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Copper is a potent inhibitor of the propensity for human ProIAPP$_{1-48}$ to form amyloid fibrils *in vitro*

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Abstract

Background: The amyloidogenic peptides IAPP and ProIAPP$_{1-48}$ are implicated in β cell death in type 2 diabetes mellitus. While the mechanism of their deposition in vivo is unknown we have shown that in vitro metals can both accelerate, for example Al(III), and inhibit, for example Cu(II), their formation of amyloid.

Methods: We have used a combination of thioflavin T fluorescence (ThT) and transmission electron microscopy (TEM) to investigate the potency with which Cu(II) prevented human ProIAPP$_{1-48}$ from forming β sheets of amyloid fibrils both in the absence and presence of significant molar excesses of Al(III) or Zn(II).

Results: Cu(II) prevented ProIAPP$_{1-48}$ from forming fibrillar materials with β sheet structure at all concentrations above equimolar to peptide. At equimolar Cu(II) to ProIAPP$_{1-48}$ fibrillar-like materials were observed by TEM though these were not ThT-positive. Significant excesses of the competitive metals Al(III) and Zn(II) were unable to influence these effects of Cu(II).

Conclusions: Cu(II) was shown to be a potent inhibitor of amyloid formation by ProIAPP$_{1-48}$ and its potency was unaffected by significant excesses of either Al(III) or Zn(II). If the propensities for IAPP and ProIAPP to form amyloid are central to the aetiology of cell death in type 2 diabetes mellitus then the availability of Cu(II) to prevent amyloidogenesis may be a critical factor for future therapy.

Background

Islet amyloid polypeptide (IAPP or amylin) is an highly amyloidogenic peptide which is implicated in the death of β cells in the pancreas in type 2 diabetes mellitus (T2DM) [1]. It is a product of the metabolism of ProIAPP which upon incomplete enzymatic processing can also yield ProIAPP$_{1-48}$. This, possibly aberrant, form of ProIAPP is also amyloidogenic, is found co-localised with IAPP in amyloid deposits in the pancreas and is implicated in β cell death in T2DM [2-7].

Metals may have a role in amyloidogenesis *in vivo* [8] and are known to influence *in vitro* amyloid fibril formation of IAPP [9-11] and ProIAPP$_{1-48}$ [12]. Cu(II) prevents amyloidogenic peptides from forming β sheet structures [13-15] and also abolishes the β sheet conformation of preformed amyloid fibrils [16,17]. It remains equivocal as to whether the propensity for Cu(II) to prevent amyloidogenic peptides from forming β sheets is dependent upon the Cu(II) to peptide ratio and whether the potency of this effect is influenced by competitive cations. To this end, herein we have investigated how the Cu(II) to peptide ratio affects the formation of β sheets by ProIAPP$_{1-48}$ and whether the presence of either Al(III) or Zn(II) is an additional influence on this process.

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Methods

ProIAPP$_{1-48}$ was synthesised by standard FMOC-based solid phase methods using an Applied Biosystems 433A synthesiser and thereafter purified by RP HPLC, using water/acetonitrile mixtures buffered with 0.1% TFA, on a POROS 20R2 column. Peptide content was confirmed by quantitative amino acid analysis and lyophilised aliquots were stored at -80°C until required. Thawed peptide was dissolved in ultra pure water (conductivity < 0.067 μS/cm) to give stocks of *ca* 200 μM which were used to prepare treatments of either 20 or 50 μM peptide in modified KH buffer [15] at pH 7.4. This medium includes the biological buffer PIPES which unlike many other biological buffers is not known for any significant interactions with metal ions [18]. It also includes *ca* 1 mM of both of the physiologically important divalent metals Ca^{2+} and Mg^{2+}, both of which influence the aggregation properties of amyloidogenic peptides [19,20], which should be, but are not, included in all *in vitro* studies of *in vivo* amyloid aggregation. Metals (Al(III), Cu(II), Zn(II)) were added to treatments from certified stock solutions (Perkin-Elmer, UK) which guarantees that the metals are added to media as the free metal cations which is particularly important for the sparingly soluble $Al^{3+}_{(aq)}$ [21]. Metals were present in media prior to the addition of peptide except where Cu(II) was added to treatments containing preformed amyloid fibrils. EDTA was added to treatments from freshly prepared 0.1M stock solutions. All final concentrations of peptide, metals and EDTA are given in table titles and figure legends. All treatments were incubated at 37°C and β

