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Published in:
Twenty Second International Conference on Miniaturized Systems for Chemistry and Life Sciences (µTAS 2018) - Conference proceedings

Publication date:
2018

Document Version:
Submitted manuscript

Link to publication

Citation for published version (APA):
GROWTH AND SEPARATION OF CRYSTALS AND PROTEIN AGGREGATES IN ACOUSTOFLUIDICS
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ABSTRACT
Microfluidic devices offer a well-controlled environment for crystal nucleation and growth. Important parameters are mixing and the associated shear. Incomplete or insufficient mixing results in a wide crystal size distribution and might affect the morphology and purity of the product. Bulk acoustic waves offer the possibility to quickly mix two liquid streams and to manipulate particles in the microfluidic channel. In this contribution, fast mixing of two liquid streams by acoustic streaming is demonstrated. Miconazole nitrate crystals were grown in the presence of the acoustic field, once a desired size is reached the crystals focus rapidly on the pressure node. Growth and focusing of amyloid fibrils in the microfluidic channel has also been demonstrated.

KEYWORDS: acoustofluidics, crystallization, amyloidosis

INTRODUCTION
Mounting evidence suggests that shear flows influence the aggregation behavior of proteins [1]. Shear influences both the nucleation and crystal growth [2,3]. Acoustic streaming is a great methodology to create lateral convection in otherwise laminar flow operated channels. It offers a good strategy to reduce mass transfer resistance dramatically [4]. It could be expected that the shear patterns associated with the vortex flows of acoustic streaming will affect the crystallization/nucleation process greatly. Small particles or crystals are only affected by the Stokes drag forces and will flow along with the vortices. Larger crystals on the other hand will experience a radiation force and will rapidly focus at either the node or anti-node of the standing pressure wave in the channel [5].

In the present contribution a proof of concept has been obtained for the crystal formation and growth in presence of the acoustic field. Fast nucleation of a small pharmaceutical molecule (Miconazole nitrate) was performed in the presence of acoustic streaming. The formed crystal nuclei were allowed to grow under the same conditions. Once a desired size was reached, the crystals were focused on the pressure node and separated from the main flow. Within a broader study on the influence of controlled flow on protein aggregation, we were able to visualize and focus clusters of amyloid fibrils from lysozyme. This is of great relevance since the formation of amyloid fibrils underlies a wide range of human disorders, including neurodegenerative and cardiovascular diseases like Alzheimer and cardiac amyloidosis.

EXPERIMENTAL
Rectangular microfluidic channels were etched in a silicon wafer. A width and thickness of respectively 375 µm and 310 µm were fabricated. Two inlets and outlets were provided and connected to syringe pumps by means of capillaries. A standing pressure wave was generated inside the channel with a piezo-ceramic element (PZT) having a resonance frequency at 2.0 MHz (Figure 1). Coupling of the microfluidic chip to the PZT was done by a thin glycerol layer. An in-house built PMMA holder ensured a good connection between the chip, PZT and Nanoports. The applied voltage on the PZT was generated by a frequency generator and amplified by a RF power amplifier.

Visualization of all the experiments was done using an inverted fluorescent microscope in combination with a CCD camera. First mixing experiments were performed to quickly mix the solvent with the antisolvent. Water containing a fluorescent salt and non-fluorescent DI water were brought into contact in the microfluidic chip. Mixing was initiated by actuating the PZT at a frequency of 1.93 MHz. The recorded movies were analyzed with ImageJ software. The intensity of each pixel was related to the fluorescence intensity and eventually to the efficiency of the mixing.

For the crystallization experiments miconazole nitrate was dissolved in dimethylsulfoxide (DMSO) (5 w/w%) and brought in the microfluidic channel at a flowrate of 2 µl/min. Water was used as an antisolvent and pumped
through the channel at a flowrate of 8 µl/min. Crystal formation was monitored in absence and presence of the acoustic field. Crystals reaching a cut-off size were focused in the center of the microfluidic channel.

