Journal of Diabetes Science and Technology
Volume 4, Issue 6, November 2010

SYMPOSIUM

The Nature of Amyloid-like Glucagon Fibrils

Jesper Søndergaard Pedersen, Ph.D.

Abstract

Protein aggregation and formation of amyloid fibrils is a phenomenon usually associated with proteotoxicity and degenerative diseases, such as type 2 diabetes, Alzheimer's disease, and prion diseases. However, several protein and peptide hormones are known to have a high propensity to form amyloid-like fibrils *in vitro* raising concerns about safety and stability of pharmaceutical protein solutions. Comprehensive understanding of the aggregation mechanisms is an important prerequisite to the design of strategies to prevent fibril formation. Detailed kinetic, spectroscopic, and morphological studies have revealed that glucagon can form several types of fibrils that differ at the level of molecular packing of the peptide. Each type forms through distinct nucleation-dependent aggregation pathways influenced by solution conditions and can be self-propagated by seeding. An increasing number of functional amyloid-like structures have been discovered in nature, and it has recently been proposed that an amyloid-like state of glucagon may be utilized by the pancreatic α-cells as *in vivo* storage form. This article reviews the current state of our knowledge about the nature of the different types of amyloid-like glucagon fibrils, the mechanisms by which they form, and discusses implications for formulation strategies and the safety of glucagon pharmaceuticals.

J Diabetes Sci Technol 2010;4(6):1357-1367

Conformational States of Glucagon

Glucagon is a secreted 29-residue peptide hormone that is synthesized primarily in the pancreatic α-cells.[1] Glucagon has found extensive use as an emergency treatment of insulin-induced hypoglycemia[2] as well as a muscle-relaxing pretreatment for endoscopy of the gastrointestinal tract.[3] Ever since the first purifications in the 1950s,[4] it became clear that glucagon can exist in at least three different conformational states: α-helical in crystals and trimers,[5,6] random-coil monomer,[7] or β-sheet-rich in gels and fibrils[8] (**Figure 1**). Because its isoelectric point is between 7.5 and 8.5, glucagon has a low solubility around neutral pH,[9] increasing under acidic or alkaline conditions. The α-helical content of glucagon can be increased by addition of lipid vesicles,[10-12] or by increasing peptide concentration leading to dynamic formation of soluble trimers or hexamers.[10-12] However, high concentrations also increase rate of fibril formation at both acidic and alkaline pH.[13,14] Because of the high

Author Affiliation: Department of Biochemistry, Molecular Biology, and Cell Biology, Rice Institute for Biomedical Research, Northwestern University, Evanston, Illinois

Abbreviations: (Aβ) amyloid-β-peptide, (CD) circular dichroism, (CyD) cyclodextrin, (EM) electron microscopy, (HP-βCyD) hydroxypropyl-β-cyclodextrin, (M-βCyD) methyl-β-cyclodextrin, (NMR) nuclear magnetic resonance, (QSA) quantitative seeding assay, (ThT) thioflavin T, (T_m^{app}) apparent thermal dissociation midpoint, (Trp) tryptophan, ($t_{threshold}$) threshold time

Keywords: aggregation, amyloid, cyclodextrins, drug formulation, excipients, fibrils, glucagon

Corresponding Author: Jesper Søndergaard Pedersen, Ph.D., BMBCB, Northwestern University, 2205 Tech Drive, Hogan 2-100, Evanston, IL 60208; email address *jsp@phage.dk*

propensity for amyloid fibril formation, pharmaceutical glucagon [e.g. GlucaGen® HypoKit® (Novo Nordisk, Princeton, NJ) or Glucagon™ (Eli Lilly and Company, Indianapolis, IN)] is currently supplied as a powder to be dissolved in supplied water immediately before use. Despite the +5 charge of glucagon at acidic pH in the resulting solution, the dissolved peptide gradually aggregates and eventually forms amyloid-like fibrils, which share the intermolecular cross β-pleated sheet architecture with amyloid protein deposits found in histological samples from amyloidosis patients.[15]

Amyloid–Disease-Related and/or Functional Structures

The word amyloid is usually associated with degenerative diseases such as type 2 diabetes mellitus, Alzheimer's disease,[16] and prion diseases.[17,18] Amyloid formation can result in loss of natural protein function, as well as toxic gain-of-function.[19] However, pre-fibrillar oligomeric structures of many proteins appear to have a higher toxicity than large amyloid aggregates,[20] possibly because of larger surface-to-mass ratios.[21] It has been suggested that the formation of large amyloid-like deposits may, at least in some cases, represent an active mechanism by which cells can protect themselves against smaller, more cytotoxic aggregates.[22,23] Indeed, amyloid structures may not be generally cytotoxic, as an increasing number of functional amyloid structures are being reported, involved in biofilm formation, melanin anchoring, reduction of interfacial tension, and coating of spores.[24,25] It has been proposed that glucagon could be stored in the α-helical trimeric form inside the pancreatic α-cells,[6] possibly stabilized by divalent cations such as Zn^{2+} as seen for insulin.[29] However, recent studies suggest that glucagon and several other peptide and protein hormones may be stored as functional amyloid-like structures inside pituitary secretory granules.[30] In line with this, these hormones exhibit a high propensity to form amyloid of relatively low stability allowing them to revert to functional state upon secretion.[31] However, several studies indicate that the aggregates formed by glucagon can be particularly toxic,[32] exceeding that of other peptide hormones and the amyloid-β-peptides (Aβ),[30] which appears inconsistent with a functional role of glucagon amyloid. However, in contrast to the single native protein fold, several amyloid-folds can be generated from the same sequence,[33-36] and recent studies indicate that toxicity depends greatly on the structure of the protein aggregate.[37,38] This means that toxicity could arise from a particularly toxic type of glucagon fibril formed under the given conditions. We have recently demonstrated that glucagon is able to form at least five different types of amyloid-like fibrils that can be propagated by seeding in a strain-specific manner,[12,28,39,40] as previously known for prions.[41,42] Each fibril type can be identified from a unique combination of specific characteristics, including thioflavin T (ThT) staining, circular dichroism (CD) spectrum fingerprints, thermostability and morphology in electron microscopy (EM) (see **Figure 2** and **Table 1**), suggesting that the fibrils differ at the level of molecular packing of glucagon.[39,43,44]