sheet formation was followed using both ThT fluorescence [15] and transmission electron microscopy [16]. These are established methods for identifying β sheet formation in dilute peptide solutions in the micromolar concentration range. Polarising microscopy and fluorescence microscopy with ThT were used to identify and characterise spherulites [12,22].

Results

Low concentration (20 μM) of ProIAPP$_{1-48}$

At a peptide concentration of 20 μM ProIAPP$_{1-48}$ slowly formed ThT-positive amyloid producing fluorescence of 10 (2.6) and 42 (12.8) AU after 24h and 21 days respectively (Table 1). In the presence of a sub-stoichiometric concentration of Cu(II), 10 μM, ThT fluorescence was unchanged after 24h (13 (2.2) AU) and significantly lower after 21 days (29 (3.3) AU). At equimolar Cu(II) to peptide ThT fluorescence was significantly lower after both 24h (6 (5.2) AU) and 21 days (8 (3.1) AU). When the Cu(II) to peptide ratio was 2.0, 5.0 or 10.0 ThT fluorescence was effectively zero at both time points (Table 1). In the absence of added Cu(II) the presence of a 10-fold excess of Al(III) (200 μM) resulted in significantly higher ThT fluorescence at both 24h (46 (21.5) AU) and 21 days (75 (23.2) AU). However, the additional presence of only 10 μM Cu(II) reduced ThT fluorescence significantly at both 24h (7 (1.5) AU) and 21 days (12 (0.7) AU) and at higher concentrations of Cu(II) ThT fluorescence was effectively zero (Table 1). The ThT fluorescence of 20 μM ProIAPP$_{1-48}$ in the presence of a 10-fold excess of Zn(II) (200 μM) was unchanged by the presence of this metal and the fluorescence was significantly lower in the additional presence of 10 μM Cu(II) and effectively zero for Cu(II):peptide ratios > 1.0 (Table 1). When these experiments were repeated for Cu(II):peptide ratios of 0, 0.5, 0.75, 1.0, 1.5 and 2.0 and all treatments were incubated for 7 days the presence of Cu(II) effectively prevented the formation of ThT-positive amyloid at equimolar (20 μM) or higher concentrations (Table 2).

High concentration (50 μM) of ProIAPP$_{1-48}$

ProIAPP$_{1-48}$ formed ThT-positive amyloid more rapidly and more extensively at a peptide concentration of 50 μM giving fluorescence of 29 (10.9), 81 (19.6) and 216 (21.9) AU after 0, 24 and 72h respectively (Table 3). TEM of samples incubated for 24h showed dense 'plaques' of fibrils (mean diameter 5.6 (0.80) nm n=50) (Figure 1a). Addition of Cu(II) to a final concentration of 200 μM to preformed fibrils at 72h reduced ThT fluorescence by 80% within 150 s (Table 3). When 50 μM ProIAPP$_{1-48}$ was incubated in the presence of an equimolar concentration of Cu(II) the ThT fluorescence at each timepoint was effectively zero (Table 3). However, TEM of samples incubated for 24h showed fibrillar material (Figure 1d) though individual fibrils had a 'fuzzy' appearance (mean diameter 7.5 (1.23) nm n=50). Addition of EDTA, a divalent metal ion chelator, to a final concentration of 1mM to preparations incubated for 72h increased ThT fluorescence from 8 (3.8) to 72 (24.4) AU within 150 s (Table 3). When 50 μM ProIAPP$_{1-48}$ was incubated in the presence of a 5-fold excess of Cu(II) ThT fluorescence was zero at each time point and TEM showed no fibrillar materials. However, addition of EDTA to a final concentration of 1mM to preparations incubated for 72h increased their ThT fluorescence from 0 to 26 (5.9) AU within 150 s (Table 3).