In the broader context of protein aggregates, amyloid fibrils from lysozymes were grown overnight in the microfluidic channel and without the presence of an acoustic field. Once the fibrils were formed, a flowrate of 4 µl/min was set and the acoustic field was activated.

![Figure 1: Schematic representation of microfluidic channel: The microfluidic channel is connected to a piezo ceramic element vibrating at 1.93 MHz. Actuation is done with a frequency generator. The vibrations are transferred to the channel wall, creating a standing pressure wave (thicknesses are not to scale). The generated vortex flow is shown in the enlarged picture.](image)

**RESULTS AND DISCUSSION**

Mixing of two water streams due to the presence of an acoustic field was visualized by a camera. Figure 2a shows snapshots at different times at a fixed observation position (16 mm downstream the contact area) during the mixing process, overlaid with the concentration profile. In this case the flow rate was set to 5 mm s⁻¹ and the actuation voltage to 15 V<sub>pp</sub>. At t = 0.60 s the channel already had a mixing efficiency of η = 0.94. The mixing efficiency has been measured for a range of applied voltages and flowrates (Fig. 2b). Actuation at voltages lower than 10 V<sub>pp</sub> does not induce any visible mixing. For a linear velocity of 5 mm s⁻¹ a mixing of already η = 0.65 is reached at 10 V<sub>pp</sub> actuation. Complete mixing (η = 0.94) is reached at an actuation voltage of 15 V<sub>pp</sub> for both flowrates of 5 mm s⁻¹ and 10 mm s⁻¹. To our knowledge such fast mixing in a microfluidic channel with bulk acoustic waves has not been demonstrated yet.

![Figure 2: a) Top view of microfluidic channel. Shown is the mixing at a flow rate of 5 mm/s and an actuation of 15 V<sub>pp</sub>. A mixing efficiency of 94% is reached after 0.60 seconds. b) Mixing efficiency in function of applied voltage for two different flow rates, 5 mm s⁻¹ (♦) and 10 mm s⁻¹ (□). At lower voltages the water streams mix better for a low flow rate of 5 mm s⁻¹. The time needed for complete mixing is around 1 s and 2 s for flow rates of 5 mm s⁻¹ and 10 mm s⁻¹, respectively.](image)

Crystallization of miconazole nitrate was visualized in the microfluidic channel. In absence of the acoustic field but presence of flow, small needle like crystals appeared a short time after incubation. These crystals focused rapidly (1.28 s) upon activation of the acoustic field at a voltage of 10 V<sub>pp</sub> (Fig. 3a). As a substitute for crystals, the focusing
velocity was measured for 5 µm fluorescent polystyrene particles. A migration velocity that decreases as the node position is approached is observed (Fig. 3b). This is in accordance with the literature [6]. Moreover, the obtained crystal morphology is significantly different compared to crystals grown in batch. In the microfluidic reactor, small needle like crystals are observed in contrast with star shaped aggregation of needles (sea urchin morphology) obtained in batch (Fig. 3c). In the broader study of controlled shear on protein aggregates, the grown amyloid fibrils from lysozymes were focused on the pressure node upon activation of the acoustic field (Fig. 3d, 3e).

Figure 3: a) Focusing of small needle like miconazole nitrate crystals. b) Radial focus velocities of 5 µm particles as a function of five different voltages (see legend). A migration velocity that decreases as the node position is approached is observed. c) Miconazole nitrate crystals with a sea urchin morphology (left) and needle morphology (right). d) Focusing of amyloid fibrils in microfluidic channel. e) TEM micrograph of amyloid fibrils.

CONCLUSION

We have demonstrated the use of bulk acoustic wave to quickly mix two liquid streams in order to start crystal nucleation and growth. The same bulk acoustic wave have elegantly been applied to focus the formed crystals in the center of the microfluidic channel. Next to crystals, amyloid fibrils have been grown and separated in the microfluidic channel. Growth and separation by bulk acoustic wave of crystals or amyloid fibrils in microfluidic channels has to our knowledge never been achieved before.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the European Research Council for the support through the ERC Starting Grant EVODIS.

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