Published in final edited form as:

ChemBiochem. 2019 January 18; 20(2): 270–275. doi:10.1002/cbic.201800553.

Determination of Aqueous Two Phase System Binodals and Tie-lines by Electrowetting-on-Dielectric Droplet Manipulation

Taisuke Kojima [Dr.],

The Wallace H Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory School of Medicine, 950 Atlantic Drive NW, Atlanta 30332 (USA)

Chu-Chi Lin [Mr.],

Department of Mechanical Engineering, National Taiwan University, No.1, Sec. 4, Roosevelt Rd., Taipei 10617 (Taiwan)

Shuichi Takayama [Dr.], and

The Wallace H Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory School of Medicine, 950 Atlantic Drive NW, Atlanta 30332 (USA)
takayama@gatech.edu

Shih-Kang Fan [Dr.]

Department of Mechanical Engineering, National Taiwan University, No.1, Sec. 4, Roosevelt Rd., Taipei 10617 (Taiwan) skfan@fan-tasy.org

Professor Chi-Huey Wong's 70th Birthday

Abstract

Handling aqueous two phase systems (ATPSs) formed by liquid-liquid phase separation (LLPS) relies on the accurate construction of binodal curves and tie-lines, which delineate polymer concentrations required for phase separation and depict the properties of the resulting phases, respectively. Various techniques to determine binodal curves and tie-lines of ATPSs exist, but most rely on manual pipetting of relatively large volumes of fluids in a slow and tedious manner. To overcome these disadvantages, this paper describes microscale droplet manipulation by electrowetting-on-dielectric (EWOD) for determination of ATPS binodals and tie-lines. EWOD enables automated handling of droplets in an optically-transparent platform that allows for *in situ* droplet observation. Separated phases are clearly visible and volumes of each phase are readily determined. Additionally, in considering the molecular crowding present in living cells, this work examines the role of a macromolecule in prompting LLPS. These results show that EWOD-driven droplet manipulation effectively interrogates phase dynamics of ATPSs and macromolecular crowding in LLPS.

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EWOD-enabled droplet manipulation integrated with ATPSs quantifies phase dynamics of various ATPS solutions through binodals and tie-lines. *In situ* observation of droplets in the

presence of background macromolecules reveals macromolecular crowding effects on the phase dynamics and can potentially interrogate physiological LLPS prevalent in living cells.

Keywords

EWOD; Droplet Manipulation; ATPS; Phase Diagram; Molecular Crowding

Introduction

Aqueous two phase systems (ATPSs) are comprised of two immiscible polymers or polymer-salt combinations dissolved in water at appropriate concentrations.^[1] The two separated phases form a distinct interface with interfacial tensions that can be many orders of magnitude smaller than air-water and organic solvent-water interfaces.^[2] Many molecules and particles preferentially partition to one of the two phases depending on their physicochemical properties making ATPSs useful for separation of biomolecules^[3] and nanomaterials.^[4] The all-aqueous environments also make the systems useful for cell patterning,^[5] immunoassays,^[6] and bio-microanalysis.^[7] The liquid-liquid phase separation (LLPS) phenomenon underlying ATPSs is also the basis for formation of membrane-less compartments in living cells.^[8] Thus, ATPSs can be used as model systems to study biochemical properties of membrane-less organelles and protocells.^[9] One key property of ATPSs that needs to be quantified for all of these applications and studies include the minimal concentrations of the phase forming polymers and salts required for LLPS. Plots of these minimal concentrations are called binodal curves. For concentrations of polymers and salts above the binodal curve, it is important to be able to predict the expected volumes and chemical composition of each phase that results from LLPS, as well as the interfacial tension between the phases. These properties of each of the two phases as well as the interfacial tension between the two phases can be derived from tie-lines that link two specific points along the binodal curve.

Conventional methods for binodal curve and tie-line determination employ manual dilution of concentrated phase-forming polymers through extensive pipetting followed by waiting for phase separation, then determining the volumes, weighing the mass of the resulting phases, or measuring the interfacial tension between the phases. Several techniques have been developed for microscale determination of ATPS binodals using dehydration,^[10] microfluidics,^[11] and manual serial dilution.^[12] The previously-reported microscale techniques are useful for binodal determination but are less useful for determination of tie-lines and phase compositions. Here, we describe the use of a convenient microscale method for binodal curve and tie-line determination using electrowetting-on-dielectric (EWOD).