The presence of a 10-fold excess of Al(III) (500 μM) accelerated fibril formation by 50 μM ProIAPP$_{1-48}$ producing fluorescence of 29 (16.8), 177 (85.5) and 230 (71.9) AU after 0, 24 and 72h respectively (Table 3). TEM of samples incubated for 24h showed dense deposits of fibrillar materials (mean diameter 6.7 (0.84) nm n=50) (Figure 1b). Addition of Cu(II) to a final concentration of 200 μM to preformed fibrils at 72h reduced ThT fluorescence by 60% within 150s (Table 3). When 50 μM ProIAPP$_{1-48}$ and 500 μM Al(III) were incubated in the presence of 50 μM Cu(II) the ThT fluorescence at each timepoint was effectively zero (Table 3). However, TEM of samples incubated for 24h again showed fibrillar-like material which was 'fuzzy' in appearance (mean diameter 7.3 (1.83)

Table 1. The influence of the ratio of Cu(II) to ProIAPP$_{1-48}$ on ThT fluorescence of 20 μM ProIAPP$_{1-48}$ in the absence and presence of 200 μM Al(III) or Zn(II) after 24h or 21 days. Mean and SD are given , n=3.

Cu(II)-ProIAPP$_{1-48}$ Ratio		0.0	0.5	1.0	2.0	5.0	10.0
ProIAPP$_{1-48}$ Only	24h	10(2.6)	13(2.2)	6(5.2)	4(2.0)	0(0.0)	0(0.0)
	21d	42(12.8)	29(3.3)	8(3.1)	5(1.5)	0(0.0)	0(0.0)
ProIAPP$_{1-48}$ + Al(III)	24h	46(21.5)	7(1.5)	5(3.7)	4(0.3)	1(0.2)	5(1.1)
	21d	75(23.2)	12(0.7)	13(3.8)	5(0.5)	5(2.0)	5(0.5)
ProIAPP$_{1-48}$ + Zn(II)	24h	14(1.9)	8(0.6)	6(3.9)	1(0.8)	0(0.0)	3(3.7)
	21d	36(3.4)	17(8.3)	12(5.4)	8(2.8)	1(0.2)	0(0.0)

Table 2. The influence of the ratio of Cu(II) to ProIAPP$_{1-48}$ on ThT fluorescence of 20 μM ProIAPP$_{1-48}$ in the absence and presence of 200 μM Al(III) or Zn(II) after incubation for 7 days. Mean and SD are given , n=3.

Cu(II)-ProIAPP$_{1-48}$ Ratio	0.0	0.5	0.75	1.0	1.5	2.0
ProIAPP$_{1-48}$ Only	50(7.4)	9(1.4)	9(0.9)	5(0.6)	4(0.5)	2(0.7)
ProIAPP$_{1-48}$ + Al(III)	58(12.0)	13(4.0)	13(2.4)	8(1.5)	6(1.7)	8(1.6)
ProIAPP$_{1-48}$ + Zn(II)	26(1.7)	6(1.2)	3(1.6)	5(2.3)	3(2.0)	2(2.3)

Table 3. The influence of equimolar and 5-fold excess Cu(II) on ThT fluorescence of 50 μM ProIAPP$_{1-48}$ in the absence and presence of a 10-fold excess of Al(III) or Zn(II) after 0, 24 and 72h incubation at 37°C. After 24 and 72h either Cu(II) (to give 200 μM) or EDTA (to give 1 mM) was added to each preparation and ThT fluorescence measured after 150s. Mean and SD are given, n=3.