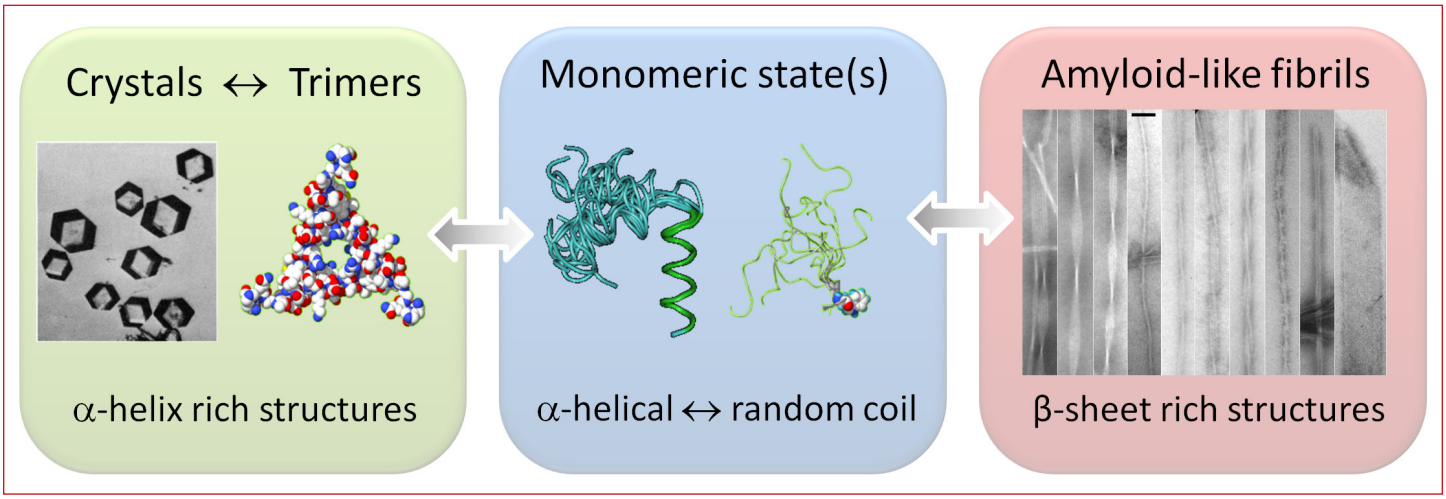

Figure 1. Conformational states of glucagon ranging from α-helical crystals and trimers, α-helical or random-coil monomers to several types of β-sheet-rich amyloid-like fibrillar states (see text for details). Picture of glucagon crystals,[4] coordinates for trimer, and α-helical and random coil glucagon taken from the pdb data base entries 1gcn,[6] 1KX6,[26] and 1NAU.[27] Electron microscopy image gallery of glucagon fibrils with 50-nm scale bar.[28]

All Types of Glucagon Fibrils Form Reversibly and Show Differences in Stability

The CD spectrum of different types of glucagon fibrils exhibit unique fingerprint features that distinguish them (**Figure 2A**), even though the secondary structure information is not straightforward to interpret.[12,39] The stability of different fibrils can be compared by monitoring the changes in CD spectrum during gradual temperature ramping giving reproducible thermal melting mid-points (T_m^{app}) for most types of fibrils (**Figure 2B**). Type A fibrils that form at high glucagon concentrations represent the least stable fibril type characterized so far, with a low melting midpoint (T_m^{app} <32 °C), compared to type $B_{agitated}$ fibrils (T_m^{app} = 55 °C) that form at low glucagon concentrations.[39]

In contrast to the +5 predicted for monomeric glucagon at pH 2.5, the net charge of glucagon in type $B_{agitated}$ fibrils appears to be zero.[46] Consequently, the stability of type $B_{agitated}$ fibrils is highly dependent on pH, and they can be dissociated instantly by decreasing the pH to 1.1.[39] In contrast, type S fibrils are very stable in acid, but T_m^{app} is highly dependent on salts in the solution increasing with 22 °C for every tenfold increase in salt concentration (**Figure 2C**). Divalent sulfate anions are particularly stabilizing to type S fibrils, and addition of only 1 mM Na_2SO_4 (7:1 ratio glucagon at 0.5g/liter or 0.144 mM) gives them a selective growth advantage

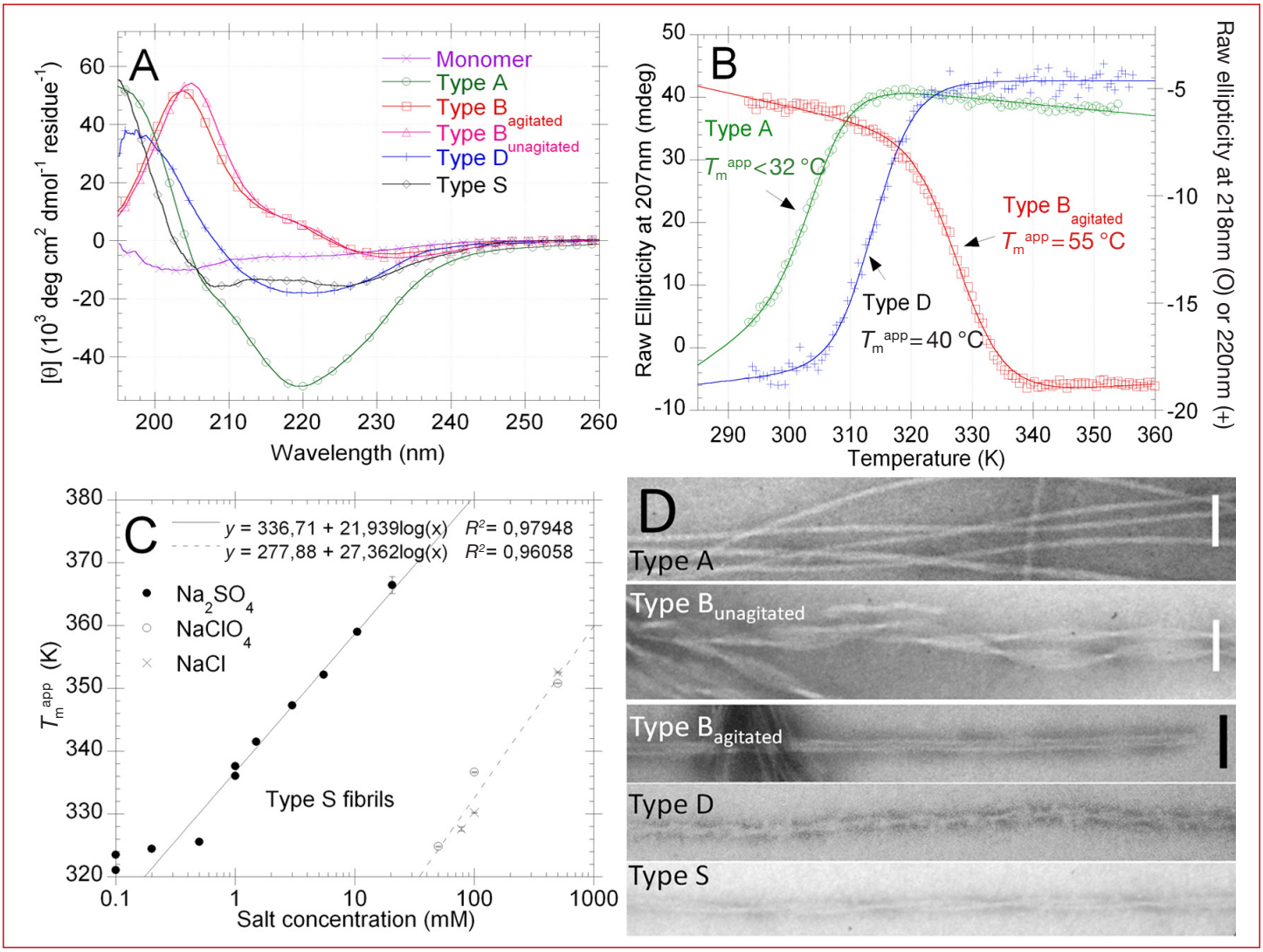

Figure 2. Properties of different types of glucagon fibrils. (**A**) Comparison of CD-spectra of different glucagon fibril types showing unique fingerprint features.[45] (**B**) Stability of fibrils can be estimated for comparative purposes by monitoring changes in CD during temperature ramping at constant speed (90 °C/h, 0.025 g/liter sonicated fibrils, 25 mM glycine/HCl pH 2.7).[39] (**C**) Stability of type S fibrils is highly dependent on salt concentrations and SO_4^{2-} anions are particularly stabilizing to the fibrils.[40] (**D**) Comparison of fibril morphology of different types of glucagon fibrils as viewed in electron microscopy. Scale bar is 50 nm.[45]

over other fibril types.[40] This suggests that the positive charges of glucagon become incorporated into type S fibrils, shielded by the interaction with counter ions from the solution.[40,43] Consequently, type S fibrils dissociate rapidly when pH is increased beyond neutral, most likely because of the electrostatic repulsion between the negative charges of the SO_4^{2-} and the deprotonated N-terminus and the three asparagine residues.[40] On the other hand, type B fibrils remain intact and also form *de novo* at pH 9.5.[45]

