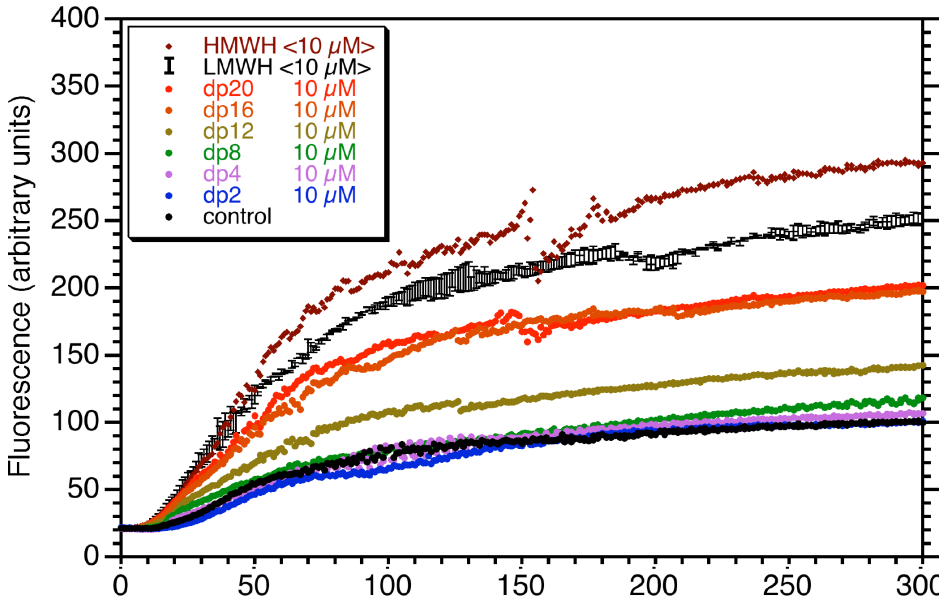
Amyloidogenic proteins

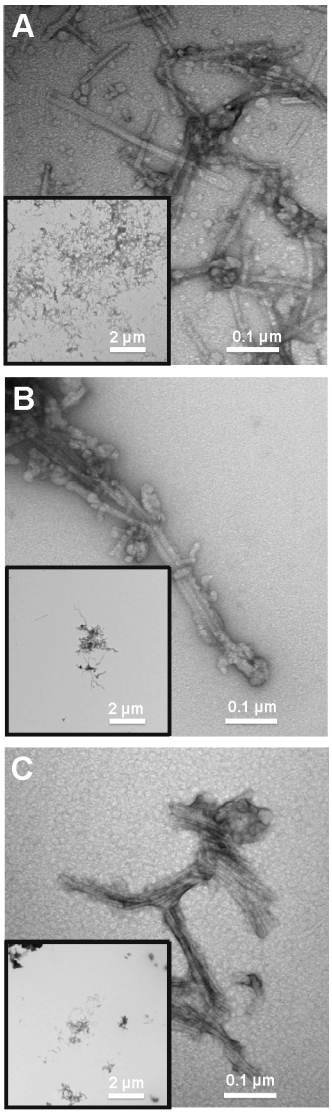
Our research on amyloids is focused on four molecules involved in human disease: amylin, an endocrine hormone involved in type 2 diabetes; α-synuclein, a protein involved in Parkinson's disease; SEVI, an amyloid involved in HIV infectivity and Aβ, the peptide responsible for Alzheimer's disease.

Amylin - Amylin is an endocrine hormone that helps regulate blood glucose levels and controls appetite [1]. In patients with type 2 diabetes, amylin misfolds into amyloid plaques. The plaques, or soluble oligomer precursors, are implicated in the destruction of the pancreatic β-cells that make insulin and amylin in the end stages of type 2 diabetes [2]. We determined the NMR structure of amylin in a micelle bound state [3]. The structure offers clues as to how amylin aggregates could disrupt membrane function. We used total internal reflection fluorescence microscopy (TIRFM) to image the growth of individual amylin fibrils and to study the role of secondary nucleation (growth from pre-existing fibrils) in fibrillogenesis [4]. We have done extensive studies on the pH dependence of fibrillogenesis and used kinetic data to characterize the pK_a values of the two ionizable sites of amylin (His18 and the N-terminal α-amino group) in the fibrils [5]. The studies are physiologically relevant because amylin is stored in the β-cell secretory granules at pH 5.5, conditions that hinder fibril formation but is released into the extracellular matrix at pH 7.4, which favors fibril formation [6]. The pH dependence of amylin fibrillogenesis is in large part due to the ionization of His18. Fibril growth occurs readily when His 18 is in its neutral state but is hindered when His18 is charged, due to electrostatic repulsion between adjacent monomers in the fibril. In addition to fibril growth kinetics, the protonation of His 18 affects fibril morphology and