Cu(II)-ProIAPP$_{1-48}$		**0**	**0**	**1:1**	**1:1**	**5:1**	**5:1**
			+Cu(II)		+EDTA		+EDTA
ProIAPP$_{1-48}$	**0h**	29(10.9)		5(1.6)		0	
	24h	81(19.6)	40(2.5)	6(4.2)	60(17.7)	0	30(7.7)
	72h	216(21.9)	41(8.8)	8(3.8)	72(24.4)	0	26(5.9)
ProIAPP$_{1-48}$+Al(III)	**0h**	29(16.8)		13(5.0)		3(4.6)	
	24h	177(85.5)	138(92.1)	3(1.4)	65(31.2)	0	36(13.1)
	72h	230(71.9)	91(50.7)	11(16.0)	76(24.8)	0	25(3.5)
ProIAPP$_{1-48}$ + Zn(II)	**0h**	11(5.5)		0		0	
	24h	30(6.0)	25(9.7)	1(0.9)	52(14.3)	0	21(6.1)
	72h	83(17.4)	31(9.4)	1(2.0)	78(33.0)	0	21(4.6)

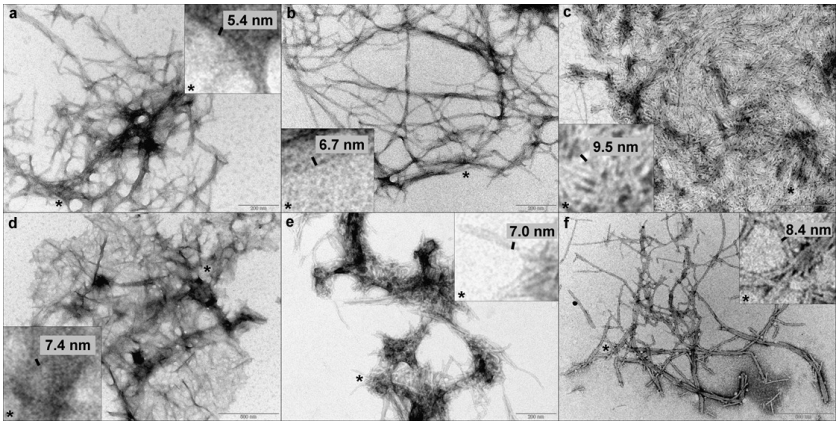

Figure 1a-f. Transmission electron microscopy of 50μM ProIAPP$_{1-48}$ in physiological medium at pH 7.4 following incubation for 24h at 37°C in the presence of various combinations of metals. a – ProIAPP$_{1-48}$ only; b – ProIAPP$_{1-48}$ + 500 μM Al(III); c – ProIAPP$_{1-48}$ + 500 μM Zn(II); d - ProIAPP$_{1-48}$ + 50 μM Cu(II); e - ProIAPP$_{1-48}$ + 500 μM Al(III) + 50 μM Cu(II); f - ProIAPP$_{1-48}$ + 500 μM Zn(II) + 50 μM Cu(II). Asterisk identifies the position of the magnified insert. Each insert shows a fibril diameter in nm. Scale bars; a,b,e – 200 nm; c,d,f – 500 nm.

nm n=50) (Figure 1e). Addition of EDTA to a final concentration of 1mM to preparations incubated for 72h increased ThT fluorescence from 11 (16.0) to 76 (24.8) AU within 150s (Table 3). When 50 μM ProIAPP$_{1-48}$ and 500 μM Al(III) were incubated in the presence of 250 μM Cu(II) ThT fluorescence was zero at each time point and TEM showed no fibrillar materials. However, addition of EDTA to a final concentration of 1mM to preparations incubated for 72h increased the ThT fluorescence from 0 to 25 (3.5) AU within 150s (Table 3).

