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Focusing of Microcrystals and Liquid Condensates in Acoustofluidics

Pierre Gelin ^{1,*}, Joris Van Lindt ², Anna Bratek-Skicki ², Sander Stroobants ², Marzena Krzek ² , Iwona Ziemecka ¹, Peter Tompa ², Wim De Malsche ¹ and Dominique Maes ^{2,*}

¹ Department of Chemical Engineering, μ Flow Group, Vrije Universiteit Brussel, Brussels 1050, Belgium; Iwona.ziemecka@vub.be (I.Z.); Wim.De.Malsche@vub.be (W.D.M.)

² Structural Biology Brussels, Vrije Universiteit Brussel, Brussels 1050, Belgium; joris.van.lindt@vub.be (J.V.L.); Anna.Brater-Skicki@vub.be (A.B.-S.); sander.stroobants@vub.be (S.S.); Marzena.Krzek@vub.be (M.K.); peter.tompa@vub.be (P.T.)

* Correspondence: pierre.gelin@vub.be (P.G.); dominique.maes@vub.be (D.M.); Tel.: +32-26293617 (P.G.); +32-26291852 (D.M.)

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Abstract: Manipulation of high-density materials, such as crystals and liquid condensates, is of great importance for many applications, including serial crystallography, structural and molecular biology, chemistry, and medicine. In this work, we describe an acoustic technique to focus and harvest flowing crystals and liquid condensates. Moreover, we show, based on numerical simulations, that the acoustic waves can be used for size-based particle (crystals, droplets, etc.) separation. This is an essential technological step in biological research, medical applications, and industrial processes. The presented technology offers high precision, biocompatibility, ease of use and additionally, is non-invasive and inexpensive. With the recent advent of X-ray Free Electron Laser (XFEL) technology and the associated enormous importance of a thin jet of crystals, this technology might pave the way to a novel type of XFEL injector.

Keywords: microcrystals; liquid-liquid phase separation; microfluidics; acoustic radiation; particle separation; focusing; serial crystallography; free electron laser; crystal harvesting

1. Introduction

Microfluidic technologies benefit from a high surface to volume ratio, well-defined residence time, and precise control over mass and heat transfer. This allows for the optimization of crystallization conditions and the formation of high-quality crystals [1–3]. Moreover, microfluidic technology offers exceptional control for the investigations of biological phenomena. Finally, microfluidics allows to precisely manipulate material inside the microfluidic device. With the advent of the free electron laser (FEL) sources, high-quality X-ray data can be extracted from crystals measuring only a few micrometers (2–5 μ m). However, moving these crystals from a conventional crystallization experiment to the XFEL beam remains challenging [4,5]. Many different devices, such as optical tweezers [5], acoustic wave-based devices [4,6–8], and robotic devices [9,10], have been made to automate the transfer of microcrystals from the growth plate to the X-ray beam. These techniques are sophisticated, expensive, and might create unwanted forces which can compromise the quality of the data set and alter the structure of the crystal. Moreover, they manipulate crystals initially at rest. Given the importance of jet technology to XFEL crystal injection, the inability to manipulate moving crystals has been a limitation in acoustic implementation at XFEL sources [6–8]. In contrast with previous methods, acoustofluidic devices allow for X-ray analysis to be performed in flowing streams, thereby eliminating the crystal mounting step that might be detrimental to the quality of the crystal and the data [11].

Recent discoveries in cell biology also attracted the attention of scientists to liquid-liquid phase separation (LLPS), which is often observed in the test tube and is thought to occur in eukaryotic cells. LLPS is a process which allows different components, above their saturation concentration, to de-mix from the homogeneous solution state to form concentrated liquid droplets. When they form in cells, they are considered to constitute membraneless organelles (MLOs), also termed biomolecular condensates [12]. MLOs are necessary for normal cellular physiology; however, it was shown that they are also involved in neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [13–16]. Recently it has been shown that bacteria may also contain MLOs [17]. Manipulation of these high-density liquid condensates, especially focusing them, can create opportunities for new experiments, and thus lead to new insights in the pathology and treatment of these type of diseases.