Intrinsic Tryptophan Fluorescence Can Be Used to Monitor the Formation of Glucagon Fibrils

A variety of methods have been used to monitor the aggregation of proteins into fibrils, ranging from simple sample turbidity readings to X-ray diffraction and calorimetry.[47] *In situ* ThT fluorescence[48] is a frequently used method that allows high-throughput monitoring of amyloid fibril formation kinetics. However, because some types of fibrils stain very poorly with this dye, ThT emission cannot be used as a general quantitative measure for the extent of fibril formation.[39] Glucagon contains a single tryptophan (Trp) residue,[49] a particularly useful internal spectroscopic probes whose fluorescence spectrum reports on the local environment of the side chain.[50] Fibril formation changes the emission spectrum of glucagon Trp-25, in most cases a dramatic blue-shift in emission maximum from around 352 nm for monomer to as low as 318 nm for type B fibrils is observed,[39] indicating embedding of the side-chain in a hydrophobic environment. Such spectral changes can be used to quantify the degree of glucagon fibril formation, even for fibril types that do not stain with ThT. Moreover, the fluorescence spectrum also contains information about local structure and solvent accessibility of Trp,[43] which in combination with other techniques played a key role in the discovery of different types of fibrils and the mechanisms by which they form.[39]

Fibrils Grow Exponentially from Unfavorable Thermodynamic Nuclei

The elongated shape of fibrils implies that they grow linearly by addition of molecules to their ends.[51,52] Exponential growth of fibrils can be achieved via secondary pathways, such as branching, breaking or heterogeneous nucleation (catalysis of nucleation by existing fibrils), which result in a continuous increase of the number of fibril ends that can accept monomers at a rate proportional to fibril mass present at any given time.[53] Formation of individual types of glucagon fibrils can be studied by adjusting solution conditions to favor growth of that particular type fibril (**Table 1**), such as relatively low glucagon concentrations together with agitation for type $B_{agitated}$ (see **Figure 3**). Quantitative

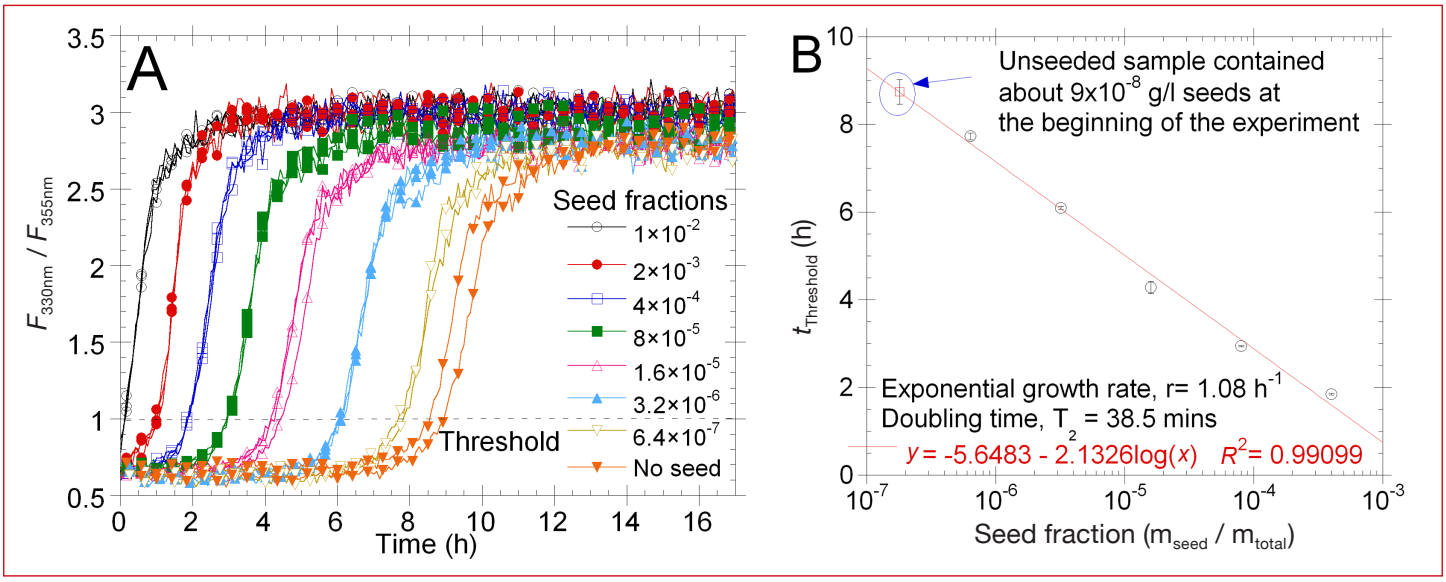

Figure 3. Quantitative seeding assay for formation of type $B_{agitated}$ fibrils from 0.5 g/liter glucagon in 50 mM glycine/HCl pH 2.5 with 3-second agitation every 10 minutes using Trp-25 emission signal upon excitation at 295 nm. (**A**) Seeded fibrillogenesis assay with a two-fold dilution series of preformed fibrils. Fibril formation is detected as a shift in the ratio between emission signals at 330 nm and 355 nm. (**B**) Linear correlation between $t_{threhold}$ and the seed fraction demonstrates that fibrils grow exponentially and the slope of the curve indicates that fibrils have a doubling time of about 38.5 minutes under the given conditions. The hypothetical concentration of seeds or fibril nuclei at time t=0h (when glucagon was dissolved) can be estimated from the curve.[39]

Table 1.
Summary of Reported Fibril Structures for Glucagon, and References in which the Structures are Investigated

Fibril type	"Recipe" growth conditions[a]	Characteristics	ThT staining	References
A	High (>5 g/liter) glucagon, 50 mM glycine pH 2.5 low agitation	Metastable single protofilament fibrils.	High	12,39,44,54
$B_{unagitated}$	<0.5 g/liter glucagon, 50 mM glycine pH 2.5 low agitation	Branching twisted fibrils.	Low	12,44,55
$B_{agitated}$	<0.5 g/liter glucagon, 50 mM glycine pH 2.5 or 9.5 vigorous agitation	Non-twisted fibrils consisting of two or more parallel protofilaments. Growth by fragmentation. Charge neutral in fibrillated state.	Low	28,39,46
D	<0.5 g/liter glucagon, 50mM glycine pH 2.5 + 150-250 mM Cl^-	Twisted, tightly packed fibrils. Unusual X-ray diffraction pattern with peaks at 5.32 and 6.36Å in addition to the classical 4.78Å.	Low	39,43
S	<0.5 g/liter glucagon, 0.01 N HCl, 1 mM SO_4^{2-}	Twisted mature fibrils. Dramatically stabilized sulfate salts.	High	40,43

[a] The prevailing structure of fibrils formed may be influenced by the source of glucagon as well as properties of surfaces.