The presence of a 10-fold excess of Zn(II) slowed fibril formation by 50 μM ProIAPP$_{1-48}$ producing fluorescence of 11 (5.5), 30 (6.0) and 83 (17.4) AU after 0, 24 and 72h respectively (Table 3). TEM of samples incubated for 24h showed dense mats of fibrillar material as well as single fibrils (mean diameter 9.5 (2.07) nm n=50) (Figure 1c). Addition of Cu(II) to a final concentration of 200 μM to preformed fibrils at 72h reduced ThT fluorescence by 60% within 150s (Table 3). When 50 μM ProIAPP$_{1-48}$ and 500 μM Zn(II) were incubated in the presence of 50 μM Cu(II) the ThT fluorescence at each timepoint was effectively zero (Table 3). However, TEM showed the presence of fibril-like materials under these conditions in spite of the lack of ThT fluorescence (mean diameter 8.5 (1.30) nm n=50) (Figure 1f). Addition of EDTA to a final concentration of 1mM to preparations incubated for 72h increased ThT fluorescence from 1 (2.0) to 78 (33.0) AU within 150s (Table 3). When 50 μM ProIAPP$_{1-48}$ and 500 μM Zn(II) were incubated in the presence of 250 μM Cu(II) ThT fluorescence was zero at each time point and TEM showed no fibrillar materials. However, addition of EDTA to a final concentration of 1mM to preparations incubated for 72h increased the ThT fluorescence from 0 to 21 (4.6) AU within 150s (Table 3).

The influence of a 10-fold excess of either Al(III) or Zn(II) on the rate at which 50 μM ProIAPP$_{1-48}$ formed ThT-positive amyloid was measured over 6h and Al(III) was found to significantly accelerate this process while Zn(II) slowed fibril formation (Figure 2). When each of these preparations was incubated for 28 days at 37°C and thereafter observed using polarising microscopy numerous spherulites of varying diameter, 10-100 μm, were observed though only in the presence of Al(III). Spherulites were observed singularly as well as in clusters (Figure 3a-c). When these spherulites were viewed by fluorescence microscopy (Olympus WVB filter, Ex: 400-440 nm, Em: 475-700nm) in the presence of ThT they showed positive ThT fluorescence confirming the presence of β sheets in their structure (Figure 3d-f).

Discussion

We have confirmed that in the absence of added metal human ProIAPP$_{1-48}$ forms amyloid fibrils readily at both 20 and 50 μM (Tables 1-3; Figure 1a; Figure 2). Addition of Cu(II) to a 4-fold excess to preformed fibrillar material rapidly reduced its ThT fluorescence (Table 3) and confirmed the known ability of Cu(II) to abolish/reverse β sheet structure in amyloid deposits [16,17]. The presence of Al(III) at 10-fold excess to ProIAPP$_{1-48}$ accelerated fibril formation and particularly in the short term (Figure 2). Fibrils formed in the presence of Al(III) (Figure 1b) were significantly wider in diameter (mean diameter 6.7 (0.84) nm n=50) than for ProIAPP$_{1-48}$ only preparations (mean diameter 5.6 (0.80) nm n=50). The addition of excess Cu(II) rapidly reduced the ThT fluorescence of fibrillar ProIAPP$_{1-48}$ though to a lesser extent than was seen for ProIAPP$_{1-48}$ amyloid formed in the absence of Al(III) (Table 3). This suggested that Al(III)-induced fibrillar ProIAPP$_{1-48}$ might be more stable to disruption by Cu(II) than deposits formed from the peptide only. ProIAPP$_{1-48}$ also formed spherulites in the presence of Al(III) (Figure 3a-c) but not in any other treatment. This confirmed our previous observation [12] and we were additionally able to show using fluorescence microscopy that the Al(III)-induced spherulites were composed of β sheets of ProIAPP$_{1-48}$ (Figure 3d-f). ThT fluorescence suggested that a 10-fold excess of Zn(II) slowed the rate at which ProIAPP$_{1-48}$ formed amyloid fibrils (Tables 1-3; Figure 2). However, TEM evidence appeared to contradict these findings as dense mats of fibrillar-like materials were formed in preparations which gave relatively low ThT fluorescence (Figure 1c). The fibrils formed in the presence of Zn(II) (mean diameter 9.5 (2.07) nm n=50) were almost twice the diameter of those formed by ProIAPP$_{1-48}$ only (mean diameter 5.6 (0.80) nm n=50) and this clear difference in the morphology of Zn(II)-induced fibrillar materials might explain the difference in ThT binding and hence ThT fluorescence?

