Review

Structure analysis of short peptides by analytical ultracentrifugation: Review

Tsutomu Arakawa[1,*], Takako Niikura[2], Yoshiko Kita[3], Fumio Arisaka[4]

[1] *Alliance Protein Laboratories, 3957 Corte Cancion, Thousand Oaks, CA, USA;*
[2] *Department of Neurology, Georgetown University, Washington DC, USA;*
[3] *Department of Pharmacology, Keio University Schoold of Medicine, Tokyo, Japan;*
[4] *Department of molecular bioporocessing, Tokyo Institute of Technology, Yokohama, Japan.*

ABSTRACT: Short peptides are potential drug candidates for pharmaceutical and biotech industries. Short peptides are natural ligands for numerous G-protein coupled receptors (GPCR) and hence constitute a large number of drug candidates. Synthetic short peptides are also extensively developed as agonistic or antagonistic ligands that function in a similar manner to antibodies, soluble receptors and protein ligands. Characterization of the peptides in solution is often performed in the presence of organic solvents, which can presumably generate the structure bound to the target surface and also enhance the solubility of the peptides. Analytical ultracentrifugation (AUC) technique should provide information on the state of self-association of the peptide in solution. Its application for short peptides has been far less than the applications for proteins. We believe that AUC should be used to show the associated state of the peptides, as reviewed in this paper.

Keywords: Analytical ultracentrifugation, sedimentation, peptide, aggregation, disordered structure

1. Introduction

Requirement of high quality analysis of protein solution has lead to extensive applications of analytical ultracentrifuge (AUC) for pharmaceutical proteins (*1-5*). This technology measures the homogeneity of proteins in solution, in particular the amount of aggregated proteins and their sizes (*1-5*). It appears that the same scrutiny has not been extended to the low molecular weight peptides, with a few exceptions (*6-9*), while peptides constitute a major part of drug developments (*e.g.*, peptide mimetics developed by Affymax Inc., USA). Many G-protein coupled receptors (GPCR) use short peptides as a natural ligand (*10-12*). Synthetic short peptides are developed as an agonistic or antagonistic ligand that targets various cell surface receptors, protein ligands and cell surface markers in a similar manner that antibodies are used (*14,15*). At first glance, AUC may not be a versatile technology for short peptides, as they would not sediment due to their small mass as in proteins (*16*). We show here that AUC is informative for the analysis of short peptides in solution using a neuroprotective peptide, Humanin and ADNF9, as a model.

Humanin and ADNF9 are 24 and 9 amino acid peptides and protect neurons from various cell toxic stresses (*17-19*). The mechanism of their protective effects is not clear, in part due to lack of information on the conformational state in solution. We have used AUC to characterize the self-association of these peptides in different aqueous solutions and shown that their biological activities must be evaluated in the context of the solution structure (*8,9,20-22*). Here we review such data to demonstrate the value of AUC for peptide research and development.

2. Principle of AUC

Numerous publications are available for the operative principle of AUC (*1-3,23*). It uses centrifugal force to sediment (or float) the solutes whose density differs from the medium. Proteins and peptides generally have a higher density than aqueous medium and hence sediment toward the bottom of the cell (Figure 1). The sedimentation of the solutes (here peptides) is monitored by absorbance (or refractive index), while sedimenting, as a function of cell position and time (see middle row of Figure 1). The rate of the sedimentation of the solute is a function of the mass and shape. A typical sedimentation profile of Humanin (HN) peptide is shown in Figures 2A and B. Each curve

**Address correspondence to:*
Dr. Tsutomu Arakawa, 3957 Corte Cancion, Thousand Oaks, CA 91360, USA.
e-mail: tarakawa2@aol.com

