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Review

Prion propagation *in vitro*: are we there yet?

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Prion diseases are caused by proteinaceous pathogens termed prions. Although the details of the mechanism of prion propagation are not fully understood, conformational conversion of cellular prion protein (PrP^{C}) to misfolded, disease-associated scrapie prion protein (PrP^{Sc}) is considered the essential biochemical event for prion replication. Currently, studying prion replication *in vitro* is difficult due to the lack of a system which fully recapitulates the *in vivo* phenomenon. Over the last 15 years, a number of *in vitro* systems supporting PrP^{C} conversion, PrP^{Sc} amplification, or amyloid fibril formation have been established. In this review, we describe the evolving methodology of *in vitro* prion propagation assays and discuss their ability in reflecting prion propagation *in vivo*.

Key words: prion disease, prion, cellular prion protein, disease-associated scrapie prion protein, *in vitro* conversion, *in vitro* prion amplification, prion infectivity

Introduction

Prion diseases, also known as transmissible spongiform encephalopathies, are fatal neurodegenerative disorders including Creutzfeldt-Jakob disease in humans, scrapie in sheep, chronic wasting disease in cervids, and bovine spongiform encephalopathy in cattle. The only known component of the infectious prion particle is the disease-associated isoform of the prion protein designated PrP^{Sc}.[1] PrP^{Sc} replication is facilitated in a nucleic acid free manner, in which the causative agent functions as a template to convert the normal cellular prion protein, PrP^{C}, into its infectious isoform.[2] The conversion process appears to be triggered by interaction of PrP^{Sc} with PrP^{C}.[3] When PrP^{C} is converted to PrP^{Sc}, it undergoes a major biochemical alteration from an α-helical to a ß-sheet conformation.[3,4] PrP^{C} is easily hydrolyzed by proteinase K (PK) digestion, while similar treatment on PrP^{Sc} leaves a PK-resistant core termed PrP27-30.

Conversion of PrP^{C} to PrP^{Sc} has been successfully reproduced in cell-based and animal systems in which PrP^{Sc} was propagated and prion infectivity was maintained.[5,6] Several *in vitro* conversion assays have been introduced over the past 15 years to investigate how PrP^{C} is conformationally altered by PrP^{Sc}. However, molecular conversion in various cell-free systems failed to completely reproduce the proposed prion conversion process. Although close, none of the *in vitro* systems perfectly simulate prion propagation. Conversion of PrP^{C} to PrP^{Sc} seems to be difficult in most cell-free reactions unless many other molecules besides PrP isoforms were also present.

The continuous evolution of *in vitro* assays mimicking the conditions of prion conversion and propagation is under progress. In the following sections, we attempt to review all of the *in vitro* conversion assay systems available in an unbiased manner and discuss how they have contributed in answering the important questions in the field of prion biology. The detailed conditions utilized in each methodology are summarized in Table 1.

Initial Development of *In Vitro* Conversion

The initial development of an assay to reconstitute the PrP conversion process *in vitro* began in Prusiner's laboratory.[7] Prusiner and colleagues attempted to convert chimeric mouse/hamster MHM2 PrP expressed in N2a cells or metabolically labeled PrP^{C} of ScN2a cells in the presence of either exogenous or endogenous PrP^{Sc} by incubating overnight. They also attempted to convert Syrian hamster (SHa) PrP^{C} synthesized by cell-free translation systems supplemented with microsomal membranes prepared

from scrapie-infected SHa brain cells. Despite the novel idea behind these approaches, protease-resistant MHM2 PrP (PrP-res), radio-labeled PrP-res, and SHaPrP-res were not formed by the assays. Even though all experiments gave negative results, it is apparent that these experimental processes sparked ideas that would soon lead to the establishment of a successful *in vitro* conversion assay.

Table 1. Summary of *in vitro* assays for PrP^C conversion and PrP-res formation.