In this contribution, we propose the use of standing bulk acoustic waves to manipulate flowing dense material, either crystals or liquid condensates, formed in microfluidic devices. In the presence of a standing pressure wave, crystals or high-density droplets will experience a radiation force arising from the scattering of the pressure wave. Depending on their material properties, they will migrate towards the pressure node or antinode (Figure 1c). The migration velocity towards the pressure (anti)node is called the radiation velocity [18]. The idea of manipulating solid materials using bulk acoustic waves has already been applied on polymer microbeads and on cells [19–22]. A common application of standing acoustic wave is the size-based separation hereof [19,23]. While exposure to acoustic energy is capable of causing damage, the gentleness of the radiation force and the biocompatibility of bulk acoustic waves on living cells has been documented [24–26].

Figure 1. (a) Schematic representation of an acoustofluidic channel: A piezoceramic element is vibrating at 1.93 MHz. Vibrations are transferred to the channel wall, creating a standing pressure wave. (b) Picture of an acoustofluidic chip, showing two inlets and two outlets together with the acoustic zone. (c) The behavior of large particles ($>2\ \mu\text{m}$) in an acoustofluidic device. “Hard” particles focus on the pressure node while “soft” particles focus on the pressure antinodes. (d) The behavior of small particles ($<2\ \mu\text{m}$) in an acoustofluidic device.

In this contribution, we demonstrate the possibility to use bulk acoustic waves to manipulate flowing crystals of active pharmaceutical compounds (API) and proteins as well as liquid condensates. The radiation force is applied to focus dense biological material at the pressure node of a microfluidic channel. This technology might set a new path in the field of serial crystallography and in XFEL experiments. Additionally, a proof of concept for size-based separation of crystals is shown numerically.

2. Materials and Methods

2.1. Microfluidic Chip Design and Set-Up

Rectangular microfluidic channels ($375\ \mu\text{m} \times 310\ \mu\text{m}$ and $375\ \mu\text{m} \times 210\ \mu\text{m}$) were etched in a silicon wafer with a total thickness of $525\ \mu\text{m}$. The channels were sealed with an anodically bonded borosilicate glass lid. Two inlets and outlets were provided and connected to syringe pumps (KDS200, KD Scientific Inc. Holliston, MA, USA) by means of capillaries. A standing pressure wave was generated in the microfluidic channel with a piezo-ceramic element (PZT) ($30\ \text{mm} \times 20\ \text{mm} \times 1\ \text{mm}$, APC International Ltd. Mackeyville, PA, USA). To ensure a good energy transfer from the PZT to the chip, a thin glycerol layer served as a coupling layer. An in-house built PMMA holder provided a good connection between the microfluidic chip, PZT, and fluidic connectors. Schematic representation and a picture of the acoustofluidic chip are shown in Figure 1. The PZT was driven by a frequency generator (AFG1062, Tektronix UK Ltd. Bracknell, United Kingdom) around 2 MHz. The applied voltage was amplified by an RF power amplifier (210L, Acquitek S.A.S) with a power output of 10 Watt. The voltage amplitude across the PZT was monitored with an oscilloscope (TBS1104, Tektronix UK Ltd. Bracknell, United Kingdom).

