Biomaterials 101 (2016) 96–107

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

# Nanoscale effects in dendrimer-mediated targeting of neuroinflammation

Elizabeth Nance [a, d, 1], Fan Zhang [b, d], Manoj K. Mishra [d], Zhi Zhang [a], Siva P. Kambhampati [d], Rangaramanujam M. Kannan [b, c, d, e, f, *], Sujatha Kannan [a, d, e, f, **]

[a] Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
[b] Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD 21218, USA
[c] Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD 21218, USA
[d] Center for Nanomedicine, Department of Ophthalmology, Wilmer Eye Institute Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA
[e] Hugo Moser Research Institute, Kennedy-Krieger Institute, Baltimore, MD 21205, USA
[f] Kennedy Krieger Institute – Johns Hopkins University for Cerebral Palsy Research Excellence, Baltimore, MD 21287, USA

ARTICLE INFO

*Article history:*
Received 20 February 2016
Received in revised form
20 May 2016
Accepted 24 May 2016
Available online 26 May 2016

*Keywords:*
Nanoparticle
Dendrimer
Neuroinflammation
Brain injury
Glia

ABSTRACT

Neuroinflammation, mediated by activated microglia and astrocytes, plays a key role in the pathogenesis of many neurological disorders. Systemically-administered dendrimers target neuroinflammation and deliver drugs with significant efficacy, without the need for ligands. Elucidating the nanoscale aspects of targeting neuroinflammation will enable superior nanodevices for eventual translation. Using a rabbit model of cerebral palsy, we studied the *in vivo* contributions of dendrimer physicochemical properties and disease pathophysiology on dendrimer brain uptake, diffusion, and cell specific localization. Neutral dendrimers move efficiently within the brain parenchyma and rapidly localize in glial cells in regions of injury. Dendrimer uptake is also dependent on the extent of blood-brain-barrier breakdown, glial activation, and disease severity (mild, moderate, or severe), which can lend the dendrimer to be used as an imaging biomarker for disease phenotype. This new understanding of the *in vivo* mechanism of dendrimer-mediated delivery in a clinically-relevant rabbit model provides greater opportunity for clinical translation of targeted brain injury therapies.



## 1. Introduction

Neurological diseases account for 13% of the global burden of disease and, as a result, more than $760 billion a year is spent trying to treat them [1]. Drugs that are used to treat the injured or diseased brain also take about 35% longer to be developed for use in humans compared to drugs for any other type of disease [2]. This is because (i) transport of drugs and drug delivery vehicles across the blood-brain-barrier (BBB) is difficult to achieve and (ii) injury is often diffuse, making it difficult for therapeutics to reach target cells even if administered locally [3,4]. In addition, common hallmarks of neurological disease such as inflammation, excitotoxicity, and impaired fluid flow (vascular and cerebrospinal) lead to a heterogeneous, dynamic, complex environment, even within the same disease spectrum. Nanotechnology-based strategies to target specific cells involved in brain disease can potentially slow disease progression and promote repair and regeneration, enabling normal development and maturation of the brain [5]. More specifically, selective targeting of common disease hallmarks such as neuroinflammation, which has recently been elucidated as a key mediator in autism, cerebral palsy (CP), stroke, traumatic brain injury (TBI), and Alzheimer's, to name a few, has the potential to delay the onset of disease and provide a longer therapeutic window for treatment [6].

Dendrimer-based platforms have become promising carriers for targeting inflammation [7]. Phosphorus dendrimers have been used to track macrophage polarization and fate in the presence of spinal or peripheral nerve injury [8,9], inhibit neutrophil

* Corresponding author. Center for Nanomedicine, Department of Ophthalmology, Wilmer Eye Institute Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA.
** Corresponding author. Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.
*E-mail addresses:* krangar1@jhmi.edu (R.M. Kannan), skannan3@jhmi.edu (S. Kannan).
[1] Current address: Department of Chemical Engineering, University of Washington, Seattle WA, 98195.

http://dx.doi.org/10.1016/j.biomaterials.2016.05.044
0142-9612/

recruitment in lung inflammation [10], and deliver therapeutics to inflammatory cells in models of arthritis [11]. Poly(amidoamine) (PAMAM) dendrimers (size ~3–12 nm) have well-defined size, branching architecture, and a high density of tailorable surface functional groups and have been shown to cross the impaired BBB in animal models [12,13]. Boridy et al. have shown celastrol incorporated PAMAM dendrimers can mediate inflammatory signaling and cytokine release from microglia in the chronically inflamed brain [14]. Systemically administered hydroxyl-modified, generation-4 (G4-OH) PAMAM dendrimers have shown significant accumulation, following systemic administration, in a rabbit model of CP [13], a mouse model of adult ischemic stroke [15], a mouse model of neonatal stroke [10], a canine model of hypothermic circulatory arrest induced brain injury [16], a rat model of retinal degeneration [17], and a primate model of ischemic optic neuropathy [18]. More importantly, when these dendrimers accumulated in specific cells that mediate inflammation, such as microglia and astrocytes, they led to profound effects when conjugated with *N*-acetyl cysteine (D-NAC), including dramatic improvement of phenotype [13], arrest in neurological injury [10], and neurological repair [16]. Given the strong medical need for optimizing therapeutic delivery to overcome biological barriers, reduce off-site toxicity, and achieve efficacy, it is important to explore the *in vivo* mechanism of how these PAMAM dendrimers, with no targeting ligands, selectively localize in cells that mediate neuroinflammation.