Conversion Method	Conversion Buffer	Incubation	Sonication/ Agitation	PrP^C Source	PrP^{Sc} Source	Percent Converted/ Amplified	Infectivity	Reference
Mixing	PBS with protease inhibitors	37°C ≤ 24 hr		Lysate of N2a cells expressing MHM2 PrP^C	PrP27-30 purified from prion-infected mouse brains	0%		7
Metabolic Radiolabeling	PBS with protease inhibitors	37°C ≤ 24 hr		Lysate of ScN2a cells expressing [^{35}S]-PrP^C	Endogenous PrP^{Sc} of ScN2a cells	0%		7
Microsomal Membranes	20 mM Tris buffer, pH 7.5	25°C 1 hr		[^{35}S]-PrP^C synthesized by cell-free translation systems	Microsomal membranes from scrapie-infected hamster brain cells.	0%		7
Cell-Free Conversion	0.75 M GdnHCl, 130 mM NaCl, 10 mM Tris-HCl, pH 7.0	20°C 22 hr		[^{35}S]-PrP^C expressed in mouse fibroblast cells	Brain-derived PrP^{Sc} treated with 2 -3 M GdnHCl for 5 h at 37°C	10-20% of PrP^C converted	No	8
Cell-Lysate Conversion	50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.5%SDS	37°C 48 hr		Lysate of CHO cells expressing MHM2 PrP^C	Brain-derived mouse PrP^{Sc}	Successful, not quantified		24
PMCA	PBS with 0.05% Triton X-100, 0.05% SDS, protease inhibitors	37°C 10-72 hr	40 sec sonication	Normal, uninfected crude brain homogenate	Prion-infected crude brain homogenate	~ 20 -100 fold increase of PrP^{Sc}	Yes	26, 27
PMCA under non-denaturing conditions	PBS with 1% Triton-X 100, 0.5 mM EDTA	37°C 16-48 hr	Continuous agitation, 800 rpm	Purified brain -derived PrP^C	PrP27-30	~10-fold increase of PrP^{Sc}	Yes	29, 30
rPrP-PMCA	PBS with 0.05-0.1% SDS, 0.05-0.1% Triton X-100	37°C 24 hr	40 sec sonication	rPrP^C expressed in *E.coli*	Purified PrP^{Sc} or crude homogenate of prion-infected brains	~10% of rPrP^C converted; fold increase of PrP^{Sc} not quantified		34
QUIC	PBS with 0.05% SDS, 0.05% Triton X-100	45°C 46 hr	10 sec agitation, every 2 min	rPrP^C expressed in *E.coli*	Prion-infected crude brain homogenate	Variable, sensitive to environmental conditions		35
Autocatalytic Conversion Assay	β-oligomer : sequential dilution with 5 M urea, 20 mM NaOAc‡, 0.2 M NaCl, pH 3.7, and with 1 M urea, 20 mM NaOAc, 0.2 M NaCl, pH 5.5	20°C 16 hr		rPrP^C expressed in *E.coli*	None			36
	amyloid fibril : identical buffer to generate β-oligomer†	37°C 10-72 hr	Continuous agitation, 600 - 900 rpm	rPrP^C expressed in *E.coli*	None		Yes	38, 40

† The buffer system has been improved for the recent studies in which buffer containing 1 M GdnHCl, 2.4 M urea, and 150 mM NaCl, pH5.0-6.8 was used [37-39]. ‡ NaOAc: sodium acetate.

Cell-Free Conversion Assay

A milestone was reached by Caughey and colleagues when the first PrP-res was formed in an *in vitro* assay termed cell-free conversion.[8] This method utilized guanidine hydrochloride (GdnHCl)-treated PrP^{Sc} purified from prion-infected brains and radio-labeled PrP^C derived from mouse fibroblast cells. When a large excess of PrP^{Sc} was incubated with small amounts of PrP^C, autoradiography of the PK-digested sample indicated that 10-20% [^{35}S]-PrP^C was converted into [^{35}S]-PrP-res.[9,10,11] Although slowly becoming out-dated with the introduction of more modern techniques, the cell-free conversion assay has become the best characterized *in vitro* conversion system available, and it has been modified on multiple occasions to better answer different questions associated with the molecular mechanism of PrP^{Sc} replication.

