Photocatalytic degradation of prions using the photo-Fenton reagent

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Summary Prions are proteinaceous infectious agents postulated to be the causative agents of a group of fatal neurodegenerative diseases known as transmissible spongiform encephalopathies (TSEs). A known iatrogenic transmission route of TSEs to humans occurs via prion-contaminated surgical instruments or biological materials. Prions, unlike most common pathogens, exhibit an extraordinary resistance to conventional decontamination procedures. We have recently demonstrated that the application of TiO2-based heterogeneous photocatalytic oxidation is able to significantly reduce prion infectivity. The present study investigates the potential of a homogeneous photocatalytic method, based on the photo-Fenton reagent, to degrade prion proteins. We show that the photo-Fenton reagent efficiently degrades not only recombinant prion proteins, but also the total protein amount from brain preparations of naturally or experimentally infected species and PrPSc (PrP scrapie) contained in sheep scrapie brain homogenates. © 2008 The Hospital Infection Society. Published by Elsevier Ltd. All rights reserved.

Introduction

Prion diseases or transmissible spongiform encephalopathies (TSEs) comprise a group of fatal degenerative diseases of the central nervous system affecting both humans and animals.1 Human TSEs are categorised into acquired, sporadic and
genetic disorders. Nosocomial transmission has been observed after medical treatments with prion-infected surgical tools or by the use of infected biological materials from affected individuals.

Prions contained in liquid waste or on contaminated surgical instruments and devices cannot be fully inactivated by procedures commonly used to inactivate viruses and bacteria. The World Health Organization guideline about the use of single-use disposable surgical items in patients with confirmed or suspected TSEs can only be seen as a transient solution. First, it is cost-ineffective; second, asymptomatic carriers cannot be excluded as potential sources of the infectious agent. This is of importance, considering the long incubation time of the disease and the variety of tissues which may harbour infectivity. Although prion diseases in humans are rare, the risk of an iatrogenic transmission cannot be tolerated by any health authority. Thus, prion inactivation or decontamination of surgical tools remains a prominent topic of prion disease research.

Recently, our group demonstrated that prion infectivity persists over years even upon exposure to environmental conditions. Effective treatment of liquid waste originating from facilities that handle prion-infected or possibly infected materials such as biochemical laboratories, hospitals, abattoirs and farms limits the bioavailability of the pathogen and significantly decreases the risk of interspecies transmission. The application of a novel oxidation process, in particular the titanium dioxide (TiO2)-mediated photocatalytic oxidation, can significantly reduce prion infectivity. Heterogeneous photocatalysis belongs to the advanced oxidation processes (AOPs), a group of physicochemical oxidation methods used mainly for the decontamination of water and air polluted with organic compounds. The different AOPs share a basic characteristic, the generation and use of powerful transitory species, principally the free hydroxyl radicals (OH·), the strongest oxidative oxygen-based species in nature. Together with other highly oxidant species (e.g. peroxide radicals) they easily attack organic pollutants non-selectively, resulting in their complete mineralisation. Moreover, photocatalytic oxidation mainly mediated by TiO2, in the presence of artificial or solar light, has been reported in several cases to successfully inactivate viruses, and pathogenic bacteria both in air and liquid media.

The photocatalytic degradation of proteins involves several chemical and photocatalytic stages and a great number of intermediates. Previous studies have proposed mechanisms of oxidative cleavage mediated by OH·, which are able to attack the main and the side chains of proteins, producing molecules with lower molecular weight and finally CO2.

The application of Fenton or Fenton-like reactions to protein degradation and particularly prions has been reported. We aimed to determine whether a modification of the classical Fenton system, the so-called 'photo-Fenton reagent' (Fe3+/H2O2/UV-A, visible light), which also belongs to the AOPs, has the potential to oxidise prions.

Methods

Substrates for photo-Fenton treatment

Bovine serum albumin (BSA) was obtained from Sigma–Aldrich (Athens, Greece). Recombinant proteins, i.e. 10×His (10 histidine)-tagged bovine and ovine prion proteins (PrPs) were expressed in E. coli BL21 (DE3) cells. Denatured proteins were purified from inclusion bodies by affinity chromatography. Before photocatalytic treatment, aliquots of each protein were precipitated by mixing with excess of methanol. The pellets were resuspended by vortexing in the photo-Fenton mixture described below.

