



Optimized experimental pre-treatment strategy for temporary inhibition of islet amyloid polypeptide aggregation

Madison Q. Ferguson, Maria C. DeRosa *

Department of Chemistry, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, K1S 5B6, Canada

ARTICLE INFO

Keywords:

Islet amyloid polypeptide
Aggregation
Fibril
Type 1 diabetes
Type 2 diabetes
Thioflavin T assay
Transmission electron microscopy

ABSTRACT

Islet amyloid polypeptide (IAPP) is a neuroendocrine hormone from pancreatic β -cells. Misfolded, aggregated IAPP is believed to be toxic to islet cells and amyloid deposits in the pancreas are pathological hallmarks of type 2 diabetes. Rapid fibrillization of this peptide makes it difficult to study in its soluble form, impeding a better understanding of its role. In this study, a variety of popular pretreatment methods were tested for their ability to delay aggregation of IAPP, including solutions of hexafluoroisopropanol, sodium hydroxide, hydrochloric acid, phosphate buffered saline, ammonium hydroxide, as well as tris buffer at different pH and containing either calcium (II), zinc (II), or iron (II). Aggregation was assessed using the thioflavin T fluorescence assay as well as by transmission electron microscopy. Tris buffer at pH 8.1 containing Zn(II) was found to have the best balance of temporary inhibition of aggregation and biological relevance.

1. Introduction

Islet amyloid polypeptide (IAPP), also known as amylin, is a 37-amino acid residue co-synthesized and secreted with insulin from β -cells in the pancreas. IAPP aids in the secretion of glucagon from α -cells in the pancreas to the blood stream and has a secondary role of being a gastric emptying as well as a satiety agent [1–4]. The aggregation of IAPP is implicated in the pathogenesis of type 2 diabetes (T2DM) due to its toxicity in beta cells and evidence of IAPP amyloid plaques in humanized rodent models for T2DM [1,4,5]. Furthermore, evidence that IAPP levels are elevated in type 1 diabetes (T1DM) patients and that it acts as an islet autoantigen suggests it may also play a role in T1DM [6]. Notably, IAPP aggregates form rapidly in islets transplanted into T1D recipients and may be implicated in islet graft dysfunction [7].

Amyloidogenesis of IAPP is a self-driven process where misfolded IAPP will preferentially aggregate. This process initiates when two or more mostly unstructured monomers misfold into a beta-sheet conformation yielding cytotoxic oligomers, then progress exponentially to a fibrillar form. The fibril form can insert in the phospholipid bilayer leading to cell apoptosis, specifically of β - cells (Fig. 1a). IAPP perturbation of the phospholipid bilayer is among many factors that may explain its cytotoxicity to β - cells [5].

Further study is needed in order to decipher the exact mechanism of IAPP aggregation and to elucidate its potential role in T1DM and T2DM.

Unfortunately, the rapid aggregation of the peptide upon dilution can make these studies difficult. Nevertheless, there are many examples of exogenous ligands (e.g. ions, small molecules [8], polymers, and nanoparticles [9]) that have been identified as inhibitors of amyloid polypeptide (APP) as well as IAPP fibrillization through a variety of pathways [10–14]. Notably, metal ion solutions have been shown to redirect aggregation of amyloids off-pathway leading to oligomeric forms with varying impact on toxicity. For example, iron solutions were found to induce a more cytotoxic form of APP by impeding aggregation [12]. Whereas, specifically for IAPP, it has been shown that zinc at the same concentration does not elicit these toxic effects [15]. Zinc is found in relatively high concentrations within the pancreas, and has been shown to have a potential effect in delaying aggregation *in vitro* [16,17].

A simple, biologically inert, pretreatment strategy is needed in order to delay, but not prevent, IAPP aggregation and permit longer term experiments to determine mechanism of aggregation and the effectiveness of new inhibitors. In this work, multiple pretreatment methods used to suppress the aggregation of similar amyloid polypeptides were applied to IAPP (Fig. 1 b) [3,18–24]. Moreover, these methods were compared to some new approaches in order to optimize a pretreatment method for IAPP studies. All the methods were assessed by transmission electron microscopy (TEM) and the thioflavin T assay (ThT). ThT is a fluorescent dye that is the gold standard for identifying and quantifying the formation of structurally ordered fibrils over a long period of time

* Corresponding author.

E-mail address: maria.derosa@carleton.ca (M.C. DeRosa).

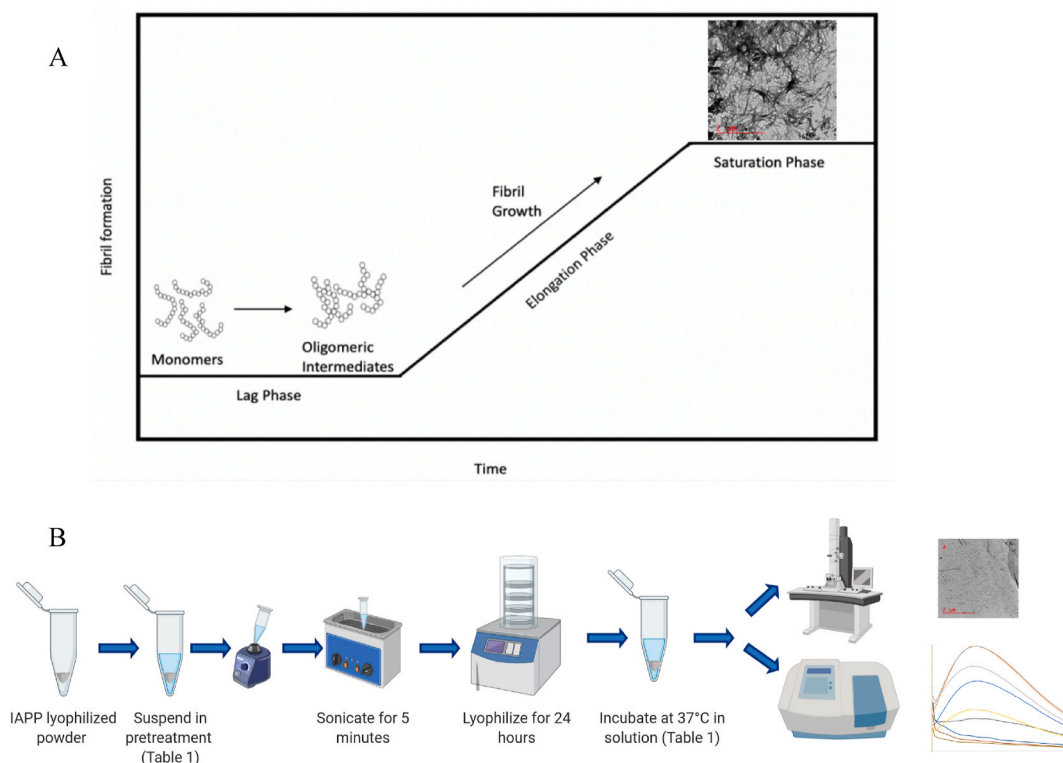


Fig. 1. a) Illustration of human IAPP fibril formation. Oligomers form from misfolded monomers, changing their conformational state and leading to cytotoxicity. The elongation phase is where oligomers extend and aggregate into fibrils, eventually reaching a saturation phase where the majority of the IAPP have formed into fibrils. b) Scheme outlining the experimental protocol used in this work for suppressing IAPP aggregation. Figure prepared in Biorender.