Caughey's group made two major modifications for the cell-free conversion assay. First, GdnHCl was substituted with either KCl or NaCl to generate radio-labeled PrP-res under more physiological conditions. A number of studies preferentially chose KCl over NaCl under GdnHCl-free conditions, implicating KCl may be more suitable.[10,12,13,14,15] Although successful, the overall efficiency of the reaction under these conditions was reduced 25-50% in comparison to reactions containing GdnHCl.[16] The second major

modification was the establishment of the solid-phase cell-free conversion assay using non-isotopic material such as biotinylated PrP^C.[17,18,19] This format incorporates a 96-well plate for high-throughput conversion.[17] Following the attachment of the partially purified PrP^{Sc} or scrapie-positive microsomes on the plate surface, conversion of PrP^C was carried out with or without GdnHCl treatment over a period extended up to 48 hr. Enzyme-conjugated avidin allowed biotinylated PrP-res to be detected by either Western blot analysis or directly on the plate in an ELISA-like fashion.[17] Scrapie-positive microsomes converted ~20% of PrP^C into PrP-res conformation, while only ~10% of PrP^C was converted into PrP-res with partially purified PrP^{Sc}. These achievements were successful in creating an environment for cell-free conversion that was more similar to physiological conditions and more applicable to rapidly screen large numbers of compounds inhibiting both binding and conversion.[17,18,19]

Other groups have attempted to replace the PrP^C substrate purified from mammalian cells with the protein generated by baculovirus-infected insect cells or bacteria in cell-free conversion.[20,21,22] Iniguez *et al*. was able to convert radio-labeled PrP^C expressed in insect cells to PrP-res via the GdnHCl method.[21] Kirby *et al*. demonstrated that, upon incubation with partially purified PrP^{Sc}, the bacterially expressed and refolded [^{35}S]-PrP^C was successfully converted into PrP-res under GdnHCl-free conditions.[22] Similarly, Eiden *et al*. generated PrP-res after slightly modifying the conditions to eliminate the use of radio-active material by utilizing L42 epitope (W144Y)-tagged PrP^C expressed in *E. coli*.[20] Since these PrP^C substrates were generated in non-mammalian cells, post-translational modification states of these proteins were not identical to native PrP^C. Despite the glycosylation differences, conversion efficiency was not significantly altered from the original assay, suggesting that post-translational modification did not appear to influence conversion efficiency under these experimental conditions.

Cell-free conversion has several limitations even after the improvements described above. In this system, the concentration of the PrP^{Sc} seed must be 50-fold higher than PrP^C to obtain the formation of PrP-res.[8] Although cell-free conversion simulates several critical aspects of *in vivo* replication, unrealistic stoichiometry between PrP^C and PrP^{Sc} indicated that conversion in this system did not reflect the continuous PrP^{Sc} formation *in vivo*.[15] Furthermore, PrP-res generated by cell-free conversion was inadequate to transmit the disease in bioassay. Although cell-free conversion initiated by hamster-adapted scrapie Sc237 prions converted the chimeric mouse/hamster MH2M PrP^C into PrP-res, this product did not cause disease in > 550 days after challenging transgenic mice expressing MH2M PrP^C. This argues that the acquisition of protease resistance *in vitro* was not sufficient for the propagation of infectivity.[23]

Cell-Lysate Conversion Assay

Saborio *et al*. introduced a system termed the cell-lysate conversion assay.[24] This method describes incubating lysate of Chinese hamster ovary cells over-expressing MHM2 PrP^C with a 10-fold molar excess of PrP27-30, which is only one-fifth of the molar excess of PrP^{Sc} required for the cell-free conversion assay. Interestingly, conversion was unsuccessful with purified MHM2 PrP^C that was incubated with a 10-fold molar excess of PrP27-30; however, the addition of PrP^C-depleted cell lysate recovered the production of MHM2 PrP-res. This result supports the hypothesis that some unidentified factors available in the lysate play a role in the conversion process. Although the molar excess of PrP^{Sc} required was significantly decreased, this system still has similar problems as those described for cell-free conversion.