2.2. Preparation: Pharmaceutical Compound, Protein, Liquid Condensate

A broad spectrum of biological compounds was introduced in the acoustofluidic channel. As a reference for small active pharmaceutical ingredients (API), miconazole nitrate was chosen. Miconazole nitrate, kindly offered by Ajinomoto Omnicem N.V., was dissolved in dimethyl sulfoxide (DMSO) (276855-1L, anhydrous >99.9%, Sigma Aldrich, Schnelldorf, Germany) until a concentration of 5 w/w%. Deionized (DI) water was used as an antisolvent. Trace amounts of fluorescein sodium salt (FITC) (F6377-100G, Sigma Aldrich) were added to this solution to enable visualization under the fluorescence microscope (IX71, Olympus Corporation, Shinjuku, Japan). Lysozyme was chosen as a model protein. A 50 mM sodium acetate buffer was prepared with deionized water and adjusted to a pH of 4.5. Hen egg white lysozyme (89833, Thermo Fisher Scientific) was dissolved in the prepared buffer to a concentration of 72 mg/ml and used as a stock solution. Before experiments, the lysozyme solution was filtered with a $0.1\ \mu\text{m}$ PTFE syringe filter to remove impurities. A sodium chloride stock solution of 200 mg/ml was used in the same buffer as precipitant. A trace amount of FITC was added to this solution for visualization purposes. High-density liquid droplets were formed using heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2) which belongs to a protein family involved in RNA metabolism. hnRNPA2 is a disordered and very dynamic protein which samples a broad ensemble of conformations. The used protein consists of 155 amino acids representing a low complexity domain (LCD) of full-length hnRNPA2. The induction of LLPS of the low-complexity domain (LCD) of this protein will be published elsewhere. In brief, the LCD of the hnRNPA2 protein was expressed in *Escherichia coli* BL21, purified under denaturing conditions (3 M urea) and stored in a high pH buffer (0.01 CAPS, pH 11) at a concentration of 0.3 mg/ml. For visualization purposes, hnRNPA2 LCD was labeled with Dylight®488 and was mixed with non-labeled hnRNPA2 LCD at a ratio of 1:200.

2.3. Experimental Procedure

All experiments were visualized with an inverted fluorescence microscope connected to a CCD camera (C9100-13, Hamamatsu Photonics, Hamamatsu, Japan). For the crystallization experiment with an API, the miconazole nitrate solution was brought in the microfluidic channel at a flow rate of $2\ \mu\text{L}/\text{min}$. DI water with trace amounts of FITC was used as an antisolvent and pumped through the channel at a flow rate of $8\ \mu\text{L}/\text{min}$, giving a total flow velocity of $1.4\ \text{mm}/\text{s}$. Once complete mixing of the solvent with the anti-solvent was reached, crystal formation was monitored in the absence of the acoustic field. Upon observation of small miconazole nitrate crystals, the acoustic field was turned on, and the formed crystals were focused on the pressure node. The applied voltage was equal to

10 V_{p-p} . For the protein crystallization experiment, the lysozyme and sodium acetate stock solution were mixed until a final concentration of 35 mg/ml and 65 mg/ml, respectively. Preparation of the supersaturated solution was done at an elevated temperature (45 °C) to avoid crystallization outside the microfluidic channel. The supersaturated solution was pumped in the microfluidic channel and was then stopped to allow crystals to grow. Once crystals were observed, the acoustic field was turned on (7 V_{p-p}), and the formed crystals were focused in the center of the channel. Phase separation of the high-density droplets of the hnRNPA2 LCD solution was induced by concentrating the protein five times and decreasing the pH from 11.0 to 7.0 with 0.5 M MES buffer. LLPS high-density droplets were observed in the absence of the acoustic field at a flow velocity of 143 $\mu\text{m/s}$. Focusing of the droplets was achieved by activating the acoustic field.

2.4. Numerical Simulations

By providing a quantitative description of the radiation force, we show that it has different effects on particles of different hardness and size, that is, it can be used for their separation and manipulation. The radiation force for a spherical particle is given by [18]:

