SUPPLEMENTARY INFORMATION

Preparation of protein solutions

Glucose Isomerase (GI) from *Streptomyces rubiginosus* was purchased from Hampton Research (USA). Ovalbumin from *Gallus gallus*, β-lactoglobulin and hemoglobin from *Bovine* and bovine pancreatic trypsin inhibitor (BPTI) were purchased from Sigma (Germany). These proteins were selected according to the individual dipole moment, which was calculated using the Protein Dipole Moments Server[1].The unit of the dipole moment is Debye (D, 1 D=1×10^{-18} statC·cm). All buffers and precipitant solutions (SI Table 1) were filtered through a 0.2 μm or 0.45 μm filter (SARSTEDT, Germany). Protein solutions were centrifuged for 60 minutes at 16000 × g and 4 ℃ before use. Concentrations were measured applying a Nanodrop ND-2000 (Thermo-Scientific, Germany).

Table 1. Solution conditions and molecular parameters of proteins and precipitants

Protein	Glucose Isomerase	Ovalbumin	BPTI	β-lactoglobulin	Hemoglobin
Dipole moment (D)	1082	1061	941	594	201
Mw (kDa)	43.23	42.86	6.5 (58aa)	18.4	64.5 (tetramer)
Isoelectric point	5.0	5.19	10.5	4.76	6.8
Protein buffer	10 mM MES pH 6.5, 500 mM NaCl	150 mM K_2HPO_4-KH_2PO_4 pH 7.4	50 mM NaAc pH 4.5	1×PBS pH 7.4	50 mM Tris-HCl pH 9.0
Initial concentration of protein solution (mg/ml)	27	50	40	25	50
Precipitant	12% PEG 20000	3 M $(NH_4)_2SO_4$	700 mM KSCN	25% PEG 3350	9% PEG 6000
Mixing ratio	1:1	1:1	1:1	1:1	1:1

Setups for producing pulsed electric fields

Two EF setups were assembled to apply pulsed electric fields (EFs) to protein suspensions. The one shown in SI Fig. 1a**,** is platinum (Pt) wire EF setup, consisted of a rectangular plastic piece in the middle with a round cell covered by two rectangular glass plates and two inserted Pt wires with 0.3 mm diameter, 1.5 cm length and a resistance of approx. 22.5 mΩ at 20 ℃ (Sigma, Germany). The other one shown in SI Fig. 1b, called parallel conductive glass (PCG) EF setup, consisted of a rectangular plastic piece in the middle with a round cell covered by two rectangular conductive glasses, which are indium tin oxide (ITO) coated with dimensions of 2 cm × 2 cm and a surface resistance of 3.2-4.8 mΩ (Sigma, Germany). The ITO coated surfaces of the two glass plates were positioned inwards and facing towards each other. The sample container capacity of each EF setup is approximately 45 μl. The setup was connected to a KWD-808 instrument (Jiangsu, China) which can generate defined pulsed electric fields, as shown in SI Fig.1c. The pulse-width (τ) of the basic waveform component in each waveform was 0.6±0.15 ms. The maximum pulse amplitude in each waveform outputted on 500 Ω load ($V_{max\text{-}500\Omega}$) during this experiment was 20 V. Waveform 1 is composed of equidistant identical pulses. In the waveform 2, bunches of rectangular pulses alternatingly appeared at high frequency and low frequency. Rectangular pulses appeared in the first half period of waveform 3 with a uniform amplitude, but no pulses output in the second half period. The pulse amplitude of the pulse in the waveform 4 rises up in the first half period and falls down with identical pulses in the second half period. In the waveform 5, the amplitude of the basic pulse rises up in the first half pulse pattern period with no output pulse in the second half period.