Protein Misfolding Cyclic Amplification (PMCA)

Soto and colleagues established PMCA that utilizes cyclic bursts of sonication to convert PrP^C into a protease-resistant, infectious PrP^{Sc}-like product under a stoichiometric condition in which PrP^C is in excess.[25] This system was composed of a mixture of prion-infected brain homogenate (IBH) diluted in a >1000 fold excess of normal, uninfected brain homogenate (NBH). Each PMCA cycle allowed amplification of PrP^{Sc} during the 1 hr incubation at 37˚C and disruption of aggregated PrP^{Sc} by five 1 sec sonication pulses. Incubation facilitated conversion and aggregation of PrP isoforms, while sonication multiplied the number of small aggregates available to induce PrP^{Sc} conversion. Analysis of the samples that underwent 0, 5, 10, 20, or 40 PMCA cycles demonstrated that the amount of newly generated PrP^{Sc} was directly proportional to the number of cycles conducted. The newly formed PrP^{Sc} constituted > 95% of total PrP^{Sc} after 5 amplification cycles.[25]

A major change in PMCA was achieved by the incorporation of a programmable sonicator and a 96-well plate format, which enabled high through-put assays.[26] In this PMCA, each round consisted of 20 cycles with a 40 sec sonication every 30 min. Upon completion of each round, a small aliquot of the amplified samples were taken and diluted 10-1000-fold into fresh NBH to carry out the subsequent rounds of PMCA. Serial PMCA was shown to be continued suc-

cessfully even after the original PrP^{Sc} seeds were diluted up to 10^{55} –fold. This suggests that PrP^{Sc} could be replicated infinitely *in vitro*. Furthermore, the products of serial PMCA preserved characteristics of the original PrP^{Sc} seed such as electrophoretic mobility, glycosylation pattern, amino acid composition, PK resistance, Fourier transform infrared spectroscopy profile, electron microscopy profile, heat-resistance profile, and resistance to denaturation by GdnHCl.

More importantly, unlike previous *in vitro* conversion methods, the PrP^{Sc} generated by PMCA was found to be infectious. When serial PMCA products were inoculated, animals succumbed to disease. It appears that infectivity of serial PMCA was due to newly synthesized PrP^{Sc} since the original PrP^{Sc} seeds were diluted beyond the minimum infectious level. Although infectious, the *in vitro* generated PrP^{Sc} product exhibited longer incubation periods in animals than an equal amount of brain-derived PrP^{Sc}. This suggests that PMCA is less robust in generating infectious prion particles than *in vivo* systems. Nonetheless, prion strain properties of brain-derived PrP^{Sc} appeared to be conserved in the PMCA product by exhibiting indistinguishable clinical signs and vacuolation pattern. In addition, the pathogenecity of *in vitro* generated PrP^{Sc} appeared to be stable upon serial transmission.[27]

The PMCA assay has a strong up-side, but it still has a few drawbacks. The success of PMCA was specifically influenced by the prion strains and the PrP^{C} substrate, which requires optimization of ultrasound strength and length of sonication in a case by case manner for maximum amplification.[28] Similar to the cell-lysate conversion assay, PMCA appears to require the presence of unknown factors available in the brain homogenate. Inferiority of PMCA-generated prion particle to its natural counterpart in transmitting disease may be hindered by sonication and the presence of detergents, which might denature cellular protein factors or disrupt the native mechanism for the *in vivo* conversion of PrP^{C} to PrP^{Sc}. However, problems associated with this assay seem relatively minor in comparison to the previous methods described for *in vitro* conversion.

PMCA under Non-Denaturing Conditions

Supattapone modified the PMCA technique by omitting the use of sonication and anionic detergent sodium dodecyl sulfate because either process could potentially denature cellular protein factors and alter the normal biochemical reactions required for conversion *in vivo*.[29] This assay was performed with 1:50 dilution of 10% (w/v) IBH into NBH. A conversion reaction incubated for 16 hr at 37˚C with continuous shaking produced ~6-fold increase in PrP-res compared to the PrP^{Sc} seed, while incubation for > 48 hr under the same conditions produced > 10-fold increase in PrP-res.[29] Generation of PrP-res was also dependent on temperature as more products were detected in the assay conducted at 37˚C in comparison to 25˚C and 4˚C. The introduction of the non-denaturing method was significant because fundamental properties of PrP^{Sc} formation involved in cellular cofactors could be studied, which was not permitted with the method described by Soto's group.