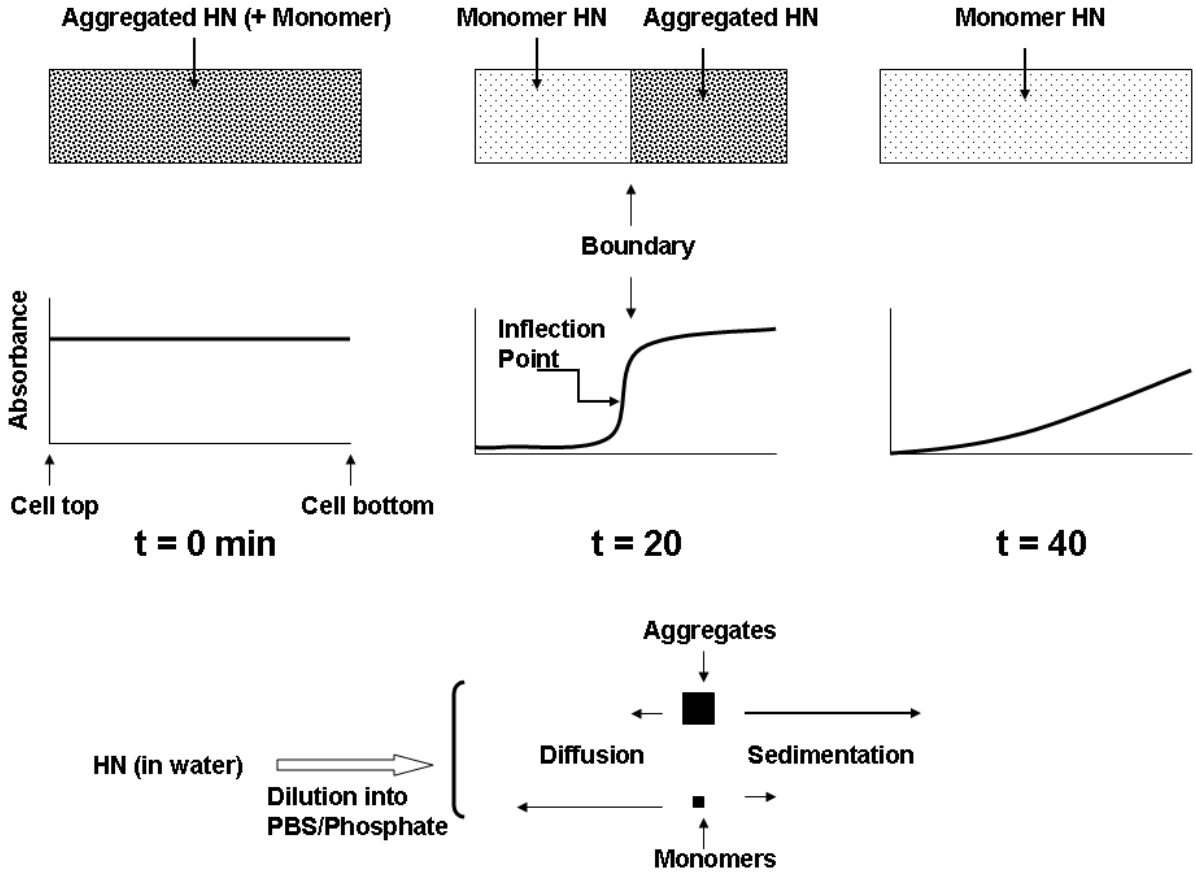

Figure 1. Schematic illustration of sedimentation profile. First row shows the distribution of monomeric and aggregated HN in the centrifuge cell as a function of sedimentation time. Second row shows the absorbance profile of the cell at different sedimentation time. Third row shows the results of dilution of HN dissolved in water into buffers.

corresponds to UV absorbance at different positions in the centrifuge cell. As the peptide sediments, the curve moves toward the bottom of the cell (from left to right), reflecting sedimentation of the peptide. The shape of the curve indicates the homogeneity (or heterogeneity) of the sample. If it contains one species (for example, all in monomer), then it should show an inflection point, as depicted in Figure 1 (see middle row). If the sample contains many different forms (*e.g.*, monomer, dimer, *etc.*), then the curve shows a broad boundary or multiple inflection points. Analysis of the curve gives the information about the distribution of various self-associated species of the peptide samples in solution.

3. Sedimentation analysis of example peptides

Preparation of peptide samples appears to contribute to the outcome of the self-association of HN peptides and possibly other peptides. As far as Humanin peptides are concerned, the standard procedure for their biological analysis has been to dissolve them first in water and then dilute into the assay media (*17*). In fact, wild type HN peptide is insoluble when directly dissolved in phosphate-buffered saline (PBS) (*20*). HN and HN derivatives are fully soluble, upon visual inspection, in water. Although it would be interesting to know the self-association of the HN peptides in water, hydrodynamic technologies, including AUC, cannot be applied to such system due to unscreened charges on the peptides: the addition of electrolytes screens the charges and makes the peptides behave normally in hydrodynamic measurements. While insoluble upon direct dissolution of the HN in PBS, the samples once dissolved in water lead to an apparently transparent solution when diluted into PBS or other aqueous buffers. It should be noted that apparent transparency does not mean that they are in functional structure or state in buffer solution. This appears to be the case for HN.