EWOD^[13] allows precise and automated droplet manipulation and enables pipette-free droplet dispensing, mixing, and splitting.^[14] Such characteristics facilitate various biomedical applications including PCR, immunoassay, cell patterning, and proteomics, and initiated the field of droplet-based digital microfluidics (DMF).^[15] Recently, an EWOD-based droplet manipulation method was reported to be useful for on-chip ATPS formation and mixing to study molecular extraction.^[16] We hypothesize that droplet manipulation using EWOD could be further extended to determine ATPS binodals and tie-lines. In addition to the commonly-appreciated advantages of EWOD, such as automated dilution and mixing, this study also takes advantage of the ability to image parallel plate-confined phase separated droplets directly in the device for accurate volume determination, and accelerated phase coalescence, where small droplets of one phase dispersed across the other phase rapidly merge upon droplet movement. Binodal curves and volume ratio of DEX – PEG are obtained directly from observing droplets after dilutions or evaporation-mediated concentration. Tie-lines and each phase composition are determined using a mathematical fitting of the volume ratio data.^[17] We specifically study dextran (DEX) and polyethylene glycol (PEG) solutions as they are among the most commonly used and well-characterized ATPSs.

In this work, digital microfluidic (DMF) devices are fabricated using conventional methods of photolithography, etching, and deposition.^[18] EWOD-driven contact angle changes of an ATPS droplet in a DMF device were measured. Stock PEG, DEX, and PBS solutions were separately dispensed from dedicated reservoirs in the device where air was surrounded to facilitate droplet operations. Individual PEG, DEX, and PBS droplets were generated by splitting from the stock solution reservoirs. Different concentrations of PEG and DEX were created by serial dilution with PBS droplets or by evaporation. A binodal state was defined by a stable liquid-liquid interface after droplet movement-induced coalescence of the phases. Multiple binodal concentrations were determined using different mixing ratios of the DEX – PEG droplets, and a binodal curve and tie-lines were estimated using the same device. Finally, DEX – PEG solutions were prepared with various concentrations of Ficoll as a non-interacting macromolecule and the resulting shifts of the binodal curve evaluated.

Results and Discussion

EWOD-enabled droplet manipulation and characterization of a DEX – PEG ATPS

We first validated EWOD-driven droplet manipulation in a DMF device. Sessile droplets of DEX and PEG solutions were dispensed on an open-surface DMF device and contact angle

changes of the solutions were measured as a function of applied 1 kHz AC voltage (Figure 1 and Scheme S1A). The contact angle of both solutions decreased after 20 V and the droplet shifted and became flattened as the voltage increased. Subsequent droplet manipulation used driving voltages of 35-40V and splitting voltages ~50V based on these results. A contact angle change of a DEX droplet submerged in a PEG solution was also characterized on the DMF device (Figure S1 and Movie S1). The negligible contact angle change infers that manipulation of the DEX droplet submerged by the PEG solution is rather challenging probably due to the small difference in electrostatic properties of PEG and DEX solutions.^[19] Conversely, droplet manipulation at an air-droplet-substrate interface provides droplet maneuverability and facile operation. The results of the sessile drop experiments demonstrate the feasibility of manipulating ATPS droplets by EWOD at the air-droplet-substrate interface in the DMF device.

Next, we demonstrated ATPS formation, dissolution, and reformation by sequential droplet coalescence, dilution, and evaporation (Figure 2 and Movie 1). DEX, PEG, and PBS droplets were split from their respective reservoirs and merged to form DEX – PEG ATPSs in the parallel-plate DMF device (Scheme 1 and Figure 2A). A clear liquid-liquid interface appeared after merging DEX and PEG droplets, indicating ATPS formation (Figure 2B and E). Dilution of the ATPS droplet by merging the PBS droplet disrupted the interface and eventually resulted in a single-phase (homogeneous) droplet (Figure 2C and F). The interface dissolution indicated that the DEX – PEG concentration of the droplet decreased below the binodal concentration. Interestingly, subsequent evaporation of the droplet in the device increased DEX – PEG concentrations resulting in tiny DEX droplets emerging near the air – droplet interface (Figure 2D and G). This was due to formation of concentration gradients upon evaporation that increased DEX – PEG concentrations near the droplet periphery and caused local phase separation (Figure S10). The tiny droplets disappeared after droplet moving-enhanced mixing (Movie 2). The observed local microphase separation is attributed to fast evaporation near the droplet boundary where DEX – PEG concentration locally increased above the binodal concentration while the global concentration still remained below the critical concentration. Such local microphase separation is reminiscent of spatiotemporally-regulated, sub-cellular local LLPS, but without evaporation through often ill-defined mechanisms that nonetheless have important biofunctional implications.^[20]

