Original Article

The integrity of chemically treated plasmid DNA as a chemical-based choice for prion clearance

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1. Introduction

Regenerative medical products manufactured by culturing cells carry the risk of developing transmissible spongiform encephalopathy (TSE) due to ruminant-derived materials such as bovine serum that are often applied in the media. Since the products are composed of living cells, it is difficult to remove or inactivate adventitious agents such as prions and viruses during the manufacturing process. Therefore, it is necessary for the raw materials to be free of adventitious agents prior to production in order to prevent contamination to the final product. When we manufacture regenerative medical products using raw materials derived from humans and animals, we need to adopt the “Standard for Biological Raw Materials” in order to ensure the safety of such raw materials. According to the “Standard for Biological Raw Materials”, the “raw material” specifically means a tissue collected from humans or animals, an extract or a pooled thing such as a tissue/a body fluid [3]. These are used as starting materials to produce the raw materials used in the manufacture products such as pharmaceuticals, medical devices, and regenerative medicine, and then the “Standard for Biological Raw Materials” clearly outlines the range of the acceptable raw materials associated with each product [3].

Taking human insulin used for production of regenerative medicine as an example, the insulin is the raw material for producing regenerative medicine products, in other words an auxiliary material. This auxiliary material must meet the requirements of the
“Standard for Biological Raw Materials”. Therefore, the raw materials used to produce the insulin as the auxiliary material, specifically the peptone contained in the culture medium of E. coli need to meet the requirements of the “Standard for Biological Raw Materials” [2,3]. In contrast, the raw materials used in the construction of a cell bank, which includes cells such as E. coli that produce human insulin (recombinant protein) included as medium components in cell culture steps, are exempt from this regulation [3]. Since bovine insulin (recombinant protein) included as medium components in the product.

Example plasmid DNA on the raw material for the regenerative medical materials.

In cases involving the use of plasmid DNA, there are still some issues associated with the existence of ruminant-derived peptones in medium components used for the replication of plasmid DNA in E. coli. Because we could not confirm the absence of prions when the origin of peptones is unknown and is part of the medium of E. coli, we could not use the plasmid DNA as a raw material (Fig. 1). In order for plasmid DNA to meet the requirements of the “Standard for Biological Raw Materials”, it is necessary that peptones is not made of animal-derived components, or that it be treated by high temperature and alkali solution. Currently, peptones that are made of non-animal-derived raw materials are various derived of microbe or plants, specific examples include soybean, milk, yeast and the likes.

Furthermore, an infection with prions is different from conventional infections because the hypothesized role of proteins as adventitious agents is not caused by organisms with nucleic acids such as bacteria or viruses but by the conformational change from the isoform of native proteins to misfolded proteins [4–6]. Consequently, general sterilization methods, which have a significant effect on microbes and viruses (i.e., autoclave at 121 °C for 15 min or UV-ray irradiation), are not often effective against prions, so prion-specific sterilization methods are required in the medical field for items such as surgical instruments, medical devices, and blood products [7–9]. The previously reported methods of removing or inactivating prions have been designed for surgical instruments or medical devices but not for plasmid DNA. Additionally, chemical reagents such as sodium hydroxide (NaOH) or chlorine for inactivation have been found to corrode the raw materials [7].

At any rate, it is not clear whether the potency of plasmid DNA is maintained by adding chemicals to the plasmid DNA. The plasmid DNA was used as an example in this study, and chemical treatments were performed using chemical reagents in the methods that were reported to inactivate prions [1]. Therefore, we examined the potency of plasmid DNA that could be maintained from chemical reagents.