The improvement made to this PMCA method was to remove the additional factors present in the brain homogenate. This version of modified PMCA utilized PrP27-30 as seeds to convert mature, mammalian PrP^{C} partially purified from brain homogenate by detergent solubilization along with immunopurification. Continuous shaking of the mixture of PrP27-30 and PrP^{C} molecules at a molar ratio of 1:250 yielded ~2-fold PrP-res amplification. Supplementation of polyanionic compounds such as synthetic poly A^{+} RNA in this reaction dramatically increased PrP-res formation to ~ 10-fold, which are levels equivalent to those obtained with the crude brain homogenate.[30,31] Interestingly, even more vigorous PrP-res formation was achieved if sonication was applied to the protocol.[30] In addition, the PrP-res product generated from this modified version of PMCA under non-denaturing conditions has been indicated to be infectious; however, the *in vivo* study has not been described in entirety.[31] Because this protocol uses purified PrP^{C} and PrP^{Sc} for conversion, it may represent one the most effective assays for identifying co-factors that play a role in PrP^{Sc} propagation.

On the basis of earlier success,[30] Supattapone's group recently applied a periodic sonication, instead of continuous agitation, to their modified PMCA to increase the conversion rate. Suggesting its essential role in this revised method, no periodic sonication resulted in failure of PrP-res formation. Under this condition, incubation of PrP27-30 and PrP^{C} highly purified by a combination of several chromatographic steps along with synthetic poly A^{+} RNA molecules resulted in efficient PrP-res formation.[32,33] Surprisingly, even in the absence of PrP27-30 seeds, purified PrP^{C} supplemented with synthetic poly A^{+} RNA propagated PrP-res, implicating *de novo* generation of PrP^{Sc}.[32] Similar to seeded PMCA products, *de novo* generated PrP^{Sc} was infectious when inoculated into animals and exhibited almost equivalent infectivity, neuropathological characteristics, and clinical symptoms to natural prions found in the diseased brain.[32] This method of PMCA has the most simplistic requirements for the formation of infectious PrP^{Sc}.

Recombinant PMCA and Quaking-Induced Conversion (QUIC)

Caughey and colleagues recently reported a protocol that uses recombinant (r) PrP as a substrate to amplify PrP-res in PMCA, which is referred to as rPrP-PMCA.[34] This method slightly modified the conditions of conventional PMCA established by Soto and colleagues. The modification includes an incubation disrupted by less frequent sonication over a period of 24 hr. When rPrP prepared from transformed *E. coli* was seeded by either crude homogenate or purified PrP^{Sc} derived from prion-infected brains, rPrP-PMCA allowed amplification of rPrP-res. This product was distinguishable from the other species of rPrP-res spontaneously formed by rPrP self-aggregation due to the molecular size differences. Complication with spontaneous rPrP self-aggregation can be avoided by addition of Triton X-100. The optimized rPrP-PMCA demonstrated a sensitive ability to convert rPrP to rPrP-res only with a minute amount of (ag –fg) PrP^{Sc} seeds. In fact, two rounds of PMCA using this protocol were sufficient to amplify PrP^{Sc} from the cerebral spinal fluid of animals at the terminal stage of prion disease. This system eliminates the involvement of brain homogenate-associated factors while allowing incorporation of diversely manipulated PrP substrate.

The QUIC assay was derived from the rPrP-PMCA procedure.[35] QUIC exchanged the use of sonication with automated tube shaking to induce the conversion of $rPrP^{C}$ to PrP-res. QUIC was able to detect prions at a sensitivity level similar to rPrP-PMCA. QUIC has several advantages over conventional PMCA with its speed, sensitivity, simplicity, and ease of duplication. However, rPrP-res generated from rPrP-PMCA or QUIC have not been tested *in vivo* for infectivity.

Autocatalytic Conversion Assay

Baskakov developed a novel *in vitro* system referred to as the autocatalytic conversion assay. The principle of this assay heavily relies on selective refolding of denatured rPrP in the absence of PrP^{Sc}. In essence, rPrP denatured by urea or GdnHCl was directed to induce two types of β-sheet-rich, non-native PrP molecules designated β-oligomers and amyloid fibrils.[36,37,38] The β-oligomers generated by the autocatalytic conversion procedure retained resistance to PK treatment. Interestingly, the β-oligomers could be converted into an amyloid fibril by further incubation with continuous shaking.[36,38] However, amyloid fibril formation did not require preformed β-oligomers but could be independently generated by continuous shaking under identical conditions in which β-oligomers were formed.[37,38]