Table 1

Description of pretreatment and testing strategies investigated in this study. Identification name is used to describe the pretreatment along with testing solution used for that given analysis.

Identification name	Pretreatment	Testing solution
HCl	2 mM HCl	PBS pH 7.4
NaOH	2 mM NaOH	
NH ₄ OH	10% NH ₄ OH	
PBS	PBS pH 7.4	
HFIP		
Zn 8.1 HFIP	Neat HFIP	2 mM Zn in Tris pH 8.1
Zn 7.8 HFIP		2 mM Zn in Tris pH 7.8
Zn 7.4 HFIP		2 mM Zn in Tris pH 7.4
Ca 8.1 HFIP		2 mM Ca in Tris pH 8.1
Ca 7.8 HFIP		2 mM Ca in Tris pH 7.8
Ca 7.4 HFIP		2 mM Ca in Tris pH 7.4
Fe 8.1 HFIP		2 mM Fe in Tris pH 8.1
Fe 7.8 HFIP		2 mM Fe in Tris pH 7.8
Fe 7.4 HFIP		2 mM Fe in Tris pH 7.4
Zn 8.1		2 mM Zn ²⁺ in Tris buffer pH 8.1
Ca 8.1	2 mM Ca ²⁺ in Tris buffer pH 8.1	2 mM Ca in Tris pH 8.1
Fe 8.1	2 mM Fe ²⁺ in Tris buffer pH 8.1	2 mM in Tris H 8.1

[25,26]. Our studies indicate that a pretreatment consisting of HFIP and Zn(II) ion at pH 8.1 was the most effective pretreatment strategy for delaying IAPP aggregation and could be used in future work to find new aggregation inhibitors.

2. Experimental

2.1. Materials

IAPP was purchased from New England Peptide. All other reagents

were purchased from Sigma-Aldrich (Oakville, On, Canada). Pretreatment solutions of 100 mM Tris-HCl (either pH 8.1, 7.8, or 7.4), 100 mM NaCl, and 2 mM of metal dichloride, HCl, NaOH, NH₄OH, HFIP, or PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄) were prepared in house.

2.2. IAPP pretreatments and testing

0.5 mg of lyophilized IAPP from 0.1% TFA was suspended in 1 mL of one of the pre-treatment solutions at room temperature (see Table 1). Solutions were vortexed and placed onto sonicator for 5 min, placed under a stream of argon, removing volatile organics, for 5 min, then were freeze-dried using a lyophilizer at -50°C for 24 h (See Fig. 1b).

Pretreated, lyophilized IAPP samples were then re-dissolved into testing solutions to monitor aggregation (Table 1). Pretreated lyophilized IAPP from hexafluoroisopropanol (HFIP) 10% ammonium hydroxide (NH₄OH) [20], 2 mM Hydrochloric acid (HCl) [19], PBS pH 7.4 [20], 2 mM sodium hydroxide (NaOH) [19], were all suspended in 5 mL of PBS buffer pH 7.4 solution. The remaining pretreated HFIP samples were dissolved in 5 mL of the following solutions: 2 mM Ca (II) chloride in Tris buffer (pH 7.4, 7.8 and 8.1), 2 mM Zn (II) chloride in Tris buffer (pH 7.4, 7.8 and 8.1) [15], 2 mM Fe (II) [12] chloride in Tris buffer (pH 7.4, 7.8 and 8.1). Pretreated lyophilized IAPP from 2 mM Ca (II) chloride, 2 mM Zn (II) chloride and 2 mM Fe (II) chloride in Tris buffer pH 8.1 were all re-dissolved in 5 mL of their same respective solution. Note that salts are not removed during lyophilization step and therefore they are present in final solution. Different pH conditions were assessed from pH 7.4 to 8.1, which is the approximate range found in beta cells of the pancreas [27, 28]. pH of all solutions was checked before and after the experiment and no changes were noted. All solutions were placed into an incubator at 37°C. 100 µL of each solution was extracted every 24 h for testing by fluorescence and TEM until end of testing at 192 h.

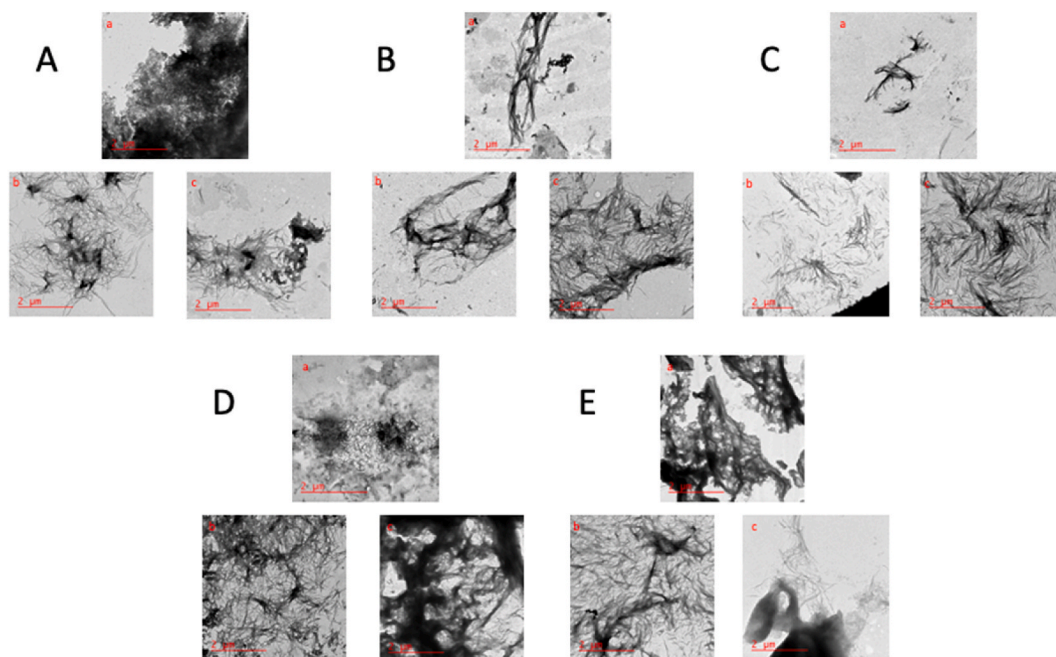


Fig. 2. 25 μM IAPP pretreated in HCl (A), PBS (B), NaOH (C), HFIP (D), NH₄OH (E), suspended in 5 mL pH 7.4 PBS solution drop casted onto TEM grids and stained using 1% uranyl acetate solution. 60 min (a), 120 h (b), 192 h (c) incubation time at 37°C prior to drop casting. All scale bars are 2 μM.