2. Materials and methods

2.1. Plasmid DNA

Plasmid DNA was originally purchased as a pAcGFP1-C1 vector from Takara Clontech. The vector is a full-length 4.7 kb that contains the kanamycin/neomycin resistance gene, human cytomegalovirus (CMV) immediate early promoter, and the Aequorea coerulescens green fluorescent protein (GFP) gene. Transformation was performed using E. coli strain DH5-alpha competent cells (Takara-bio, 9057) according to the instruction manual as follows: 100 µl of competent cells and 10 ng of plasmid DNA were gently mixed, kept on ice for 30 min, heated at 42 °C for 45 s, and left on ice for 2 min. Up to a total of 1 ml of additional SOC medium (Takara-bio, 9057) was incubated at 37 °C in an incubator-shaker for 1 h. The mixture was spread onto an LB plate with a final 30 µg/ml kanamycin application (Sigma–Aldrich, K4000) and was incubated at 37 °C overnight. The next day, LB broth (Sigma–Aldrich, L7275; composed of 10 g/l enzymatic digest of casein [tryptone], 5 g/l yeast extract [low sodium], 5 g/l sodium chloride, and 2 g/l invert binding agents) with kanamycin was incubated for 20–21 h and was purified using NucleoBond Xtra Midi (Takara-bio, Clontech). The plasmid DNA was eluted and prepared by elution buffer (5 mM Tris/HCl, pH8.5).

2.2. Inactivation

For the purpose of inactivating prions, we selected four chemical reagents: 7% guanidine hydrochloride (Gdn-HCl), 3M guanidine-thiocyanate (Gdn-SCN), 3M Trichloroacetic acid (TCA), and 3% sodium dodecyl sulfate (SDS), which were prepared from 8 mol/l guanidine hydrochloride solution (Nacalai), 6 mol/l guanidine-thiocyanate solution (Nacalai), trichloroacetic acid solution 100 w/v% (Nacalai), and 10% SDS solution with DNase/RNase-free water (Invitrogen). A mixture with 50 µl of the plasmid DNA and 450 µl of the chemical reagent was kept for 2 h at room temperature, except for 3% SDS, which was additionally boiled for 3 min at 100 °C. The plasmid DNA treated with chemical reagents was precipitated with ethanol, and it was made soluble by elution buffer (5 mM Tris/HCl, pH8.5) (Fig. 2). The plasmid DNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

2.3. Transfection

HEK293 (JCRB9068) cells were obtained from the National Institutes of Biomedical Innovation, Health and Nutrition (JCRB Cell Bank, Osaka, Japan). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Nacalai) with 10% fetal bovine serum (GE HyClone) in 5% CO₂ at 37 °C for 5 days. HEK293 cells were seeded at...
1.25 × 10^5 cells per well in 24-well plates (Iwaki) in 0.5 ml of culture medium and cultured overnight. The next day, in order to transfect the plasmid DNA into HEK293 cells, a diluted DNA (Gdn-HCl, Gdn-SCN, SDS) was added to each well (500, 250, or 50 ng/well) in Opti-MEM (Invitrogen). The DNA-Lipofectamine LTX Reagent complex was incubated for 30 min at room temperature and added to each of the wells containing cells. The cells were incubated at 37 °C in a CO₂ incubator for 2 days post-transfection. The cells were photographed using the inverted microscope (Leica, DM IL LED) with phase contrast and a monochrome camera (Leica, DFC3000G). In order to identify the expression of GFP protein, the image was taken using the excitation filter of 460 nm–500 nm. These photographs were processed using the image analysis software (Leica Application Suite X, version 3.4.2.18368).

2.4. Flow cytometry

The transfected and non-transfected cells collected from 24-well plates were analyzed using a BD FACS Calibur (BD Biosciences, FL1: 530 nm) after passing through 35-μm nylon mesh without staining cells. The data were analyzed utilizing BD CellQuest Pro software (BD Biosciences).