Cu(II) potently inhibited amyloid fibril formation by ProIAPP$_{1-48}$ (Tables 1-3). Sub-stoichiometric concentrations of Cu(II) reduced fibril formation significantly while equimolar concentrations and above were sufficient to practically prevent the formation of ThT-positive amyloid. It was of interest that at equimolar Cu(II) to peptide and despite the lack of ThT fluorescence, TEM showed the presence of fibrillar-like materials (Figure 1d) and that the individual fibrils were wider (mean diameter 7.5 (1.23) nm n=50) and had a 'fuzzy' appearance in comparison to those found in the absence of Cu(II) (Figure 1a). Importantly these non-ThT binding/fluorescent fibrils were not present when Cu(II) was present to 5-fold excess where the peptide was precipitated as amorphous, non-distinct deposits. The addition of EDTA to a concentration of 1 mM resulted in significant increases in ThT fluorescence and especially so at equimolar Cu(II) to peptide (Table 3) and helped to confirm that binding of Cu(II) by ProIAPP$_{1-48}$ was responsible for preventing the peptide from forming amyloid fibrils with a β sheet structure. The potency with which Cu(II) prevented ProIAPP$_{1-48}$ from forming amyloid was not ameliorated at all by the additional presence of significant excesses of the potentially competitive metals Al(III) and Zn(II) (Tables 1-3). Again at equimolar Cu(II) to ProIAPP$_{1-48}$ and despite almost no ThT fluorescence TEM showed fibrillar-like materials in the presence of both Al(III) (Figure 1e) and Zn(II) (Figure 1f) while at 5-fold excess Cu(II) no fibrillar material was formed. The presence of a significant excess of Al(III) or Zn(II) had no influence upon the propensity for Cu(II) to inhibit the formation of β sheets of ProIAPP$_{1-48}$ which suggested that the peptide bound Cu(II) with great avidity and at one or more sites which were definitely distinct from Al(III) and also likely different to Zn(II). There are no previous data on metal binding by ProIAPP and only very few for IAPP. Both ProIAPP and IAPP include the same intramolecular disulphide (Cys2-Cys7 IAPP) bridge and this is a likely target for disruption by both Cu(II) and Zn(II) [23]. Data on the influence of Zn(II) on IAPP amyloidogenesis are equivocal [10,11] and such differences might be explained by the suggestion of both high and low affinity binding sites involving histidine [24]. However, herein for ProIAPP$_{1-48}$ lower ThT fluorescence in the presence of 0.50 mM Zn(II) was accompanied by extensive formation of amyloid-like fibrils of significantly wider diameter which may suggest that Zn(II) binding of ProIAPP$_{1-48}$, and pos-

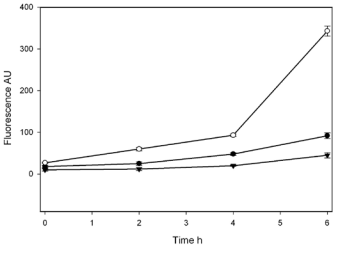

Figure 2. The rate of formation of ThT-positive ProIAPP$_{1-48}$ amyloid for; solid circle – 50 μM ProIAPP$_{1-48}$ only; open circle – 50 μM ProIAPP$_{1-48}$ + 500 μM Al(III); solid triangle - 50 μM ProIAPP$_{1-48}$ + 500 μM Zn(II). Mean and SD are plotted, n=3.