Effects of heparin fragment length on amylin fibrillization, where dp = number of saccharide monomers. LMWH – size heterogeneous low molecular weight fraction with dp12 to dp18. HMWH – size heterogeneous high molecular weight fraction with dp18 to dp90.

cytotoxicity of amylin towards β-cells [5]. In separate studies, we have used a quenched hydrogen exchange technique developed in my lab [7] to examine the location and stability of hydrogen-bonded secondary structure in amylin fibrils [8]. Finally, we have looked at the effects of the glycosaminoglycan heparin on amylin fibrillization, since amylin plaques *in situ* are complexed with the glycosaminoglycan chains of heparan sulfate proteoglycans [9,10]. We found that heparin enhances fibrillization in a manner that depends on the length of the polysaccharide fragments [11]. We used nuclear magnetic resonance (NMR) to establish that the negatively charged heparin fragments bind to the positively charged N-terminal half of amylin. We used FRET to determine that heparin associates with amylin fibrils rather than enhancing fibrillization catalytically. We used TIRFM to show that fluorescein-labeled heparin is co-localized with amylin fibrils. The mechanism of binding appears to involve charge complementation between the negatively charged heparin helix structure and the positively charged cross-β sheet structure of amylin fibrils. To see how heparin affects the biological function of amylin, we used a fluorimetric assay of cytotoxicity towards a mouse model of pancreatic β-cells. With heparin fragments longer than 20 saccharides, cell death was similar to when amylin was added alone. By contrast, short heparin fragments of 2 to 8 saccharides protect against cytotoxicity [11]. The observation is intriguing since heparin fragments or similar charged molecules could be developed as drugs to treat type 2 diabetes.

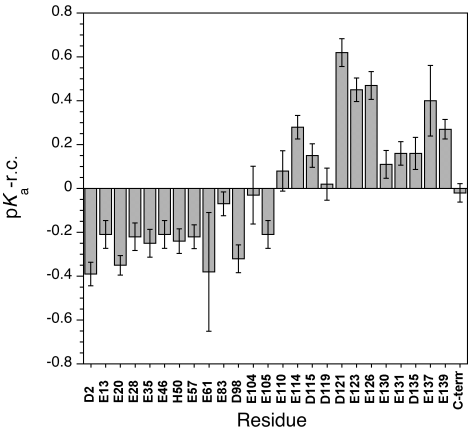


TEM images of SEVI fibrils at 150,000x magnification. Control experiment showing SEVI fibrils grown in the absence of metals (**A**), in the presence of 1 mM $ZnCl_2$ (**B**), and in the presence of 1 mM $CuCl_2$ (**C**). The insets for each panel show images obtained at a lower magnification of 11,000x to illustrate the decrease in the amount of fibrils formed when metals are present. While the amount of fibrils is decreased in the presence of metals, fibril morphology is conserved.

<u>SEVI</u> - SEVI is a naturally-occurring 40-residue peptide fragment of prostatic acid phospahtase identified in a screen of human semen compounds [12]. The peptide forms amyloid fibrils that increase the infectivity of HIV up to 10^5-fold, by facilitating the fusion of HIV virions with target cells [12]. We have studied the effects of metals on SEVI fibrillogenesis [13]. Metals such as Zn^{2+} and Cu^{2+}, which are histidine ligands, inhibit fibril formation at concentrations of 10-1000 μM whereas Mg^{2+} and Ca^{2+} have no effect. We used NMR to show that Zn^{2+} and Cu^{2+} bind to the two histidine residues at positions 3 and 23 in SEVI monomers. The metals appear to stabilize residual α-helix