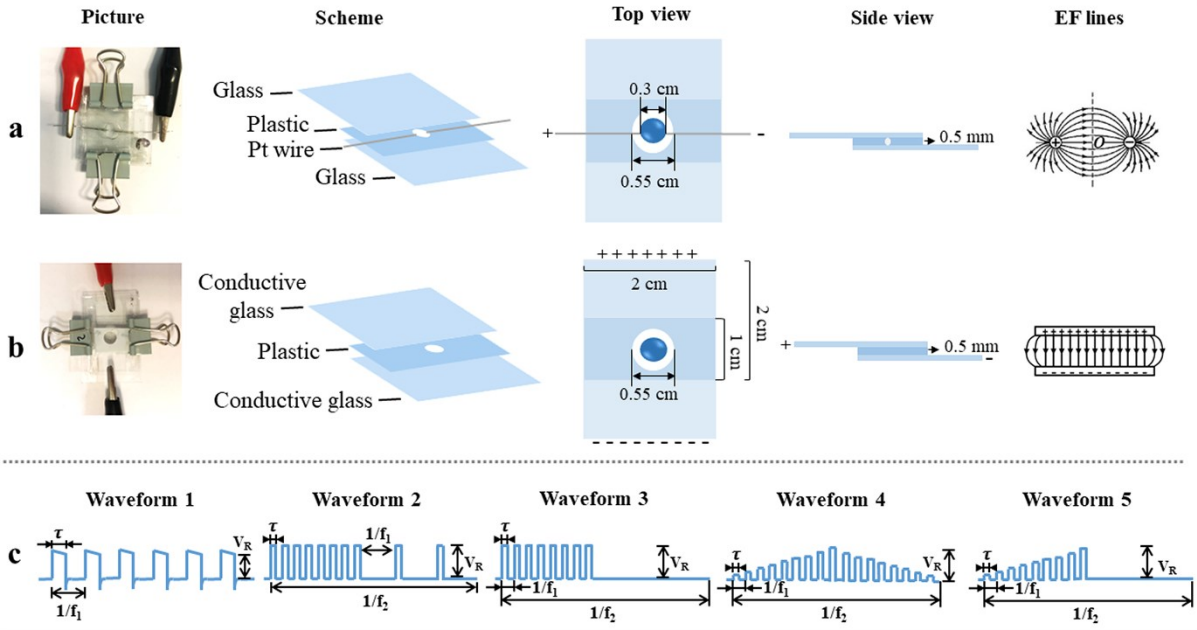

Figure 1 Scheme and photographic pictures of (a) the Pt EF setup and (b) the PCG EF setup. The direction and distribution of corresponding EF lines to each EF setup is shown in the left bottom of each scheme. (c) Diagrams of five EF waveforms, the maximum output pulse amplitude (V_R) depends on the resistance (R) of the load.

Methods and experimental setup applied to monitor protein LLPS applying optical microscopy

Droplets with 1:1 protein to precipitant volume ratio and a total volume of 3 µl were sealed with two glass cover slides. Then, they were subsequently exposed to EFs and monitored over time by a Leica M205C Microscope with an adapted cold light source. Glucose isomerase (GI), ovalbumin, bovine pancreatic trypsin inhibitor (BPTI) and β-lactoglobulin shown in SI Table 1 were selected and investigated applying five different EF waveforms ($V_{max\text{-}500\Omega}$=20 V) with the Pt EF and the PCG EF setup, respectively. Conditions of protein solutions and corresponding precipitants used for the experiments are summarized in SI Table 1. To evaluate the morphologies of LDCs nearby the anode and nearby the cathode of the PCG EF setup, the pictures were recorded from the anode side (+) and the cathode side (-), respectively, by turning the PCG EF setup upwards.

Polarized and depolarized dynamic light scattering (DLS/DDLS) experiments

To study the dynamic assembly and the internal order of protein liquid dense clusters applying DLS and DDLS, an instrument providing a laser wavelength of 532 nm and a laser output power of 100 mW was utilized (SI Fig. 2. XtalConcepts, Germany). The instrument allows to acquire parallel DLS and DDLS measurements, each data acquisition of which was for 20 s with 120 s waiting time performed at 20 ℃. The appropriate viscosity values were considered to calculate the hydrodynamic radii. A transparent cuvette (101.015-QS, Hellma Analytics, Germany), with 3 mm × 3 mm inner cross-section and 21 mm height was used as the sample container. As shown in SI Fig. 2, two Pt wires were inserted at two opposite corners of the cuvette to generate electric fields. In preparation, 20 µL of GI solution at 7.5 mg/ml was mixed with 20 µL of 12% PEG 20000 and pipetted into a cuvette. The sealed cuvette was placed in the cuvette holder of the DDLS instrument and an EF with waveform 4 ($V_{max\text{-}500\Omega}$=20 V) was applied up to 5 h.

