# Effective Separation of Tetramer and Aggregate in Acidic Transthyretin Amyloidogenic Protein using CHT™ Ceramic Hydroxyapatite Media

## Abstract

The efficient purification of amyloidogenic proteins and their unstable derivatives remains a significant challenge due to their inherent tendency to aggregate under adverse conditions. In this study, we successfully isolated both the tetrameric and aggregated forms of the acidic amyloidogenic protein transthyretin using a single-step purification approach with ceramic hydroxyapatite media. Collectively, these results highlight the potential of ceramic hydroxyapatite as a purification media for the rapidly expanding field of amyloidosis medical research.

## Introduction

Transthyretin (TTR) is an aggregation-prone protein that functions as a transporter of thyroxine and retinol-binding protein. Mutations in TTR are associated with the generation of amyloid fibrils and the development of TTR-related amyloidosis (ATTR)[1]. ATTR-related cardiomyopathy (ATTR-CM) and polyneuropathy (ATTR-PN) are progressive systemic diseases caused by dissociation of the stable tetrameric TTR form to its monomer, followed by misfolding and self-assembly to amyloid aggregates[2]. FDA approval (2019) of Pfizer's TTR stabilizers VYNDAQEL and VYNDAMAX for the treatment of the ATTR-CM[3] and the very recently launched WAINUA™ (eplontersen) for ATTR-PN by Ionis-AstraZeneca[4] demonstrate an excellent medical progress and renewed focus to a previously scarcely explored rare disease field. Isolation of amyloidogenic components of TTR cascade for medical research purposes is hampered by challenges in regulating the in vitro rate of TTR dissociation and hence its propensity to aggregate as well as their closely matching biochemical properties (pI, hydrophobicity) arising from their shared peptide backbone. The theoretical pI of TTR has been calculated to 5.2 thus it is classified as acidic protein. Herein we have effectively generated TTR aggregates in vitro and employed a novel ceramic hydroxyapatite purification method to isolate the tetrameric and aggregated TTR versions for research purposes[5].

## Ceramic Hydroxyapatite

Ceramic Hydroxyapatite is a spherical macroporous form of hydroxyapatite known for its physical and chemical robustness. Functioning as both the ligand and support matrix, its multimodal resin features metal affinity interactions with calcium sites (C-sites) and cation exchange interactions with phosphate sites (P-sites). These sites allow for unique and precise separation of an extremely broad range of therapeutic modalities with excellent results on recombinant proteins and acidic enzymes[6]. At typical operative conditions (pH 6.5 - 7.5), acidic proteins are negatively charged, thus primarily expected to bind to the calcium site whilst being repelled from the phosphate site. The affinity of TTR for calcium has been described[7], leading to the assumption that tetrameric form can bind to ceramic hydroxyapatite under suitable conditions. A combination of sodium chloride (NaCl) and sodium phosphate (NaP) gradients were explored herein, to effectively bind and elute both the tetrameric and aggregate versions of TTR using ceramic hydroxyapatite media.

## CHT Ceramic Hydroxyapatite Type I Media*

Table 1. CHT Ceramic Hydroxyapatite Type I Media Characteristics**

| | |
|---|---|
| Functional groups | $Ca^{2+}$, $PO_4^{3-}$, OH- |
| Particle size | 20, 40, and 80 µm (nominal) |
| Recommended linear flow rate | 50 –1,000 cm/hr |
| Operating pH range | 6.5 – 14 |
| Sanitization | 1–2 N NaOH |
| Autoclavability (121°C, 20 min) | Yes |
| Packing density (g/ml packed bed) | 0.63 g/ml |
| Dynamic binding capacity | >25 mg lysozyme/g |
| Nominal pore diameter | 600–800 Å |
| Maximum operating pressure | 100 bar (1,500 psi) |

** CHT Ceramic Hydroxyapatite Type I Media is manufactured by HOYA Technological Corporation and distributed globally by Bio-Rad Laboratories, Inc. Hercules, CA USA.*