$$F_{\text{rad}} = 4\pi\phi a^3 k_0 E_{\text{ac}} \sin(2k_0 y) \quad (1)$$

with a as the radius of the particle, k_0 the angular phase number, y the channel width, E_{ac} the acoustic energy, and ϕ the acoustic contrast factor. The contrast factor defines how “hard” a particle is compared to the surrounding liquid. A “hard” ($0 < \phi$) particle will migrate towards the pressure node while a “soft” ($\phi < 0$) particle will migrate towards the pressure antinode (Figure 1c). It is important to mention that particles smaller than around 2 μm are not affected by the radiation force but by drag forces (Figure 1d). These arise due to the vortex flow of the surrounding liquid in the presence of the acoustic wave [18,20]. To gain more insight in the size dependence of the radiation force, simulations were carried out in COMSOL® Multiphysics. As a substitute for crystals, polystyrene particles were used for their known acoustic properties. The numerical model used has been described elsewhere [27]. Briefly, the first order acoustic wave was calculated followed by the second order time-average flow. The amplitude of the wall displacement was set to 0.25 nm. The Particle Tracking interface was used to visualize the trajectory and velocity of polystyrene particles. Radiation velocity of different particle size was simulated varying between 4 μm and 10 μm .

3. Results and Discussion

3.1. Crystal Handling in Microfluidic Channel

The radiation force drives material with a positive contrast factor towards the pressure node of a standing pressure wave according to Equation (1). Rising the local density of crystals or high-density droplets by focusing them in a specific location increases the signal to noise ratio significantly. This facilitates the detection and could enable easier characterization of the material of interest. This is especially of relevance in current serial crystallography experiments where a thin jet is brought in the X-ray beam. Focusing of miconazole and lysozyme microcrystals is shown in Figure 2. Miconazole nitrate crystals were grown in the microfluidic channel until an average size of $15 \pm 3 \mu\text{m}$ ($n = 17$) and focused rapidly upon activation of the acoustic field (Figure 2a, Supplementary Materials: Video S1). At an applied voltage of 10 V_{p-p} , the crystals reach their equilibrium position in 1.28 seconds. Note that the radiation velocity can be increased by raising the applied voltage [28]. Moreover, the obtained crystal morphology was significantly different compared to crystals grown in batch. In the microfluidic reactor, small needle-like crystals were observed in contrast with a sea urchin morphology (aggregation of needles) obtained in batch.

Figure 2. (a) Focusing of miconazole nitrate crystals ($15 \pm 3 \mu\text{m}$). Upon activation of the acoustic field, crystals are focused in the center of the microfluidic channel. Time needed to focus is 1.28 seconds at a driving voltage of $10 V_{p-p}$. (b) Focusing of lysozyme microcrystals ($30 \pm 13 \mu\text{m}$) in the center of the channel, the time needed to focus is 1.22 seconds at a driving voltage of $7 V_{p-p}$.

Focusing of lysozyme microcrystals is shown in Figure 2b. An average crystal size was measured to be $30 \pm 13 \mu\text{m}$ ($n = 17$). At an applied voltage of $7 V_{p-p}$, lysozyme crystals were completely focused within 1.22 seconds. As can be noticed, the migration velocity depends strongly on the crystal size. This follows directly from the fact that the radiation force is proportional to the particle diameter (Equation (1): $F_{\text{rad}} \propto a^3$) and can also be seen in the focusing of lysozyme crystals (Supplementary Materials: Video S2). This size dependence of the radiation force offers a powerful tool for the separation of crystals based on their size (see Section 3.2). Recent progress in X-ray structural characterization techniques (XFEL) allows for the collection of high-quality data from micron-sized crystals ($2\text{--}5 \mu\text{m}$). The amount of crystals needed, however, is extremely high. In most setups, a jet of micron-sized crystals is injected in the X-ray beam. Bulk acoustic waves allow to focus flowing crystals in a thin jet and as such this technology can much increase the efficiency of data acquisition. Moreover, the crystal detection can be performed in situ eliminating the manual handling of micron-sized crystals. It is important to note that the radiation force pushes material towards the central plane. To focus material on a line in the exact center of the channel, a second focusing step has to be performed in which an additional standing pressure wave has to be created between the top and bottom of the microfluidic channel [29]. This can easily be done by matching the height of the channel with the width of the channel. Crystals being focused in the center of a channel maintain their liquid mobility by preventing fouling to the surfaces. Fouling is a common showstopper in the pharmaceutical industry [30] as well as in XFEL injectors [31]. Typically, only a few minutes of data collection can be done before the injector must be removed and cleaned. The use of bulk acoustic waves could not only allow efficient data acquisition but also prevent this enormous clogging problem.

