Neuroscience Letters 602 (2015) 126–132

Contents lists available at ScienceDirect

Neuroscience Letters

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

Peptide-functionalized polymeric nanoparticles for active targeting of damaged tissue in animals with experimental autoimmune encephalomyelitis

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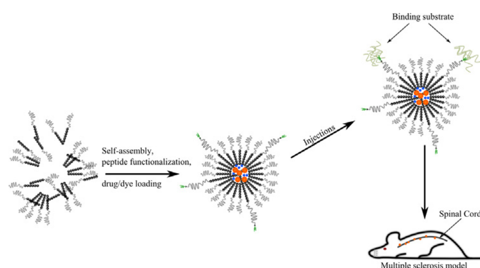
HIGHLIGHTS

- Leaky blood vessels accompany many CNS pathologies, including multiple sclerosis.
- Fibrinogen and nidogen are progressively up-regulated after disease onset.
- Targeted delivery can reduce “off-target” effects.
- Peptide modified polymeric nanoparticles can target blood clots and ECM molecules.
- Fibrin targeting nanoparticles demonstrate increased binding to lesion sites.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 30 May 2015
Received in revised form 23 June 2015
Accepted 24 June 2015
Available online 30 June 2015

Keywords:
Spinal cord
Multiple sclerosis
Nanoparticles
Targeted delivery

ABSTRACT

Increased permeability of blood vessels is an indicator for various injuries and diseases, including multiple sclerosis (MS), of the central nervous system. Nanoparticles have the potential to deliver drugs locally to sites of tissue damage, reducing the drug administered and limiting associated side effects, but efficient accumulation still remains a challenge. We developed peptide-functionalized polymeric nanoparticles to target blood clots and the extracellular matrix molecule nidogen, which are associated with areas of tissue damage. Using the induction of experimental autoimmune encephalomyelitis in rats to provide a model of MS associated with tissue damage and blood vessel lesions, all targeted nanoparticles were delivered systemically. *In vivo* data demonstrates enhanced accumulation of peptide functionalized nanoparticles at the injury site compared to scrambled and naive controls, particularly for nanoparticles functionalized to target fibrin clots. This suggests that further investigations with drug laden, peptide functionalized nanoparticles might be of particular interest in the development of treatment strategies for MS.
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http://dx.doi.org/10.1016/j.neulet.2015.06.049
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1. Introduction

Increased permeability of blood vessels is a classical biomarker for different types of injuries and diseases, including those of the central nervous system (CNS), where the blood brain and blood spinal cord barrier usually prevents the passage of most drugs that have been delivered systemically [1,2]. Furthermore, diseased blood vessels are associated with areas of tissue pathology in multiple sclerosis (MS), an inflammatory, demyelinating disease, and might represent a suitable target for local drug delivery [3]. In many instances, systemic administration of large quantities of therapeutic agents is required in order to obtain the expected biological effect. However, this approach results in "off-target" distribution of therapeutic molecules, and can produce undesired side effects such as systemic or neurotoxicity [4]. Nanoparticles (NP) represent an interesting drug delivery vehicle since they have been shown to accumulate naturally at sites of leaky vasculature. However, significant uptake in the reticuloendothelial systems such as liver and spleen has also been observed and active targeting aims at improving the efficiency of drug delivered through molecular recognition of unique markers [5,6]. Many approaches to target vasculature are focused on cell markers on activated endothelium, vascular smooth muscle cells, or inflammatory cells, which only provide limited targeting as they are heterogeneous and transiently expressed. Extracellular matrix molecules (ECM) and fibrin represent suitable targets because they are up-regulated in the diseased spinal cord for a prolonged period of time and are associated with blood vessels at lesioned tissue [3].

In this study, we demonstrate the progressing up-regulation of fibrin and nidogen/entactin-1 in an experimental animal model of MS and show enhanced binding to these targets of peptide functionalized poly(ethylene glycol)-*block*-polycaprolactone (PEG-*b*-PCL) nanoparticles (NPs) *ex vivo* and *in vivo* compared to non-functionalized or scrambled-peptide control NPs. This minimally invasive technique has the potential to increase drug concentration at the site of injury, diminish the undesirable effects of systemic delivery, and to increase efficacy of hydrophobic drugs.