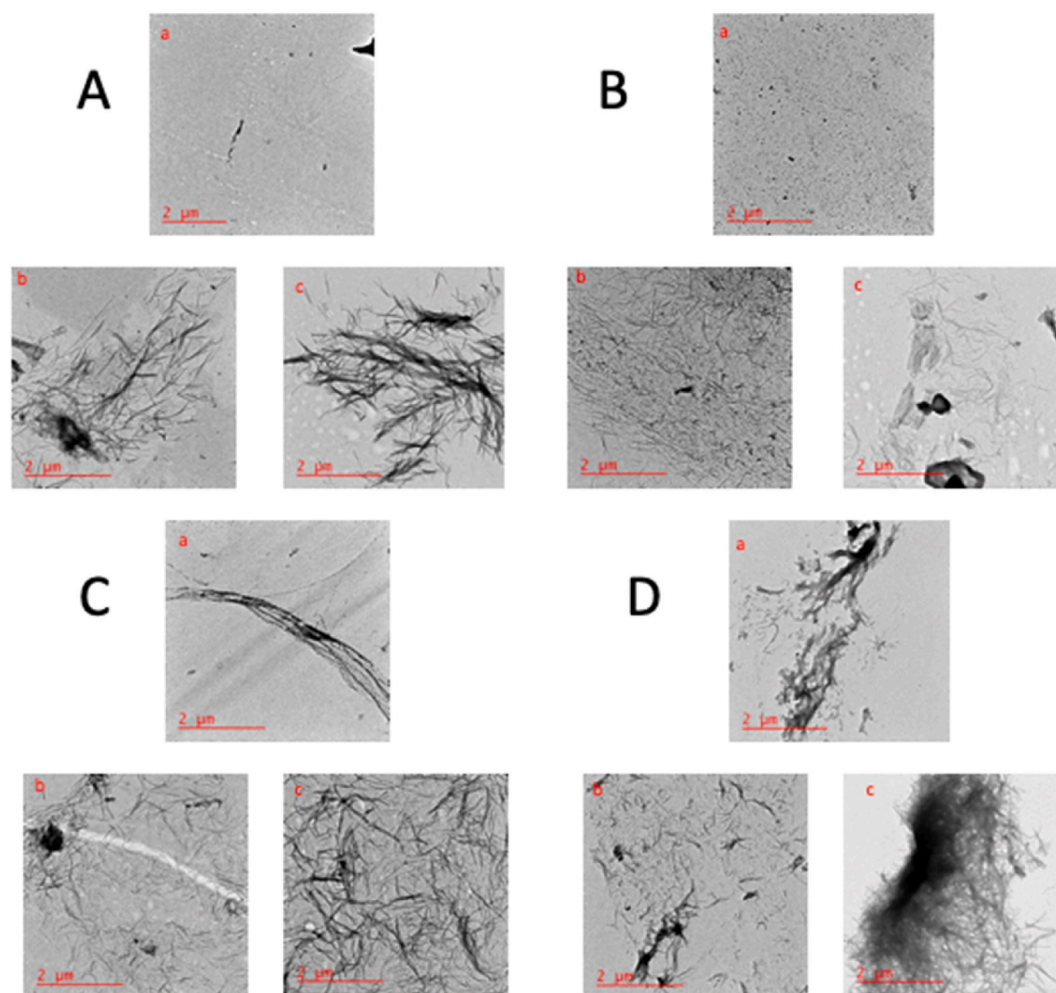


Fig. 3. 25 μM IAPP pretreated with Ca 8.1 (A), HFIP (B,C,D), all lyophilized then suspended in 5 mL of Ca pH 8.1 solution (A,B), Ca pH 7.8 solution (C) Ca pH 7.4 solution (D), drop casted onto TEM grids and stained using 1% uranyl acetate solution. 60 min (a), 120 h (b), 192 h (c) incubation time at 37°C prior to drop casting. All scale bars are 2 μM.

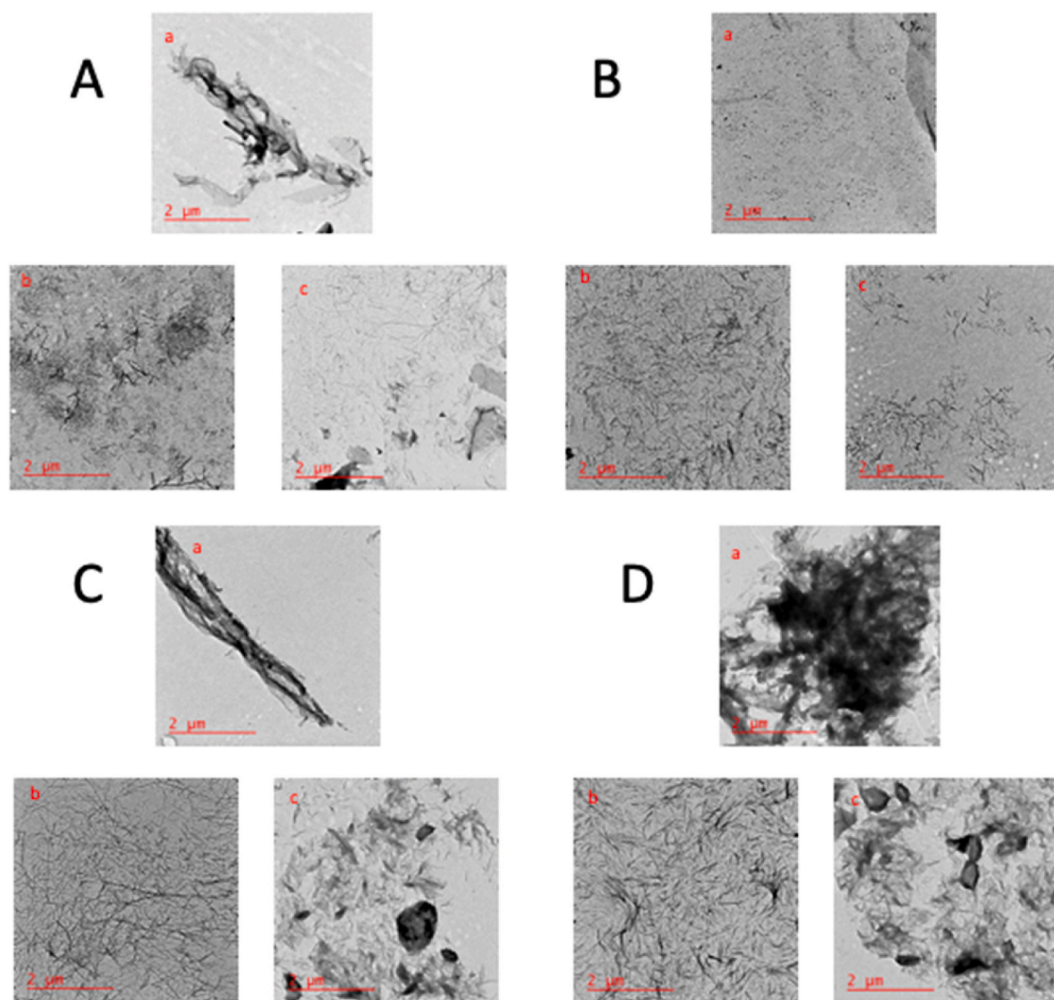


Fig. 4. 25 μ M IAPP pretreated with Zn 8.1 (A), HFIP (B,C,D), all lyophilized then suspended in 5 mL of Zn pH 8.1 solution (A,B), Zn pH 7.8 solution (C) Zn pH 7.4 solution (D), drop casted onto TEM grids and stained using 1% uranyl acetate solution. 60 min (a), 120 h (b), 192 h (c) incubation time at 37°C prior to drop casting. All scale bars are 2 μ M.