2.5. Western blot

The protein solution (10 μg) was prepared using a polyacrylamide gel (12% Bis-Tris Gel, Invitrogen), and was then transferred on a PVDF membrane. Anti-GFP rabbit antibody (abcam; ab183734, 1:10,000) or anti-β-actin rabbit antibody (Cell Signaling; #4970, 1:1000) was used as the primary antibody with anti-rabbit IgG HRP-linked antibody (Cell Signaling; #7076, 1:1000) as the secondary antibody. The target proteins were detected with ECL Reagent (Cell Signaling) and a ChemiDoc Touch Imaging System (Bio-Rad).

2.6. DNA integrity (agarose gel electrophoresis and bioanalyzer)

Restriction enzyme digestion was performed on the plasmid DNA with EcoR1 (Takara-bio). So as to separate the sizes of the DNA fragments, the concentration of agarose (Nippon Gene) in a gel was prepared at 0.8%. The DNA samples were added with dye-loaded DNA fragments, and electrophoresis was performed at 100 V for 30 min after loading a molecular weight ladder (1 kbp, Nacalai) or the DNA samples into the lane of the gel. The post-electrophoresis gel was dipped into DNA staining solution (Atto, 1,10,000) and diluted TBE buffer (Atto) at room temperature for 15 min, and the gel was analyzed by a ChemiDoc Touch Imaging System (Bio-Rad). Additionally, the plasmid DNA digested by EcoR1 was measured size and integrity using the Bioanalyzer instrument (Agilent, 2100 Bioanalyzer) and DNA 7500 kit (Agilent).

3. Results and discussion

“Regarding the Implementation of the Standards for Biological Ingredients” indicates methods to apply when using raw materials derived from biological sources (excluding plants) [3]. For heat-resistant substances, methods involving autoclaving or chemicals are indicated (e.g., after autoclaving at 121 °C for 30 min in 1 mol/l NaOH, wash, rinse with water, and employ conventional sterilization), while for non-heating resistant substances, one of the methods involving chemical reagents is indicated (i.e., cover with 2 mol/l NaOH or undiluted sodium hypochlorite, incubate for 1 h, and then rinse with water) [3]. Despite the differences in these methods, the effect of prion inactivation is known to be somewhat consistent in the results [8]. Tateishi et al. treated the mouse-adapted CJD Fukuoka-1 strain with alkali and acid (NaOH, HCl, or formic acid), chaotropic ions (Gdn-HCl, Gdn-SCN, or TCA), de-naturants (SDS), and organic solvents (phenol), and they then showed that chemical reagents, formic acid (60% or 80%), 7M Gdn-HCl, 3M Gdn-SCN, 3M TCA, 3% SDS, and phenol (50% or 80%), could generate a 7-log reduction [1]. Therefore, we chose four chemical reagents (Gdn-HCl, Gdn-SCN, TCA, or SDS) that we could easily obtain from among chemical reagents that would completely block prion transmission and are relatively easy to handle. In order to ascertain the agreement between the plasmid DNA treated by chemicals and the plasmid DNA untreated by chemicals, we evaluated the following points: 1) the condition of plasmid DNA (i.e., integrity of DNA and nucleotide sequencing) and 2) the function of plasmid DNA (i.e., protein production with transfecting into HEK293 cells) (see Table 1).