*** Referenced from Bio-Rad Bulletin 5667*

## Materials and Methods

Recombinant wild-type TTR (TTRwt) was expressed and purified from E. Coli cells and provided by Eri Chatani, University of Kobe, Japan. TTRwt exists at its natively folded tetrameric state at the neutral pH. Dissociation of TTRwt was performed via acidic partial denaturation that is known to dramatically increase the aggregation rate, allowing for in vitro mimicking of the amyloidogenesis event[8]. Dynamic light scattering (DLS) analysis to verify aggregation presence was performed with a Horiba SZ100 equipment, under standard, polydisperse model conditions. The pH of the protein sample was then adjusted to 6.5 by dilution in 10 mM NaP pH 7.5 to allow efficient binding with ceramic hydroxyapatite. Chromatography purification was conducted on an NGC system (Bio-Rad) using a 4.6 mm diameter x 35 mm length column, filter size 10 µm packed with CHT Ceramic Hydroxyapatite Type I 20 µm media (0.35 g), flow rate 1 ml/min in bind and elute mode. The buffer composition varied according to the protocol employed and is individually described. Verification of isolated components was performed via size exclusion chromatography analysis using a Tosoh TSKGel Super SW3000 4.6 X 300 mm column and 100 mM Na2SO4 in 100 mM NaP pH 6.7 (flow 0.25 ml/min, 30 min) in Hitachi LaChrom Elite HPLC.

## Results & Discussion

### TTRwt tetrameric elution from CHT Ceramic Hydroxyapatite Media is NaP-dependent.

In normal pH conditions, TTRwt tetrameric elution from ceramic hydroxyapatite media appears to be phosphate-dependent (Figure 1A) since application of an increased concentration of NaCl does not lead to its elution (Figure 1B). These data confirm eligibility of TTR binding to calcium site of ceramic hydroxyapatite.

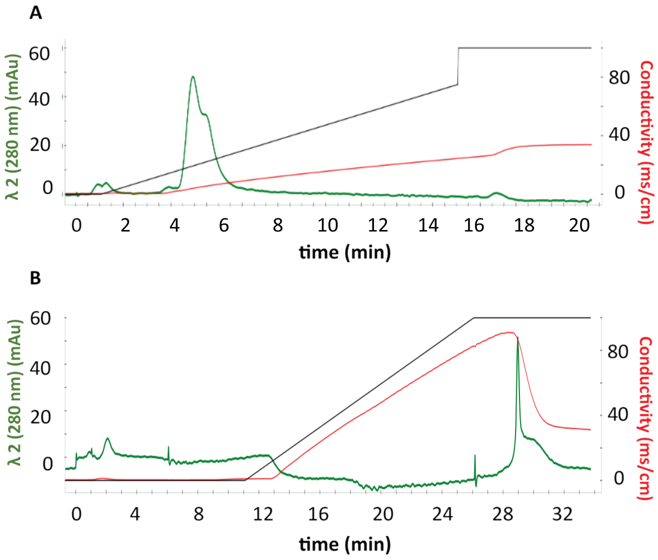

**Figure 1:** Chromatograms demonstrating the binding and elution profile of TTRwt on ceramic hydroxyapatite media. **(A)** phosphate gradient (equilibration: 3 mM NaP, 5 min, gradient: 3 mM -300 mM NaP, 15 min, wash: 400 mM NaP, 5 min), demonstrating successful elution of TTR at approximately 30 mM NaP **(B)** NaCl gradient (equilibration: 3 mM NaP, 5 min, gradient: 3 mM NaP - 10 mM NaP plus 1M NaCl, 15 min, wash: 400 mM NaP, 5 min), demonstrating no elution of TTR throughout the NaCl gradient.

## Aggregates suitable for CHT Ceramic Hydroxyapatite Media separation are relatively homogeneous and do not exceed 300 nm in diameter.

To investigate the binding and elution limitations of ceramic hydroxyapatite media with respect to aggregates, TTR aggregates of varying sizes were generated through acidic partial denaturation. The optimized protocol involved dissolving lyophilized TTR in 10 mM acetate buffer pH 4 and incubating it at 25°C for 5 – 7 days. Under these conditions, the resulting aggregates exhibited a relatively narrow size distribution with a median diameter of approximately 100 nm (Figures 2A and 2B). Modifications to this protocol, such as the addition of 50 mM NaCl (Figure 2C) or further acidification to pH 2 followed by incubation at 4°C for 7 days (Figure D), intended to accelerate and enhance aggregate formation, resulted in significant protein loss due to rapid degradation. These conditions also produced more heterogeneous particle populations with median diameters exceeding 100 nm (Figure 2C) or oversized fibers greater than 1000 nm (Figure 2C), which were unable to penetrate the 10 µm pores of the CHT Ceramic Hydroxyapatite Media column. In both cases, the aggregate sizes corresponding to molecular weights over 2000 kDa exceeded the handling capacity of ceramic hydroxyapatite media purification.