Prion-infected brain tissue from different species including human (sporadic Creutzfeldt–Jakob disease, sCJD), cow (bovine spongiform encephalopathy, BSE), sheep (scrapie), hamster (263K scrapie) and mouse (79A scrapie and 301 V BSE) was also used in the study. Brain homogenates (10% w/v) were prepared in phosphate-buffered saline (pH 7.4), 0.5% w/v sodium deoxycholate, 0.5% w/v Igepal (a non-ionic detergent) and 5 mM phenylmethanesulphonylfluoride. A 20% w/v sheep scrapie brain homogenate was similarly prepared. The initial total protein amount of the recombinant proteins and of the different brain homogenates was estimated by the Bradford assay.

Photocatalytic experiments

Analytical grade FeCl3·6H2O and H2O2 were purchased from Alfa-Aesar (Karlsruhe, Germany) and Merck Chemicals (Athens, Greece) respectively. Experiments were performed at room temperature in uncapped disposable 1.5 mL plastic tubes and the reaction mixture was gently stirred during the photocatalytic treatment. Irradiation was carried out using two parallel 8 W blacklight blue
fluorescent tubes, mounted in standard 8 W fluorescent tube holders (TLD 8 W/08, Phillips, Athens, Greece), 5 cm above the irradiated solution surface. The light intensity at this position, in the UV-A region of 340–400 nm, was measured using a Photometer/Radiometer PMA 2100 (Solar Light Co., Glesnide, Pensilvania) equipped with a UV-A detector and found to be 6.1 ± 0.3 mW/cm².

The final reaction volume for each tube was 50 μL. BSA or recombinant PrPs were photocatalytically treated for 150 min in the presence of UV-A with 12 μg/mL Fe³⁺ and 500 μg/mL H₂O₂ at pH 3.0 adjusted with 1 N HClO₄. After the treatment the equivalent of 3 μg of the initial protein content was loaded per lane on a 12% sodium dodecyl sulphate (SDS) acrylamide gel and stained with Coomassie Brilliant Blue R250.15 Samples of whole-brain homogenates were diluted in distilled water and treated with 500 μg/mL Fe³⁺ and 5000 μg/mL H₂O₂ at pH 3.0 in the presence of UV-A. Samples were analysed on a 12% SDS acrylamide gel. Total proteins were stained with silver nitrate, whereas PrPs were detected by western blot. Control reactions were handled similarly, but in combinations in which at least one of the photo-Fenton components (Fe³⁺/H₂O₂/UV-A, visible) was absent.

Semiquantification of PrP immunodetection

In order to assess the sensitivity limit of PrP detection in this study, serial dilutions of the sheep scrapie homogenate were subjected to immunoblotting as described below. The initial load of 1800 μg was serially diluted in 2-fold steps down to 0.88 μg brain equivalents (Figure 1A).

Electrophoresis and immunoblotting

SDS–polyacrylamide gel electrophoresis (PAGE)-analysed samples of BSA and recombinant prion proteins were stained with Coomassie Brilliant Blue. Gels were scanned and analysed densitometrically as described below.

Degradation of proteins contained in brain homogenates was visualised by the more sensitive silver staining. PrP degradation was monitored by western blotting. Sheep scrapie brain homogenates were analysed by SDS–PAGE and proteins were transferred on to polyvinylidene fluoride (PVDF) membranes. Blots were probed with the anti-PrP monoclonal antibody 8G8 (recognising amino acids 111–118 of the human PrP) diluted 1:3000 from 1 mg/mL stock in blocking buffer (PBS containing 0.1% v/v Tween 20 and 5% w/v skimmed milk). As secondary antibody, a rabbit antimouse—horseradish peroxidase conjugate (Pierce, Athens, Greece) diluted 1:5000 in blocking buffer was used. Prion proteins were visualised both with the enhanced chemiluminescence (ECL, Pierce) and with the more sensitive SuperSignal West Femto Substrate (Pierce), able to detect femtograms of protein, according to the manufacturer’s instructions.

Densitometry

Densitometric measurements were performed with the ImageJ software (version 1.37v, available at http://rsb.info.nih.gov/ij/) through the analysis of multiple film exposures to ensure that comparisons were made within the linear range of the film. As relative band density (R) we define:

\[ R = \frac{a(t=x)}{a(t=0)} \]

where \( a \) is the absolute value of the area under the curve created by the protein band at the investigated time point.