In contrast to solid crystals, liquid condensates formed by LLPS behave like liquid droplets. These droplets are initially small and over time grow in size. In our experiments, we were able to focus both small ($4 \mu\text{m}$) and large ($24 \pm 7 \mu\text{m}$; $n = 9$) droplets proving that even liquid-like particles can be focused and manipulated with the use of acoustics (Figure 3, Supplementary Materials: Video S3, and Supplementary Materials: Video S4). The small droplets refer to droplets formed at the beginning of the experiment while the large droplets are droplets obtained after 1 h. Manipulation of these high-density phases in acoustics enables one to increase the signal to noise ratio allowing to visualize

small droplets which were not visible in the absence of the acoustic field. Therefore, this method offers a very simple and useful tool for droplet characterization and preparation for further experiments, such as focusing, droplet fusion, sorting, measurements of formation/maturation kinetics, and medium exchange. It is of particular importance for the density phases involved in many physiological and pathological processes, which might lead to designing a new class of diagnostic tools and/or therapies.

Figure 3. Focusing of high-density droplets. Upon activation of the acoustic field, droplets are focused in the center of the microfluidic channel, with a driving voltage of 25 V_{p-p}. (a) Focusing of small droplets in the center of the channel, (b) focusing of large droplets.

3.2. Numerical Simulations

As mentioned above, the radiation force and related radiation velocity strongly depend on the particle size (Equation (1)), allowing the separation of particles or crystals based on their size. To gain more insight in typical time and velocity values related to these phenomena, numerical simulations were performed to calculate the radiation force on polystyrene particles in water. The effect of the particle diameter on the radiation velocity was investigated as well. The size dependency of the radiation force is shown in Figure 4. As expected, the largest particles (10 μm) have the highest radiation velocity (5.5 mm/s maximum) and the smallest particles (4 μm) a low radiation velocity (0.9 mm/s maximum). Particles of 4 μm and 5 μm have a velocity difference of 0.5 mm/s. This difference in radiation velocity is sufficient to separate a particle mixture in different streams, each containing one particle size. It should be noted that the radiation velocity is not constant over the width of the channel. A decrease in velocity is observed closer to the node, which is in agreement with Equation (1).

Figure 4. Radiation velocity of polystyrene particles along the width of the channel for different particle diameter (see legend). Data were obtained by COMSOL simulations.

In the pharmaceutical industry, crystal size has an important influence on the product behavior; for example, during the formulation or the dissolution and disintegration rate in the body. Miconazole nitrate, for example, requires different crystal sizes for different formulations, such as creams or powders. After the synthesis of an active pharmaceutical compound and purification by crystallization, additional cumbersome operations are often needed to obtain the desired crystal size. Acoustic separation of the desired crystal size is a fast and reliable technology which can easily be implemented in a production process. In this work, we present only a proof of concept for the particle separation based on their size. In a typical setup, microcrystals of different sizes would be inserted in the microfluidic channel at the outside edges, separated by an inert stream in the center (see Figure 1b). Upon activation of the acoustic field and with the correct process parameters chosen (flow velocity, voltage, number of outlets, etc.), the largest crystals focus rapidly in the center of the channel, where they can be extracted. The smaller crystals, however, have not focused yet and can be extracted from different outlets. Experiments devoted to the separation phenomenon are being conducted in our lab and will be presented and discussed in detail in a future publication.