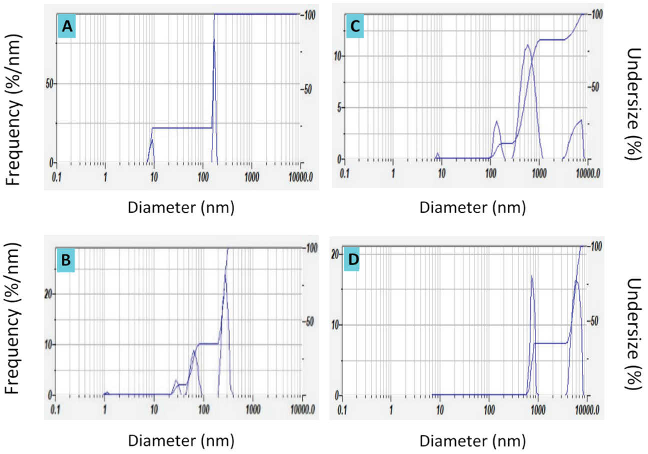

**Figure 2:** DLS evaluation of generated TTR aggregates upon different conditions. **(A)** typical smaller particles with sizes between 10-300 nm indicating presence of tetramer and oligomer or **(B)** variable particle sizes not overcoming the median diameter of 100 nm, **(C)** Median particle size shifted above 100 nm **(D)** Formation of particles with diameter over 1000 nm denotes typical amyloid structures.

## Aggregation range formation 25-50% is reaction-dependent and of average size 600 kDa.

Verification of the % of aggregation was obtained via subjecting 10 µg of treated TTR under HPLC- gel filtration chromatography. Typical conditions as shown below:

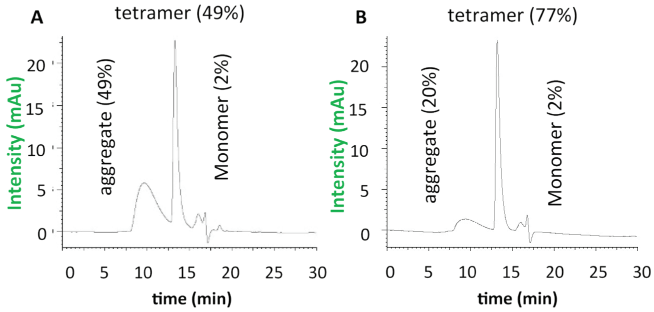

**Figure 3:** HPLC analysis of aggregate formation. Incubation of 1 mg/ml TTR in 10 mM acetate buffer pH 4 for 5-7 days yielded either **(A)** equal amount of aggregate/tetramer plus 2% monomer or **(B)** prevalent tetramer with significant amount of aggregate plus 2% monomer. Aggregate range formation was approximately kept between 100-1,200 kDa.

## Generated aggregation can be efficiently eluted from CHT Ceramic Hydroxyapatite Media column via a desalting phosphate gradient.

The first attempt to separate tetrameric and aggregate forms of TTR via the standard phosphate and NaCl gradients led to successful elution of tetramer but no aggregate, similar to Figure 1. Since it has been demonstrated that NaCl gradient does not affect tetrameric elution, a salting-in binding step in low phosphate was employed to enhance affinity between acidic protein and calcium site and allow for successful binding of tetramer plus aggregate[9]. Tetramer remains bound during the salt-in process and is eluted at around 20 mM NaP (beginning NaP gradient) in the presence of high (1M) NaCl. Aggregation elution is achieved via a NaP gradient (10-300 mM) with a simultaneous salting-out process (Figure 4).

