1
Power Ultrasound – a Means to Promote and Control Crystallization in Biotechnology

LINDA J. McCAUSLAND* AND PETER W. CAINS

C3 Technology, Accentus plc, Harwell Business Centre, Oxfordshire, OX11 0QJ, UK

Introduction

Ultrasound is very familiar as a diagnostic tool in medical and biological operations. In recent years, it has also been increasingly used as a power input source to intensify chemical synthesis and processing. Sonochemistry, the application of ultrasound to promote and modify chemical reactions, was a subject of intensive research interest a decade or so ago (Cains *et al.*, 1998), and recent advances in equipment development have made its implementation viable at industrial manufacturing scale (Cains *et al.*, 2004). Ultrasound has also been applied to promote physical processes, such as phase mixing and atomization, across a range of industries, including fine chemicals, food processing, and petrochemical production. Its use in deactivating and deagglomerating bacterial clusters and flocs has also been reported recently (Joyce *et al.*, 2003).

The past ten years has seen important advances in the application of power ultrasound to biomolecular crystallization, and we now provide a short review on these advances. Most of this 'sonocrystallization' has been with small and medium-sized molecular entities (McCausland *et al.*, 2001a; McCausland and Cains, 2002a,b), where crystallization takes place relatively readily under mild conditions of supersaturation. Working with larger molecules, such as proteins and polypeptides, and with very soluble compounds, such as sugars (McCausland *et al.*, 2001b), is more complex because of the difficulties in inducing crystal nucleation, and the large supersaturation driving forces that are developed as a consequence.

There is considerable interest in controlling the crystallization and crystal properties of substances such as carbohydrates and proteins, in particular because the crystallizations under conventional conditions are very difficult to carry out and control (see, for example, Chayen and Saridakis, 2002). As part of a progressive development towards more challenging applications, ultrasound has been success-

*To whom correspondence may be addressed (linda.mccausland@accentus.co.uk)

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fully applied to the crystallization of sugars and amino acids, and we review some of these successes here. Of potentially huge significance is the possibility for applying ultrasound to the crystallization of proteins.

Power ultrasound and crystallization

Power ultrasound applications generally employ sonic frequencies in the range 20–130 kHz, well below the MHz range used for diagnostic applications. This is because the requirements are fundamentally different: the effects of power ultrasound depend critically on the induction of cavitation events, while diagnostic ultrasound ideally induces no cavitation to prevent biological damage from tissue disruption or free radical production. As a general rule, power density delivery is greater at lower frequency, and for this reason most of our work has utilized a frequency of 20 kHz, slightly above the normal audible threshold around 18 kHz.

The phenomenon of acoustic cavitation and its role in inducing crystallization have been described in detail elsewhere (McCausland and Cains, 2002a; Joyce *et al.*, 2003; Cains *et al.*, 2004). Crystallization is usually analysed in terms of the fundamental processes of nucleation and crystal growth (Mullin, 2001). These occur sequentially in any crystallization process, but they can be very difficult to decouple for fundamental investigations. There is considerable evidence that acoustic cavitation induces nucleation (McCausland *et al.*, 2001a,b; McCausland and Cains, 2002a,b; Joyce *et al.*, 2003), although the exact physical mechanism via which this occurs is still incompletely understood. For small molecule crystallizations, these researchers have demonstrated that ultrasonic cavitation induces nucleation to an extent related to the duration and intensity (power density) at which ultrasound is delivered, and also reduces or eliminates induction times.

Acoustic cavitation occurs above a threshold level of ultrasonic power input (Young, 1989), and this threshold must be exceeded throughout a significant proportion of the working liquid for the technique to be effective. The threshold varies with the liquid (solvent) medium and with the physical properties of the solution, including viscosity and vapour pressure, but in our experience a minimum power density in the region of 35 $W.L^{-1}$ is usually required. The probe and bath systems commonly used to supply ultrasound will deliver these intensities locally close to the transducer source, but they will not deliver a uniform power density throughout the liquid volume. Ultrasonic probes produce very high intensities focused longitudinally from the tip, but solutions insonated by a probe need to be stirred to prevent material being trapped in 'dead' zones located laterally from the tip. Baths need to be mixed in their depth direction, because the transducer source is located in the base and intensity will fall away with height. In recent years, a new range of equipment has been developed (Cains *et al.*, 2004) to enable effective operation above threshold levels at a range of scales from a few mL to thousands of litres.