seeding assays (QSA), where a monomeric solution is seeded with a dilution series of preformed fibrils, can be used to reveal kinetic properties of the mechanism by which they were formed. The linear correlation between the logarithm of the amount of seeds added and the lag-time or threshold time ($t_{threshold}$) clearly demonstrates that the fibrils grow exponentially even through the period where the amount of fibrils is below the detection limits (**Figure 3B**). The rate of exponential growth under the given conditions, in this case a doubling time of 38.5 minutes, can be accurately calculated from the slope of the curve. The $t_{threshold}$ for the unseeded experiment can be used to estimate nucleation rates. Although nucleation in principle occurs throughout the experiment, the exponential growth of fibrils from early nucleation events contribute to the majority of the fibrils formed, minimizing the effect of later nucleation events.[53] Notably, the lag-time is highly reproducible even for the unseeded experiment,[39] which suggests that glucagon fibril nucleation is not a single stochastic event, but can be considered as an unfavorable thermodynamic equilibrium between monomers (0.5 g/liter) and type $B_{aggregated}$ nuclei/seeds (<9 × 10^{-8} g/liter) at the given conditions. It is often stated that amyloid formation proceeds via a complex multistep mechanism involving several intermediate states.[56] However, several studies designed to detect intermediate states during formation of glucagon fibrils using size exclusion chromatography,[32] fast field fractionation,[57] dynamic light scattering,[12] nuclear magnetic resonance (NMR)[58] and small angle X-ray scattering,[54] did not detect accumulation of any oligomeric intermediate species other than the aforementioned α-helical trimers. Thus it appears that complexity arises not from obligatory intermediates, but from the competition between multiple aggregation pathways.[28,34,45]

Different Fibril Types Form via Distinct Pathways

Structural investigations of the individual glucagon fibril types can reveal the secondary pathway by which they achieve exponential growth. Time-lapse total internal reflection fluorescence microscopy demonstrates directly that type $B_{unagitated}$ fibrils grow via branching under conditions without agitation[55] (**Figure 4A**). The branching generates new fibril ends that can accept monomers resulting in slow exponential growth.[53] Agitation or stirring has been reported to accelerate fibril formation of a number of proteins, including insulin,[59] prions,[60] Aβ,[36] and glucagon.[39] Electron microscopy pictures demonstrate progressive shortening of type $B_{agitated}$ glucagon fibrils formed under agitated conditions (**Figure 4B**), suggesting

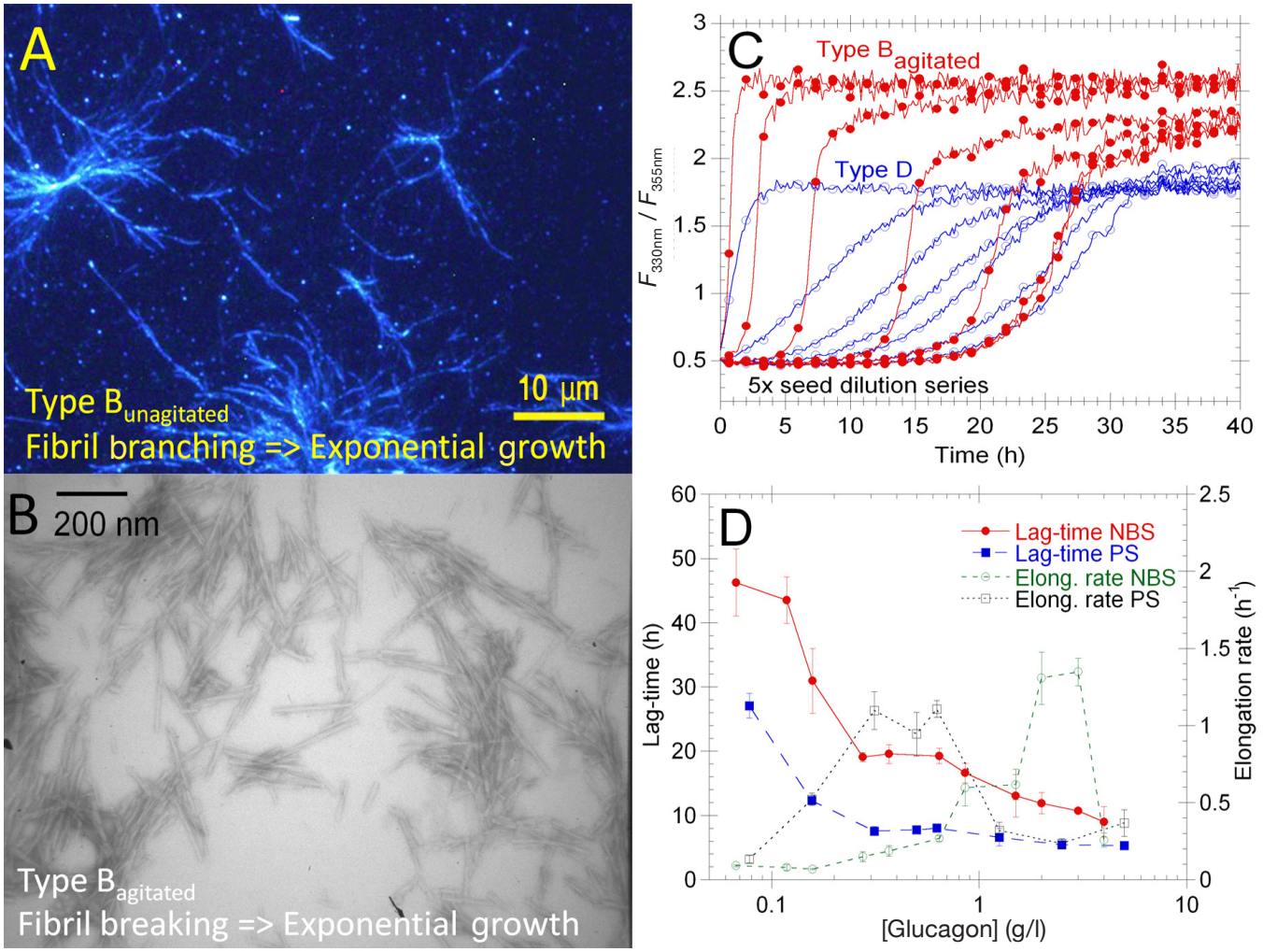

Figure 4. The secondary pathways leading to exponential growth of glucagon fibrils in 50 mM glycine/HCl buffer at pH 2.5. (**A**) Total internal reflection fluorescence microscopy image from a time series demonstrating that branching of type $B_{unagitated}$ increases the number of fibril ends under unagitated conditions.[55] (**B**) Electron microscopy image from a time series demonstrating progressively shorter fibrils, indicating continuous increase in number of fibril ends due to breaking of type $B_{agitated}$ fibrils under agitated conditions.[39] (**C**) Time-courses of Trp fluorescence during seeding of 1 g/liter glucagon with 5-fold dilution series, starting at 1%, of type $B_{agitated}$ (●) or type D (○) shows different significant difference both spectrum and curve shape. At low seeding fractions of type Bagitated significant amounts of type A fibrils form transiently, resulting in a lowered F330nm/F355nm fluorescence ratio at low seeding fractions.[39] (**D**) Effect of surface properties on glucagon fibril formation kinetics of glucagon at variable concentrations in 50 mM glycine/HCl pH 2.5 with 3-second agitation every 10 minutes, using either polyethylene oxide non-binding surface (NBS™, Corning, Lowell, MA) or standard polystyrene (PS Nunc) plates. Lag times and elongation rates (signifying the maximum slope of the growth curve) were determined using the previously described formula.[39,61]