First, we examined the initial concentration and the amount of the chemically treated plasmid DNA. The DNA yield using ethanol precipitation after chemical treatment, except for TCA treatment, tended to increase based on initial concentration and amount of DNA that was treated by Gdn-HCl, Gdn-SCN, and SDS. When the DNA concentration is 300 ng/μl, it is high for the DNA yield to treat Control, Gdn-SCN and DDS. TCA treatment can hardly recover DNA. Gdn-HCl has a tendency to have a slightly lower recovery than Gdn-SCN and SDS (Table 2). Thus, the chemically treated plasmid DNA was precipitated by ethanol, and the purified plasmid DNA was named first DNA, and the plasmid DNA that amplified and purified the first DNA in E. coli was named second DNA (Fig. 2). The mutation and deletions in DNA induced by the chemicals are known.
Validating that plasmid DNA do not have any the mutation and the deletion due to the chemicals used, the first DNA was examined the nucleotide sequence using the sequence equipment. The second DNA was also examined to show that the chemically treated plasmid DNA could be amplified in E. coli without problem. Next, we ascertained the DNA fragment from the band of agarose gel electrophoresis and bioanalyzer. The DNA fragment was near 4.7 kb in length and the band was present, with the exception of TCA treatment. Moreover, as a result of bioanalyzer’s electropherogram, a peak was observed at aligned migration time 82s in Gdn-HCl, Gdn-SCN and SDS except for TCA (Fig. 3). In contrast, when the band was absent from the DNA fragment, it was suggested that the plasmid DNA was disrupted by TCA. TCA exposure was previously reported to result in chromosomal anomalies such as chromosome break, fragments, and chromatid exchanges [10]. Furthermore, the band was also present in the second DNA of the chemical reagents, Gdn-HCl, Gdn-SCN, and SDS (Fig. 3).

When the first and second DNA were transfected into HEK293 cells, which existed in the production of green fluorescence protein (Supplementary Fig. 1, Fig. 4), flow cytometry analysis showed that the population of chemically treated GFP-positive cells were equal at 49.97% and 52.68% in the first DNA and second DNA of the control group of 500 ng/well (Supplementary Figs. 1 and 2). Alternatively, when the different lot of plasmid DNA were transfected into HEK293 cells, the rate of GFP positive cells tend to increase depending on amount of plasmid DNA in the second DNA (Figs. 4–6).

*Fig. 3.* Integrity of DNA fragmentation using agarose gel electrophoresis and Bioanalyzer. The DNA fragmentation, pAcGFP1-C1 vector, has a length of 4731 bp. The ethanol precipitation was performed with a group of chemical reagents (Gdn-HCl, Gdn-SCN, TCA, or SDS) and in a group (Control) to which elution buffer was added instead of chemical reagents. The plasmid DNA without ethanol precipitation was named “Before.” The lower peak (aligned migration time, 35s) or upper peak (aligned migration time, 88s) of bioanalyzer’ electropherogram is marker. (A) Integrity of DNA fragmentation using agarose gel electrophoresis (Lot. C). (B) Integrity of chemically treated DNA fragmentation using Bioanalyzer (Lot. C). (C) Integrity of original DNA fragmentation using Bioanalyzer.

| Reagents | Characteristics          |
|----------|--------------------------|
| Gdn-HCl  | mRNA isolation, chaotropic ion |
| Gdn-SCN  | mRNA isolation, chaotropic ion |
| TCA      | Caustic agent, chaotropic ion |
| SDS      | Protein denaturant, anionic detergent |

| Table 1  |
|------------------|------------------|
| The characteristics of four chemicals used in inactivation. |
| Reagents | Characteristics          |
|----------|--------------------------|
| Gdn-HCl  | mRNA isolation, chaotropic ion |
| Gdn-SCN  | mRNA isolation, chaotropic ion |
| TCA      | Caustic agent, chaotropic ion |
| SDS      | Protein denaturant, anionic detergent |

| Table 2  |
|------------------|------------------|
| The processing of plasmid DNA. (a) The initial concentrations and amounts of plasmid DNA before chemical treatments. (b) The DNA yield of plasmid DNA using ethanol precipitation after chemical treatment. The DNA yield was calculated using the amount of DNA after ethanol precipitation (μg) ÷ the initial amount of DNA (μg) × 100. |
| (a) |
| Concentration (ng/μl) | Amount (μg) |
| Lot. A | 47.4 | 2.37 |
| Lot. B | 100.0 | 5.0 |
| Lot. C | 300.0 | 15.0 |
| (b) |
| DNA yield (%) | Control | Gdn-HCl | Gdn-SCN | TCA | SDS |
| Lot. A | 27.43 | 17.72 | 34.68 | 5.99 | 31.81 |
| Lot. B | 86.56 | 71.88 | 80.8 | 2.08 | 85.52 |
| Lot. C | 99.12 | 88.36 | 105.28 | 1.68 | 104.84 |
Fig. 3. (continued)
In an analysis of the nucleic acid sequence of the first DNA in Gdn-HCl, Gdn-SCN, and SDS, DNA fragments found by agarose gel electrophoresis showed no gene mutation (data not shown). These results indicate that plasmid DNA could withstand the chemicals used for prion inactivation.