4. Conclusions

We have shown the possibility to use standing bulk acoustic waves to manipulate flowing crystals inside microfluidic channels, and have evoked the possibility to use this technology within current XFEL technology. Current serial crystallography methods suffer from an extremely low efficiency, where the hit rate of the X-ray beam is typically less than 1%. Increasing the local density of crystals in a thin jet can lead to a much more efficient data acquisition. Moreover, the use of bulk acoustic waves could also prevent the detrimental clogging problem of XFEL injectors. This technology could bring fundamental changes to the design of XFEL injectors.

We also showed the possibility to focus the high-density droplets formed by LLPS, which is a recently emerging topic with high relevance in molecular cell biology. This observation allows for the use of experimental techniques that previously lacked a high signal to noise ratio and might lead to designing a new class of diagnostic tools and/or therapies.

Acoustofluidics is a technology that can easily be implemented in a sample preparation process. Moreover, this technology is biocompatible, ensuring product integrity. The size dependency of the radiation force has been demonstrated numerically on polystyrene particles. This gives a proof of

concept for the possibility to separate crystals based on their size. Size-based crystals separation will be investigated in detail in a future publication.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4352/9/3/120/s1>, Video S1: Focusing of miconazole nitrate crystals, Video S2: Focusing of lysozyme crystals, Video S3: Focusing of small high-density droplets, Video S4: Focusing of large high-density droplets.

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References

- Shi, H.H.; Xiao, Y.; Ferguson, S.; Huang, X.; Wang, N.; Hao, H.X. Progress of crystallization in microfluidic devices. *Lab Chip* **2017**, *17*, 2167–2185. [\[CrossRef\]](#) [\[PubMed\]](#)
- Gerard, C.J.J.; Ferry, G.; Vuillard, L.M.; Boutin, J.A.; Ferte, N.; Grossier, R.; Candoni, N.; Veessler, S. A Chemical Library to Screen Protein and Protein-Ligand Crystallization Using a Versatile Microfluidic Platform. *Cryst. Growth Des.* **2018**, *18*, 5130–5137. [\[CrossRef\]](#)
- Zhang, S.; Gerarda, C.J.J.; Ikni, A.; Ferry, G.; Vuillard, L.M.; Boutin, J.A.; Ferte, N.; Grossier, R.; Candoni, N.; Veessler, S. Microfluidic platform for optimization of crystallization conditions. *J. Cryst. Growth* **2017**, *472*, 18–28. [\[CrossRef\]](#)
- Guo, F.; Zhou, W.; Li, P.; Mao, Z.; Yennawar, N.H.; French, J.B.; Huang, T.J. Precise Manipulation and Patterning of Protein Crystals for Macromolecular Crystallography Using Surface Acoustic Waves. *Small* **2015**, *11*, 2733–2737. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wagner, A.; Duman, R.; Stevens, B.; Ward, A. Microcrystal manipulation with laser tweezers. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2013**, *69*, 1297–1302. [\[CrossRef\]](#) [\[PubMed\]](#)
- Cuttitta, C.M.; Ericson, D.L.; Scalia, A.; Roessler, C.G.; Teplitsky, E.; Joshi, K.; Campos, O.; Agarwal, R.; Allaire, M.; Orville, A.M.; et al. Acoustic transfer of protein crystals from agarose pedestals to micromeshes for high-throughput screening. *Acta Crystallogr. Sect. D* **2015**, *71*, 94–103. [\[CrossRef\]](#) [\[PubMed\]](#)
- Roessler, C.G.; Agarwal, R.; Allaire, M.; Alonso-Moriet, R.; Andi, B.; Bachega, J.F.R.; Bommer, M.; Brewster, A.S.; Browne, M.C.; Chatterjee, R.; et al. Acoustic Injectors for Drop-On-Demand Serial Femtosecond Crystallography. *Structure* **2016**, *24*, 631–640. [\[CrossRef\]](#) [\[PubMed\]](#)
- Mafuné, F.; Miyajima, K.; Tono, K.; Takeda, Y.; Kohno, J.; Miyauchi, N.; Kobayashi, J.; Joti, Y.; Nango, E.; Iwata, S.; et al. Microcrystal delivery by pulsed liquid droplet for serial femtosecond crystallography. *Acta Crystallogr. Sect. D* **2016**, *D72*, 520–523. [\[CrossRef\]](#) [\[PubMed\]](#)
- Jacquemet, L.; Ohana, J.; Joly, J.; Legrand, P.; Kahn, R.; Borel, F.; Pirocchi, M.; Charraut, P.; Carpentier, P.; Ferrer, J.-L. A new highly integrated sample environment for protein crystallography. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2004**, *60*, 888–894. [\[CrossRef\]](#) [\[PubMed\]](#)
- Deller, M.; Rupp, B. Approaches to automated protein crystal harvesting. *Acta Crystallogr. Sect. F* **2014**, *70*, 133–155. [\[CrossRef\]](#) [\[PubMed\]](#)
- Gerard, C.J.J.; Ferry, G.; Vuillard, L.M.; Boutin, J.A.; Chavas, L.M.G.; Huet, T.; Ferte, N.; Grossier, R.; Candoni, N.; Veessler, S. Crystallization via tubing microfluidics permits both in situ and ex situ X-ray diffraction. *Acta Crystallogr. Sect. F Structural Biol. Commun.* **2017**, *73*, 574–578. [\[CrossRef\]](#) [\[PubMed\]](#)
- Hyman, A.A.; Weber, C.A.; Jülicher, F. Liquid-liquid phase separation in biology. *Annu. Rev. Cell Dev. Biol.* **2014**, *30*, 39–58. [\[CrossRef\]](#) [\[PubMed\]](#)