2.3. Fluorescence testing

50 μ M thioflavin T (ThT) was dissolved into 100 μ L of sample solution and allowed to incubate at room temperature for 45 min before reading. Excitation $\lambda = 445$ nm emission $\lambda = 483$ nm Fluorolog fluorescence spectrophotometer (Horiba Jobin Yvon, Edison, NJ, USA) the average of 3 scans were plotted against one another for aggregation comparison analysis. Each sample was analyzed every day for eight days.

2.4. Transmission electron microscopy (TEM)

5 μ L of sample solution was drop casted onto a 200-copper mesh TEM grid FCF200-CU 50 (Electron microscopy science) then 1 μ L of 98% depleted 1% uranyl acetate (Polysciences) solution was applied to grid for 10 s interaction then removed. Grids were thoroughly washed with water and allowed to dry for 48 h prior to analysing using Technai G2 F20 TEM at the Carleton University Nano-imaging Facility.

3. Results

3.1. Transmission electron microscopy analysis

TEM was performed to qualitatively determine the relative shape and size of aggregates formed from each pretreatment. (See

representative images in Figs. 4–7).

Pretreatment tests of HCl, PBS, NaOH, HFIP, and NH_4OH (see Table 1 for pretreatment description) all yielded large fibrils within 60 min suspended in solution (Fig. 2). HFIP showed smaller aggregates on day one, although at day five and day eight mass amounts of large fibrils were observed. HCl, PBS, NaOH, and NH_4OH all showed similar-sized fibrils across all days with slight increases observed from day one to day five.

Fig. 3 examines treatments that involved Ca(II) ion. On day one, fewer aggregates were observed in Ca 8.1 and Ca 8.1 HFIP testing when compared on Ca 7.8 HFIP or Ca 7.4 HFIP. By day five and day eight, the degree of fibrilization was relatively consistent across all these treatments indicating that there is an initial inhibition of aggregation, but that aggregation is not completely halted. This is desirable for a pretreatment strategy that could be used in the discovery of new inhibitors.

Results from Zn(II) ion containing treatments can be visualized in Fig. 4. Zinc ion is found in the mM level in pancreatic beta cells [29] and has been observed to increase IAPP aggregation at atypical concentrations and decrease aggregation at physiological concentrations [30,31]. Only Zn 8.1 HFIP shows a noticeable reduction in aggregation of fibrils on day one while allowing for aggregation to proceed but at a slower rate through day five and day eight testing. All other zinc treatments (Zn 8.1, Zn 7.8 HFIP and Zn 7.4 HFIP) showed larger aggregates on day one progressing through day five, increasing in fibril complexity and size.

Fe(II) treatments are shown in Fig. 5. Interestingly, iron pretreatment

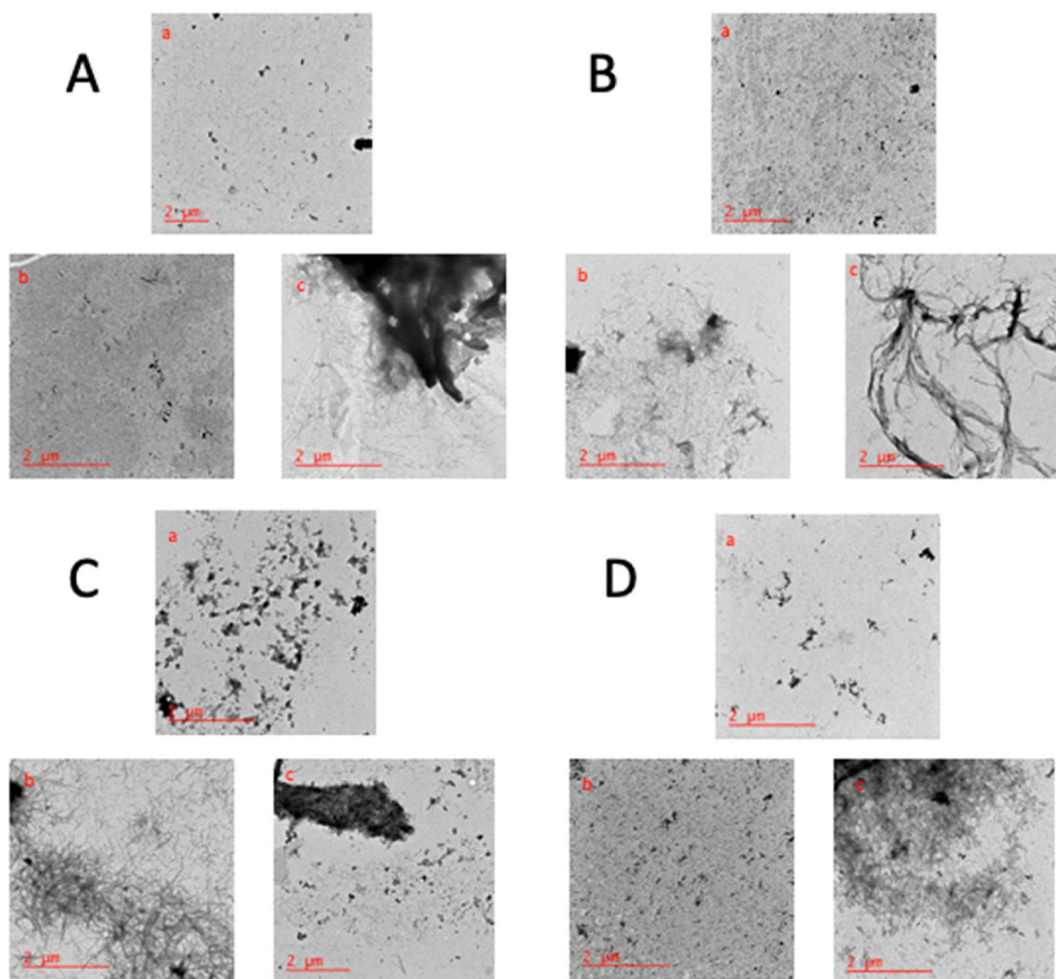


Fig. 5. 25 μM IAPP pretreated with Fe 8.1 (A), HFIP (B,C,D), all lyophilized then suspended in 5 mL of Fe pH 8.1 solution (A,B), Fe pH 7.8 solution (C) Fe pH 7.4 solution (D), drop casted onto TEM grids and stained using 1% uranyl acetate solution. 60 min (a), 120 h (b), 192 h (c) incubation time at 37°C prior to drop casting. All scale bars are 2 μM .