When normal prion proteins change into transmissible abnormal prion proteins and these altered proteins accumulate in the brain, prion disease develops. Prion disease, which is known as BSE in cows, affects both humans and animals. Infection is suspected to occur through the intake of the bovine parts containing BSE, and the Japanese regulatory authority has paid careful attention to ensure the safety of raw materials such as beef or gelatin from bovine sources in the fields of animal husbandry, food, and medicine. Moreover, the route of transmission in the medical field is expected to occur through surgical instruments, medical devices, blood products, and biopharmaceuticals [11,12].

Prions are known to have strong resistance to formalin fixation and heat, unlike conventional adventitious agents [13]. To inactivate BSE-affected cattle tissues by dry heat devices using a conventional sterilization method takes 20 min at 600 °C or higher [14]. Not surprisingly, substances that can withstand temperatures of 600 °C or higher are limited. One report showed that the reactivity of inactivation using the EU recommended autoclave procedure for prions (133 °C, 3 Bar, 20 min) differed among the prion strains [15].

A problematic source of secondary infection of prions is surgical instruments used in brain surgery. There are reports of prion inactivation that was assumed during the chemical or autoclave sterilization of surgical or medical equipment, which involved prion proteins attached to stainless steel and glass tubes [16–18]. Other groups reported that they evaluated anion-exchange membrane chromatography in order to remove prions from clinical-grade culture media containing human or fetal calf serum involving therapeutic recombinant proteins [19,20]. However, those methods require careful monitoring of the surgical instruments so that they are not harmed by the sodium hydroxide solution [8]. It seems that chemical reagents used in this study were originally intended for surgical instruments. When the methods used to chemically treat plasmid DNA are used on other objects, excluding surgical instruments, it is believed that there is no problem in performance, with the exception of TCA treatment.
Fig. 4. Transfection plasmid DNA into HEK293 cells. GFP protein transfecting plasmid DNA into HEK293 cells using Lipofectamine. The plasmid DNA was treated with chemical reagents and precipitated by ethanol (Leica Microsystems; magnification, 100×; scale bars, 100 μm). NC is only HEK293 cells, not transfected with plasmid DNA. (A) Transfection first plasmid DNA into HEK293 cells (Lot. B). (B) Transfection second plasmid DNA into HEK293 cells (Lot. D). (C) Transfection original plasmid DNA into HEK293 cells.
Fig. 5. GFP positive rate of HEK293 cells transfected with the plasmid DNA using Lipofectamine. Control is only precipitated by ethanol without chemically treatment. NC is only HEK293 cells, not transfected with plasmid DNA. (Leica Microsystems; magnification, 100 ×; scale bars, 100 μm). (A) The first DNA was treated with chemical reagents and precipitated by ethanol (Lot. B). (B) The second DNA was amplified and purified from the first DNA in E. coli (Lot. D). (C) The original DNA which was not amplified in E. coli and was purchased the plasmid DNA, transfected into HEK293 cells.
4. Conclusions

It is necessary to carefully consider the inactivation method based on the substance because of the risk of corrosion or destruction of the substance. We examined plasmid DNA and performed chemical treatment with reagents that produced a 7-log reduction among many prion inactivation methods. Our findings suggest that it is possible for plasmid DNA to withstand chemical treatment for inactivation.

Declaration of competing interest

The authors declare no conflicts of interest in association with this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2020.05.005.

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