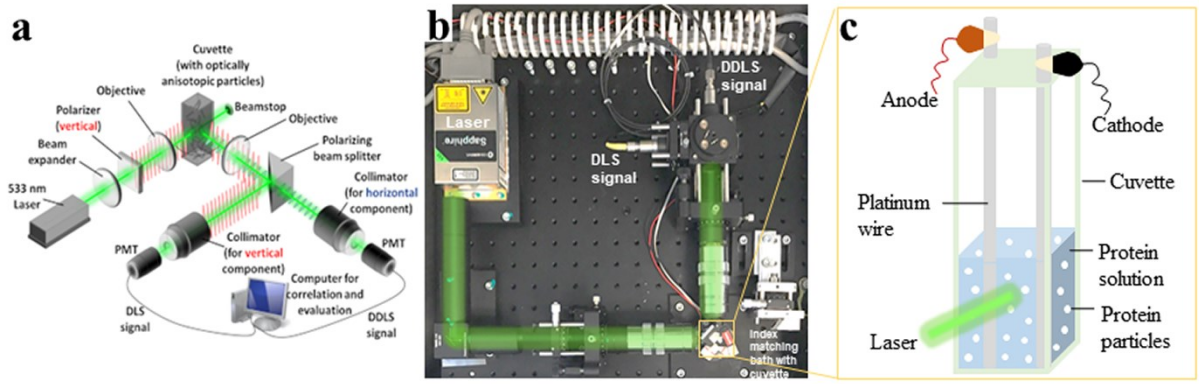

Figure 2 (a) Schematic[2] and (b) photographic representation of the DDLS instrument. (c) Scheme of the cuvette used for the DDLS experiments with two Pt wires.

Sample preparation for Transmission Electron Microscopy

A volume of 2 µl of GI protein solution was mixed with 2 µl of 12% (w/v) PEG 20000 in the platinum wire EF setup and treated with waveform 4 ($V_{max\text{-}500\Omega}$=20 V) for 5 hours. Subsequently, the sample was centrifuged at 3000 × g for 5 minutes to separate GI LDCs. The supernatant was exchanged with 10 µl 12% PEG 20000 and centrifuged for 5 minutes at 3000 × g and 20 ℃. The supernatant was exchanged with 20 µl cross-linking solution (5 mM MES pH6.5, 250 mM NaCl, 6 % PEG 20000 and 2.5 % (v/v) glutaraldehyde), which was mixed with LDCs gently then incubated at 20 ℃ for 48 h to stabilize LDCs. After incubation the cross-linked sample was again centrifuged at the same conditions and the supernatant was replaced with 10 µl of ultrapure water to wash out most of the buffer components after another centrifugation step. Finally, the LDCs were resuspended in 5-10 µl fresh ultrapure water (depending on the amount of LDCs pellets) for negative staining to increase the contrast of LDCs. For negative staining, a carbon coated 3.05 mm copper grid (300 mesh) (PLANO GmbH, Germany) was used. Before loading the sample, the grid was glow-discharged for 30 s at 25 mV (GloQube Plus, Quorum). The respective sample solution was pipetted onto the grid and blotted with Whatman paper after 30 s, followed by two times grid washing with 10 µl ultrapure water. For staining, 10 µl of 2 % (w/v) uranyl acetate in ultrapure water was used and pipetted onto the grid. Afterwards the grid was incubated on the droplet surface for 30 s, the uranyl acetate liquid was blotted with Whatman paper and finally the grid was dried on the Whatman paper. A Jeol JEM-2100 Plus transmission electron microscope operated at 200 keV was used for the imaging experiments.

Methods and experimental procedure of Circular dichroism spectrum

CD spectroscopy was applied to investigate the secondary structure content of selected proteins with distinct solvents composition under different EF conditions using a JASCO-815 spectropolarimeter (JASCO, Japan), applying in parallel EFs with five different pulse waveforms, respectively. For experiments 100 µL protein solution at a concentration of 0.2 mg/ml was pipetted into a microplate (PolySorp F8-Wells Nunc-Immuno Module, VWR, Germany) with two Pt wire- electrodes attached to expose protein samples with five pulsed waveforms ($V_{max\text{-}500\Omega}$=20 V), respectively. After 1 hour of exposure, the protein samples were transferred to a quartz cuvette with 1 mm path length (Hellma-Analystics, Germany). Protein solutions used for all groups were prepared in one tube to keep the same protein concentration. CD measurements were averaged over 10 individual spectra, recorded within a wavelength range of 260 - 190 nm, with a scanning speed of 100 nm/min and a bandwidth of 1 nm. The spectra were evaluated by the Software Spectra Manager from JASCO.