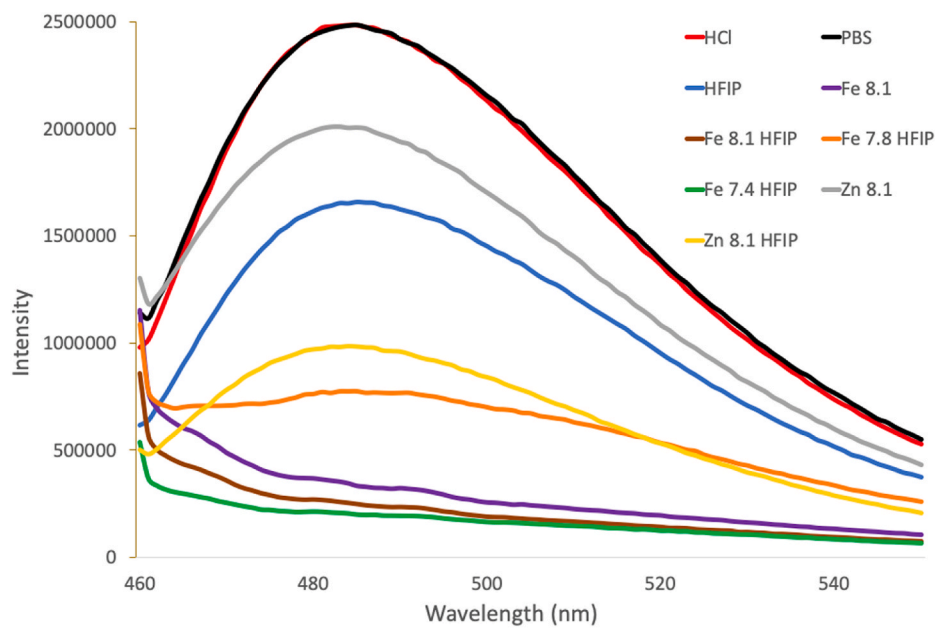


Fig. 6. 25 μM concentration of human IAPP suspended in respective buffers (see legend) at 37 °C for 192 h. Samples were treated with 50 μM ThT for 1 h and analyzed using excitation 445 nm and emission of 460 nm–550 nm.

Day 1

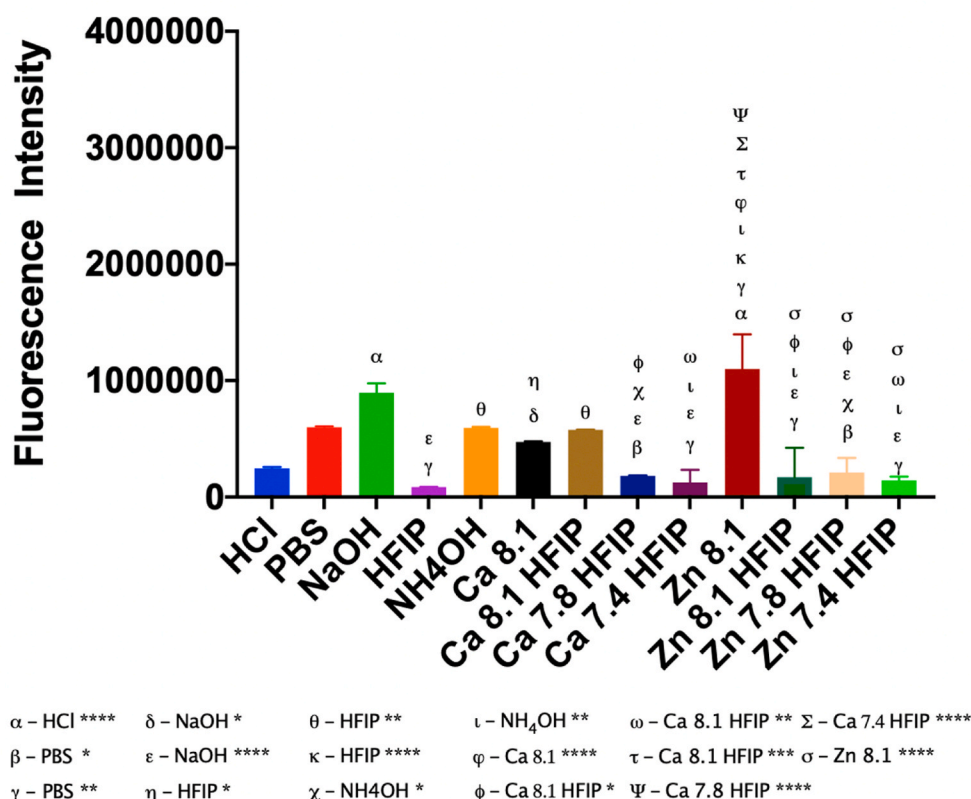


Fig. 7. 25 μ M concentration of human IAPP suspended in respective buffers at 37 °C for 60 min. Treated with 50 μ M ThT for 1 h, analyzed using excitation 445 nm and emission 483 nm. All tests were analyzed using one-way anova, Greek symbols indicate the groups where a significant difference was noted. “*” indicates $P \leq 0.05$, “**” indicates $P \leq 0.01$, “***” indicates $P \leq 0.001$ and “****” indicates $P \leq 0.0001$.

all show much smaller fibrils across all eight days. The smallest amount of fibril formation is observed in Fig. 7 A and B where Fe 8.1 and Fe 8.1 HFIP all show very small fibrils present when compared to other treatments. The relative fibril size increases slowly over eight days indicating significant delay of the aggregation process. Notably, the morphology of these aggregates are different from what was observed in the other treatments. Specifically, there are fewer large needle-like fibrils and more amorphous particulates.

3.2. Thioflavin T analysis

The ThT fluorescence assay was used to complement the TEM imaging and provide more ensemble assessment of the degree of aggregation after each treatment. Fig. 6 shows representative spectra for ThT fluorescence of the IAPP samples after treatment and incubation over 192 h. Interestingly, despite TEM evidence of fibrils in the iron-containing pretreatments, the ThT assay consistently showed no fluorescence signal for the presence of aggregates when any Fe(II) was present (See Figs. 5 and 6). This anomaly has been noted elsewhere [12] and for this reason, those treatments were excluded from the remainder of the ThT analysis.

Aggregation determined using the ThT assay (See Figs. 7–9) increased from day one to three then remained at a plateau from day three to eight for all treatments except for the Zn 8.1 HFIP and Zn pH 8.1 systems. Both Zn 8.1 HFIP and Zn 8.1 reached maximum on day four whereas Zn 8.1 HFIP had a significantly lower intensity indicating less aggregation was obtained before it reached that plateau.

Fig. 10 provides a direct comparison between our HFIP and Zn 8.1

methods. Overall Zn 8.1 HFIP pretreatment and testing solution provide the lowest overall aggregation occurring across 8 days, while striking an important balance that it does not prevent aggregation entirely. Therefore, this method is recommended for facilitating the discovery of new inhibitors.

4. Discussion

Interest in achieving a better understanding of the mechanism of IAPP aggregation and its role in pathogenesis of T2DM and T1DM is on the rise [1,15,23,31–35]. Given the propensity for the peptide to fibrillize, studies examining the non-aggregated peptide are difficult. Under conditions described by previous studies to support the non-fibril form, evidence of highly aggregated peptide was observed at the outset [36–38]. Thus, this work sought a method for treating IAPP to ensure that investigations could be done with samples that were not already in aggregated form. On the other hand, a pretreatment that led to complete inhibition of aggregation or to an off-path aggregation state that was not physiologically relevant was also not ideal. Thus, a balance was sought by qualitatively examining the degree of aggregation after treatment using TEM as well as quantifying the aggregation state using the ThT fluorescence assay.

