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Engineering theranostic nanovehicles capable of targeting cerebrovascular amyloid deposits

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ABSTRACT

Cerebral amyloid angiopathy (CAA) is characterized by the deposition of amyloid beta (Aβ) proteins within the walls of the cerebral vasculature with subsequent aggressive vascular inflammation leading to recurrent hemorrhagic strokes. The objective of the study was to develop theranostic nanovehicles (TNVs) capable of a) targeting cerebrovascular amyloid; b) providing magnetic resonance imaging (MRI) contrast for the early detection of CAA; and c) treating cerebrovascular inflammation resulting from CAA. The TNVs comprised of a polymeric nanocore made from Magnevist® (MRI contrast agent) conjugated chitosan. The nanocore was also loaded with cyclophosphamide (CYC), an immunosuppressant shown to reduce the cerebrovascular inflammation in CAA. Putrescine modified F(ab′)$_2$ fragment of anti-amyloid antibody, IgG4.1 (pF(ab′)$_2$4.1) was conjugated to the surface of the nanocore to target cerebrovascular amyloid. The average size of the control chitosan nanoparticles (conjugated with albumin and are devoid of Magnevist®, CYC, and pF(ab′)$_2$4.1) was 164 ± 1.2 nm and that of the TNVs was 239 ± 4.1 nm. The zeta potential values of the CCNs and TNVs were 21.6 ± 1.7 mV and 11.9 ± 0.5 mV, respectively. The leakage of Magnevist® from the TNVs was a modest 0.2% over 4 days, and the CYC release from the TNVs followed Higuchi's model that describes sustained drug release from polymeric matrices. The studies conducted in polarized human microvascular endothelial cell monolayers (hCMEC/D3) *in vitro* as well as in mice *in vivo* have demonstrated the ability of TNVs to target cerebrovascular amyloid. In addition, the TNVs provided contrast for imaging cerebrovascular amyloid using MRI and single photon emission computed tomography. Moreover, the TNVs were shown to reduce pro-inflammatory cytokine production by the Aβ challenged blood brain barrier (BBB) endothelium more effectively than the cyclophosphamide alone.

1. Introduction

Cerebral amyloid angiopathy (CAA) is characterized by the deposition of amyloid beta (Aβ) proteins within the walls of small to medium-sized blood vessels of the brain and leptomeninges. About 80% of Alzheimer's disease (AD) patients manifest some degree of CAA [1]. Several studies have demonstrated that AD patients with CAA show worst cognitive test performance during life [2–4]. In addition to causing cerebrovascular inflammation, CAA triggers vascular dysfunction, which is believed to accelerate AD progression [5–7]. If detected in the early stages, the vascular inflammation resulting from CAA can be treated using immunosuppressants such as cyclophosphamide [8]. However, cyclophosphamide has narrow therapeutic index and long-term cyclophosphamide administration via conventional routes is associated with severe side effects.

On the other hand, a definitive diagnosis of CAA requires pathological examination of the affected tissue, which can only be done at autopsy [9]. Computed axial tomography (CT) is commonly used to detect CAA. However, due to low sensitivity of CT, only advanced stages of CAA associated with acute stroke and massive hemorrhages can be detected. Theoretically, magnetic resonance imaging (MRI) has sufficient spatial and contrast resolution to visualize cerebrovascular amyloid deposits. However, visualization of deposits less than 35 μm in diameter will require contrast enhancement [10]. Due to the lack of effective contrast agents, clinicians currently make a probable diagnosis of CAA based on the occurrence of strictly lobar hemorrhages, particularly in

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the cortico-subcortical area, detected by T_2^*-weighted MRI [11]. Unfortunately, these hemorrhages occur in the advanced stages of CAA, when the opportunities of intervention are limited [12].

The objective of this study is to develop a theranostic nanovehicle (TNV) capable of permeating the blood brain barrier (BBB) and specifically targeting cerebrovascular amyloid deposits. By providing MRI contrast and delivering immunosuppressants to the amyloid ridden cerebrovascular tissue, the TNVs aid in the early detection of CAA and allow for pre-symptomatic treatment. In addition, using a 21.1 T (900 MHz) magnet, the strongest magnetic field available for MRI [13] the gain in signal-to-noise ratio (SNR) is increased and the biodistribution of administered Gadolinium conjugated TNVs can be visualized with increased sensitivity.

2. Materials and methods

2.1. Materials

Medium molecular weight chitosan with a degree of deacetylation around 84%, pentasodium tripolyphosphate (TPP), donor horse serum, heparin, and gentamicin sulphate were purchased from Sigma-Aldrich (St. Louis, USA). Dulbecco's modified Eagle medium and F-12 nutrient mixture 50:50 (DMEM/F-12), Ultra-pure agarose, 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide (EDC), and N-hydroxylsuccinimide (NHS) were procured from Invitrogen (Carlsbad, CA). Cyclophosphamide was purchased from MP Biomedicals (Solon, OH). Magnevist® containing 469 mg/ml gadopentetate dimeglumine (Gd-DTPA) was procured from Berlex Laboratories (Montville, New Jersey).