Supplementary Figures

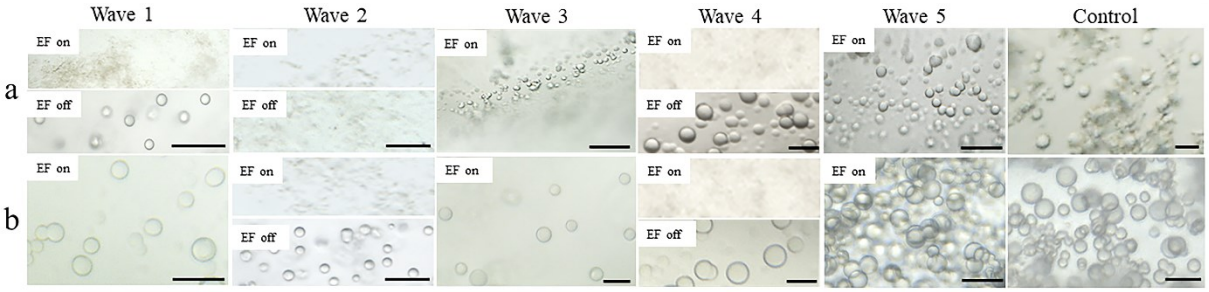

Figure 3 LDCs of ovalbumin formed with (a) the Pt EF setup and (b) the PCG EF setup. The dimensions of LDCs formed with Pt EFs are smaller compared to those formed with PCG EFs when applying waveforms 3 and 5. The phase separation was hindered when EFs of waveforms 1, 2, and 4 were applied and reversible when EFs with the waveforms 1, 4 and PCG EF with waveform 2 were switched off. Irreversibility was observed for waveform 2 of the Pt EF setup. This observation is probably explained by the high frequency and average pulse amplitude of waveform 2. The scale bars correspond all to 25 µm.

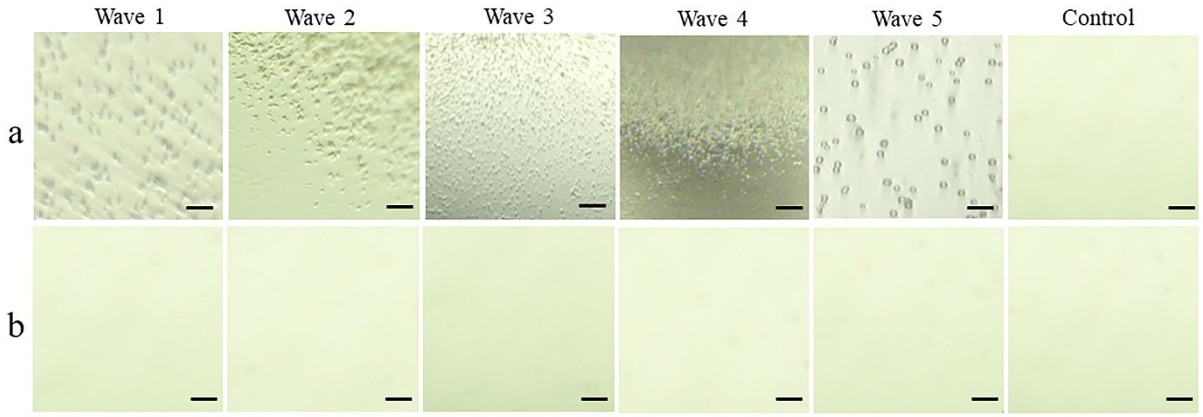

Figure 4 LDCs of BPTI formed with (a) the Pt EF setup and (b) the PCG EF setup. No LLPS was observed after mixing BPTI with KSCN at 20℃ when an EF was absent (control). Generally, the LDC formation of BPTI in the presence of SCN^- is reported to be temperature dependent,[3] therefore, the droplets in the control sample remained clear even after an incubation of 24 hours. However, LDCs appeared in the same condition after 1 hour of applying Pt wire EFs. Induction of BPTI phase separation did not appear when applying the EFs based on parallel conductive glass. All scale bars correspond to 50 µm.