Results

Photo-Fenton treatment of pure proteins

Initially, we tested the effect of the photo-Fenton reactivity on BSA, bovine and ovine PrP at an initial protein concentration of 150 μg/mL. After 150 min of photocatalytic treatment with 12 μg/mL Fe³⁺ and 500 μg/mL H₂O₂, proteins were undetectable on Coomassie Brilliant Blue-stained SDS–PAGE gels (Figure 2 A, C). Control experiments under the same conditions but in the absence of the photo-Fenton reagent showed no or partial degradation, as expected, in the case of Fe³⁺/H₂O₂ in the absence of light (Figure 2B).

Photo-Fenton treatment of infected brain homogenates

Protein oxidation, mediated by the Fe³⁺/H₂O₂/UV-A system, was also confirmed in experiments in which the whole protein content of 1% w/v brain homogenates from different species was exposed to the oxidative environment. The initial total protein amount of the different 10% w/v homogenates ranged between 1.8 and 2.1 mg/mL.

Figure 2D shows that after 2 h of UV-A illumination in the presence of 500 μg/mL Fe³⁺ and 5000 μg/mL H₂O₂ (pH 3.0), complete degradation of the total protein load was accomplished, as
Figure 1  (A) 2-fold serial dilutions of the untreated 20% w/v sheep scrapie homogenate. The amount of brain tissue loaded in each lane (in μg) is indicated on the top. Blot was developed with the West Femto substrate. (B) Degradation of PrP in relation to illumination time after densitometric analysis of proteins detected with the ECL substrate in ( ■ ) 1, ( ● ) 5 and ( ▲ ) 10% w/v sheep scrapie homogenates. (C-H) Western blots of photo-Fenton (500 μg mL⁻¹ Fe⁺³, 5000 μg mL⁻¹ H₂O₂) treated 1 (C, D), 5 (E, F) and 10% w/v (G, H) sheep scrapie homogenates for the indicated time points. The untreated sample (t: 0 min) contains 0.35, 0.9 and 1.8 mg brain equivalent respectively. Arrow: 32.5 kDa molecular mass standard.
demonstrated by silver-stained SDS–PAGE gels. Similarly to the recombinant protein control reactions, in which at least one component of the photo-Fenton system was absent, this showed no or minor protein degradation (data not shown).

Serial dilution immunoblotting was performed to estimate the minimum tissue amount required for PrP detection. A total of 7 µg of brain tissue was found to contain the minimum amount of PrP detectable by the applied protocol (Figure 1A).

In order to monitor the degradation of PrPSc, 1% w/v sheep scrapie homogenate was treated with 500 µg/mL Fe3+ and 5000 µg/mL H2O2 (pH 3.0). Samples at different time points were subjected to PrP detection by immunoblotting. As demonstrated in Figure 2D, within 2 min of treatment PrP is undetectable even when visualised with the West Femto substrate (Figure 1F, H), whereas western blots of control reactions showed no or minor decrease of the PrP signal under the same treatment conditions (Figure 3).

Discussion

Numerous studies have dealt with the oxidation of model compounds and real effluents by the Fenton reagent, a mixture of iron (II) salts and H2O2. In this technology, the ferrous and/or ferric cation decomposes H2O2, to generate powerful oxidising agents, able to degrade organic and inorganic substances. The process involves a large number of reactions including redox, complexation, precipitation, equilibrium reactions, and so on. Fenton reagent is an attractive oxidative system, which produces OH· radicals (Equation 1) in a very simple way for waste-water and soil treatment, since iron is an abundant, non-toxic element and H2O2 is easy to handle and environmentally safe.
Fe$_{3}^{3+}$ + H$_{2}$O$_{2}$ → Fe$_{2}^{3+}$ + OH$^{-}$ + OH$^{-}$. 

Fe$_{3}^{3+}$ + H$_{2}$O + hv (< 450 nm) → Fe$_{2}^{2+}$ + H$^{+}$ + OH$^{-}$.

The reaction can be greatly enhanced by UV/visible light (artificial or natural), producing additional OH$^{-}$ and leading to the regeneration of the catalyst [Equation (2), photo-Fenton reaction].