The functional structure of HN has been proposed to be a dimer, based on co-precipitation of two different forms of the peptide (*24,25*). As schematically shown in Figure 3, first, a HN peptide was conjugated to beads. When this bead was mixed with a presumably dimeric Humanin fused with immunological detection tag (HN-tag), the HN-tag was detected in the bead fraction, indicating that HN-tag was trapped by the bead through its binding to HN attached on the bead (Figure 3, line 1). When the above bead fraction containing both HN and HN-tag was incubated with excess HN, no HN-tag was observed in the bead fraction by immunostaining

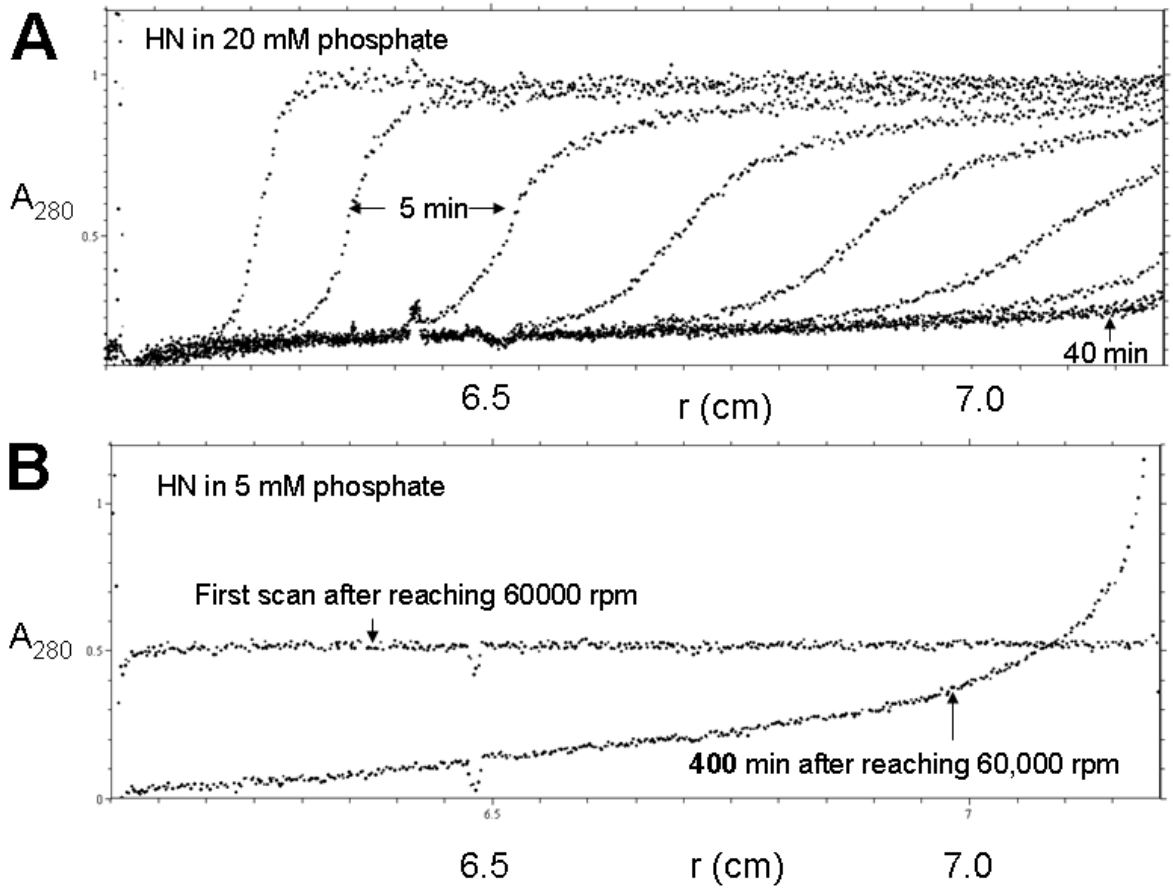

Figure 2. Sedimentation profile of HN in 20 (A) and 5 (B) mM phosphate, pH 6.0. Reformatted from ref. *22*.

(line 2). These observations were used to hypothesize that HN functions as a dimer. However, the same data can equally be explained from aggregated HN. HN extensively aggregates in PBS (*20*). Figure 3 (line 3) assumes that HN-tag also aggregates. Aggregated HN-tag may further self-associate with the HN on the bead, resulting in both HN and HN-tag aggregated on the bead. When this bead was mixed with excess HN aggregates (line 4), HN-tag aggregates are displaced with the HN aggregates, releasing HN-tag into solution. Based on this analysis, it appears that dimerization does not appear to be the only explanation for the observed co-precipitation of HN with HN-tag. If dimerization is not the mechanism of the HN function, then what is the structure of HN that exhibits neuroprotective activity.