2. Material and methods

2.1. Nanoparticle preparation

Peptides (Mimotopes) were conjugated to α-amino-ω-hydroxy terminated PEG_{5800}-*b*-$PCL_{19,000}$ (M_w/M_n: 1.4, Polymer Source) with succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate in a 10-fold molar excess (Thermo Fischer Scientific). Ellman's reagent (Thermo Fischer Scientific) was used to determine the extent of thiolation of free and aminated PEG-*b*-PCL conjugated peptides (λ = 412) [7]. The following peptide sequences were used: **naive**: none; **targeted: fibrin** NH_2-CGGGNQEQVSP-COOH; **scrambled: fibrin** NH_2-CGGGVQENQPS-COOH [8]; **targeted: laminin** NH_2-CGGGNIDPNAV-COOH; **scrambled: laminin** NH_2-CGGGIPANDNV-COOH [9]. Scrambled peptide controls were used to see if the sequence itself has binding properties, even in random order. Peptide-block copolymer conjugates were mixed with unmodified block copolymer (PEG_{2000}-*b*-PCL_{2800}, M_w/M_n: 1.15, Polymer Source, USA) to form functionalized nanoparticles. The unmodified polymer or the polymer mix was dissolved in N,N'-dimethylformamide (DMF, Sigma–Aldrich) and added to stirring 0.1 M phosphate buffered saline (PBS). The samples were dialyzed against PBS using 10 kDa molecular weight cut off snakeskin dialysis tubing (PIE68100; Thermo Fischer Scientific) and sterile-filtered with a 0.2 μm syringe filter (Whatman). Average NP size (diameter, nm), polydispersity index (PDI), and surface charge (zeta [ζ] potential, mV) were measured by dynamic light scattering (DLS, Malvern Instruments) in PBS (*n* = 3) [10]. NPs were loaded with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine or 3,3′-dioctadecyloxacarbocyanine perchlorate (DiI, DiO, respectively, Invitrogen) for visualization by dissolving the fluorescence dye with the polymer in DMF before the micelle formation. The incorporation of DiI/DiO into PEG-*b*-PCL micelles was verified spectrophotometrically (*n* = 4).

2.2. Fibrin-gels

Fibrin gels (200 μL) were prepared, as described earlier [8], by mixing the four components such that final concentrations of 3 mg/ml fibrinogen (clottability ≥75%, Sigma–Aldrich), 20 μmol $CaCl_2$ (Merck), and 1.25 NIH units/ml of thrombin (Sigma–Aldrich) were obtained with 18.75 μg/ml of DiI-laden NPs added. After gelation for 1 h at room temperature, resulting in a cross-linked fibrin network with a low modulus, the gels were soaked in PBS overnight to allow free NPs to diffuse out. The quantity of free NPs was determined with a fluorescence plate reader. Fluorescence intensity of samples were standardized to their initial solution first and then to the non-functionalized NP control (*n* = 3).

2.3. Animal model of multiple sclerosis

Female Dark Agouti rats (7–9 weeks, 150–200 g; Harlan Laboratories) were used for modeling the experimental autoimmune encephalomyelitis (EAE) as described previously [11]. Following induction, animals were examined daily for clinical symptoms of EAE [12]. All animals were housed according to NIH guidelines and the guide for the care and use of animals. Animal procedures were approved by the University of Miami Miller School of Medicine institutional animal care and use committee (IACUC).

2.4. Nanoparticle administration

To test if we would be able to identify the NP within the spinal cord after processing, an equi-mix of DiO- and DiI-laden (0.01 mg/ml), non-functionalized NPs within 1 μl of PBS, or 1 μl physiological saline (no NP control), was injected directly into the lumbar region (L5) of a health spinal cord (female Fischer 344 rats). Injections were made stereotactically using a microinjector and a Hamilton syringe, the needle was kept in place for 2 min before withdrawal.

To enable the intravenous (i.v.) administration of functionalized NPs, anesthetized Dark Agouti rats underwent jugular vein exposure as described previously [13]. DiI laden, functionalized NPs (1.5 mg/ml concentration) were administered daily over a period of 2 min (100 μl in PBS) starting at 7 days post-EAE induction for three consecutive days. All Animals were maintained for 24 h after final NP administration.

2.5. Tissue processing

Animals were deeply anesthetized at 0, 5, 10 or 15 days post-EAE induction (dpi, *n* = 8 per time point) or at the end points following i.v. (10 dpi, *n* = 5 per group) or intraspinal NP delivery (24 h, *n* = 3 per group). Immunoblotting and analysis of spinal cord homogenates (L5) was performed as described previously (*n* = 4 per time point) [14]. Primary antibodies used were goat anti-entactin/nidogen (1:1,000; R&D Systems), mouse anti-UC45 fibrin-α chain (1:1,000; GeneTex), and anti-β-actin (1:10,000; Sigma–Aldrich). Bands were visualized with HRP-conjugated secondary antibodies (1:5,000; Jackson ImmunoResearch) and levels of each of the specific bands were normalized to β-actin within each sample run in the same gel. Animals for histology were transcardially perfused with 4% PFA and the L3–L6 lumbar spinal cord excised and prepared for

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