Application to small molecules – sugars and amino acids

Sugars are difficult to crystallize by conventional methods; they form viscous syrups at high concentrations that are difficult to nucleate. *Table 1.1* summarizes a

Table 1.1. Ultrasonic crystallization of D-xylose from aqueous solutions (from McCausland *et al.*, 2001b). 25 g D-xylose dissolved in 10 mL water at 50°C, cooled at 0.2 K.min^{-1} to 20°C, then isolated. Average ultrasonic (20 kHz) intensity equivalent to ~35 W.L^{-1}

Insonation conditions	Temp. (°C) at which solid first appeared	Crystal size distribution (mass) Cumulative percentiles (μm) 10%	50%	90%
No ultrasound (control)	36	27	67	149
Insonated for 2 min starting at 46°C	43	43	106	211
Continuous insonation from start	46	Particles <10 μm		

series of results obtained for a simple cooling crystallization of D-xylose (McCausland *et al.*, 2001b) The application of 20 kHz ultrasound caused crystals to be observed at 43–46°C, while cooling had to be continued to 36°C in the case where no ultrasound was employed. The figures in the three right-hand columns of *Table 1.1* give the crystal size, in μm, below which 10%, 50% and 90% respectively of the total particle mass may be found. A 2 minute burst of ultrasound produced larger particles than the control, resulting from the growth of the crystals nucleated at this point. Continuous application throughout the crystallization produced a much larger number of nuclei, resulting in a mass of very fine crystals. These effects have been observed in other small molecule crystallizations, and it has been possible to 'tailor' the crystal size distribution and the consequent physical properties of the product by varying the insonation profile between a single short burst and continuous application throughout the crystallization (McCausland and Cains, 2002a,b; McCausland *et al.*, 2002b).

Table 1.2 summarizes a further series of tests with the disaccharides D-sucrose, D-lactose, D-maltose, and D-cellubiose. The procedure was similar to that employed in *Table 1.1*: saturated solutions were prepared at 50°C and cooled at 0.2 K.min^{-1} to 20°C, then isolated. Ultrasound (20 kHz) was applied continuously at an average energy density of around 35 W.L^{-1}. Except for D-lactose, the disaccharides were nucleated only with great difficulty where no ultrasound was applied. In all cases, the application of ultrasound gave crystal products within 10°C of the starting temperature.

Table 1.2. Ultrasonic crystallization of disaccharides from aqueous solutions (from McCausland, 2001b). Saturated solutions prepared at 50°C and cooled at 0.2 K.min^{-1}

Solute	Quantity dissolved in 10 mL water (g)	Temp. (°C) at which solid appeared Without ultrasound	With ultrasound
D-sucrose	18	<40	47
D-lactose	5.5	41	43
D-maltose	13	<20*	40
D-cellubiose	2.0	<20*	42

*No crystals appeared at 20°C.