Let us go back to sedimentation profile of HN in 20 mM phosphate, pH 6.0 (Figure 2A). The HN peptide sediments as large aggregates, as can be seen by a movement of sedimentation boundary in 5 min span (an indication of fast sedimentation). Careful examination of sedimentation pattern showed a fraction of the peptide remaining in the cell after 40 min spin at 60,000 rpm. Sedimentation profile at 40 min has a shallow slope ascending from the top to the bottom of the cell. This can be explained from the distribution of HN into aggregates and monomers (see Figure 1, bottom row). Here we assumed that the structure of HN in water (though unknown for self-association) converts to aggregates (shown a large black square) and monomers (small square) upon dilution into PBS or phosphate buffer, pH 6.0. At $t = 0$ (*i.e.*, before centrifugation), both monomers and aggregates are distributing in the cell as a homogeneous solution (see top row of Figure 1) and hence has a flat absorbance from the top to the bottom of the cell (middle row). After ~20 min centrifugation ($t = 20$) at 60,000 rpm, the aggregates sediment half-way to the bottom leaving the monomer-only phase behind. Boundary between the monomer-only phase and sedimenting aggregates is broader than depicted due to diffusion of aggregates (though slow). Monomer-only phase also should have a concentration gradient of monomers that also sediment, but at a much slow rate than aggregates (see bottom row, small arrow). At $t = 40$, all aggregates sediment to the bottom, leaving only the monomers in the cell. Slow sedimentation and fast diffusion of the monomer, as depicted in the bottom row (Figure 1), cause a shallow concentration gradient. Namely, the observed shallow slope at $t = 40$ in Figure 2A means that there is a fraction of HN that does not sediment fast enough to form a boundary. This was not observed in PBS: namely, no absorbance gradient, such as shown in Figure 1 (middle row, far right)

