Journal of Chromatography A, 1379 (2015) 1–8

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Prion protein-coated magnetic beads: Synthesis, characterization and development of a new ligands screening method☆

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ARTICLE INFO

Article history:
Received 2 August 2014
Received in revised form 1 December 2014
Accepted 3 December 2014
Available online 12 December 2014

Keywords:
Prion protein
Ligand fishing
Magnetic beads
Anti-prion compounds
Quinacrine
Screening method

ABSTRACT

Prion diseases are characterized by protein aggregation and neurodegeneration. Conversion of the native prion protein (PrP^C) into the abnormal scrapie PrP isoform (PrP^{Sc}), which undergoes aggregation and can eventually form amyloid fibrils, is a critical step leading to the characteristic path morphological hallmark of these diseases. However, the mechanism of conversion remains unclear. It is known that ligands can act as cofactors or inhibitors in the conversion mechanism of PrP^C into PrP^{Sc}. Within this context, herein, we describe the immobilization of PrP^C onto the surface of magnetic beads and the morphological characterization of PrP^C-coated beads by fluorescence confocal microscopy. PrP^C-coated magnetic beads were used to identify ligands from a mixture of compounds, which were monitored by UHPLC–ESI-MS/MS. This affinity-based method allowed the isolation of the anti-prion compound quinacrine, an inhibitor of PrP aggregation. The results indicate that this approach can be applied to not only "fish" for anti-prion compounds from complex matrixes, but also to screening for and identify possible cellular cofactors involved in the deflagration of prion diseases.

1. Introduction

Prion diseases, classified as transmissible spongiform encephalopathies (TSEs), are characterized by protein aggregation and neurodegeneration and are invariably fatal due to the lack of effective treatment or cure. These diseases affect humans (Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, kuru and fatal familial insomnia) as well as other animals, including sheep (scrapie), deer and elk (chronic wasting disease), and cattle (bovine spongiform encephalopathy) [1–3].

Conversion of the native prion protein (PrP^C) into the abnormal scrapie PrP isoform (PrP^{Sc}), which undergoes aggregation, is the critical step leading to the development of these diseases [2,4]. The isoform normally found in the cell, PrP^C, is a soluble protein rich in α-helices, and the conversion of PrP^C into PrP^{Sc} involves refolding into a structure rich in β-sheets. PrP^{Sc} is partially resistant to digestion by proteases and is insoluble in aqueous solutions [5]. Due to its insolubility, PrP^{Sc} is susceptible to accumulation, forming fibers, that tend to aggregate in the central nervous system, resulting in the degeneration of brain function [6].

Human prion diseases can occur as sporadic, genetic or infectious disorders [7]. The sporadic form of prion disease is the most common, in which no mutations have been identified in the gene that encodes PrP^C, and the two isoforms (PrP^C and PrP^{Sc}) share the same amino acid sequence [8]. The conversion mechanism of PrP^C to PrP^{Sc} is still poorly understood, and several groups have suggested that an additional unknown cofactor could initiate or modulate this conversion [8–12]. Nucleic acids bind to PrP^C and, depending on their concentration and sequence, can exert catalytic or inhibitory effects on the conversion of PrP isoforms [9].

The search for a chemotherapeutic approach has focused on screening for anti-prion compounds to block the conversion of

☆ Presented at the 41st International Symposium on High Performance Liquid Phase Separations – HPLC 2014, 10–15 May 2014, New Orleans, LA, USA.

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http://dx.doi.org/10.1016/j.chroma.2014.12.014
0021-9673/

PrP^C to PrP^{Sc} [13]. These compounds may bind directly to PrP^C and stabilize its conformation, preventing the conversion to PrP^{Sc} and inhibiting aggregation.

Several compounds and synthetic peptides have been screened as potential antiprion agents, including polyanions (e.g. sulfated glycans) [11,14,15], curcumin [16], Congo red [17], cyclic tetrapyrroles [18], and others [13]. Antimalarial compounds were also investigated as potential antiprion agents; among them, quinacrine was identified as one of the most potent compounds, with reported IC_{50} values ranging from 0.3 to 0.5 μM in neuroblastoma cells [19,20]. Quinacrine has a high affinity for a PrP carboxyl-terminal binding site (Tyr225, Tyr226 and Gln227), with a K_D ranging from 1 to 5 mM [1,21,22].

Currently, the screening process for anti-prion compounds is often conducted through in vitro assays, using cell culture models, prevention of PrP amyloid formation and competitive binding assays. However, these approaches involve evaluating single isolated compounds and cannot isolate an active compound from a complex mixture.

Assuming that the prerequisite for anti-prion activity of a compound is affinity for the prion protein [23], as has been demonstrated with quinacrine, an affinity-based assay to isolate and identify anti-prion agents in compound mixtures would be beneficial. An approach that is gaining momentum for the identification of novel ligands from complex matrix is ligand fishing [24]. In this approach, the targeted protein is immobilized onto the surface of a stationary phase, predominantly, magnetic beads, and the resulting protein-based stationary phase is used to "fish" out the active compounds from a complex mixture [24,25].