In this study, we use an *in vivo* rabbit model of CP, with features similar to CP in humans [19], to (i) characterize the impact of nanoparticle size on passage across an impaired BBB, (ii) understand how dendrimer surface functionality dictates movement in the brain parenchyma and uptake by activated microglia, and (iii) quantify dendrimer uptake and localization in the injured newborn brain as a function of disease severity. These findings are crucial for the development and improvement of disease-appropriate therapeutic nanoparticle platforms in both pediatric and adult central nervous system (CNS) disorders, where neuroinflammation is a key mediator of disease pathology [20,21].

## 2. Materials and methods

### 2.1. Materials and reagents for dendrimer synthesis and characterization

Ethylenediamine-core poly(amidoamine) (PAMAM) generation four hydroxyl-terminated dendrimer (G4-OH), amine-terminated dendrimer (G4-$NH_2$), and generation 3.5 carboxylate dendrimer (G3.5-COOH) were purchased from Dendritech Inc. (Midland, Michigan). Cy5-mono-NHS ester was purchased from Amersham Biosciences-GE Healthcare. Benzotriazol-1-yl-oxy-tripyrrolidinophosphonium hexafluorophosphate (PyBOP), 6-(Fmoc-amino)caproic acid, *N*-Fmoc-1,5-diaminopentane hydrobromide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC·HCl), Hydroxybenzotriazole (HOBt), acetonitrile (ACN), trifluoroacetic acid (TFA), triethylamine (TEA) and diisopropylethylamine (DIEA) were purchased from Sigma-Aldrich (St Louis, Missouri). Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (St. Louis, Missouri). All other reagents and solvents were used as received. Regenerated cellulose (RC) dialysis membrane (molecular weight cut-off 1 kDa or 8 kDa) was obtained from Spectrum Laboratories Inc (Rancho Dominguez, California).

### 2.2. Preparation of dendrimer-Cy5 conjugates

We functionalized hydroxyl (OH) and carboxylate (COOH) dendrimers with reactive amine groups using a suitable linker and reacted with Cy5-*N*-hydroxysuccinamide (NHS) ester to get the corresponding Cy5-conjugates. For the amine ($NH_2$) dendrimer, we directly reacted an NHS ester form of Cy5 with the PAMAM dendrimer. The methods for COOH dendrimer are provided below since they are previously unpublished, and the methods for $NH_2$ and OH dendrimer have previously been published [22,23] and are provided in brief in supplemental information.

*Carboxylate-functionalized PAMAM dendrimer-Cy5 conjugate*: We partially functionalized G3.5 carboxylate dendrimer with reactive amine groups on the surface by reacting *N*-Fmoc-1,5-diaminopentane hydrobromide with a sodium salt free form of carboxylate dendrimer using a coupling reaction method to get the Fmoc-functionalized intermediate. The methanolic solution of the sodium salt of G3.5-COOH was treated with 1.25 M methanolic HCl solution till the pH of the solution reached 7.0 in cold condition. The methanol was evaporated and redissolved in water and dialyzed against water for 6 h to remove the excess of the salt. Finally the resultant water layer was freeze dried to get the white powder as free carboxylate group on the surface of the dendrimer.

The salt free form of G3.5-COOH dendrimer (100.0 mg, 0.07 mmol) was dissolved in anhydrous DMSO under nitrogen atmosphere in a 50 mL round bottom flask at 40 °C until the solid completely dissolved into the solution. EDC·HCl (44.5 mg, 0.23 mmol) and HOBt (31.5 mg, 0.23 mmol) were dissolved in DMSO and added into the reaction mixture and stirred for 1 h under nitrogen at room temperature. *N*-Fmoc-1,5-diaminopentane hydrobromide (47.0 mg, 0.116 mmol) was dissolved in DMF with 5% DIEA and added drop wise to the reaction mixture and stirred for 24 h. The reaction mixture was dialyzed against DMF using 1 kDa cutoff dialysis membrane for 24 h and the DMF was removed under reduced pressure to obtain yellow color oily product of Fmoc-functionalized dendrimer. The Fmoc-functionalized intermediate was dissolved in anhydrous DMSO without further purification. We deprotected the Fmoc groups using a mixture of piperidine/DMF to get the amine-terminated carboxylate dendrimer. The mixture of piperidine/DMF (2:8, 5 mL) was added drop wise and the reaction mixture was stirred for 15 min under nitrogen atmosphere. The mixture of solvents was evaporated under reduced pressure and the reaction mixture was subjected to dialysis in (2:8) DMSO/DMF for 24 h. The DMF was evaporated under reduced pressure and the traces of DMF were removed by dialyzing against DI water for 24 h, and lyophilized to obtain pale yellowish powder of G3.5-COOH-$NH_2$. The product was characterized using $^1$H NMR, which suggested that ~ 3–4 molecules of linker amines were conjugated to each dendrimer. $^1$H NMR (DMSO-$d_6$): $\delta$ 1.07–1.64 (m, C$H_2$ protons of linker), 2.20–3.58 (m, C$H_2$ protons of G3.5-COOH), 4.68 (t, C$H_2$COO-C protons of G3.5-COOH), 7.84–7.96 (m, internal amide protons of G3.5-COOH).

### 2.3. Characterization of D-Cy5 conjugates

*$^1$H NMR Characterization*:

$^1$H NMR spectra of the intermediates and final dendrimer-Cy5 conjugates were recorded on a Bruker (500 MHz) spectrometer using DMSO-$d_6$ solvents. Proton chemical shifts were reported in ppm ($\delta$).

*Fluorescence spectroscopy*:

Fluorescence spectra of free Cy5, as well as all three final dendrimer-Cy5 conjugates, were recorded in methanol and in phosphate buffer [0.1 M], using a Shimadzu RF-5301 Spectrofluorometer. All measurements were done with a fixed excitation wavelength (645 nm) and emission wavelength of 662 nm.

*High Performance Liquid Chromatography (HPLC)*:

The final dendrimer-Cy5 conjugates, as well as their