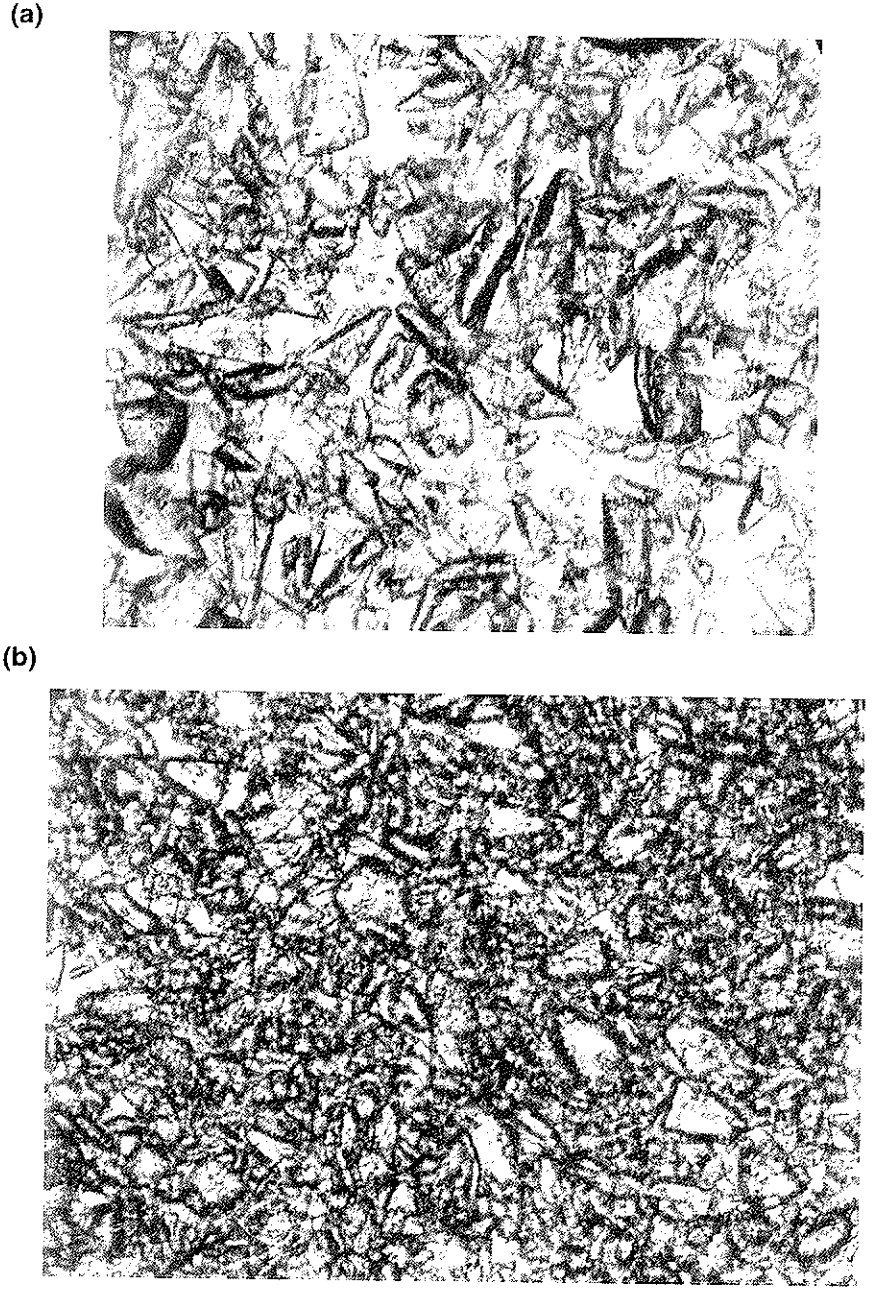

Figure 1.1. Optical micrographs (20×) of D-lactose prepared (a) without ultrasound and (b) with continuous ultrasound at 35 $W.L^{-1}$.

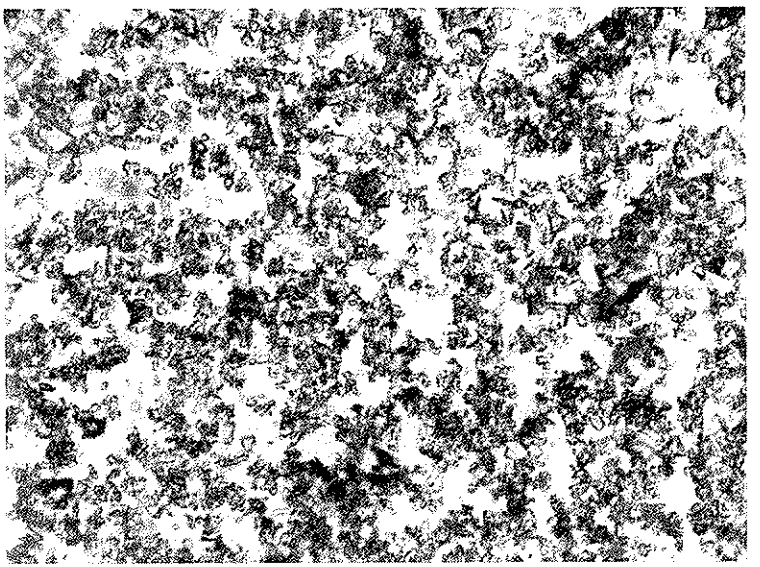

Figure 1.2. Optical micrograph (20×) of D-cellubiose prepared with continuous ultrasound at 35 $W.L^{-1}$.

Figure 1.1 shows photomicrographs of the D-lactose products at 20× magnification. Although, in this case, the application of ultrasound did not affect the cooling temperature at which the crystals appeared as much as the other cases, it resulted in a decrease in the mean particle size and a more uniform appearance of the crystals. *Figure 1.2* shows a micrograph of the D-cellubiose product obtained with ultrasound, which exhibits a relatively uniform distribution of small crystals. No crystalline product could be isolated from the corresponding non-insonated D-cellubiose solution down to a temperature of 20°C.