that this catalyzes, breaking to create more free fibril ends.[18,62] The CD spectrum of fibrils formed with and without agitation is very similar (**Figure 2A**), but their morphology in EM images is remarkably different (**Figure 2D**): Type $B_{unagitated}$ fibrils are twisted and show branching, whereas type $B_{agitated}$ form parallel pairs, suggesting that differences in packing of protofilaments is important for the ability of fibrils to branch and break, respectively. Remarkably, the $A\beta_{1\text{-}40}$ peptide forms similar self-propagating "quiescent" and "agitated" fibrils, which share the twisted and non-twisted morphologies of type $B_{unagitated}$ and $B_{agitated}$ glucagon fibrils, respectively.[36] According to solid-state NMR the packing of the $A\beta_{1\text{-}40}$ in the two fibrils types is similar, but quiescent fibrils have a triangular cross-section with a narrow central cavity created by three protofilaments, while the agitated only consist of two protofilaments.[63] Quiescent and agitated forms of insulin fibrils with different optical properties have recently been reported.[64] Moreover, the intriguing "stirred" and "rotated" types of human prion fibrils[65] suggest that different modes of agitation may specifically enhance the secondary pathways for particular fibril types, giving these selective growth advantage over other types of fibrils.[28,64]

Growth rate of type B glucagon fibrils is highest between 0.3 and 0.6 g/liter[39] and concentrations above this significantly inhibit their formation, possibly due to the formation of α-helical trimers.[12] In contrast, the relatively unstable type A fibrils grow well even at concentrations above 8 g/liter where α-helical trimers are abundant.[12,39] When growth of type A fibrils reaches equilibrium (i.e., where elongation and dissociation rates are equal) the remaining concentration of free glucagon is still sufficient, and now low enough, to allow the growth of type $B_{unagitated}$ fibrils.[45] As the more stable type B fibrils consume the remaining monomers, the dissociation rate of the unstable type A fibrils becomes larger than the elongation rate, leading to a net conversion from type A to $B_{unagitated}$ fibrils. Thus, type A fibrils act as an off-pathway reservoir that lowers glucagon concentration sufficiently to allow formation of type $B_{unagitated}$ fibrils.[45] Comparison between the development of Trp emission spectrum during seeding with type $B_{agitated}$ or type D fibrils demonstrates propagation of the spectrum of the seeding parent fibrils (**Figure 4C**), and reveal that the two form via distinct pathways.[39] Thus, one can think of the different fibril types as microbe species that grow exponentially by consuming the media nutrients (glucagon monomers) in competition with each other,[28] with the important addendum that fibril nuclei can materialize spontaneously. Accordingly, fibrils types that are incapable of exponential growth and nucleate slowly never reach our detection limits before they are outcompeted by types that do grow exponentially.

Studies on the formation of insulin and Aβ fibrils have demonstrated that the properties of surfaces that contact protein solutions can have a tremendous impact on both the rate of fibril formation and the type of fibrils formed.[35,38,66] Changing the surface properties of microtiter plates used for the glucagon fibril formation assay from untreated polystyrene to non-binding polyethylene oxide increases lag times, but also changes the shape of growth curves, suggesting that binding to surfaces may catalyze both growth and/or nucleation of specific types of glucagon fibrils (**Figure 4D**).

Strategies for Controlling Fibril Formation

Delivery of both insulin and glucagon in an automated closed-loop system is currently being pursued as a method of improving quality of life in patients with type 1 diabetes.[61,67,69] Such systems require stable liquid formulation where formation of fibrils is minimized due to potential clogging of infusion catheters in addition to potential toxicity of fibrils formed. Knowledge of the mechanisms that lead to protein fibril formation is an important prerequisite to the design of strategies to prevent it. In principle, prevention of nucleation, elongation or secondary pathways should all significantly hamper fibril formation. Having non-binding surfaces contact glucagon solutions is important (**Figure 4-D**); however, a single crack in the surface of a tube could catalyze fibril nucleation and would be enough to start exponential growth of fibrils.

The effects of various additives on growth and nucleation rates for different fibril types can be tested with QSA (**Figure 3**). Designed peptide inhibitors of amyloid structures have been tested for Aβ,[70,71] and there is evidence that this strategy might have value because purity of glucagon has great impact on fibril forming kinetics[4]; fibrils grow much faster from highly purified pharmaceutical-grade glucagon compared to samples containing several solid-state synthesis-derived impurities.[72] Moreover, addition of 1% oxidized glucagon dramatically decreases growth rate of fibrils, and introduces variability in the otherwise highly reproducible lag-time for fibril formation,[61] suggesting that glucagon analogs can interfere with fibril formation by competing with or blocking unmodified glucagon adsorption at the ends of fibrils. However, the ability of glucagon to form a multitude of different aggregates means that strategies to design inhibitors based on the structure of a particular fibril type could fail, because glucagon simply forms other types of fibril structures immune to this register poisoning. Therefore a strategy of stabilizing monomeric form, preventing it from interacting with other molecules, appears to be a much more attractive method for inhibition of fibril formation in general.

α, β, and γ cyclodextrins (CyD) are cyclic sugars consisting of 5, 6 or 7 α-D-glucosyl units, respectively, which have found extensive use as excipients in pharmaceutical formulations due to the ability of their lipophilic inner cores to bind and stabilize a variety of drug molecules.[73] The core of β-CyD has an appropriate size to fit aromatic side chains,[74] and a small change in emission spectrum indicates that β-CyDs also bind Trp-25 of glucagon, which is not observed α-CyDs (**Figure 5A**). Because of their low solubility in water, β-CyDs are often modified with various chemical groups on the "edge"-hydroxyls to increase solubility.[75] According to Trp-based kinetics, addition of hydroxypropyl-β-CyD (HP-βCyD) results in a significant increase in lag-times for the formation of both type $B_{agitated}$ and type S fibrils (**Figure 5B**), whereas addition of 12 mM methyl-β-CyD (M-βCyD) is able to block formation of type $B_{agitated}$ fibrils completely,[61]