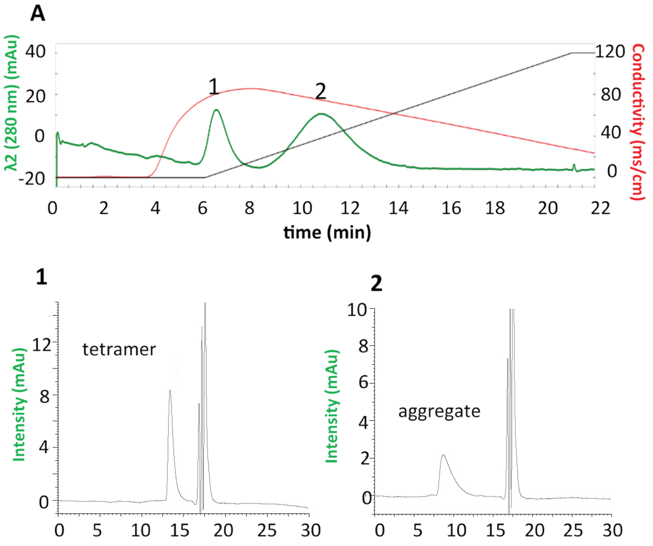

**Figure 4:** Successful separation of TTR tetramer and aggregate via desalting, phosphate gradient. **(A)** Chromatogram demonstrating elution of TTR tetramer and aggregate. Protocol employed: equilibration: 5 min, 3 mM NaP pH 6.5, binding: 5 min, 10 mM NaP plus 1 M NaCl pH 6.5, elution: 15 min gradient from 10 mM NaP plus 1 M NaCl pH 6.5 to 200 mM NaP pH 6.5 (desalting), washing: 5 min, 400 mM NaP, pH 6.5. Successful isolation of **(1)** tetramer (retention time 13.4 min) and **(2)** aggregate (retention time 8.7 min equivalent to 800kDa) from relevant peaks was confirmed via gel filtration (HPLC) analysis.

Reducing the concentration of NaCl during the phosphate gradient is critical for delaying the elution of aggregates. When NaCl is maintained at 1 M while increasing NaP concentration, aggregates tend to elute earlier, overlapping with the tetrameric form. However, this high-salt condition can enhance the separation of larger aggregates, which elute later during the high-phosphate wash step (Figure 5).

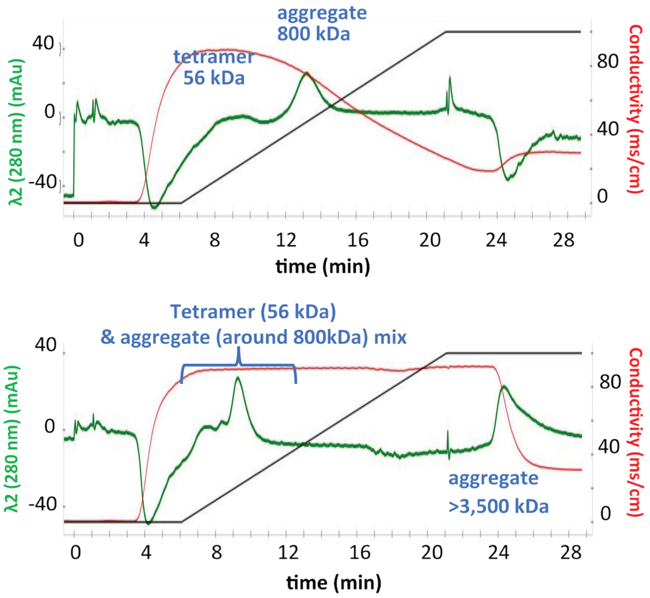

**Figure 5:** Comparison data of desalting versus high salt maintenance in NaP gradient elution of TTR using ceramic hydroxyapatite media. **(A)** Chromatogram of a typical desalting protocol to separate TTR tetramer and aggregate. Tetramer elution occurs first, with no overlapping of aggregate elution. **(B)** Chromatogram of a salting TTR mixture binding followed by a phosphate gradient in high salt. Aggregate elution overlaps with tetrameric elution whereas bigger aggregates are eliminated at high phosphate step.

If the tetrameric form elutes during the salt-in process, it is recommended to reduce the NaP concentration in the binding buffer from 10 mM to 5 mM. Although the addition of $CaCl_2$ to the binding buffer has previously been recommended to enhance retention of calcium-binding proteins on the CHT Ceramic Hydroxyapatite Media column[10], it should be avoided for amyloidogenic proteins, as it promotes additional aggregation on the column.

## Summary

Collectively, the data demonstrates that:

1. TTRwt (tetramer) binds to ceramic hydroxyapatite at low phosphate concentration (3 mM) and is eluted at approximately 30 mM NaP, whereas a NaCl gradient does not impact its elution profile, thus TTRwt binding is calcium-site dependent.

2. Aggregates larger than ~300 nm cannot be efficiently purified with ceramic hydroxyapatite media.

3. Employed aggregation protocol successfully generates tetrameric and aggregated versions of TTR with some monomers.

4. TTR, a representative acidic-calcium binding protein, can be efficiently separated by its aggregation via ceramic hydroxyapatite media on the recommended pH working range (6.5).

5. Alternative protocols may be employed depending on the aggregate's characteristics to be isolated– higher aggregate will elute more efficiently in a high phosphate presence.

## Conclusion

This novel protocol demonstrates the effective use of CHT Ceramic Hydroxyapatite Media in separating tetrameric and aggregated forms of transthyretin (TTR), highlighting its potential for purifying challenging targets such as amyloidogenic proteins. Additionally, the protocol showcases its versatility in binding and eluting acidic enzymes through buffer adjustments, overcoming its limitations in low-pH environments.

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