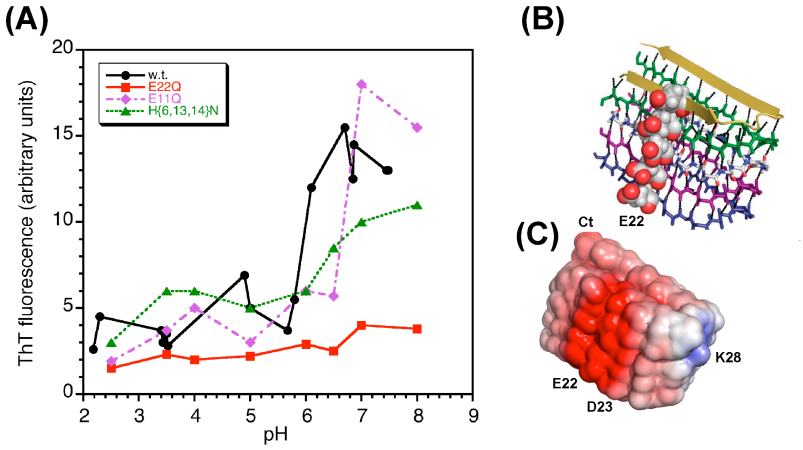
structure. This may inhibit fibrillization by lowering the concentrations of SEVI monomer conformations competent to assemble into fibrils. The observation is likely to be physiologically important because zinc (~2 mM) and copper (~8 mM) in human semen occur at large concentrations that should inhibit SEVI fibrillization [14]. By contrast, zinc and copper are present at low concentrations in vaginal fluids. In the acidic environment of the vaginal tract (pH 4), the two histidines in SEVI will be protonated and unable to bind metals. Consistently, we found that Cu^{2+} and Zn^{2+} no longer inhibit fibrillization at acidic pH. Additional work on SEVI includes the development of an *E. Coli* over-expression system that would allow us to produce isotope labeled samples of the peptide for NMR quenched hydrogen exchange experiments and for solid-state NMR structural studies of the fibrils.

α-synuclein - α-synuclein is a 140-residue protein that forms fibrillar deposit in dopaminergic neurons known as Lewy Bodies [15]. The protein is genetically linked to Parkinson's disease [16]. We have used NMR to study the hydrogen exchange properties of the protein [17] and to determine ionization constants for all 26 acidic residues in the protein [18]. The sequence of the protein has an unusual distribution of charged residues. Residues in the N-terminal 100-residues are arranged in KTKEGV repeats that are poised to form ion pairs which could help nucleate the folding of the protein into α-helical structure [19]. The C-terminal 40 residues of the protein have a high proportion of acidic residues. Electrostatic repulsion between negatively charged residues keeps the C-terminal tail unfolded and confers protection from misfolding [20]. We have done additional studies on the fibrillization of the protein. These studies have been hampered by poor reproducibility, due to the extremely slow fibrillization of α-synuclein [21]. We are exploring how a number of factors, including a heat treatment step in the purification protocol affect α-synuclein fibrillization.



Differences between αS and random coil pK_a values (p$K_{a,\alpha S}$ – p$K_{a,r.c.}$). The N-terminal domain shows systematically lowered pK_a values. The acidic residues resist protonation to keep their negative charges which participate in weak electrostatic interactions with positively charged residues in the KTKEGV repeats. By contrast, the C-terminal domain shows elevated pK_a values. The acidic residues abide protonation to lessen electrostatic repulsion between the high density of like negative charges at the C-terminus.

Aβ - For the Alzheimer's Aβ peptide we compared pK_a values of the free and SDS micelle bound states [22]. We also characterized the pH dependence of fibrillization and assigned the apparent pK_a of 6.0 to residue Glu22, using variants of the Aβ peptide that replaced individual ionizable residues [22].



Roles of charged residues in the pH dependence of Aβ fibrillization. (**A**) Comparison of the ThT fluorescence plateau of Aβ variants as a function of pH. The lines are not fits but simply included to guide the eye. (**B**) Structure of the Aβ(1–42) protofilament. The organization of 5 Aβ monomers in the protofilament is shown, and residue E22 is highlighted with CPK spheres. Note the side-chain carboxylates from E22 (red spheres) form an array of negative charges running along the length of the fibril. (**C**) Electrostatic surface of the Aβ(1–42) protofilament. Charged residues in the core of the fibril structure are labeled.

