The binding of pullulan modified cholesteryl nanogels to Aβ oligomers and their suppression of cytotoxicity

Sebastien Boridy [a], Haruko Takahashi [b], Kazunari Akiyoshi [b], Dusica Maysinger [a],*

[a] *Department of Pharmacology and Therapeutics, McGill University, McIntyre Medical Sciences Building, 3655 Promenade Sir William Osler, Montreal, Quebec H3G 1Y6, Canada*
[b] *Institute of Biomedical and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan*

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ABSTRACT

Among various hydrogels able to form monodisperse and stable nanoparticles (20–30 nm) are those with pullulan-bearing cholesteryl moieties (CHP). These nanoparticles can interact with soluble proteins through hydrophobic bonding. The objectives of this study were to investigate whether CHP nanogels would interact with oligomeric forms of the 42 amino acid variant of β-amyloid ($A\beta_{1-42}$) and if the formation of CHP-$A\beta_{1-42}$ oligomer entities will reduce cytotoxicity of $A\beta_{1-42}$ in primary cortical cells and microglial (N9) cells. By employing fluorescent CHP analogs with different charges we provide evidence that, (i) both neutral and positively charged CHP nanoparticles interact with $A\beta_{1-42}$ monomers and oligomers, (ii) neutral CHP is non-toxic, but positively charged derivatives ($CHPNH_2$) are toxic, particularly in primary cortical cultures, and (iii) binding of both monomeric and oligomeric $A\beta_{1-42}$ to CHP significantly reduces $A\beta_{1-42}$ toxicity in both the primary cortical and microglial cells. These results suggest that CHP nanogels could provide a valid complementary approach to antibody immunotherapy in neurological disorders characterized by the formation of soluble toxic aggregates, such as those in Alzheimer's disease (AD).

1. Introduction

Nanomaterials show promise as diagnostic and therapeutic tools [1]. Polymeric nanoparticles have shown particular promise in this field due to their highly favourable biocompatibility [2], their stability, and their potential for extended release as nanocarriers for targeted drug-delivery to the brain via the blood brain barrier [3]. Akiyoshi's group has made significant advances through the formulation of amphiphilic polymers with the propensity to self-aggregate, referred to as nanogels [4–6]. Monodisperse and colloidally stable nanogel particles (20–30 nm) form upon the self-assembly of partly hydrophobized (less than 5 wt%) cholesteryl group-bearing pullulans (CHP) in water (see Fig. 1). These CHP nanoparticles can form complexes spontaneously with various hydrophobic substances and soluble proteins through hydrophobic interactions [7]. In this regard, a cationic hydrogel nanoparticle ($CHPNH_2$) designed for intracellular protein delivery was developed [8].

CHP nanoparticles have protein-folding and refolding activity *ex vivo* [7,9], their behaviour being quite reminiscent of the artificial chaperone model proposed by Rozema and Gellman; for a review, see Ref. [10]. Given that the downregulation of chaperone expression and/or activity is thought to be heavily implicated in the pathogenesis of various neurodegenerative diseases [11], nanogels could be of particular value as artificial chaperones in neuro-nanomedicine. Notably, the hallmark of these disorders is the accumulation of protein aggregates intra- and extracellularly. In Alzheimer's disease (AD), these aggregates form plaques which are presently regarded as reservoirs for toxic oligomeric species of the β-amyloid peptide (Aβ) [12], and recently, Aβ oligomers have been shown to associate with senile plaques near excitatory synapses in a mouse model of AD [13]. Much of this research has focussed on the aggregation-prone 42 amino acid variant, $A\beta_{1-42}$, the product of sequential processing of amyloid precursor protein (APP) by β-secretase, followed by γ-secretase [14]. A plethora of studies indicate that it is not only the increased generation of Aβ peptides that is damaging, but rather their reduced clearance, and the accumulation of soluble protein aggregates [11,15]. Endogenous molecular chaperones are thought to play a primary role in regulating the aggregation and elimination of these toxic misfolded species, thereby preventing cell damage, especially in susceptible long-lived, post-mitotic cells, such as neurons. Hence, it is intriguing that nanogels have been shown to prevent Aβ fibrillation in vitro [16].