The rate of amyloid fibril formation was monitored by thioflavin T (ThT) fluorescence, which demonstrated that conversion rate was dependent on many parameters. Amyloid fibril formation was more rapid in neutral pH in which short fibrils similar to prion rods were formed, while an acidic pH favored the formation of long fibrils with distinct coil morphology.[38] In addition, amyloid fibril formation was delayed in the presence of higher concentrations of urea. Furthermore, providing evidence as being an autocatalytic process, the lag phase for amyloid fibril formation was significantly reduced by seeding with small amounts of pre-folded amyloid fibril.[36,37]

An improvement for the autocatalytic conversion assay was the introduction of the semi-automation.[37,39] The semi-automated assay incorporated the use of the GdnHCl-based method to convert full-length rPrP encompassing residues 23-230 into amyloid fibrils by incubating in a 96-well plate with continuous agitation. Combining the ThT fluorescence assay to this system allowed a microplate reader to monitor the amyloid fibril formation in real time. This semi-automated assay was particularly useful in studying kinetics of amyloid fibril conversion and screening potential anti-prion drugs in a high-throughput format.

The autocatalytic conversion assay has several advantages over a majority of the other *in vitro* conversion techniques. A major benefit is the complete removal of cellular factors that may be introduced into the reaction along with any kind of PrP^{C} substrates or PrP^{Sc} seeds derived from the biological material despite the level of purification. The autocatalytic induction of PrP^{C} conversion in a reaction originally devoid of PrP^{Sc} makes this system more relevant to the *in vivo* setting representing sporadic prion diseases. In addition, unlike rPrP-PMCA or QUIC, the disulfide bond remains intact to create a non-reduced form of recombinant protein for conversion, which mimics the native states of a disulfide bridge in PrP^{Sc} and PrP^{C} molecules *in vivo*.[36]

Although this method was reported as producing infectious amyloid fibrils, infectivity remained the most controversial characteristic of the amyloid fibrils generated by this assay. Prusiner and colleagues induced amyloid fibrils from recombinant mouse PrP 89-230 and used these synthetic prions to infect transgenic animals overexpressing mouse PrP 89-230.[40] These animals developed clinical symptoms and neuropathology of disease following lengthy incubation periods. However, synthetic prions were not able to transmit disease directly to wild type mice. To obtain infectivity in wild type mice, synthetic prions were

serially passaged to wild type mice only after primary transmission into transgenic mice overexpressing truncated PrP^C.[40,41] Additionally, transgenic mice expressing high levels of PrP^C were known to spontaneously develop neurological disease in the later stages of life without prion inoculation.[42] These facts make the infectious nature of synthetic prions still questionable.

Conclusion

Several different *in vitro* systems have been devised and tested for successful conversion of PrP^C or amplification of PrP^{Sc}. Using these methods, many previously unknown but fundamental aspects of prion propagation have been studied. However, we are still far away from the complete understading of the mechanistic details of the process despite the efforts reviewed in this article.

On the basis of the protein-only hypothesis, prion propagation is believed to faciliated by a biochemical event known as a conformational conversion of PrP^C to PrP^{Sc}. The ultimate goal of the *in vitro* systems is to re-create the condition that faithfully recapitulates prion propagation *in vivo*. In an ideal condition, a test tube containing both PrP isoforms only should be sufficient to reconstitute the replication process. However, the current form of *in vitro* reconsititution is not the *bona fide* system respresenting the *in vivo* phenomenon. One of the major obstacles is involved in unintended inclusion of cellular factors other than PrP isoforms. Furthermore, our limited knowledge on cofactor molecules makes it more difficult to conceive insight into what has occurred in prion propation *in vitro*.

Despite the limitation in the current form of *in vitro* conversion assays, simplicity of the systems over cell-based and animal systems has been advantageous. Utilization of these tools will slowly unwind the complicated molecular characteristics of prions such as the species barrier and strain properties. They will also be useful in validating the necessary environment for conversion and estimating the transmissibility of disease. By manipulating the systems, the application can be extended to a sensitive diagnosis of prions and a high-throughput screening of potent anti-prion reagents.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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