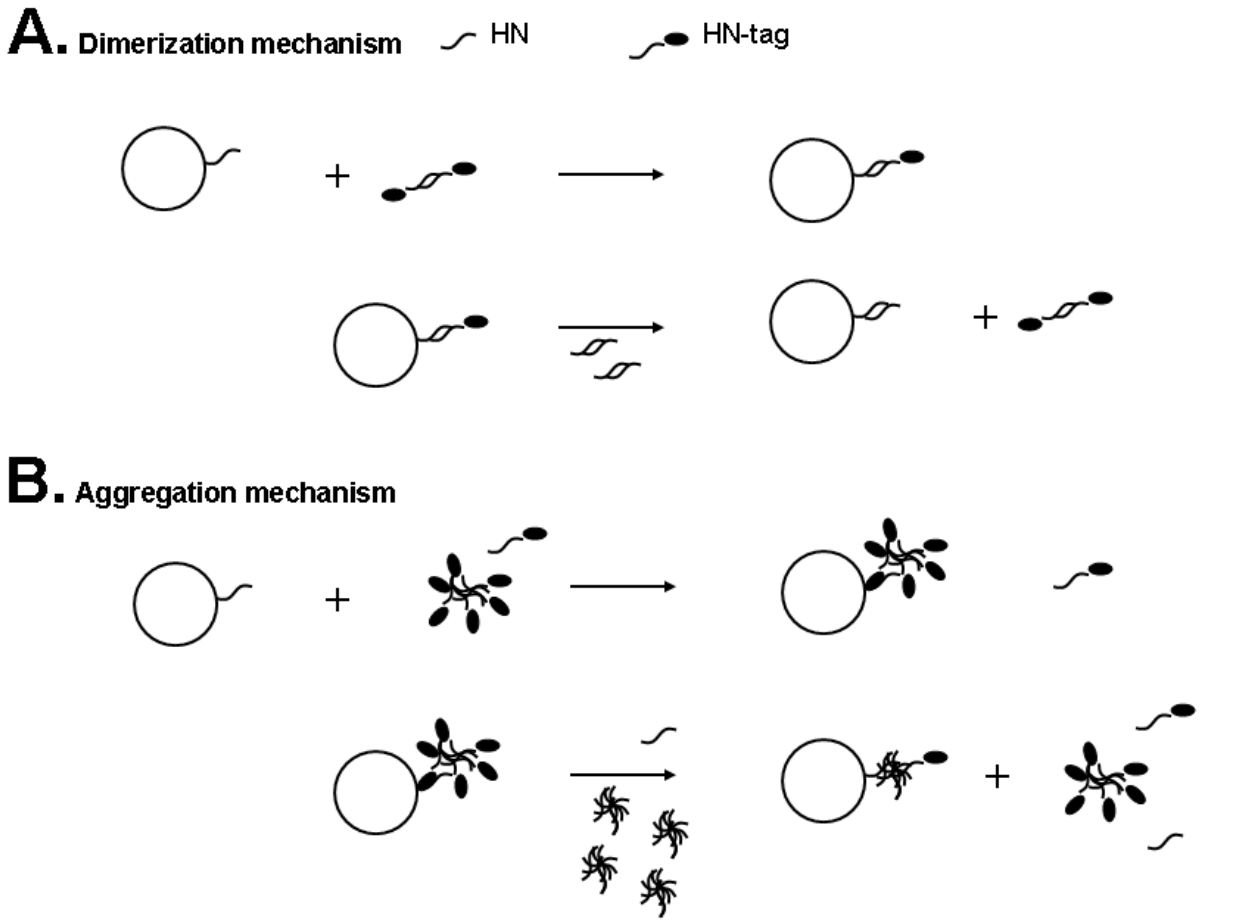

Figure 3. Schematic illustration of co-precipitation experiments. First line shows binding of dimeric HN-tag with HN on the bead. Second line shows dissociation of HN-tag in the presence of excess HN dimers. Third and fourth lines show the above binding and dissociation experiments with the aggregated HN-tag or HN.

or Figure 2A, was observed in PBS. This indicates that while a majority of the HN peptide aggregated in PBS, a fraction of HN had a smaller mass species in 20 mM phosphate, pH 6.0. Although farther away from physiological pH, the phosphate concentration was reduced to 5 mM, which increased the amount of the protein remaining in the cell after spin. Figure 2B shows the concentration gradient after 400 min spin at 60,000 rpm. Comparison with Figure 2A shows a steeper concentration gradient after 400 min spin, indicating that 5 mM phosphate clearly increased the amount of low mass species. The molecular weight of that fraction can be more clearly determined by sedimentation equilibrium.

Sedimentation equilibrium experiment is done at lower centrifugal speed and smaller cells. After reaching sedimentation equilibrium (*i.e.*, complete balance between centrifugal force and diffusion), the concentration gradient formed, such as shown in Figure 4A for HN, is used to determine the molecular weight. The observed concentration gradient in Figure 4 represents the peptide species after spinning down the aggregated species and that remain in the centrifuge cell. The molecular mass thus determined showed that the peptide in that remaining fraction is a monomer. Namely, the HN peptide either extensively aggregates or is monomeric in phosphate buffer at pH 6.0. Assuming that the aggregated species is not the functional form, the observed results suggest that the active structure is monomer.

4. HN analogs

It appears likely that the monomeric form of HN observed in 5 and 20 mM phosphate, pH 6.0, is the functional state or at least the precursor of the HN activity. We further addressed this possibility from the analysis of various HN analogs. Structure-function mutational analysis resulted in several analogs that are more active than the wild type HN. One of the analog that has a mutation of Ser14Gly had a 1,000-fold higher activity that the parent HN (*22*). One possibility may be that this analog is more soluble in PBS. Sedimentation analysis showed otherwise, as this analog also did aggregate extensively. More informative is another analog with multiple mutations that also had a similarly enhanced activity (*20*). The most significant observation for this analog is its molecular weight. Sedimentation

equilibrium analysis clearly demonstrated that this analog is a monomer in PBS and contains little aggregates. Thus, it is more likely that the monomeric state in PBS is at least the molecular species in PBS that leads to the biological function of HN, although a transition to other structures cannot be excluded under physiological conditions.

Structure-function analysis also created another analog with a single mutation of Ser7Ala, which is devoid of activity (*22*). This analog behaves similarly to HN in sedimentation analysis: *i.e.*, extensive aggregation in 20 mM phosphate, pH 6.0 and a large fraction remaining in the cell after 400 min spin at 60,000 rpm. What is remaining in the cell was determined to be a monomer similarly to HN and S14G analog: the sedimentation equilibrium data plotted in Figure 4B fit a single monomeric species. Thus, the lack of activity for this analog is not due to its aggregation state and must be due to the sequence difference.

5. Cicular dichroism

Circular dichroism (CD) is a commonly used spectroscopy for peptide samples. CD analysis showed that HN and HN analogs (both active S14G and inactive S7A analogs) are all disordered at low peptide concentrations. As shown in Figure 5, the CD spectra of the HN peptides below 0.05 mg/mL show no indication of α-helical or β-sheet structures and correspond to more or less disordered secondary structures. However, at higher peptide concentration (0.1 mg/mL), there appears to be a transition into a β-sheet structure. Such structure transition is enhanced at higher peptide concentration and must be due to extensively aggregation. Namely, it appears that the monomeric structure of HN is disordered and the aggregated structure is β-sheet.