13. Murakami, T.; Qamar, S.; Lin, J.Q.; Schierle, G.S.K.; Rees, E.; Miyashita, A.; Costa, A.R.; Dodd, R.B.; Chan, F.T.S.; Michel, C.H.; et al. ALS/FTD Mutation-Induced Phase Transition of FUS Liquid Droplets and Reversible Hydrogels into Irreversible Hydrogels Impairs RNP Granule Function. *Neuron* **2015**, *88*, 678–690. [[CrossRef](#)] [[PubMed](#)]
14. Wei, M.-T.; Elbaum-Garfinkle, S.; Holehouse, A.S.; Chen, C.C.-H.; Feric, M.; Arnold, C.B.; Priestley, R.D.; Pappu, R.V.; Brangwynne, C.P. Phase behaviour of disordered proteins underlying low density and high permeability of liquid organelles. *Nat. Chem.* **2017**, *9*, 1118–1125. [[CrossRef](#)] [[PubMed](#)]
15. Patel, A.; Lee, H.O.; Jawerth, L.; Maharana, S.; Jahnke, M.; Hein, M.Y.; Stoyanov, S.; Mahamid, J.; Saha, S.; Franzmann, T.M.; et al. A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. *Cell* **2015**, *162*, 1066–1077. [[CrossRef](#)] [[PubMed](#)]
16. Boeynaems, S.; Alberti, S.; Fawzi, N.L.; Mittag, T.; Polymenidou, M.; Rousseau, F.; Schymkowitz, J.; Shorter, J.; Wolozin, B.; Van Den Bosch, L.; et al. Protein Phase Separation: A New Phase in Cell Biology. *Trends Cell Biol.* **2018**, *28*, 420–435. [[CrossRef](#)] [[PubMed](#)]
17. Abbondanzieri, E.A.; Meyer, A.S. More than just a phase: The search for membraneless organelles in the bacterial cytoplasm. *Curr. Genet.* **2019**, 1–4. [[CrossRef](#)] [[PubMed](#)]
18. Barnkob, R.; Augustsson, P.; Laurell, T.; Bruus, H. Acoustic radiation- and streaming-induced microparticle velocities determined by microparticle image velocimetry in an ultrasound symmetry plane. *Phys. Rev. E* **2012**, *86*, 1–11. [[CrossRef](#)] [[PubMed](#)]
19. Nilsson, A.; Petersson, F.; Jönsson, H.; Laurell, T. Acoustic control of suspended particles in micro fluidic chips. *Lab Chip* **2004**, *4*, 131–135. [[CrossRef](#)] [[PubMed](#)]
20. Muller, P.B.; Rossi, M.; Marín, Á.G.; Barnkob, R.; Augustsson, P.; Laurell, T.; Kähler, C.J.; Bruus, H. Ultrasound-induced acoustophoretic motion of microparticles in three dimensions. *Phys. Rev. E* **2013**, *88*, 1–12. [[CrossRef](#)] [[PubMed](#)]
21. Petersson, F.; Nilsson, A.; Holm, C.; Jönsson, H.; Laurell, T. Separation of lipids from blood utilizing ultrasonic standing waves in microfluidic channels. *Analyst* **2004**, *129*, 938–943. [[CrossRef](#)] [[PubMed](#)]