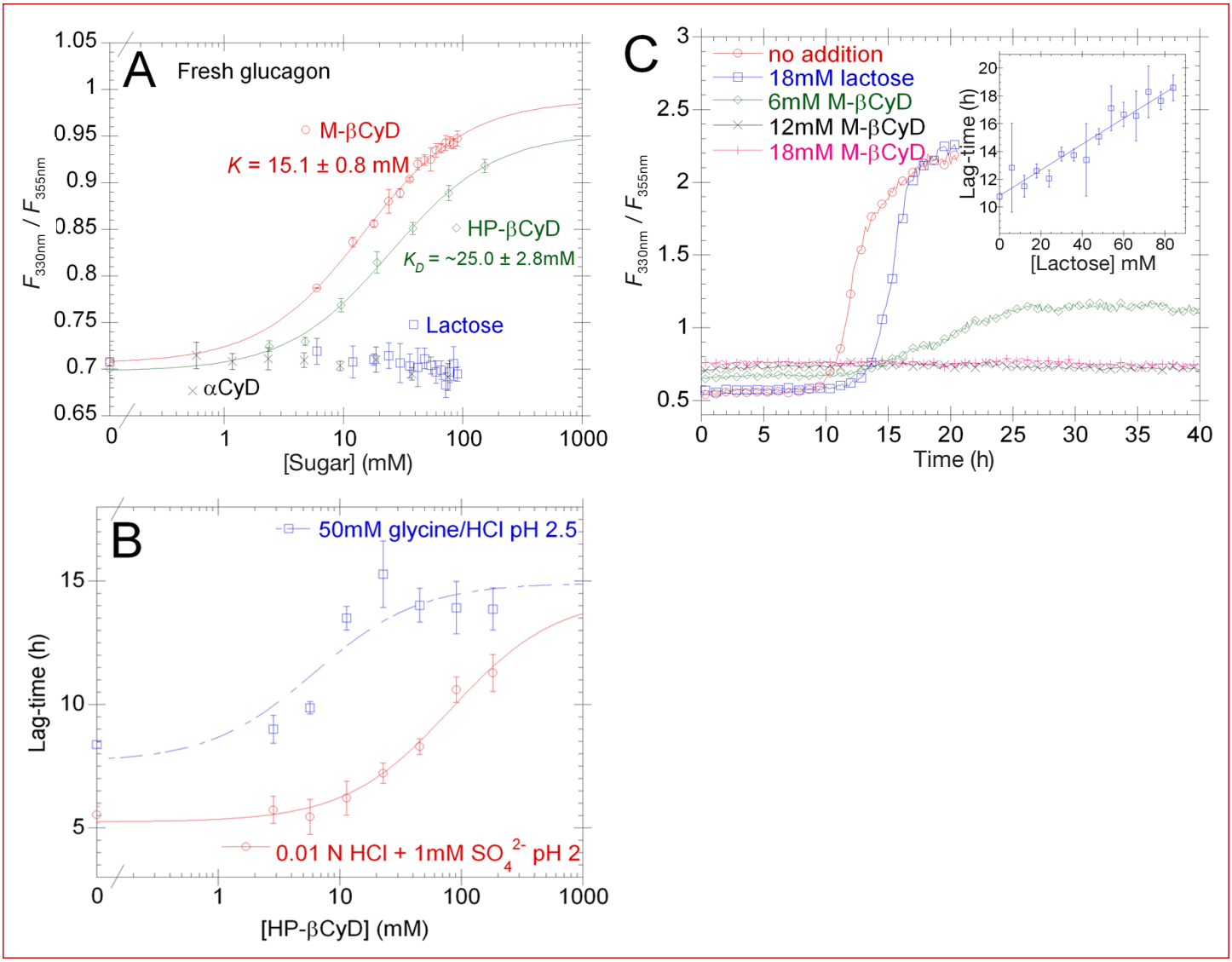

Figure 5. Effects of β cyclodextrins on the stability and fibril formation of glucagon. (**A**) Trp emission signals of 0.5 g/liter glucagon in 50mM glycine/HCl pH 2.5 show a modest blue-shift upon binding to β-CyD, which is not observed for α-CyD or lactose, suggesting that the methyl and hydroxyproply β-CyD binds Trp-25 of glucagon with a similar binding constant. (**B**) Hydroxypropyl-β-CyD (CAVASOL® W7 HP, Wacker Chemie AG) increases the lag-time of both type S and type B fibrils from 0.5 g/lliter glucagon with agitation 3 seconds every 10 minutes, but does not block fibril formation at the concentrations tested. (**C**) According to Trp emission signal, addition of 12mM or more Methyl W7 M1.8-β-CyD (CAVASOL W7 M, Wacker Chemie AG) completely suppresses formation of fibrils.[61]

even though conditions were designed to favor their formation (**Figure 5C, Table 1**). In contrast, lactose, used as excipient in the current formulation of glucagon for injection, only results in a slightly increased lag-time of fibril formation (**Figure 5B**, insert). The similar binding curves of HP- and M-βCyD to Trp-25, but dissimilar effects on fibril formation suggests that M-βCyD binds multiple aromatic side-chains on glucagon, possibly the phenylalanine-6 and/or tyrosine-10 found to be important for fibril formation.[72] Whereas HP-β-CyD is approved by the Food and Drug Administration as safe for use as an excipient in parenteral drugs,[76] the M-βCyD is not approved for injection because of its disruptive ability to extract cholesterol from cell membranes leading to hemolysis.[73,77] However, M-βCyD is approved for oral and nasal administration and has been tested as an effective excipient in glucagon formulated nasal administration.[78] Despite low stabilizing effects of γ-CyDs on human growth hormone compared to α and β-CyDs,[74] addition of γ-CyDs have been reported to have an inhibitory effect on glucagon fibril formation comparable to or exceeding β-CyDs.[79] Moreover, γ-CyDs also appear to improve the chemical stability of solid-state glucagon compared to lactose,[80] decreasing the oxidation and spontaneous decomposition of the dried peptide. Novel variants of water-soluble β-CyD and γ-CyDs are

continuously emerging,[73] and it is possible that some of the newer variants may have just the right ability to stabilize glucagon both chemically and physically, while still being safe for parenteral use.

Fibrillar Glucagon as Pharmaceutical Substance?

Even if fibril formulation of glucagon can be suppressed using the aforementioned strategies, the chemical stability of aqueous peptide solutions will always be limited because of the natural decomposition of peptides due to hydrolysis, oxidation, and deamidation, etc. These processes are expected to proceed significantly faster in monomeric states than in the less accessible stable amyloid states.[39] If glucagon is indeed stored as functional amyloid inside the α-cells,[30] it may be possible to mimic this in pharmaceutical formulation, perhaps by copying the natural structure using a seed propagation. With the large variety of possible fibrillar packings available to glucagon, it is possible that other fibril types may actually be more suitable for pharmaceutical formulations if practical challenges of the potentially increased viscosity leading to clogging of tubing can be overcome. The ideal candidate fibril type would naturally be non-toxic when injected, have high solubility and stability under formulation conditions, but still dissociate readily under physiological conditions to allow rapid activity. The most likely candidate of the currently described fibril types is probably type S fibrils. These fibrils can be formed reliably and reproducibly and are very soluble and stable under acidic conditions supplemented with sulfate. However, upon removal of sulfate or change in pH to neutral, such as found in blood, the fibrils dissociate instantly.[40] Future experiments are naturally required to test the safety of different types of glucagon fibrils.

Funding:

This work was funded by Aalborg University, Aalborg, Denmark, Novo Nordisk, Bagsværd, Denmark, and Carlsberg Research Foundation, Copenhagen, Denmark.

Acknowledgements:

Dr. Hans Aage Hjuler, and coworkers at Novo Nordisk A/S are acknowledged for funding as well as providing generous amounts of high quality glucagon. Drs. Christian Rischel and James Flink are thanked for fruitful collaborations and stimulating discussions. Dr. Christian Beyschau Andersen, Prof. Daniel Otzen and the rest of the protein biophysics group at Aarhus University are acknowledged for inspiring scientific conversations about the nature of glucagon fibrils and comments on this manuscript. Dr. Carmen Krammer is acknowledged for critical comments and proofreading.