Future directions: An area of interest is to look for inhibitors of fibrillization and cytotoxicity. To this end we will study variants of the amylin hormone sequence that is involved in type 2 diabetes to introduce charged residues, designed to interfere through electrostatic repulsion with fibril extension or with the binding of oligomeric forms of the peptide to membranes. We are also planning to explore derivatives of the short heparin polysaccharides that we have shown protect against amylin cytotoxicity towards β-cells [11]. Cytotoxicity assays offer a powerful method to study how various solution conditions or inhibitors affect fibrillization. Yet the physical basis of cytotoxicity remains poorly understood. There is considerable debate on whether oligomeric forms of amylin or fibrils are responsible for cytotoxicity [2,23-25]. We plan to address this by seeing how cytotoxicity varies as a function of the amount of time amylin is aggregated. We will also use our recently acquired expertise in fluorescence microscopy [4] to see how amylin interacts with single β-cells. Using ThT or fluorescently tagged amylin to visualize the fibrils and a dye sensitive to cell viability, we can look at whether cell death requires contact between the cells and fibrils and what cellular processes (e.g. membrane integrity, mitochondrial function) are affected in the presence of amylin. Finally, in a project funded by an NSF-Graduate Research Fellowship to my doctoral student Sarah Sheftic we want to address the question of how fibrils are formed when multiple components are involved. We will use kinetic assays to determine how Aβ fibrillization depends on the ratio of the Aβ40 and Aβ42 peptides and on the presence of the NAC fragment of α-synuclein (residues 61-95), which is found as a

component of Alzheimer's plaques. We will use TIRF microscopy in conjunction with fluorescently tagged peptides to see if Aβ40/Aβ42 peptides and Aβ40/NAC co-localize in fibrils formed when multiple components are present in the reaction.

Recent publications:

Alexandrescu, A.T. & Croke, R.L. (2008) "NMR of Amyloidogenic proteins", in Protein Misfolding (O'Doherty C.B., and Byrne, A.C eds.) Nova Publishers, Hauppauge, NY. ISBN 978-1-60456-881-3

Amylin

Alexandrescu, A.T. (2013) "Stability of hydrogen-bonded secondary structure in amylin fibrils monitored by quenched hydrogen exchange experiments", *in preparation*

Jha, S., Snell, J, Sheftic, S.R., Alexandrescu, A.T. (2013) "The pH dependence of amylin fibrillization", *in preparation*

Jha, S., Patil, S.M., Gibson, J., Nelson, C.E., Alder, N.N. & Alexandrescu, A.T. (2011) "Mechanism of amylin fibrillization enhancement by heparin", *J. Biol. Chem. 286,* 22894-22904.

Patil, S.M., Mehta, A., Jha, S., & Alexandrescu, A.T. (2011) "Heterogeneous amylin fibril growth mechanisms imaged by total internal reflection fluorescence microscopy", *Biochemistry 50, 2808-2819.*

Patil, S.M., Xu, S., Sheftic, S. & Alexandrescu, A.T. (2009) "Dynamic alpha-helix structure of micelle-bound amylin", *J. Biol. Chem. 284*, 11982-11991

α-synuclein

Croke, R.L., Patil, S.M., Quevreaux, J., Kendall, D.A. & Alexandrescu, A.T. (2011) "NMR determination of pK_a values in a-synuclein". *Protein Science 20*, 256-269.

Croke, R.L., Sallum, C.O., Watson, E., Watt, E.D. & Alexandrescu, A.T. (2008) "Hydrogen exchange of monomeric a-synuclein shows unfolded structure persists at physiological temperature and is independent of molecular crowding in *Escherichia coli*". *Protein Science 17,* 1434-1445.

Aβ

Sheftic, S.R., Croke, R.L., LaRochelle, J.R., & Alexandrescu, A.T. (2009) "Electrostatic contributions to the stabilities of native proteins and amyloid complexes." *Methods in Enzymology 466*, 233-258.

SEVI

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