A variety of treatments were investigated in this study. On balance, the zinc treatments, in particular Zn 8.1 HFIP, showed the most promise. Smaller and fewer aggregates were observed initially when compared to the other samples, where upon day eight analysis aggregation of both were comparable to all other tests. This suggests that the Zn(II) ion might play an inhibitory role in the initial few days of testing but does

Day 5

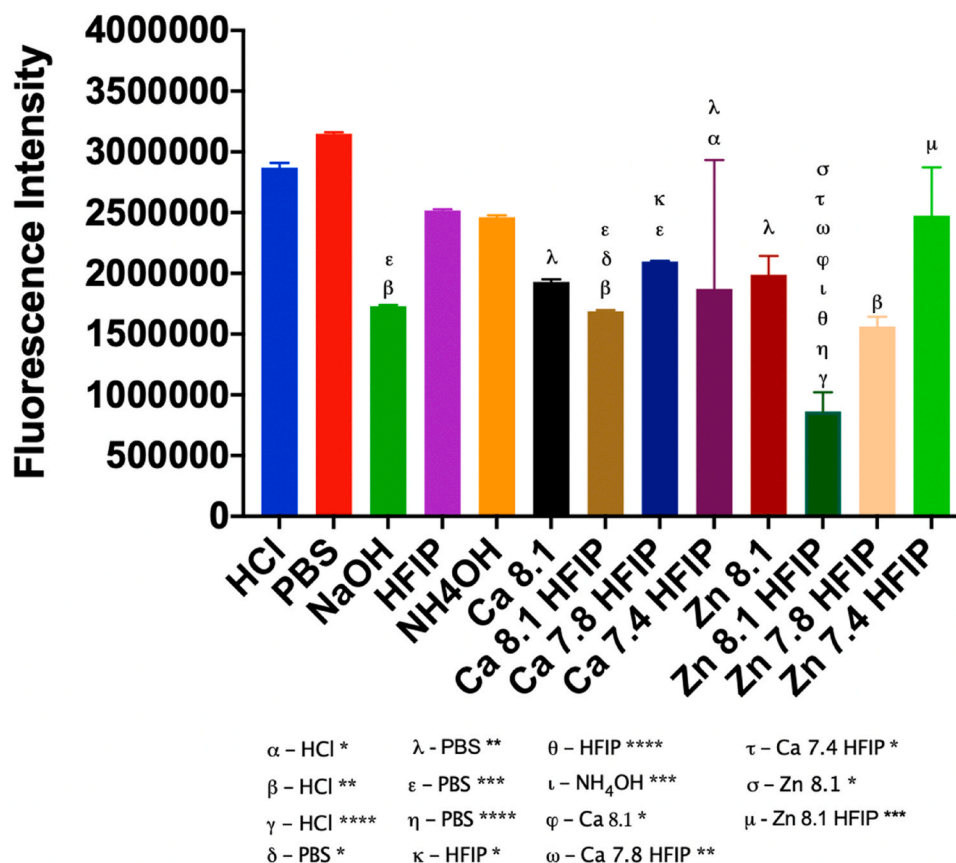


Fig. 8. 25 μ M concentration of human IAPP suspended in respective buffers at 37 °C for 120 h. Treated with 50 μ M ThT for 1 h, analyzed using excitation 445 nm and emission 483 nm. All tests were analyzed using one-way anova, Greek symbols indicate the groups where a significant difference was noted. “*” indicates $P \leq 0.05$, “***” indicates $P \leq 0.01$, “****” indicates, $P \leq 0.001$ and “*****” indicates $P \leq 0.0001$.

not permanently alter the peptide in such a way that aggregation is completely blocked. In contrast, the Fe(II) containing treatment strategies eventually led to similar sized large fibrils, however, far fewer of them and their morphology appeared to be different. It has been reported in other work that iron pauses IAPP aggregation in a highly cytotoxic oligomeric form, which may account for the differences noted here and preclude it from use as an appropriate pre-treatment [12,39]. Aggregation of IAPP is believed to be driven by an amyloidogenic region from Serine (Ser) 20-Ser29 where alanine 25, Ser 28, and Ser 29 are present. Indeed, in the rodent version of IAPP, which does not aggregate, all three of these residues are replaced with Pro. Zn(II) ion is believed to bind with the imidazole ring of histidine (His) 18 [15,25,40]. Notably, His 18 is the only residue in the N-terminus region which is different from the rodent version of IAPP [32]. Fe(II) and Ca(II) can also interact with histidine to varying degrees which may correlate with the degree of inhibition observed [40].

Other gold standard methods of pretreatment were taken into consideration for this study but then ultimately not used. Many methods use HFIP to suspend the peptide followed by filtration through a filter size of 0.22 μ m–0.45 μ m [41–43]. To avoid peptide loss, we instead investigated methods that did not require filtration. Overall, this experiment has allowed for the investigation of 17 different commonly used pretreatment methods with reports that aggregation is delayed. The most common pretreatment used for IAPP include HFIP, which in our hands has been shown to induce the highest aggregates across days

2–8. Alternatively, these investigations have shown that Zn 8.1 HFIP is suitable for delayed aggregation of IAPP but never preventing aggregation.

Understanding the mechanism and path towards aggregation of IAPP can help elucidate the role IAPP plays in the pathogenesis towards T2DM and T1DM. Pretreatment of IAPP with HFIP followed by suspension in Zn 8.1 solution appears to strike the optimal balance that allows for analysis of aggregation from initial monomeric form and onward. This method will allow for more fruitful study of IAPP to help unravel its function and impact.

5. Conclusion

In this study, 17 approaches for the pretreatment of IAPP were investigated for their inhibition of aggregation of the peptide. Overall, the initial treatment with HFIP, followed by incubation in a 2 mM Zn(II) containing Tris buffer solution at pH 8.1 yielded the optimal scenario of short term inhibition of aggregation of IAPP. This method will allow researchers to better investigate the path of IAPP aggregation as well as assess the effects of other inhibitory compounds.

Declaration of competing interest

None.