22. Fornell, A.; Garofalo, F.; Nilsson, J.; Bruus, H.; Tenje, M. Intra-droplet acoustic particle focusing: Simulations and experimental observations. *Microfluid. Nanofluidics* **2018**, *22*, 1–9. [[CrossRef](#)]
23. Laurell, T.; Petersson, F.; Nilsson, A. Chip integrated strategies for acoustic separation and manipulation of cells and particles. *Chem. Soc. Rev.* **2007**, *36*, 492–506. [[CrossRef](#)] [[PubMed](#)]
24. Böhm, H.; Anthony, P.; Davey, M.R.; Briarty, L.G.; Power, J.B.; Lowe, K.C.; Benes, E.; Gröschl, M. Viability of plant cell suspensions exposed to homogeneous ultrasonic fields of different energy density and wave type. *Ultrasonics* **2000**, *38*, 629–632. [[CrossRef](#)]
25. Vanherberghen, B.; Manneberg, O.; Christakou, A.; Frisk, T.; Ohlin, M.; Hertz, H.M.; Önfelt, B.; Wiklund, M. Ultrasound-controlled cell aggregation in a multi-well chip. *Lab Chip* **2010**, *10*, 2727–2732. [[CrossRef](#)] [[PubMed](#)]
26. Wiklund, M. Acoustofluidics 12: Biocompatibility and cell viability in microfluidic acoustic resonators. *Lab Chip* **2012**, *12*, 2018–2028. [[CrossRef](#)] [[PubMed](#)]
27. Muller, P.B.; Barnkob, R.; Jensen, M.J.H.; Bruus, H. A numerical study of microparticle acoustophoresis driven by acoustic radiation forces and streaming-induced drag forces. *Lab Chip* **2012**, *12*, 4617. [[CrossRef](#)] [[PubMed](#)]
28. Barnkob, R.; Augustsson, P.; Laurell, T.; Bruus, H. Measuring the local pressure amplitude in microchannel acoustophoresis. *Lab Chip* **2010**, *10*, 563–570. [[CrossRef](#)] [[PubMed](#)]
29. Dong, Z.; Rivas, D.F.; Kuhn, S. Acoustophoretic focusing effects on particle synthesis and clogging in microreactors. *Lab Chip* **2019**, *19*, 316–327. [[CrossRef](#)] [[PubMed](#)]
30. Da Silva, J.J.; Wanzeller, M.G.; de Almeida Farias, P.; da Rocha Neto, J.S. Development of circuits for excitation and reception in ultrasonic transducers for generation of guided waves in hollow cylinders for fouling detection. *IEEE Trans. Instrum. Meas.* **2008**, *57*, 1149–1153. [[CrossRef](#)]
31. Calvey, G.D.; Katz, A.M.; Schaffer, C.B.; Pollack, L. Mixing injector enables time-resolved crystallography with high hit rate at X-ray free electron lasers. *Struct. Dyn.* **2016**, *3*, 1–19. [[CrossRef](#)] [[PubMed](#)]