* Corresponding author. Tel.: +1 514 398 1264; fax: +1 514 398 6690.
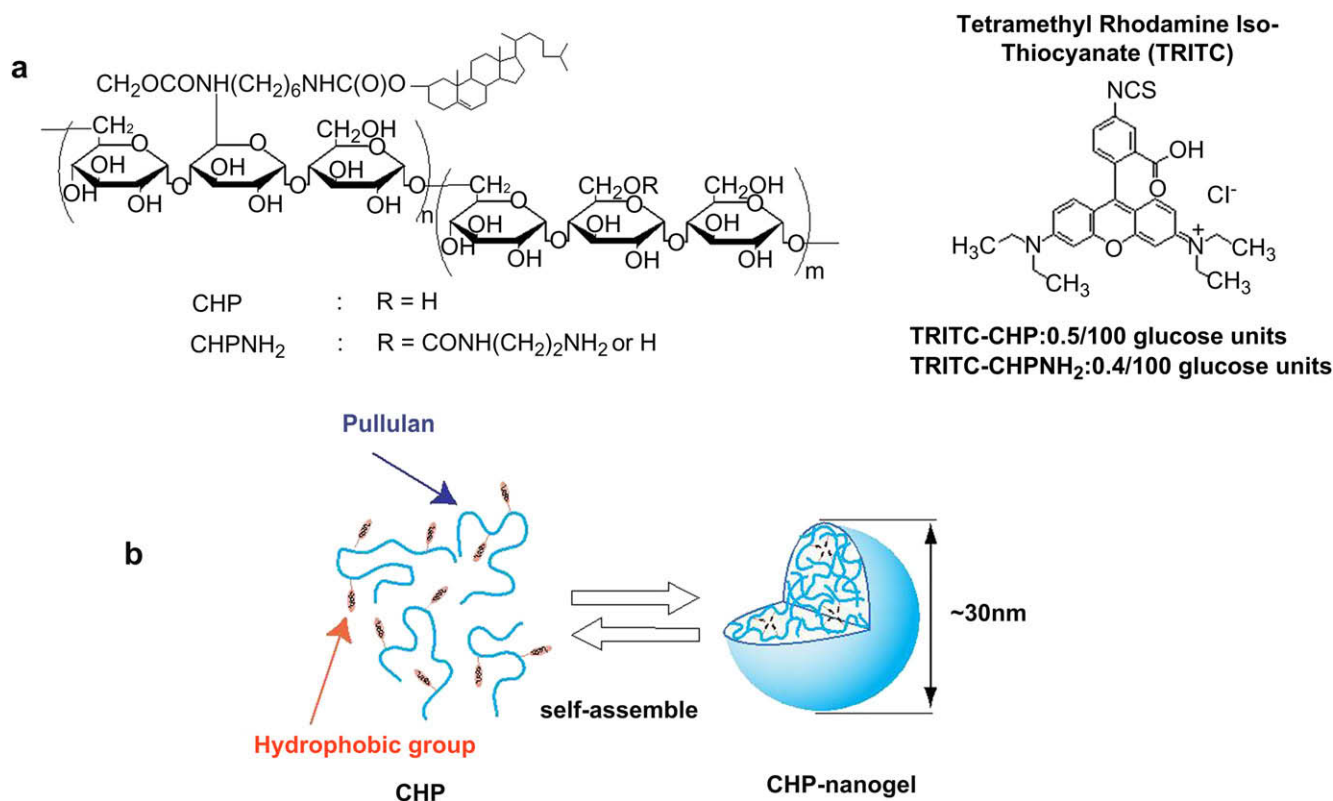*E-mail addresses:* dusica.maysinger@mail.mcgill.ca, dmaysing@pharma.mcgill.ca (D. Maysinger).

Fig. 1. Cholesterol-bearing pullulan (CHP). a, Structure of cholesterol-bearing pullulan (CHP) and cationic CHP (CHPNH₂). Note the presence of cholesterol residues. Also note the structure of TRITC and the abundance of TRITC units per 100 glucose units of TRITC-tagged CHP and CHPNH₂ compounds. b, The self-association of these residues is driven by hydrophobic interactions, which in turn drives the self-association of these polymeric nanoparticles into nanogels.