6. Perspective

Sedimentation analysis is a standard technology for aggregation analysis of therapeutic proteins. Such aggregation analysis is essential for pharmaceutical proteins, as aggregated proteins may cause immunogenicity. A small amount of protein aggregates, *e.g.*, 1-5%, can generate antibodies against therapeutic proteins: numerous examples exist for such immunogenicity problems: *e.g.*, bone morphgenic proteins (*26*), monoclonal antibody (*27*), tumor necrosis factor antagonist (*28*) and growth hormone (*29*). Sedimentation analysis is used to quantitatively show

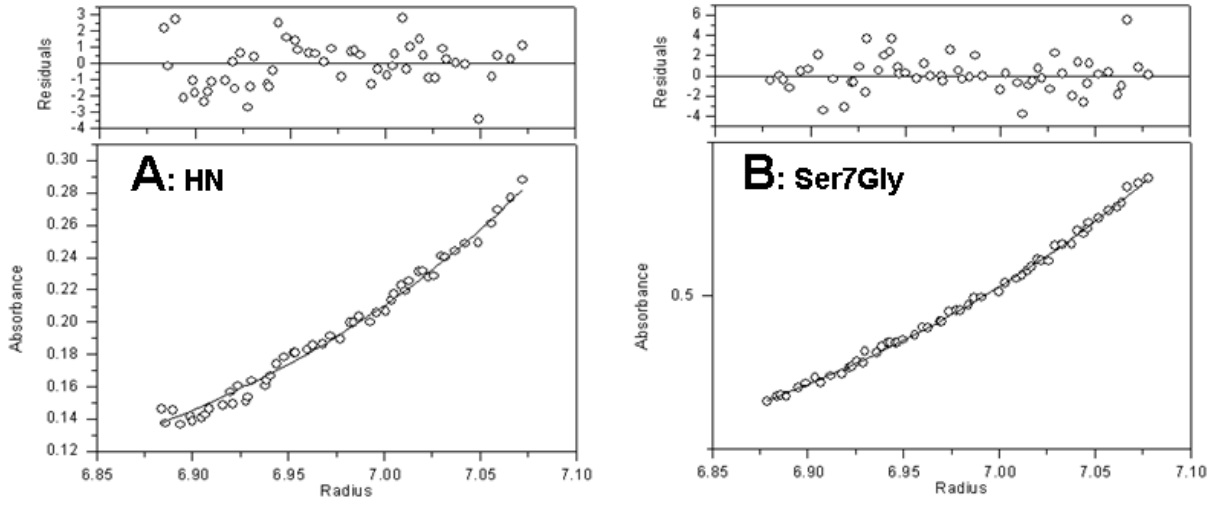

Figure 4. Sedimentation equilibrium of HN (A) and Ser7Ala analog (B) in 5 mM phosphate, pH 6.0. The data represent the peptide left in the cell after centrifugation. Reformatted from ref. *22*.

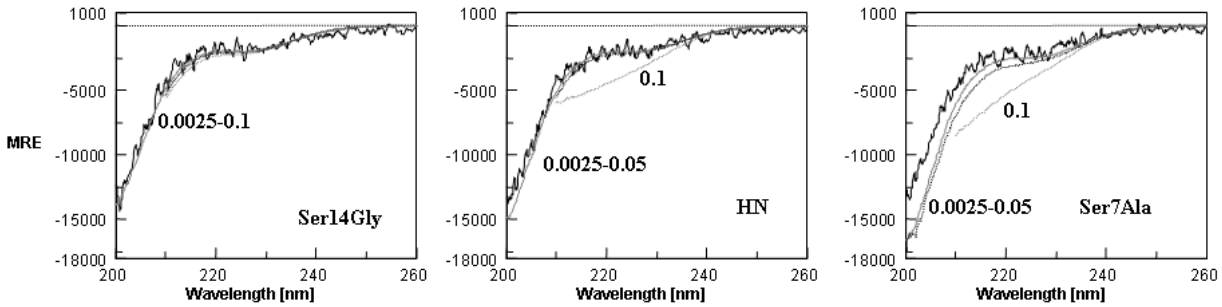

Figure 5. Circular dichroism spectra of HN, Ser14Gly analog and Ser7Ala analog in 5 mM phosphate, pH 6.0. Reformatted from ref. *22*.

the amount of aggregated species in the pharmaceutical proteins. Size exclusion chromatography is also used for this purpose with a caveat that SEC columns often non-specifically bind proteins, leading to incorrect estimate of aggregate content. AUC has no such problems. It does not appear, however, that AUC is also commonly used for therapeutic peptides. Due to small mass, it would be difficult to determine the aggregation if the peptides distribute into the monomers and small oligomers. However, certain less soluble peptides, such as HN, may be subjected to AUC analysis for structure-function analysis. In the case of HN, it was made clear that the original dimer hypothesis was unlikely and such interpretation occurred due to the tendency for HN to heavily self-associate in buffer solutions.