References:

1. Jiang G, Zhang BB. Glucagon and regulation of glucose metabolism. Am J Physiol Endocrinol Metab. 2003;284(4):E671-8.
2. Elrick H, Witten TA, Arai Y. Glucagon treatment of insulin reactions. N Engl J Med. 1958;258(10):476-80.
3. Hradsky M, Stockbrugger R, Dotevall G, Ostberg H. The use of glucagon during upper gastrointestinal endoscopy. Gastrointest Endosc. 1974;20(4):162.
4. Staub A, Sinn L, Behrens OK. Purification and crystallization of glucagon. J Biol Chem. 1955;214(2):619-32.
5. King MV. A low-resolution structural model for cubic glucagon based on packing of cylinders. J Mol Biol. 1965;11:549-61.
6. Sasaki K, Dockerill S, Adamiak DA, Tickle IJ, Blundell T. X-ray analysis of glucagon and its relationship to receptor binding. Nature. 1975;257(5529):751-7.
7. Panijpan B, Gratzer WB. Conformational nature of monomeric glucagon. Eur J Biochem. 1974;45(2):547-53.
8. Gratzer WB, Beaven GH, Rattle HW, Bradbury EM. A conformational study of glucagon. Eur J Biochem. 1968;3(3):276-83.
9. Onoue S, Yamamoto K, Kawabata Y, Hirose M, Mizumoto T, Yamada S. Novel dry powder inhaler formulation of glucagon with addition of citric acid for enhanced pulmonary delivery. Int J Pharm. 2009;382(1-2):144-50.
10. Swann JC, Hammes GG. Self-association of glucagon. Equilibrium studies. Biochemistry. 1969;8(1):1-7.
11. Gratzer WB, Creeth JM, Beaven GH. Presence of trimers in glucagon solution. Eur J Biochem. 1972;31(3):505-9.
12. Andersen CB, Otzen D, Christiansen G, Rischel C. Glucagon amyloid-like fibril morphology is selected via morphology-dependent growth inhibition. Biochemistry. 2007;46(24):7314-24.
13. Beaven GH, Gratzer WB, Davies HG. Formation and structure of gels and fibrils from glucagon. Eur J Biochem. 1969;11(1):37-42.
14. Moran EC, Chou PY, Fasman GD. Conformational transitions of glucagon in solution: the alpha to beta transition. Biochem Biophys Res Commun. 1977;77(4):1300-6.
15. Glenner GG, Eanes ED, Bladen HA, Linke RP, Termine JD. Beta-pleated sheet fibrils. A comparison of native amyloid with synthetic protein fibrils. J Histochem Cytochem. 1974;22(12):1141-58.
16. Masters CL, Beyreuther K. Pathways to the discovery of the Abeta amyloid of Alzheimer's disease. J Alzheimers Dis. 2006; 9(3 Suppl):155-61.
17. Prusiner SB. Molecular biology of prion diseases. Science. 1991;252(5012):1515-22.

18. Aguzzi A, Haass C. Games played by rogue proteins in prion disorders and Alzheimer's disease. Science. 2003;302(5646):814-8.
19. Winklhofer KF, Tatzelt J, Haass C. The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. Embo J. 2008;27(2):336-49.
20. Glabe CG. Common mechanisms of amyloid oligomer pathogenesis in degenerative disease. Neurobiol Aging. 2006;27(4):570-5.
21. Wyttenbach A. Role of heat shock proteins during polyglutamine neurodegeneration: mechanisms and hypothesis. J Mol Neurosci. 2004;23(1-2):69-96.
22. Caughey B, Lansbury PT. Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. Annu Rev Neurosci. 2003;26:267-98.
23. Cohen E, Bieschke J, Perciavalle RM, Kelly JW, Dillin A. Opposing activities protect against age-onset proteotoxicity. Science. 2006;313(5793):1604-10.
24. Otzen DE, Nielsen PH. We find them here, we find them there: Functional bacterial amyloid. Cell. Mol. Life Sci. 2008;65(4612): 910-927.
25. Fowler DM, Koulov AV, Balch WE, Kelly JW. Functional amyloid--from bacteria to humans. Trends Biochem Sci. 2007;32(5):217-24.
26. Braun W, Wider G, Lee KH, Wuthrich K. Conformation of glucagon in a lipid-water interphase by 1H nuclear magnetic resonance. J Mol Biol. 1983;169(4):921-48.
27. Ying J, Ahn JM, Jacobsen NE, Brown MF, Hruby VJ. NMR solution structure of the glucagon antagonist [desHis1, desPhe6, Glu9] glucagon amide in the presence of perdeuterated dodecylphosphocholine micelles. Biochemistry. 2003;42(10):2825-35.
28. Pedersen JS, Otzen DE. Amyloid a state in many guises: survival of the fittest fibril fold. Protein Sci. 2008;17(1):2-10.
29. Epand RM. Cation-induced conformational change in glucagon. Mol Pharmacol. 1982;22(1):105-8.
30. Maji SK, Perrin MH, Sawaya MR, Jessberger S, Vadodaria K, Rissman RA, Singru PS, Nilsson KP, Simon R, Schubert D, Eisenberg D, Rivier J, Sawchenko P, Vale W, Rick R. Functional amyloids as natural storage of peptide hormones in pituitary secretory granules. Science. 2009;325(5938):328-32.
31. Maji SK, Schubert D, Rivier C, Lee S, Rivier JE, Riek R. Amyloid as a depot for the formulation of long-acting drugs. PLoS Biol. 2008;6(2):e17.
32. Onoue S, Ohshima K, Debari K, Koh K, Shioda S, Iwasa S, Kashimoto K, Yajima T. Mishandling of the therapeutic peptide glucagon generates cytotoxic amyloidogenic fibrils. Pharm Res. 2004;21(7):1274-83.
33. Chien P, Weissman JS, DePace AH. Emerging principles of conformation-based prion inheritance. Annu Rev Biochem. 2004;73:617-56.
34. Kodali R, Wetzel R. Polymorphism in the intermediates and products of amyloid assembly. Curr Opin Struct Biol. 2007;17(1):48-57.
35. Dzwolak W, Smirnovas V, Jansen R, Winter R. Insulin forms amyloid in a strain-dependent manner: an FT-IR spectroscopic study. Protein Sci. 2004;13(7):1927-32.
36. Petkova AT, Leapman RD, Guo Z, Yau WM, Mattson MP, Tycko R. Self-propagating, molecular-level polymorphism in Alzheimer's beta-amyloid fibrils. Science. 2005;307(5707):262-5.
37. Campioni S, Mannini B, Zampagni M, Pensalfini A, Parrini C, Evangelisti E, Relini A, Stefani M, Dobson CM, Cecchi C, Chiti F. A causative link between the structure of aberrant protein oligomers and their toxicity. Nat Chem Biol. 2010;6(2):140-7.
38. Yoshiike Y, Akagi T, Takashima A. Surface structure of amyloid-beta fibrils contributes to cytotoxicity. Biochemistry. 2007;46(34):9805-12.
39. Pedersen JS, Dikov D, Flink JL, Hjuler HA, Christiansen G, Otzen DE. The changing face of glucagon fibrillation: structural polymorphism and conformational imprinting. J Mol Biol. 2006;355(3):501-23.
40. Pedersen JS, Flink JM, Dikov D, Otzen DE. Sulfates dramatically stabilize a salt-dependent type of glucagon fibrils. Biophys J. 2006;90(11):4181-94.
41. Prusiner SB. Prions. Proc Natl Acad Sci U S A. 1998;95(23):13363-83.
42. Toyama BH, Kelly MJ, Gross JD, Weissman JS. The structural basis of yeast prion strain variants. Nature. 2007;449(7159):233-7.
43. Jeppesen MD, Hein KL, Nissen P, Westh P, Otzen D. A thermodynamic analysis of fibrillar polymorphism. Biophys Chem. 2010; in press.
44. Andersen CB, Hicks MR, Vetri V, Vandahl B, Rahbek-Nielsen H, Thogersen H, Enghild JJ, Serpell LC, Rischel C, Otzen DE. Glucagon fibril polymorphism reflects differences in protofilament backbone structure. J Mol Biol. 2010;397(4):932-46.
45. Pedersen JS, Andersen CB, Otzen D. Amoloid structure - one but not the same: the many levels of glucagon polymorphism. Febs J. 2010;DOI: 10.1111/j.1742-4658.2010.07888.x (Forthcoming).
46. Jeppesen MD, Westh P, Otzen DE. The role of protonation in protein fibrillation. FEBS Lett. 2010;584(4):780-784.
47. Morris AM, Watzky MA, Finke RG. Protein aggregation kinetics, mechanism, and curve-fitting: a review of the literature. Biochim Biophys Acta. 2009;1794(3):375-97.
48. LeVine H, 3rd. Quantification of beta-sheet amyloid fibril structures with thioflavin T. Methods Enzymol. 1999;309:274-84.
49. Bromer WW, Staub A, Diller ER, Bird HL, Sinn LG, Behrens OK. The amino acid sequence of glucagon. J Am Chem Soc. 1957;79:2794-8.
50. Vivian JT, Callis PR. Mechanisms of tryptophan fluorescence shifts in proteins. Biophys J. 2001;80(5):2093-109.
51. Ban T, Hamada D, Hasegawa K, Naiki H, Goto Y. Direct observation of amyloid fibril growth monitored by thioflavin T fluorescence. J Biol Chem. 2003;278(19):16462-16465.
52. Ferkinghoff-Borg J, Fonslet J, Andersen CB, Krishna S, Pigolotti S, Yagi H, Goto Y, Otzen DE, Jensen MH. Stop-and-go kinetics in amyloid fibrillation. Available from Nature Proceedings <http://hdl.handle.net/10101/npre.2010.4279.1>.
53. Ferrone F. Analysis of protein aggregation kinetics. Methods Enzymol. 1999;309:256-74.
54. Oliveira CL, Behrens MA, Pedersen JS, Erlacher K, Otzen D, Pedersen JS. A SAXS study of glucagon fibrillation. J Mol Biol. 2009;387(1):147-61.
55. Andersen CB, Yagi H, Manno M, Martorana V, Ban T, Christiansen G, Otzen DE, Goto Y, Rischel C. Branching in amyloid fibril growth. Biophys J. 2009;96(4):1529-36.
56. Kelly JW. The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. Curr Opin Struct Biol. 1998;8(1):101-6.
57. Hoppe CC, Nguyen LT, Kirsch LE, Wiencek JM. Characterization of seed nuclei in glucagon aggregation using light scattering methods and field-flow fractionation. J Biol Eng. 2008;2:10.
58. Svane AS, Jahn K, Deva T, Malmendal A, Otzen DE, Dittmer J, Nielsen NC. Early stages of amyloid fibril formation studied by liquid-state NMR: the peptide hormone glucagon. Biophys J. 2008;95(1):366-77.

