Investigation of insulin nucleation kinetics under oscillatory flow mixing

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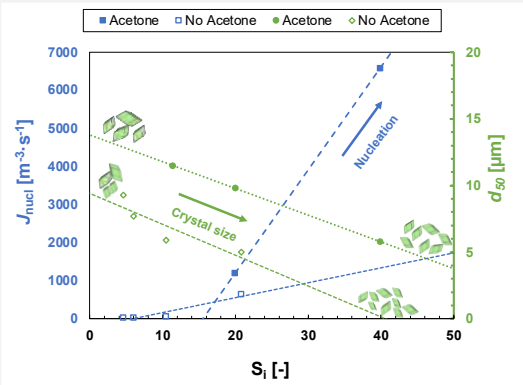

Crystallization can represent a cost-effective and scalable alternative for protein separation and purification. However, it is still not widely implemented in biopharmaceutical industry due to limited understanding of the underlying phenomena.

Herein, insulin crystallization was investigated in an oscillatory flow reactor in the presence and absence of acetone. The results show the impact of both supersaturation (i.e., insulin concentration) and acetone on nucleation kinetics and crystal size distribution (CSD). As supersaturation increases, the nucleation rate increases and mean crystal size decreases. In its turn, acetone allows faster nucleation, a narrower CSD and larger mean crystal size. The kinetic parameter *A* derived from the classical nucleation theory (CNT) also indicate the acceleration of the kinetics of molecular attachment in the presence of acetone.

These findings contribute to the better understanding of insulin crystallization mechanism under oscillatory flow mixing.

Introduction

Protein therapeutics have become an important segment of biopharma due to their increasing use to treat diseases [1]. While chromatography has been the main bioseparation method, the future of the industry relies on its ability to develop more cost-effective and scalable separation techniques. In this context, protein crystallization requires no costly equipment and consumables, handles high process volumes and protein titers, and yields highly pure products in a single-step [2]. Despite its huge potential, barriers to the industrial adoption of crystallization as a bioseparation method remain. This is namely due to the complexity of the occurring phenomena and the unavailability of generalized crystallization strategies in the case of proteins [3].

Objectives

The present work aims to develop a unique platform for protein crystallization based on oscillatory flow technology [4]. The target system is insulin, the first crystalline protein to be approved for therapeutic uses [5]. However, limited research has been conducted on agitated/sheared crystallization of insulin, in particular exploring crystallization kinetics. Therefore, insulin batch crystallization assays were carried out in a meso scale oscillatory flow reactor provided with smooth periodic constrictions (OFR-SPC) coupled to a spectrometer for *in-situ* measurements of solution turbidity. Herein, the influence of supersaturation (i.e., insulin concentration) and presence of acetone on nucleation kinetics and crystal size distribution (CSD) were assessed.

Methods

The experimental set-up is shown in Figure 1. The mixing intensity is controlled by the oscillation frequency (*f*) and amplitude (x_0), fixed at 1.83 Hz and 5.3 mm, respectively. Isothermal batch insulin crystallization trials were carried out at 20 ºC and pH 5.7 during 150 min. Experiments started by injecting simultaneously equal volumes of insulin (0.3 to 5.0 mg.mL^{-1} in 20 mM HCl) and precipitant (7.50 mM $ZnCl_2$, 75.0 mM trisodium citrate and 0% or 30% (v/v) acetone in 20 mM HCl) solutions in the meso OFR-SPC.

The solution turbidity was monitored over time through UV-Vis spectrophotometry ($\lambda = 400$ nm) to estimate the induction time (t_{ind}) and then determinate the nucleation rate (J_{nucl}) following the classical nucleation theory (CNT). Insulin solubility was measured, and crystal yield was estimated. For this, insulin concentration in solution was measured by UV-Vis spectrophotometry, at 280 nm and at 562 nm through BCA protein assay, in the absence and presence of acetone, respectively. The collected suspensions were observed under an optical microscope coupled to a camera to measure CSD. A minimum of 500 crystals were measured (Feret diameter) using Image J software to ensure a representative population of crystals with a 90% confidence interval.

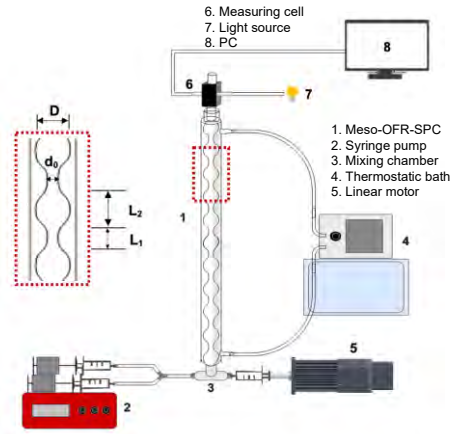

Figure 1. Schematic representation of the experimental set-up and characteristic dimensions of the meso-OFR-SPC [4]: $D = 3$ mm, $d_0 = 1.6$ mm, $L_1 = 6$ mm, and $L_2 = 13$ mm.

