably consistent among the three CP and control kits. The HPLC estimation of FITC-G4-OH in each kit was reproducible. This HPLC estimation corresponds well with the histological data where colocalization of dendrimer-FITC is seen with activated microglia and astrocytes that are confined to the periventricular white matter regions and the hippocampus, with relative sparing of the cortex in the endotoxin animals.

Microglia constitute 10% of the total cells in the brain and play a pivotal role in immune surveillance function [27]. Microglia constantly survey their local surrounding with their highly motile processes by endocytosis of nutrients and by clearing cellular debris [28,29]. Under pathological conditions, ramified microglia rapidly transform into an activated form changing to an amoeboid and rounded shape, and can migrate to the site of injury following

a chemotaxis signal [30,31]. The phagocytic capacity of activated microglia can vary based on their phenotype [32–34]. Microglia, and astrocytes to a lesser extent, are known to express scavenger receptors (also known as multiligand receptors or pattern recognition receptors) on their surface that facilitate internalization of substances such as apoptotic cells, polysaccharides, polynucleotides and bacteria [35]. The expression of these receptors may be developmentally regulated and can be upregulated in the presence of LPS or proinflammatory cytokines [36,37]. *In vitro* studies have demonstrated that activated microglia are capable of taking up nanoparticles [38,39]. We have previously shown that when activated with LPS, microglial cells actively take up dendrimers, with peak intracellular concentrations being achieved within 1–2 h after exposure [40]. Although microglia are well known to possess endocytotic, micropinocytotic and phagocytotic abilities, it is not clear whether astrocytes have these same capabilities. *In vitro* studies on astrocytes activated with LPS have demonstrated that they are capable of phagocytosis of fluorescent beads with an average size of 30 nm [41]. Astrocytes are known to phagocytose in inflammatory brain disease [42] and are involved in phagocytosis in anterograde degeneration [43]. By contrast, injection of biodegradable microspheres in the stria of mice demonstrated that microspheres less than 7.5 μm were taken up by activated microglia but not astrocytes [44]. We noticed similar results in our control animals where a subpopulation of microglia takes up the dendrimers with no localization in the astrocytes. In animals with neuroinflammation, since both microglia and astrocytes are activated, it is possible that the dendrimers are taken up by both these cell populations. We hypothesize that the PAMAM dendrimers (without targeting ligands) distribute to the ventricles through the CSF and are then taken up by the activated microglial cells and astrocytes in the periventricular regions (FIGURE 8). The ability of activated microglia and hypertrophic astrocytes to efficiently scavenge or phagocytose may facilitate increased uptake of the dendrimers by these cells in the newborns with neuroinflammation with limited uptake of FITC-G4-OH by oligodendrocytes or other neuronal cells. Hence, newborn kits with a phenotype of CP show higher uptake of FITC-G4-OH into astrocytes and microglia cells compared with healthy ones, indicating a differential uptake of the dendrimer, which may be due to activation of these cells in the presence of inflammation. Further studies to track the cellular uptake and localization of the dendrimers over time along with phagocytosis- or endocytosis-dependent labeling would help provide a better understanding of the distribution of dendrimers.

Conclusion

Understanding the intrinsic biodistribution and targeting potential of nanomaterials (*in vivo*) has a significant impact on the design of nanotherapeutic approaches. In this study we have demonstrated that neutral dendrimers localize in activated microglia and astrocytes in the presence of neuroinflammation. These results indicate the prospective use of dendrimers as effective drug and gene delivery vehicles, with a potential for targeting therapy in neuroinflammatory conditions, such as Alzheimer's, multiple sclerosis, Parkinson's disease and CP [201,202].

Future perspective

The localization of dendrimers in activated microglia and astrocytes may have broad implications in the treatment of several neuroinflammatory and neurodegenerative disorders where involvement of these cells have been implicated, such as in CP, Alzheimer's, multiple sclerosis and Parkinson's disease. We envision that in the future drugs and genes for manipulating specific pathways in activated microglia and astrocytes can be delivered in a targeted manner for suppression of neuroinflammation and attenuation of diseases that are currently considered 'incurable'.

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Figure 1. Synthesis of FITC-G4-OH conjugate

(A) G4-OH (Mw: 14,215 Da) was reacted with (B) FITC to form (C) FITC-G4-OH conjugate.

DMAP: *N*-dimethyl amino pyridine; DMSO: Dimethyl sulphoxide; EDC:

Ethyl(dimethylaminopropyl) carbodiimide; FITC: Fluorescein isothiocyanate; FITC-G4-

OH: Fluorescein-labeled neutral polyamidoamine dendrimer.

Figure 2. Purity of FITC-G4-OH conjugate was confirmed by high-performance liquid chromatography using florescent detector ($\lambda_{\text{ex}} = 495 \text{ nm}$; $\lambda_{\text{em}} = 521 \text{ nm}$)

The FITC-G4-OH conjugate (see FIGURE 1C) showed a single peak at 17.5 min in the high-performance liquid chromatogram indicating absence of free fluorescein isothiocyanate, which elutes at 25 min.

FITC-G4-OH: Fluorescein-labeled neutral polyamidoamine dendrimer.

Figure 3. Biodistribution of FITC-G4-OH in the brain after injection into the subarachnoid space in postnatal day 1 endotoxin and control kits

Increased uptake of FITC-G4-OH was seen in the periventricular regions in the endotoxin kits (top panel), and with no obvious uptake in the controls (bottom panel) at 24 h postinjection. Scale bar is 400 μm for LV and CC.