59. Banga AK, Mitra R. Minimization of shaking-induced formation of insoluble aggregates of insulin by cyclodextrins. J Drug Target. 1993;1(4):341-5.

60. Collins SR, Douglass A, Vale RD, Weissman JS. Mechanism of prion propagation: amyloid growth occurs by monomer addition. PLoS Biol. 2004;2(10):e321.

61. Pedersen JS. *In Vitro* Studies of Amyloid-Like Protein Fibrils Using Glucagon as Model System [PhD Thesis]. Aalborg: Aalborg University; 2006.

62. Hall D, Edskes H. Silent prions lying in wait: a two-hit model of prion/amyloid formation and infection. J Mol Biol. 2004;336(3): 775-86.

63. Paravastu AK, Leapman RD, Yau WM, Tycko R. Molecular structural basis for polymorphism in Alzheimer's beta-amyloid fibrils. Proc Natl Acad Sci USA. 2008;105(47):18349-54.

64. Loksztejn A, Dzwolak W. Vortex-induced formation of insulin amyloid superstructures probed by time-lapse atomic force microscopy and circular dichroism spectroscopy. J Mol Biol. 2010;395(3):643-55.

65. Makarava N, Baskakov IV. The same primary structure of the prion protein yields two distinct self-propagating states. J Biol Chem. 2008;283(23):15988-96.

66. Sluzky V, Tamada JA, Klibanov AM, Langer R. Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. Proc Natl Acad Sci U S A. 1991;88(21): 9377-81.

67. Steil GM, Rebrin K, Darwin C, Hariri F, Saad MF. Feasibility of automating insulin delivery for the treatment of type 1 diabetes. Diabetes. 2006;55(12):3344-50.

68. Castle JR, Engle JM, El Youssef J, Massoud RG, Yuen KC, Kagan R, Ward WK. Novel use of glucagon in a closed-loop system for prevention of hypoglycemia in type 1 diabetes. Diabetes Care. 2010.

69. El-Khatib FH, Russell SJ, Nathan DM, Sutherlin RG, Damiano ER. A bihormonal closed-loop artificial pancreas for type 1 diabetes. Sci Transl Med. 2010;2(27):27.

70. Findeis MA. Peptide inhibitors of beta amyloid aggregation. Curr Top Med Chem. 2002;2(4):417-23.

71. Sato T, Kienlen-Campard P, Ahmed M, Liu W, Li H, Elliott JI, Aimoto S, Constantinescu SN, Octave JN, Smith SO. Inhibitors of amyloid toxicity based on beta-sheet packing of Abeta40 and Abeta42. Biochemistry. 2006;45(17):5503-16.

72. Pedersen JS, Dikov D, Otzen DE. N- and C-terminal hydrophobic patches are involved in fibrillation of glucagon. Biochemistry. 2006;45(48):14503-12.

73. Challa R, Ahuja A, Ali J, Khar RK. Cyclodextrins in drug delivery: an updated review. AAPS PharmSciTech. 2005;6(2):E329-57.

74. Otzen DE, Knudsen BR, Aachmann F, Larsen KL, Wimmer R. Structural basis for cyclodextrins' suppression of human growth hormone aggregation. Protein Sci. 2002;11(7):1779-87.

75. Uekama K, Hirayama F, Irie T. Cyclodextrin drug carrier systems. Chem Rev. 1998;98(5):2045-76.

76. Irie T, Uekama K. Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. J Pharm Sci. 1997;86(2):147-62.

77. Thompson DO. Cyclodextrins--enabling excipients: their present and future use in pharmaceuticals. Crit Rev Ther Drug Carrier Syst. 1997;14(1):1-104.

78. Sakr FM. Nasal administration of glucagon combined with dimethyl-[beta]-cyclodextrin: Comparison of pharmacokinetics and pharmacodynamics of spray and powder formulations. International Journal of Pharmaceutics. 1996;132(1-2):189-194.

79. Matilainen L, Larsen KL, Wimmer R, Keski-Rahkonen P, Auriola S, Jarvinen T, Jarho P. The effect of cyclodextrins on chemical and physical stability of glucagon and characterization of glucagon/gamma-CD inclusion complexes. J Pharm Sci. 2008;97(7):2720-9.

80. Matilainen L, Maunu SL, Pajander J, Auriola S, Jaaskelainen I, Larsen KL, Jarvinen T, Jarho P. The stability and dissolution properties of solid glucagon/gamma-cyclodextrin powder. Eur J Pharm Sci. 2009;36(4-5):412-20.