This interconversion of ferrous and ferric ions may continuously catalyse the generation of OH$^{-}$, thus eliminating the need for further addition of ferrous salts. These reactions are the primary forces of the photochemical self-cleaning of atmospheric and aquatic environment.

In the present study the use of the Fe$_{3}^{3+}$/H$_{2}$O$_{2}$ system, as previously mentioned, leads according to Equations (1) and (2) to the production of OH$^{-}$, which attack proteins and other organic molecules such as nucleic acids, lipids and carbohydrates (data not shown) present in the brain homogenate preparations.

Figure 2 demonstrates a significantly faster degradation rate of BSA compared with bovine or ovine PrP under the same experimental conditions. This result could be attributed to the nature of the three different protein molecules. BSA is completely soluble in aqueous media in contrast to the hydrophobic recombinant prion proteins which tend to form aggregates. Thus, the main chain and the side chains of BSA molecules are more likely to be approached and attacked by OH$^{-}$ compared with the hydrophobic sites of the recombinant PrP molecules, which limit the interaction with the oxidising species.

It must be noted that, whereas recombinant prion proteins are not glycosylated, as PrP$^{Sc}$ they form
aggregates and are still a good indicator for prion degradability.

Silver-stained gels in Figure 2D suggest that the photocatalytic treatment causes not only the elimination of PrP, but of the entire protein load. The fact that OH· non-specifically oxidises and destroys organic molecules present in a tissue homogenate, suggests that it would cause the indistinguishable degradation of any kind of organic component that might be involved in TSE pathogenesis. The extent of degradation depends only on the ratio of the initial organic load to the amount of the photo-Fenton reagent.

Photo-Fenton-mediated homogeneous photocatalytic oxidation of PrP, present in sheep scrapie brain homogenates, has been investigated (Figure 1). Treatment of 1% w/v scrapie-infected brain tissue with 500 μg/mL Fe3+ and 5000 μg/mL H2O2 (pH 3.0) eliminates PrP immunoreactivity in <2 min (Figure 1D), reducing the initial PrP content at least 50-fold. Even in the case of the 10% w/v homogenate, PrP was efficiently degraded under the same conditions within 5 h of treatment. In this case, the reduction of initial PrP content exceeded 250-fold (Figure 1H). Control reactions (Figure 3) verify that efficient degradation of PrP occurs only when all components of the photo-Fenton system are present. Figure 3B and D shows the enhanced sensitivity of the West Femto substrate. It must be stressed that the amount of PrP and of organic residues adsorbed on contaminated surgical instruments, or even in liquid waste, is remarkably lower than in a 10% w/v homogenate. This fact provides indications for even shorter treatment times in scaled-up applications.

Laboratory-scale photocatalytic treatment of sheep scrapie homogenates is shown to be effective, concerning prion degradation, as detected by immunoblotting. Nonetheless, further research is required for the development and exploitation of photo-Fenton in a possible application, e.g. for routine decontamination of surgical instruments or biochemical waste. Certain advantages of this photocatalytic process render it a promising tool for the future development of a mature application. Mild operational conditions such as room temperature, atmospheric pressure, user and environmentally safe reagents are required. The cost of equipment, reagents and energy is low, suitable for routine use in healthcare facilities. It also has the potential to be successfully scaled up. As shown in studies with other types of organic pollutants, the results obtained after scaling up are in good agreement with those in laboratory scale. Moreover, the OH· radicals can easily attack soluble, insoluble or even immobilised organic targets. Some of the OH· radicals are generated, according to Equation (1), in the absence of illumination. The contribution of the illumination to the process is, mainly, the regeneration of the catalyst [Fe2+, Equation (2)]. Thus, direct illumination of contaminated surfaces is not necessary to achieve degradation of the absorbed organic residues.

In conclusion, the current study demonstrates the potential of the photo-Fenton reagent to effectively degrade prions, through the oxidative attack of powerful transitory species. Indeed, optimisation and validation of protocols is required before this process could evolve to a mature application, along with more sensitive detection methods for the routine assessment of instrument cleaning. However, the effectiveness and the simplicity of the technique, the mild operational conditions and the low cost of the reagents and equipment, render it a promising tool for prion inactivation.

Conflict of interest statement
None declared.

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References