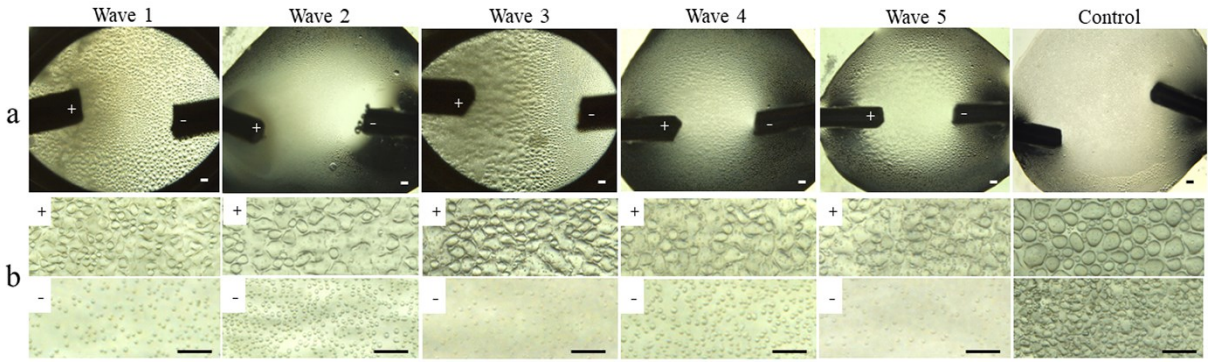

Figure 5 LDCs of β-lactoglobulin formed with (a) the Pt EF setup and (b) the PCG EF setup. No apparent morphology differences between LDCs induced by different EF waveforms were observed. The dimensions of LDCs nearby the anode were larger than those formed nearby the cathode, due to the overall negative net charged β-lactoglobulin. The size and shape of LDCs nearby the cathode were uniform. The scale bars are all 200 µm.