We have applied AUC for other short peptides, ADNF9 (*8*) and NAP (*9*). These are also 9 and 8 amino acid neuroprotective peptides. It was apparent that these are more soluble judged from the dissolution kinetics in PBS. AUC analysis demonstrated that these peptides are monomeric. CD analysis showed that their structures are disordered as in HN. They showed no indication of β-sheet structure at higher peptide concentration, consistent with no apparent aggregation even at increased concentration. Value of AUC for structure-function analysis was also demonstrated for the neuropeptide head activator containing 11 amino acids (*30*) and the neuropeptide Y containing 13 amino acids (*31*). Although earlier studies suggested a dimer for both the full length head activator peptide and the shorter fragment as a functional structure, AUC analysis revealed otherwise: while the full length was a monomer, the fragment extensively aggregated (*30*). The monomeric neuropeptide Y self-associated into oligomers in 40% trifluoroethanol, a helcal inducing organic solvent (*31*). In conclusion, AUC can be used for characterization of short peptides that should help understand the mechanism of their biological functions and avoid confusion about the solution structure responsible for the activities. With further clinical developments of peptides, AUC may also find greater applications for quality control of the therapeutic peptides.

References

1. Philo J. Is any measurement method optimal for all aggregate sizes and types? AAPS J. 2006; 8:E564-E571.
2. Berkowitz SA. Role of analytical ultracentrifugation in assessing the aggregation of protein biopharmaceuticals. AAPS J. 2006; 8:E590-E605.
3. Liu JD, Andya SJ, Shire A. A critical review of analytical ultracentrifugation and field flow fractionation methods for measuring protein aggregation. AAPS J. 2006; 8: E580-E589.
4. Pekar A, Sukumar M. Quantitation of aggregates in therapeutic proteins using sedimentation velocity analytical ultracentrifugation: Practical consideration that affect precision and accuracy. Anal Biochem. 2007; 367:225-237.
5. Gabrielson JP, Randolph TW, Kendrick BS, Stoner MR. Sedimentation velocity analytical ultracentrifugation and SEDFIT/c(s): Limits of quantitation for a monoclonal antibody system. Anal Biochem. 2007; 361:24-30.
6. Mok YF, Howlett GJ. Sedimentation velocity analysis of amyloid oligomers and fibrils. Methods Enzymol. 2006; 413:199-217.
7. Constantino HR, Culley H, Chen L, Morris D, Houston M, Roth S, Phoenix MJ, Foerder C, Philo JS, Arakawa T, Eidenschink L, Andersen NH, Brandt G, Quay SC. Development of calcitonin salmon nasal spray: similarity of peptide formulated in chlorobutanol compared to benzalkonium chloride as preservative. J Pharm Sci. 2009; in press.
8. Arakawa T, Niikura T, Tajima H, Arisaka F, Kita Y. Structure analysis of activity-dependent neurotrophic factor 9 by circular dichroism and sedimentation equilibrium. J Mol Neurosci. 2007; 33:262-267.
9. Arakawa T, Niikura T, Arisaka F, Kita Y. Short neuroprotective peptides, ADNF9 and NAP, are structurally disordered and monomeric in PBS. Int J Biol Macromol. 2009; 45:8-11.
10. Wise A, Jupe SC, Rees S. The identification of ligands at orphan G-protein coupled receptors. Annu Rev Pharmacol Toxicol. 2004; 44:43-66.
11. Mertens I, Vandingenen A, Meeusen T, De Loof A, Schoofs L. Postgenomic characterization of G-protein coupled receptors. Pharmacogenomics. 2004; 5:657-672.
12. Spiegel AM, Weinstein LS. Inherited diseases involving G proteins and G protgein-coupled receptors. Annu Rev Med. 2004; 55:27-39.
13. Sankararamakrishnan R. Recognition of GPCR by peptide ligands and membrane compartments theory: structural studies of endogenous peptide hormones inmemmbrane environment. Biosci Rep. 2006; 26:131-158.
14. Macdougall IC. Hematide, a novel peptide-based erythopoiesis-stimulating agent for the treatment of anemia. Curr Opin Investig Drugs. 2008; 9:1034-1047.
15. Pan Y, Angell YM, Green JM, Wright KL, Miguel J, Schatz PJ, Holmes CP. High-affinity FGFR2 binding peptides derived from the native epitope of the KGF ligand. Adv Exp Med Biol. 2009; 611:511-512.
16. Schuck P, MacPhee CE, Howlett GJ. Determination of sedimentation coefficient for small peptides. Biophys J. 1998; 74:466-474.
17. Hashimoto Y, Niikura T, Tajima H, *et al*. A rescue factor abolishing neuronal cell death by a wide spectrum of familial Alzheimer's disease genes and Abeta. Proc Natl Acad Sci U S A. 2001; 98:6336-6341.
18. Brenneman DE, Gozes I. A femtomolar-acting neuroprotective peptide. J Clin Invest. 1996; 97:2299-2307.
19. Arakawa T, Niikura T, Arisaka F, Kita Y. Activity-dependent neurotrophic factor, ADNF, determines the structure characteristics of Colivelin, a fusion protein of ADNF9 and Humanin analog. J Pept Sci. 2008; 14:631-636.
20. Arisaka F, Niikura T, Arakawa T, Kita Y. The structure analysis of Humanin analog, AGA-(C8R)HNG17, by circular dichroim and sedimentation equilibrium: comparison with the parent molecule. Int J Biol Macromol. 2008; 43:88-93.