2.2. Animals

B6SJLF1/J mice were obtained from Harlan Laboratories Inc. (Madison, WI) at 6–8 weeks of age and maintained in a virus-free, light and temperature controlled barrier environment until 12–14 months old. The animals were provided with standard pellets diet and water ad libitum. All procedures with the mice were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Mayo Institutional Animal Care and Use Committee.

2.3. In vitro BBB models

For amyloid targeting experiments, human cerebral microvascular endothelial cell (hCMEC/D3) monolayers were cultured on the Transwell® inserts (Costar, Cambridge, MA) coated with 0.01% rat-tail collagen (type 1), as described previously [14]. The inflammation studies were conducted using bovine brain microvascular endothelial cell (BBMVEC) monolayers grown on Transwell® inserts collated with 0.01% rat tail collagen and 0.01% bovine fibronectin [15]. High transendothelial electrical resistance values, which are characteristic of well-formed polarized endothelial monolayers, were ascertained using 'chopstick' electrodes attached to a Millicell-ERS meter (Millipore Corp., Bedford, MA). Monolayers with mean transendothelial electrical resistance (TEER) values greater than 170 Ω per cm^2 were selected for this study.

2.4. Preparation of TNVs

2.4.1. Modification of F(ab′)$_2$ fragments of IgG4.1

The F(ab′)$_2$ portion of IgG4.1 was cationized with putrescine (1,4-diaminobutane) using carbodiimide chemistry [16]. The pF(ab′)$_2$4.1 thus formed was concentrated in Vivaspin® with 30-kDa molecular weight cut off (MWCO) filter.

2.4.2. Preparation of Magnevist®-conjugated chitosan

MRI contrast agent, Magnevist®, was attached to chitosan as described previously [17,18]. The Magnevist® conjugated chitosan solution thus obtained was lyophilized for later use. The presence of amide bond that signifies the successful conjugation of Magnevist® to chitosan was verified by Fourier-transformed infrared spectroscopy (FTIR).

2.4.3. TNV formulation

Cyclophosphamide was entrapped in the nanocore made from Magnevist®-conjugated chitosan and TPP using ionic gelation method, described previously [15]. Surface of the nanocore was conjugated with pF(ab′)$_2$4.1 using carbodiimide chemistry to form the TNVs. The control chitosan nanoparticles (CCNs) were prepared in a similar fashion except that they were conjugated with bovine serum albumin (BSA). Albumin binds to a variety of proteins and was shown to act as a carrier protein for Aβ proteins. The BSA conjugated control nanoparticles were employed to demonstrate the specificity of TNV binding to amyloid deposits in comparison to the nonspecific interactions promoted by BSA conjugated control nanoparticles.

2.4.4. Radioiodination of TNVs

The TNVs and CCNs were labeled with carrier-free $Na^{125}I$, using the chloramine-T procedure as described previously [19]. Free ^{125}I was separated from the radiolabeled TNVs by dialysis against 0.01 M phosphate-buffered saline at pH 7.4.

2.5. TNV characterization

2.5.1. Particle size and zeta potential of TNVs

TNV morphology was assessed using a multimode scanning probe atomic force microscope (AFM, Veeco Metrology Inc., Plainview, NY). The mean hydrodynamic diameter of TNVs dispersed in distilled water was determined using photon correlation spectroscopy (Particle sizer; Brookhaven Instruments, NY, US), whereas the zeta potential was determined using laser doppler anemometry (Zeta Potential Analyzer, Brookhaven Instruments, NY, US).

2.5.2. Encapsulation and release of cyclophosphamide from TNVs

To quantify the amount of cyclophosphamide incorporated in TNVs, physical mixtures of cyclophosphamide and lyophilized blank nanoparticles made from Magnevist®-conjugated chitosan were prepared in the ratio of 1:1, 1:4 and 1:9, respectively. The IR spectra (500–4000 cm^{-1}) for each component, the physical mixtures, and the TNVs were acquired using FTIR spectrophotometer (PerkinElmer Life and Analytical Sciences, Connecticut). Cyclophosphamide absorbance peaks that are distinct from the blank nanoparticles were selected, and a standard curve of the peak height versus cyclophosphamide percentage was constructed. The percentage of unknown amount of cyclophosphamide in the TNVs was determined from the standard curve.

To evaluate cyclophosphamide release from the TNVs, 30 mg of lyophilized TNVs were re-suspended in 3 ml distilled water and placed in a dialysis bag (MWCO = 12.5 kDa), which was then immersed completely in a trough containing 50 ml PBS maintained at 37 °C. The trough contents were stirred constantly at 150 rpm, sampled at predetermined time intervals (0, 0.25, 0.5, 0.75, 1, 2, 3, 6, 14, 18 h), and was replaced with the same volume of fresh PBS. The amount of cyclophosphamide in the collected samples was determined using the high performance liquid chromatography (HPLC) method reported previously [20]. Gadolinium (Gd) content in TNVs was determined as per the previously published Arsenazo III colorimetric method [21,22].

2.6. Ability of TNVs to bind to Aβ fibrils

ELISA and quartz crystal microbalance-dissipation (QCM-D) methods were used to investigate the ability of TNVs to bind to pre-