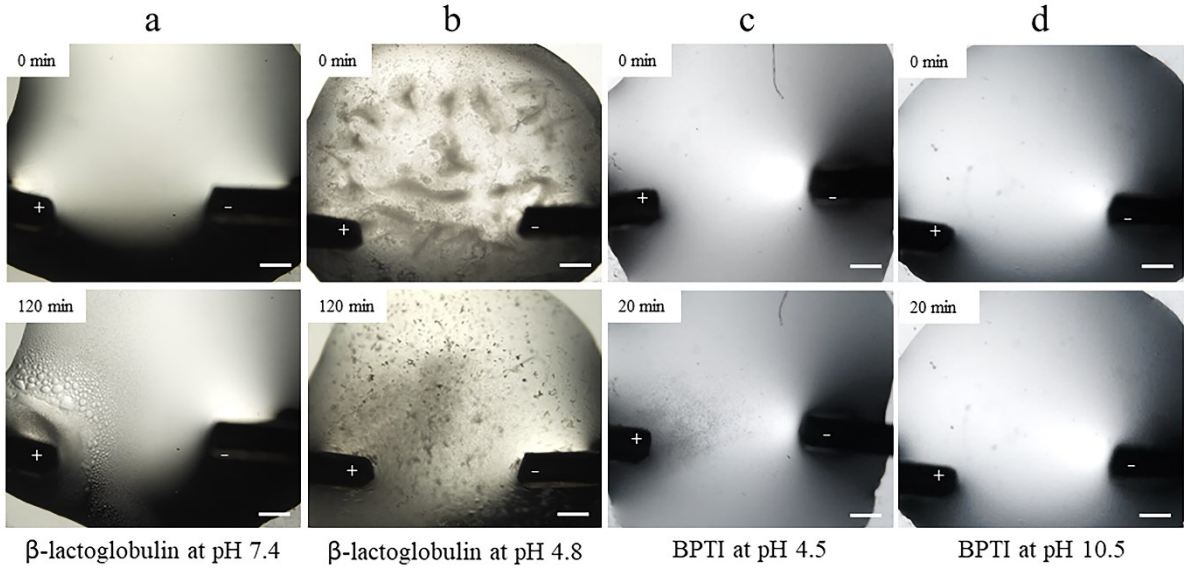

Figure 6 Images show protein migration and phase separation of (a) β-lactoglobulin at pH 7.4, (b) β-lactoglobulin at pH 4.8, (c) BPTI at pH 4.5, and (d) BPTI at pH 10.5 in a Pt EF of waveform 3 ($V_{max\text{-}500\Omega}$=20 V). Scale bars correspond all to 300 µm. In images b and d, β-lactoglobulin (pI=4.76) and BPTI (pI=10.5) solutions were at their isoelectric point, respectively. Thereby, due to an overall net charge close to zero no protein migration was observed. Solutions of β-lactoglobulin and BPTI in images a and c were adjusted to a negative overall net charge and positive overall net charge, respectively, for which protein migration and LDCs were observed in the presence of EFs. More LDCs of positively charged BPTI appeared near the anode in image c. This unexpected result was also observed and explained by Hammadi et al. when investigating protein crystallization induced by a local electric potential.[4] It was elucidated that decamers and monomers coexist in an acidic BPTI solution and the decamers have lower speed of electrophoretic motion than the monomers, 0.32 and 0.36, respectively. Therefore, theBPTI decamer to monomer ratio increased towards the anode, which locally increased the supersaturation near the anode. Consequently, more BPTI LDCs formed in close proximity of the anode.[5] Time intervals indicated in the pictures are showing the application time of the EFs.

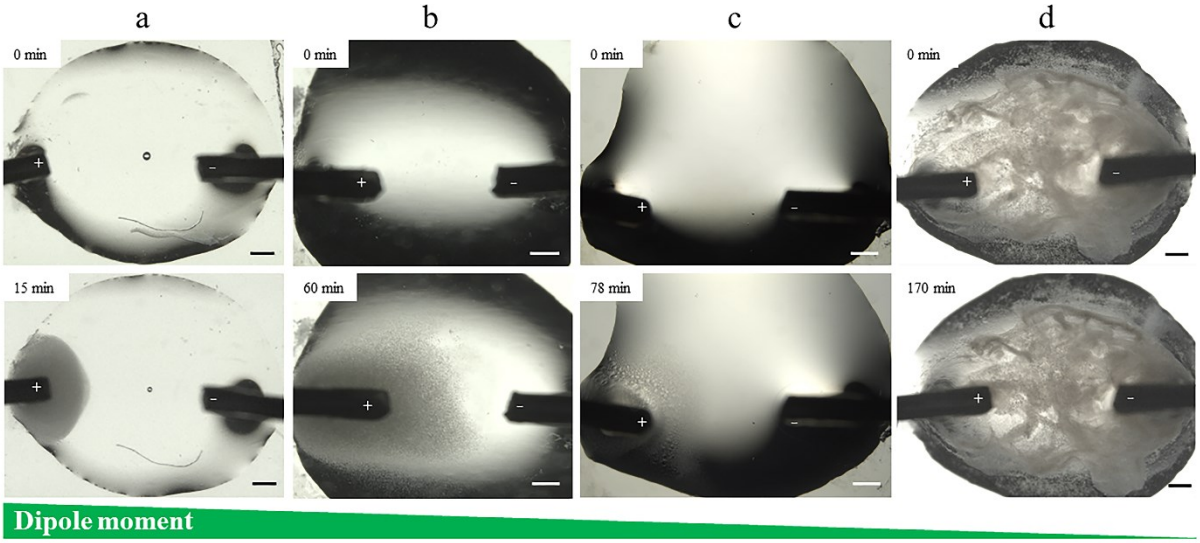

Figure 7 Protein migration and phase separation of (a) GI (dipole moment = 1082 D), (b) BPTI (dipole moment = 941 D), (c) β-lactoglobulin (dipole moment = 594 D), and (d) hemoglobin (dipole moment = 201 D) under a Pt EF with waveform 3 ($V_{max\text{-}500\Omega}$=20 V). Scale bars correspond all to 300 µm. Time intervals indicated in pictures are showing the application time of the EFs. The sample droplet conditions for each protein are summarized in SI Table 1. It can be concluded that the migration rate of proteins to either electrode sequentially increased with the increase of the characteristic dipole moment. GI, with the highest dipole moment among the four proteins, showed the highest migration rate. A dense phase within the GI solution surrounded the anode after 15 min of EF application. Hemoglobin clusters, with the lowest molecule dipole moment of 201 D, did not undergo any migration in the droplet, despite exposure to an EF for a rather long time of 170 min.

References

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