21. Kita Y, Niikura T, Arisaka F, Arakawa T. The complex structure transition of Humanin peptides by sodium dodecylsulfate and trifluoroethanol. Protein Pept Lett. 2008; 15:510-515.
22. Arisaka F, Arakawa T, Niikura T, Kita Y. Active form of neuroprotective Humanin, HN, and inactive analog, S7A-HN, are monomeric and disordered in aqueous phosphate solution at pH 6.0; No correlation of solution structure with activity. Protein Pept Lett. 2009; 16:132-137.
23. Arakawa T, Philo JS, Ejima D, Tsumoto K, Arisaka F. Aggregation analysis of therapeutic peptides, part 2: Analytical ultracentrifugation and dynamic scattering. Bioprocess Int. 2007; 5:36-47.
24. Terashita K, Hashimoto Y, Niikura T, Tajima H, Yamagishi Y, Ishizaka M, Kawasumi M, Chiba T, Kanekura K, Yamada M, Nawa M, Kita Y, Nishimoto I. Two serine residues distinctly regulate the rescue function of Humanin, an inhibitinh factor of Alzheimer's disease-related neurotoxicity: functional potentiation by isomerization and dimerization. J Neurochem. 2003; 85:1521-1538.
25. Hashimoto Y, Terashita K, Niikura T, Yamagishi Y, Ishizaka M, Kanekura K, Chiba T, Yamada M, Kita Y, Aiso S, Matsuoka M, Nishimoto I. Humanin antagonists: mutants that interfere with dimerization inhibit neuroprotection by Humanin. Eur J Neurosci. 2004; 19:2356-2364.
26. Hwang CJ, Vaccaro AR, Lawrence JP, Hong J, Schellekens H, Alaoui-Ismaili MH, Falb D. Immunogenicity of bone morphogenic proteins. J Neurosurg Spine. 2009; 10:443-451.
27. Schmidt E, Hennig K, Mengede C, Zillikens D, Kromminga A. Immunogenicity of rituximab in patients with severe pemphigus. Clin Immunol. 2009; 132:334-341.
28. Emi Aikawa N, de Carvalho JF, Artur Almeida Silva C, Bonfa E. Immunogenicity of anti-TNF-alpha agents in autoimmune diseases. Clin Rev Allergy Immunol. 2009; in press.
29. Fradkin AH, Carpenter JF, Randolph TW. Immunogenicity of aggregates of recombinant human growth hormone in mouse models. J Pharm Sci. 2009; 98:3247-3264.
30. Lai JR, Gellman SH. Reinvestigation of the proposed folding and self-association of the neuropeptide head activator. Protein Sci. 2003; 12:560-566.
31. Barnham KJ, Catalfamo F, Pallaghy PK, Howlett GJ, Norton RS. Helical structure and self-association in a 13 residue neuropeptide Y Y2 receptor agonist: relationship to biological activity. Biochem Biophys Acta. 1999; 1435:127-137.

(*Received July 24, 2009; Accepted August 8, 2009*)