The objective of the present study was to test the effectiveness of CHP nanogels to bind to Aβ monomers, and to the more deleterious Aβ oligomers, while demonstrating their ability to decrease cytotoxicity in primary mixed cortical and microglial cells in culture. CHP nanoparticles have not been investigated in the central nervous system (CNS), neither in vivo nor in primary neural cells, thus their interaction with glia and neurons are unknown. Only a few studies have reported the responsiveness of neural cells to nanomaterials, and in particular, their effects on astrocyte and microglia activation [17,18]. Astrocytes are the principal macroglial cell types of the CNS and their activation is a key element in the regulation of neural cell responses to stress and brain injuries. These star-shaped neural cells can have both positive and adverse effects on the health of neurons depending on their activation status [19]. Notably, their transformation from "resting" to activated states is associated with an upregulation of an intermediate filament specific to astrocytes known as glial fibrillary acidic protein (GFAP) [20]. Microglia are the resident macrophage population of the CNS and they are sensitive surveyors of the nervous system environment [21,22]. They respond robustly and rapidly to biological particles (e.g. viruses) and artificial nanoparticles (e.g. polymeric or metallic nanospheres) [23].

Polymeric nanoparticles are in clinical use, and several drugs in nanoformulations are currently at different stages of clinical trial, mainly for cancer therapy [24]. However, suitable nanotherapeutics for treating brain disorders are still lacking. Moreover, toxicity and specificity of nanomaterials in general are still issues of concern [17,25].

The present study shows that CHP nanogels favourably interact with Aβ monomers and oligomers leading to an enhanced viability of cells in microglia (N9) and primary cortical cultures.

2. Materials and methods

2.1. Cholesterol-bearing pullulan nanoparticles

Cholesterol-bearing pullulan (CHP) and CHP derivatives containing 17 ethylenediamine groups (CHPNH₂) were synthesized as reported previously [4,5]. CHP was substituted with 1.1 cholesteryl groups per 100 glucose units of the parent pullulan (MW = 1.0×10^5). TRITC–CHP (0.5 per 100 glucose unit) and TRITC–CHPNH₂ (0.4 per 100 glucose unit) were obtained by the reaction of CHP and CHPNH₂ with tetramethyl rhodamine B isothiocyanate (TRITC) as previously reported [8]. The nanogel suspension was prepared as follows. CHP derivative (1 mg/ml) was suspended in PBS (pH 7.4). The suspension was sonicated for 15 min with a sonicator probe and filtered through a Millipore (Jaffrey, NH, USA) filter (pore size 0.22 μm).

2.2. Cell culture and treatments

N9 microglial cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Burlington, ON, Canada) supplemented with 5% (v/v) fetal bovine serum (FBS; Gibco) and 1% (v/v) penicillin–streptomycin (Gibco). Cells were treated 24 hours after seeding. One hour prior to treatments, serum-containing media was removed, and cells were washed once and replenished with fresh serum-free media. Serum-containing controls were kept to monitor changes in cell viability due to serum withdrawal. Viability assays or live cell imaging was performed up to a maximum of 24 hours after treatment.

Primary cortical neurons and glia from 5-day-old mouse pups were isolated, mechanically and enzymatically (0.25% Trypsin; Gibco) dissociated, counted and seeded (1×10^5 cells/well) onto coated glass coverslips and grown at 37 °C and 5% CO₂ in a 24-well coated cell culture plate (Corning; Lowell, MA, USA), initially in phenol-free Dulbecco's Modified Eagle's Medium (Invitrogen, Burlington, ON, Canada) with 1 mm L-Glutamine, sodium pyruvate and 1% (v/v) PSN (Invitrogen). On the second day in vitro, cells are cultured in Neurobasal A medium without phenol red (Invitrogen) supplemented with 2% (v/v) B-27 supplement (Invitrogen), 1% (v/v) PSN (Invitrogen), and 1 mm L-Glutamine (Sigma–Aldrich (St. Louis, MO, USA)). Half of the culture media was changed every 5 days, and treatments were not given past 8 days in vitro. Coverslips are coated initially with poly-L-ornithine (100 ug/ml; Sigma), followed by laminin (0.587 ug/ml; Invitrogen). CHP (CHP, CHPNH₂) were diluted from a 1 mg/ml stock in cell culture medium for treatment. Treatments were