Results

An overview of the main results from the insulin crystallization assays carried out in the meso-OFR-SPC is given in Figure 2 and Table 1. It is possible to verify a faster insulin nucleation event with the increase of the initial supersaturation ratio (*Si*) (i.e., increase of protein concentration) both in the presence and absence of acetone, though dependence of insulin nucleation kinetics on S_i is more pronounced at higher S_i. According to the CNT, there is a strong correlation between (primary) homogeneous nucleation rate and supersaturation. As shown in Figure 2, results suggest a (primary) homogeneous nucleation mechanism at higher S_i and a (primary) heterogeneous nucleation mechanism at lower S_i.

The results also evidence the key role of acetone on the insulin nucleation kinetics. At similar S_i, nucleation rate J_{nucl} is ~ 2 times higher for insulin crystallization assays performed with acetone (Table 1). The kinetic (*A*) and thermodynamic (*B*) parameters following the CNT were derived for homogeneous nucleation (Table 1). It was verified that *A* increases ~2.5 times in the presence of acetone, indicating an acceleration of the kinetics of molecular attachment.

CSD results (Table 1) show a smaller mean crystal size (d_{50}) as S_i increases and higher mean crystal size (d_{50}) in the presence of acetone when compared to the assays without acetone. Further, narrower CSD is verified in the presence of acetone. This corroborates the nucleation kinetics results (Figure 2 and Table 1), since high J_{nucl} were verified at higher S_i leading to the formation of numerous small crystals, while at lower S_i nucleation is slower leading thus to the formation of fewer but larger crystals. Insulin crystallized under a rhombohedral shape for all the studied experimental conditions.

Regarding crystal yield, values higher than 60% were obtained. Table 1 also shows the critical impact of *Si* on crystal yield, where higher S_i led to higher crystal yields.

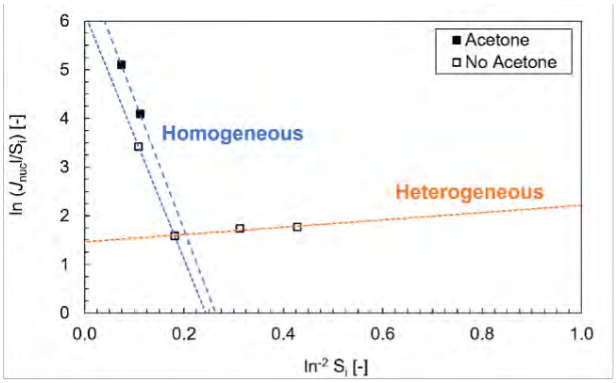

Figure 2. Overview of insulin nucleation kinetics in the meso OFR-SPC.

Conclusions

The present study describes the investigation of insulin nucleation kinetics in a meso OFR-SPC. The significant impact of the initial supersaturation ratio (i.e., insulin concentration) on insulin nucleation kinetics was confirmed. At higher supersaturations, faster nucleation kinetics were verified and consequently a higher number of smaller crystals was formed, whereas at lower supersaturations, slower nucleation kinetics were obtained leading to the formation of fewer but larger crystals. Further, it was verified that acetone accelerates the insulin nucleation kinetics and led to the formation of a more uniform insulin crystals population. In conclusion, this works gives insights into insulin crystallization mechanism under oscillatory flow mixing, which can boost the exploitation of oscillatory flow reactors for technical scale protein crystallization.

Table 1. Summary of the insulin crystallization assays performed in the meso-OFR-SPC at 20 ºC and pH 5.7.

	S_i^* [-]	Nucleation kinetics		Parameters of homogeneous nucleation		CSD		Yield [%]
		t_{ind} [s]	J_{nucl} [$m^{-3}.s^{-1}$]	A [$m^{-3}.s^{-1}$]	B [-]	d_{50} [μm]	Span [-]	
Acetone	39.9	19	6579	1.2×10^{-9}	26.8	5.8	0.7	
15%(v/v)	20.0	104	1202			9.8	0.6	
	11.4					11.5	0.7	
No acetone	20.8	196	638	4.7×10^{-10}	25.2	5.0	2.0	99.6
	10.5	2446	51			5.9	2.1	87.1
	6.0	3677	34			7.7	1.8	83.0
	4.6	4624	27			9.3	1.0	61.6

*S_i is defined as the ratio between insulin initial concentration and insulin solubility in the studied conditions.

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