Ligand fishing by derivatized magnetic particles was selected due to its versatility in the isolation of ligands from complex matrixes, such as natural product [26–30] and cell extracts [31,32], based on ligands/immobilized protein affinity. In this approach, the protein-coated magnetic beads are immersed into the complex matrix, and the non binders remain in solution or are removed by washing, while the bound ligands are eluted in an appropriate organic phase and can be identify by UHPLC–MS/MS.

To this end, we immobilized PrP^C onto the surface of magnetic beads, and the resulting protein coated beads were used for ligand fishing in a compound mixture. PrP-coated magnetic beads were characterized through fluorescence confocal microscopy. The proposed ligand fishing approach was validated by monitoring the capacity of the assay to isolate ligands from a mixture of compounds. Non-bound compounds and isolated ligands were quantified via UHPLC–ESI-MS/MS. The compound isolated from the mixture was the only one that demonstrated high inhibitory activity in aggregation formation studies, showing the correspondence between PrP^C affinity and anti-aggregation activity. Therefore, the results indicate that this approach is promising for the isolation of potential anti-prion compounds from complex matrixes, as well as of possible cofactors from cellular extracts, that could be involved in the deflagration of prion diseases.

2. Materials and methods

2.1. Reagents and chemicals

All chemicals were analytical or reagent grade and were used without further purification. Caffeine, quinacrine dihydrochloride and thiamine hydrochloride were purchased from Sigma Chemical (St. Louis, USA). Mouse recombinant prion protein (rPrP) 23–231 was expressed in *Escherichia coli* and further purified by high-affinity column refolding, as previously described [33]. Microtubes with a 2 mL capacity were obtained from Axygen Scientific (Union City, USA). The 1 μm BcMag® amine-terminated magnetic beads (50 mg/mL) were purchased from Bioclone Inc. (San Diego, CA). Ammonium acetate (≥99%), water and acetonitrile for mass spectrometry were obtained from Fluka (St. Louis, USA). Before being used in LC–MS/MS analysis, the buffer solutions were filtered through cellulose nitrate membranes (0.45 μm) provided by Phenomenex.

2.2. Apparatus

The chromatographic experiments were performed using a Shimadzu UHPLC system (Shimadzu, Kyoto, Japan), consisting of two LC 30AD pumps, an autosampler equipped with a 100 μL loop (SIL 30AC) and a UV-visible detector (SPD-M30A) interfaced to an amaZon SL ion trap mass spectrometer with electro-spray ionization as the ion source (Bruker Daltonics). The MS parameters were set at 5 L min^{-1} for the drying gas flow, 15 psi for the nebulizer pressure and 300 °C for the drying gas temperature. Data acquisition was accomplished on a Shimadzu CBM-20 A system interfaced with a computer equipped with Highstar 3.2 software (Shimadzu, Kyoto, Japan).

Far-UV Circular Dichroism – CD spectra were recorded in a Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan) at 25 °C with circular 0.10-mm-pathlength cells. Buffer spectra were subtracted from each sample spectrum, and traces were collected with four accumulations each.

Light scattering (LS) measurements were recorded on an ISSPC1 fluorometer (ISS, Champaign, IL). LS at 90° was measured illuminating the samples at 320 nm and collecting LS from 300 to 340 nm.

Spectrophotometric assays were conducted in a Shimadzu UVmini-1240 UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan).

2.3. Prion protein immobilization onto the surface of magnetic beads

Prion protein was covalently immobilized onto the surface of silica-based magnetic beads (BcMag amine-terminated magnetic beads, Bioclone), following the protocol provided by Bioclone Inc., with slight modifications. First, 9 mg of BcMag amine-terminated magnetic beads (MBs) were washed with 1 mL of 10 mM pyridine buffer pH 6.0 in a 2 mL microtube. The supernatant was discarded after magnetic separation. The MBs were suspended in 1 mL of 5% glutaraldehyde and shaken for 3 h. After magnetic separation, the MBs were washed three times with 1 mL of 10 mM pyridine buffer at pH 6.0 to remove the unreacted glutaraldehyde. The amount of PrP used in the immobilization step was optimized and the following amounts were evaluated: 0.5; 1.0; 1.5 and 2.0 mg of PrP were incubated overnight with 3 mg of MBs in 10 mM pyridine buffer at pH 6.0 under gentle rotation at 4 °C. The highest yield was obtained using 1 mg of PrP^C to 3 mg of MBs. The stability of PrP^C under these conditions was ensured by the absence of aggregate formation and isoform conversion, tested by light scattering and circular dichroism measurements. Magnetic separation was then performed and the supernatant was used to estimate the amount of immobilized PrP, based on the molar extinction coefficient of PrP (63,495 cm^{-1} M^{-1} at 280 nM [34]). The PrP-coated MBs were incubated with 1 M glycine or 1 M hydroxylamine solution for 30 min at 4 °C to quench all residual aldehyde groups. The PrP-coated MBs were washed twice with 1 mL of 5 mM ammonium acetate buffer pH 7.4 and then stored in the same buffer at 4 °C.

Control-MBs (blank experiments) were prepared through the same procedure but without the addition of PrP.

2.4. Morphological characterization

PrP was labeled with amino-reactive fluorescein isothiocyanate (FITC) prior to immobilization to evaluate the success of the