Table 1.3 shows results obtained (McCausland, 2002) from the cooling crystallization of amino acids from aqueous solutions, starting at 75°C. The concentrations of L-leucine and L-phenylalanine were slightly below the published saturation values of 3.8 g and 6.6 g, respectively. Here, 20 kHz ultrasound was applied in short 2 second bursts every 10 seconds, until solid appeared to separate from the solution. All cases show crystalline products separating at higher temperatures and lower supersaturation levels with ultrasound, compared to the non-insonated controls. The effect was most marked for L-leucine, where sub-cooling in the region of 23 K is required to induce crystallization in the non-insonated case. The results for L-phenylalanine and L-histidine are very similar to each other, and the level of reproducibility between duplicated experiments is good in all cases.

Table 1.3. Ultrasonic crystallization of amino acids from aqueous solutions. Saturated solutions prepared at 75°C and cooled at 0.2 K.min^{-1} (from McCausland, 2002)

Solute	Quantity dissolved in 100 mL water (g)	Temp. (°C) at which solid appeared	
		Without ultrasound	With ultrasound
L-leucine	3.3	52.9	66.0
		52.2	64.5
L-phenylalanine	6.2	65.2	69.1
		66.4	71.0
L-histidine	11.3	65.6	69.0
		67.0	70.1

Application to proteins

Ultrasound has been used recently to induce the separation of DNAse I from aqueous solutions as crystalline or partially crystalline particles. The objective was to develop a method to produce particles in a narrow size distribution with aerodynamic diameters in the range 3–5 μm as a model system for inhalation. The protein contained four isozymes A, B, C and D, with respective isoelectric points at pH 5.22, 4.96, 5.06 and 4.78, of which A was dominant.

Experiments were carried out with aqueous solutions at 5°C, using a pH shift to generate supersaturation. Solubilities of an unpurified DNAse I preparation were measured as a function of pH by two methods, respectively by solute addition and solvent (water) evaporation, for unbuffered solutions and for solutions buffered with 0.02 M sodium acetate. In all cases, maximum solubility is exhibited under acid conditions pH 2–4, with a sharp drop at around pH 5–6, most marked for the unbuffered solutions as a factor of 3–4. Solubilities increased gradually with increasing pH beyond 6.

Crystallizations reported in *Table 1.4* were carried out over relatively narrow pH ranges that give the largest difference in solubility. All solutions were left for 72 hours following preparation and ultrasonic treatment before the solids were separated by filtration. All solids recovered exhibited narrow particle size distributions. The first three entries in *Table 1.4* gave mean particle sizes in the region of 7 μm. For the buffered, insonated case, a slightly larger mean particle size of 10 μm was recorded, with a slightly broader size distribution. We believe this is

Table 1.4. Crystallizations of DNAse I from aqueous solutions by pH shift. 5°C. 10 mL aliquots. Insonation, 20 kHz, 70 W applied by probe, series of 5 second bursts every 15 minutes for 2 hours

Conditions	Initial DNAse concentration g/100 g solution	Initial pH	Final pH	Ultrasound
Unbuffered	2.07	2.6	4.5	No ultrasound
				Insonated
Buffered	1.08	3.8	6.3	No ultrasound
				Insonated

due to the fact that ultrasound brings on the nucleation event earlier in the crystallization, therefore the crystals have a longer growth time. It was not possible to establish whether, or to what extent, the recovered solids were crystalline. However, an activity titre showed that the reactivity of the material recovered from the insonated tests had decreased by around 50%.

The potential of using ultrasound to assist crystallization requires further development by researchers: the use of ultrasound at higher frequency – which will be less destructive – would appear a particularly fruitful path to follow.

Conclusions and further development

Power ultrasound has been demonstrated to promote nucleation in the crystallization of small molecule compounds from solution and to provide an additional means of controlling such processes. It has now been extended to small-molecule substances that form highly concentrated solutions that are difficult to nucleate, including sugars. It has also been demonstrated that the crystallization of amino acids from aqueous solutions can be promoted by ultrasound.

The potential value of ultrasound for crystallizing macromolecular materials, such as proteins, is under further investigation: the crystallization of these materials presents problems that are more complex than those involved in small molecule crystallizations. In our preliminary evaluation, we have found that ultrasound at the frequency and intensity we normally use to promote crystallization degrades activity. We are developing methods using the milder conditions of higher frequency ultrasound, which is less destructive to biological materials.

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