Day 8

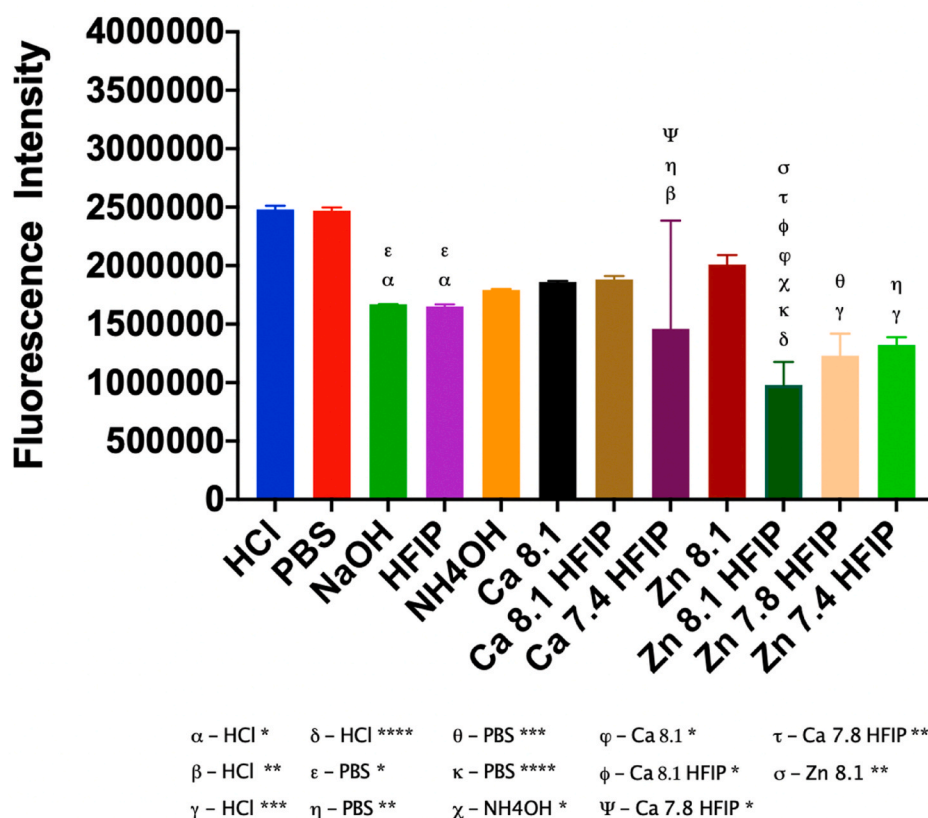


Fig. 9. 25 μM concentration of human IAPP suspended in respective buffers at 37 $^{\circ}\text{C}$ for 192 h. Treated with 50 μM ThT for 1 h, analyzed using excitation 445 nm and emission 483 nm. All tests were analyzed using one-way anova, Greek symbols indicate the groups where a significant difference was noted. "*" indicates $P \leq 0.05$, "***" indicates $P \leq 0.01$, "****" indicates, $P \leq 0.001$ and "*****" indicates $P \leq 0.0001$.

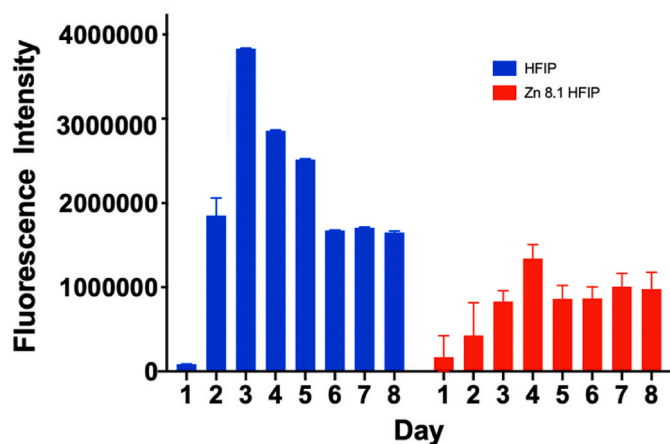


Fig. 10. 25 μM concentration of human IAPP tested ion HFIP and Zn 8.1 HFIP at 37 $^{\circ}\text{C}$ across 8 days. Samples were treated with 50 μM ThT for 1 h, analyzed using excitation 445 nm and emission 483 nm.

Acknowledgements

The authors acknowledge Carleton University and the Natural Sciences and Engineering Council for funding (Discovery Grant 224070).

References

- [1] S. Asthana, B. Mallick, A.T. Alexandrescu, S. Jha, IAPP in type II diabetes: basic research on structure, molecular interactions, and disease mechanisms suggests potential intervention strategies, *Biochim. Biophys. Acta Biomembr.* 1860 (9) (2018) 1765–1782.
- [2] M.F. Hogan, D.T. Meier, S. Zraika, A.T. Templin, M. Mellati, R.L. Hull, M. A. Leissring, S.E. Kahn, Inhibition of insulin-degrading enzyme does not increase islet amyloid deposition in vitro, *Endocrinology* 157 (9) (2016) 3462–3468.
- [3] A. Abedini, P. Cao, A. Plesner, J. Zhang, M. He, J. Derk, S.A. Patil, R. Rosario, J. Lonier, F. Song, et al., RAGE binds preamyloid IAPP intermediates and mediates pancreatic β cell proteotoxicity, *J. Clin. Invest.* 128 (2) (2018) 682–698.
- [4] Y. Kiriya, H. Nochi, Role and cytotoxicity of amylin and protection of pancreatic islet β -cells from amylin cytotoxicity, *Cells* 7 (8) (2018) 95.
- [5] D. Raleigh, X. Zhang, B. Hastoy, A. Clark, The β -cell assassin: IAPP cytotoxicity, *J. Mol. Endocrinol.* 59 (3) (2017) R121–R140.
- [6] H.C. Denroche, C.B. Verchere, IAPP and type 1 diabetes: implications for immunity, *Metabolism and Islet Transplants.* (2018).
- [7] A. Andersson, S. Bohman, L.A.H. Borg, J.F. Paulsson, S.W. Schultz, G. T. Westermark, P. Westermark, Amyloid deposition in transplanted human pancreatic islets: a conceivable cause of their long-term failure, *Exp. Diabetes Res.* 2008 (2008) 562985.
- [8] A.S. Pithadia, A. Bhunia, R. Sribalan, V. Padmini, C.A. Fierke, A. Ramamoorthy, Influence of a curcumin derivative on HIAPP aggregation in the absence and presence of lipid membranes, *Chem. Commun.* 52 (5) (2016) 942–945.
- [9] M. Wang, A. Kallinen, E.H. Pilkington, T.P. Davis, P.C. Ke, Differential effects of silver and iron oxide nanoparticles on IAPP amyloid aggregation, *Biomater. Sci.* 5 (3) (2017) 485–493.
- [10] S. Lee, A.A. Pioszak, Molecular interaction of an antagonistic amylin analog with the extracellular domain of receptor activity-modifying protein 2 assessed by fluorescence polarization, *Biophys. Chem.* 267 (2020) 106477.
- [11] R.L. Bower, L. Yule, T.A. Rees, G. Deganutti, E.R. Hendrikse, P.W.R. Harris, R. Kowalczyk, Z. Ridgway, A.G. Wong, K. Swierkula, et al., Molecular signature for receptor engagement in the metabolic peptide hormone amylin, *ACS Pharmacol. Transl. Sci.* 1 (1) (2018) 32–49.