CC: Corpus callosum; DAPI: 6-diamidino-2-phenylindole; FITC-G4-OH: Fluorescein-labeled neutral polyamidoamine dendrimer; LV: Lateral ventricle.

Figure 4. Colocalization of FITC-G4-OH in the microglial cells (lectin stain) of cerebral palsy and healthy kits following subarachnoid injection of FITC-G4-OH

(A) Lectin staining of microglia for cellular distribution of FITC-G4-OH in the brain following subarachnoid injection in postnatal day 1 cerebral palsy kits. Images show uptake of FITC-G4-OH (green) in activated microglial cells (red, Texas-red tagged lectin staining for microglia), seen as colocalization of staining in cells (arrow) around the lateral ventricle and in the corpus callosum of the newborn rabbit brain 24 h postinjection. DAPI staining of nuclei is seen in the left side of each panel. (B) Cellular distribution of FITC-G4-OH in the brain following subarachnoid injection in healthy kits (lectin staining for microglia). Images show a few microglial cells (red, Texas-red tagged lectin staining for microglia) in healthy

animals that colocalize with green FITC-G4-OH (indicated by arrows), in the periventricular region of the newborn rabbit brain at 24 h postinjection. Nuclei are identified by DAPI staining.

DAPI: 6-diamidino-2-phenylindole; FITC-G4-OH: Fluorescein-labeled neutral polyamidoamine dendrimer; LV: Lateral ventricle.

Figure 5. Colocalization of FITC-G4-OH in the brain (GFAP stain) of cerebral palsy and healthy kits following subarachnoid injection of FITC-G4-OH

(A) Cellular distribution of FITC-G4-OH in the brain following subarachnoid injection in cerebral palsy kits (GFAP staining for astrocytes cells). Images show significant uptake of FITC-G4-OH (green) in activated astrocytes (red, rhodamine-labeled GFAP staining for astrocytes), seen as colocalization of staining in the periventricular region of the newborn rabbit brain at 24 h postinjection. Nuclei are stained blue with DAPI. Arrow indicates FITC-G4-OH colocalizing with GFAP staining in activated astrocytes. (B) Cellular distribution of FITC-G4-OH in the brain following subarachnoid injection in healthy kits (GFAP staining for astrocytes cells). Images show no colocalization of FITC-G4-OH (green) with astrocytes (red, rhodamine-labeled GFAP staining for astrocytes) 24 h after subdural injection. The astrocytes are thinner and are not activated in the healthy animals. A few microglial cells appear to take up the FITC-G4-OH in the normal newborn rabbit. Nuclei are identified by DAPI staining.

DAPI: 6-diamidino-2-phenylindole; FITC-G4-OH: Fluorescein-labeled neutral polyamidoamine dendrimer; GFAP: Glial fibrillary acidic protein.

Figure 6. Cellular distribution of FITC-G4-OH in the brain following subarachnoid injection in postnatal day 1 control kits (myelin basic protein staining for oligodendrocytes cells)

Images show no colocalization of FITC-G4-OH (green) in oligodendrocytes (red, MBP staining for oligodendrocytes), DAPI for nuclear staining. Scale bar: 100 μm (top panel), 50 μm (middle panel) and 20 μm (bottom panel). Arrows indicate oligodendrocytes.

DAPI: 6-diamidino-2-phenylindole; FITC-G4-OH: Fluorescein-labeled neutral polyamidoamine dendrimer; MBP: Myelin basic protein.

Figure 7. Images following subarachnoid injection of free fluorescein isothiocyanate in the newborn rabbit

Equivalent amount of free FITC was injected and the animal was euthanized after 24 h. Astrocytes are stained with rhodamine-labeled GFAP (red). Nonspecific background staining is noted throughout the tissue. No colocalization of FITC is seen with astrocytes (A). GFAP and DAPI staining in (B); free-FITC and DAPI staining in (C). DAPI staining of nuclei seen in all slides.

DAPI: 6-diamidino-2-phenylindole; FITC: Fluorescein isothiocyanate; FITC-G4-OH: Fluorescein-labeled neutral polyamidoamine dendrimer; GFAP: Glial fibrillary acidic protein.

Figure 8. Dendrimer nanodevice injection, and the selective uptake of FITC-G4-OH in activated microglial and astrocytes in the cerebral palsy rabbit model
FITC-G4-OH: Fluorescein-labeled neutral polyamidoamine dendrimer.

Table 1

Quantitative fluorescence high-performance liquid chromatography estimation of FITC-G4-OH in newborn rabbits (endotoxin and control) 24 h after subarachnoid injection.

Serial No.	Endotoxin animal ($\mu\text{g}/\text{mg}$ tissue $\pm$ SD)		Control animal ($\mu\text{g}/\text{mg}$ tissue $\pm$ SD)	
	<i>PVR</i>	<i>Cortex</i>	<i>PVR</i>	<i>Cortex</i>
Kit 1	0.032 ± 0.004	Not detectable	0.0015 ± 0.001	Not detectable
Kit 2	0.028 ± 0.004		0.002 ± 0.001	
Kit 3	0.029 ± 0.005		0.003 ± 0.001	
Average	0.03 ± 0.004		0.002 ± 0.001	

FITC: Fluorescein isothiocyanate; FITC-G4-OH: Fluorescein-labeled neutral polyamidoamine dendrimer; PVR: Periventricular region; SD: Standard deviation.