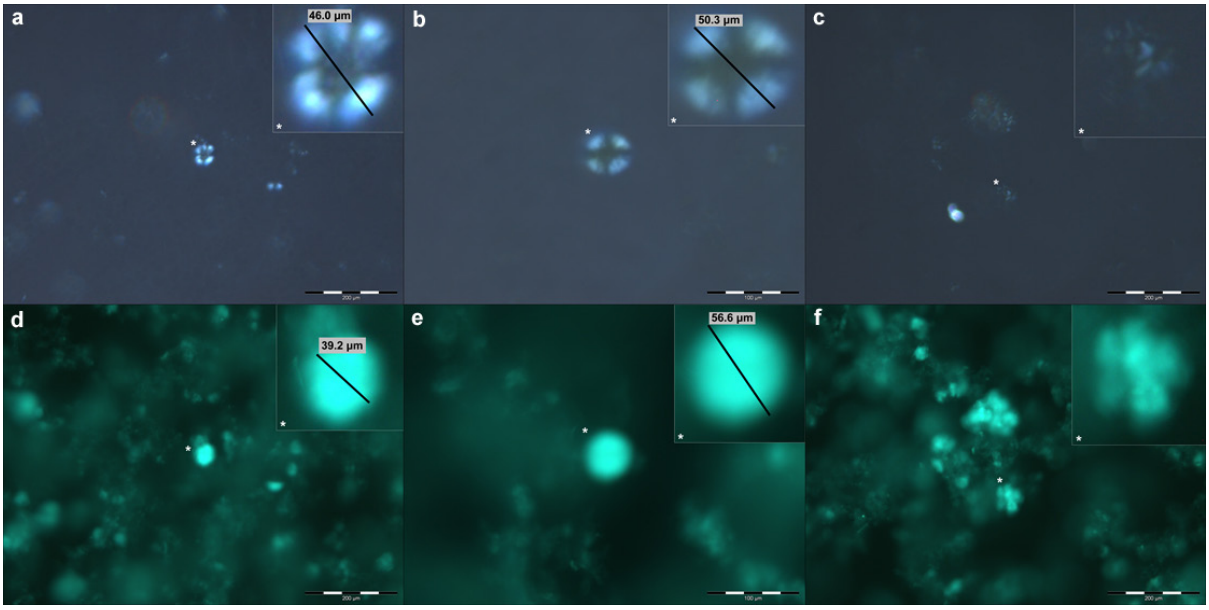

Figure 3a-f. Polarising (a-c) and fluorescence (d-f) microscopy of spherulites formed by 50 µM ProIAPP$_{1-48}$ in the presence of 500 µM Al(III) following incubation at 37°C for 28 days. a,d – single spherulite approximately 46 µm in diameter; b,e – single spherulite approximately 50 µm in diamater; c,f – cluster of spherulites. Asterisk shows the position of the magnified insert. Scale bars; a,c,d,f – 200 µm, b,e – 100 µm.

sibly IAPP, results in a different fibril morphology with a lower capacity to bind ThT and induce fluorescence. We observed similar effects for Cu(II) in that while equimolar Cu(II) prevented ProIAPP$_{1-48}$ from forming ThT-reactive amyloid this ratio of Cu(II) to peptide did not prevent the formation of amyloid-like fibrils which, as was the case for Zn(II), were of wider diameter than those formed in the absence of added metal. These ThT-negative fibrils were not formed at Cu(II):peptide ratios ≥ 2.0 which may suggest that ProIAPP$_{1-48}$, and possibly IAPP, includes at least 2 binding sites for Cu(II) and that amyloid formation is only prevented when both of these sites are occupied.

Cu(II) is both a potent inhibitor of amyloid formation and it effectively abolishes the β sheet conformation of preformed amyloid fibrils. We have now demonstrated these properties for Aβ$_{42}$ [15,16], ABri peptide [25], IAPP [10] and ProIAPP$_{1-48}$ [12] and we have shown herein that for the latter Cu(II) is effective in preventing amyloid from forming at any concentration in excess of equimolar. If amyloidogenesis of ProIAPP$_{1-48}$ is involved in the deposition of IAPP *in vivo* and subsequently the death of β cells in T2DM then, likewise for IAPP, Cu(II) should be protective and this might suggest that a disruption in Cu(II) homeostasis is involved in the aetiology of this disease. It is of speculative interest that any deficiency of Cu(II) combined with an unusually high availability of Al(III) within the environment of the pancreas would promote the deposition of IAPP and ProIAPP$_{1-48}$ and thereby potentially exacerbate β cell death.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CE – designed the experiments and wrote the manuscript. MM – carried out all the TEM. ES, BS & BI performed the experiments under the supervision of CE & MM. LW – prepared the ProIAPP$_{1-48}$. PEF – contributed to the writing of the manuscript.

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