- [12] B. Liu, A. Moloney, S. Meehan, K. Morris, S.E. Thomas, L.C. Serpell, R. Hider, S. J. Marciniak, D.A. Lomas, D.C. Crowther, Iron promotes the toxicity of amyloid β peptide by impeding its ordered aggregation, *J. Biol. Chem.* 286 (6) (2011) 4248–4256.
- [13] A. Pithadia, J.R. Brender, C.A. Fierke, A. Ramamoorthy, Inhibition of IAPP aggregation and toxicity by natural products and derivatives, *J. Diabetes Res.* 2016 (2016).
- [14] D. Sellin, L.M. Yan, A. Kapurniotu, R. Winter, Suppression of IAPP fibrillation at anionic lipid membranes via IAPP-derived amyloid inhibitors and insulin, *Biophys. Chem.* 150 (1–3) (2010) 73–79.
- [15] P. Nedumpully-Govindan, Y. Yang, R. Andorfer, W. Cao, F. Ding, Promotion or inhibition of islet amyloid polypeptide aggregation by zinc coordination depends on its relative concentration, *Biochemistry* 54 (50) (2015) 7335–7344.
- [16] L.C.S. Erthal, A.F. Marques, F.C.L. Almeida, G.L.M. Melo, C.M. Carvalho, L. C. Palmieri, K.M.S. Cabral, G.N. Fontes, L.M.T.R. Lima, Regulation of the assembly and amyloid aggregation of murine amylin by zinc, *Biophys. Chem.* 218 (2016) 58–70.
- [17] J.R. Brender, J. Krishnamoorthy, G.M.L. Messina, A. Deb, S. Vivekanandan, L. Rosa, J.E. Penner-hahn, Zinc Stabilization of Prefibrillar Oligomers of Human Islet Amyloid Polypeptide, vol. 1, 2013.
- [18] T. Takahashi, K. Tada, H. Mihara, RNA aptamers selected against amyloid β -peptide ($a\beta$) inhibit the aggregation of $a\beta$, *Mol. Biosyst.* 5 (9) (2009) 986–991.
- [19] Y. Fezoui, D.M. Hartley, J.D. Harper, R. Khurana, D.M. Walsh, M.M. Condron, D. J. Selkoe, J. Lansbury, A.L. Fink, D.B. Teplow, An improved method of preparing the amyloid β -protein for fibrillogenesis and neurotoxicity experiments, *Amyloid* 7 (3) (2000) 166–178.
- [20] T.M. Ryan, J. Caine, H.D.T. Mertens, N. Kirby, J. Nigro, K. Breheny, L. J. Waddington, V.A. Streltsov, C. Curtain, C.L. Masters, et al., Ammonium hydroxide treatment of $a\beta$ produces an aggregate free solution suitable for biophysical and cell culture characterization, *PeerJ* 1 (2013) e73.
- [21] K.U. Bilik, E. Ergüven, S. Klussmann, F. Jarosch, P.Y. Wielinga, T.A. Lutz, T. Riediger, In-vitro and in-vivo antagonistic action of an anti-amylin spiegelmer, *Neuroreport* 18 (17) (2007) 1855–1859.
- [22] Mirecka, E. A.; Feuerstein, S.; Gremer, L.; Schröder, G. F.; Stoldt, M.; Willbold, D.; Hoyer, W. β -Hairpin of Islet Amyloid Polypeptide Bound to an Aggregation Inhibitor. 1–8.
- [23] J.R. Brender, S. Salamekh, A. Ramamoorthy, Membrane disruption and early events in the aggregation of the diabetes related peptide IAPP from a molecular perspective, *Acc. Chem. Res.* 45 (3) (2012) 454–462.
- [24] S.J.C. Lee, T.S. Choi, J.W. Lee, H.J. Lee, D.G. Mun, S. Akashi, S.W. Lee, M.H. Lim, H.I. Kim, Structure and assembly mechanisms of toxic human islet amyloid polypeptide oligomers associated with copper, *Chem. Sci.* 7 (8) (2016) 5398–5406.
- [25] A. Abedini, D.P. Raleigh, The role of his-18 in amyloid formation by human islet amyloid, *Polypeptide* 44 (2005) 16284–16291.
- [26] K. Gade Malmos, L.M. Blancas-Mejia, B. Weber, J. Buchner, M. Ramirez-Alvarado, H. Naiki, D. Otzen, ThT 101: a primer on the use of thioflavin T to investigate amyloid formation, *Amyloid* 24 (1) (2017) 1–16.
- [27] P. Melamed, F. Melamed, Chronic metabolic acidosis destroys pancreas, *J. Pancreas* 15 (6) (2014) 552–560.
- [28] D.F. Magee, T.T. White, Influence of vagal stimulation on secretion of pancreatic juice in pigs, *Ann. Surg.* 161 (1965) 605–607.
- [29] T. Zhao, Q. Huang, Y. Su, W. Sun, Q. Huang, W. Wei, Zinc and its regulators in pancreas, *Inflammopharmacology* 27 (3) (2019) 453–464.
- [30] A.I. Ilitchev, M.J. Giammona, J.N. Schwarze, S.K. Buratto, M.T. Bowers, Zinc-induced conformational transitions in human islet amyloid polypeptide and their role in the inhibition of amyloidosis, *J. Phys. Chem. B* 122 (2018) 9852–9859.
- [31] J.R. Brender, K. Hartman, R. Prakash, R. Nanga, R. De, S. Bea, S. Vivekanandan, E. G. Neil, A. Ramamoorthy, Role of zinc in human islet amyloid polypeptide aggregation, *J. Am. Chem. Soc.* 12 (2007) 8973–8983.
- [32] Wiltzius, J. E. D. J. W.; Sievers, S. A.; Sawaya, M. R.; Cascio, D.; Popov, D.; Riekel, C.; Eisenberg, D. Atomic Structure of the Cross- β Spine of Islet Amyloid Polypeptide (Amylin).
- [33] E.H. Pilkington, M. Lai, X. Ge, W.J. Stanley, B. Wang, M. Wang, A. Kallinen, M. A. Sani, M.R. Whittaker, E.N. Gurzov, et al., Star polymers reduce islet amyloid polypeptide toxicity via accelerated amyloid aggregation, *Biomacromolecules* 18 (12) (2017) 4249–4260.
- [34] C. Zelus, Myricetin inhibits islet amyloid polypeptide (IAPP) aggregation and rescues living mammalian cells from IAPP toxicity, *Open Biochem. J.* 6 (1) (2012) 66–70.
- [35] P.C. Ke, P. Chen, Probing the aggregation and immune response of human islet amyloid polypeptides with ligand-stabilized gold nanoparticles, *ACS Appl. Mater. Interfaces* 11 (2019) 10462–10471.
- [36] J.R. Brender, J. Krishnamoorthy, G.M.L. Messina, A. Deb, S. Vivekanandan, C. La Rosa, J.E. Penner-Hahn, A. Ramamoorthy, Zinc stabilization of prefibrillar oligomers of human islet amyloid polypeptide, *Chem. Commun.* 49 (32) (2013) 3339–3341.
- [37] V. Armiento, K. Hille, D. Naltsas, J.S. Lin, A.E. Barron, A. Kapurniotu, The human host-defense peptide cathelicidin LL-37 is a nanomolar inhibitor of amyloid self-assembly of islet amyloid polypeptide (IAPP), *Angew. Chem. Int. Ed.* (2020) 12837–12841.
- [38] C.E. Higham, E.T.A.S. Jaikaran, P.E. Fraser, M. Gross, A. Clark, Preparation of Synthetic Human Islet Amyloid Polypeptide (IAPP) in a Stable Conformation to Enable Study of Conversion to Amyloid-like ϵ Brils vol. 470, 2000, pp. 55–60.
- [39] A. Nunes, S.M. Marques, C. Quintanova, D.F. Silva, S.M. Cardoso, S. Chaves, M. A. Santos, Multifunctional iron-chelators with protective roles against neurodegenerative diseases, *Dalton Trans.* 42 (17) (2013) 6058–6073.
- [40] P. Nedumpully-govindan, Y. Yang, R. Andorfer, W. Cao, F. Ding, Promotion or Inhibition of Islet Amyloid Polypeptide Aggregation by Zinc Coordination Depends on its Relative Concentration, 2015.
- [41] E. Godin, P.T. Nguyen, X. Zottig, S. Bourgault, Identification of a hinge residue controlling islet amyloid polypeptide self-assembly and cytotoxicity, *J. Biol. Chem.* 294 (21) (2019) 8452–8463.
- [42] H. Wang, A. Abedini, B. Ruzsicska, D.P. Raleigh, Rationally designed, nontoxic, nonamyloidogenic analogues of human islet amyloid polypeptide with improved solubility, *Biochemistry* 53 (37) (2014) 5876–5884.
- [43] X. Zhang, J.R. St Clair, E. London, D.P. Raleigh, Islet amyloid polypeptide membrane interactions: effects of membrane composition, *Biochemistry* 56 (2) (2017) 376–390.