Further evaporation increased total DEX – PEG concentrations to levels above the critical point globally and caused stable phase separation, where droplet movement accelerated coalescence but not dissolution, with clear DEX – PEG interfaces (Figure S2 and Movie 3). In evaporation-based binodal curve determination, we defined the critical concentration as this point when the locally microphase-separated droplets first became stable to droplet movement. Given the known geometry of the DMF device, we estimated the droplet volume and concentration of the DEX and PEG solutions using droplet images (Table 1). We distinguished DEX-rich phase from PEG-rich phase in the droplet image based on the contact angle of DEX phase at a DEX – PEG – Air interface in the DMF device (Scheme S1B, Figure S3, and Table S1). A DEX droplet in the PEG phase showed contact angles of more than 90° due to interfacial energy differences.^[21] The initial size and concentration of DEX and PEG solutions prior to coalescence also allowed prediction of which phase was

DEX or PEG. The results showed that we can manipulate multiple droplets and quantify phase dynamics of the DEX – PEG ATPS in the DMF device.

Binodal and tie-line determination of a DEX – PEG ATPS in a DMF device

We took advantage of high-throughput droplet manipulation and facile image-derived calculation of droplet volume and determined binodal concentrations of the DEX – PEG ATPS using the DMF device (Figure 3). Multiple droplets of DEX and PEG solutions were generated, merged, and evaporated until the droplet reached stable phase separation in the DMF device. The obtained individual critical points were fit to a binodal curve (Figure 3A and supporting information). The binodal curve determined by droplet evaporation was slightly off from the one determined by conventional bulk dilution. This discrepancy may be a result of some evaporation of stock DEX and PEG solutions in the reservoirs before droplet generation. Compared to evaporation-mediated binodal curve determination, critical points and binodal curves determined by serial DMF dilutions were more error-prone due to over-dilution by the relatively large size of the generated PBS droplets (data not shown).

Meanwhile, tie-lines were obtained using serial dilution of an ATPS droplet (Figure 3B). The volume ratio and concentration of DEX-rich and PEG-rich phases were calculated from the droplet image and used to draw a tie-line in the binodal curve. The binodal curve was extrapolated in order to obtain intercepts of the tie-line and binodal curve. The estimated phase composition is summarized in Table 2. The obtained tie-lines are comparable to those determined by a previously reported density-based measurement (Figure S4A).^[22] Tie-lines were also obtained using the droplet-evaporation method, where critical points were determined as the point at which a DEX – PEG interface became stable to droplet movement (Figure S4B and Table S2). The results show that we can simultaneously determine binodals and tie-lines in the same device in a convenient and rapid format.

Phase dynamics of ATPSs in the presence of macromolecular crowding

Johansson *et al.* predicted that background macromolecules can push down ATPS binodals and prompt phase separation through macromolecular crowding.^[23] Recent LLPS-related reports^[24] support the hypothesis but, to our knowledge, it has not been experimentally demonstrated in ATPSs. We tested the hypothesis using a 500kDa DEX – 35 kDa PEG ATPS in the presence of 400 kDa Ficoll as a LLPS-promoting background macromolecule (Figure 4). DEX and PEG droplets were generated and merged with a PBS droplet in the presence and absence of Ficoll, resulting in sub-binodal ATPS droplets at two different final concentrations (Figure 4A). As expected, the droplet solution in the absence of Ficoll became homogeneous. In contrast, in the presence of 10 w/w% Ficoll, the droplet at 1.4 w/w % DEX – 1.4 w/w% PEG showed microphase separation without a clear DEX – PEG interface while the droplet at 2.0 w/w% DEX – 2.0 w/w% PEG had a stable interface.

To understand this observation better, we determined the binodal curve of DEX – PEG ATPS in the presence of varying Ficoll concentration (Figure 4B). The results show that the binodal curves are pushed down as the Ficoll concentration increases. In the presence of Ficoll, the aforementioned two DEX – PEG concentrations were plotted near and above the pushed-down binodal curve. The tie-line of the droplet at 2.0 w/w% DEX – 2.0 w/w% PEG

concentration was also obtained (Figure S5). Interestingly, Ficoll of a smaller molecular weight (70 kDa) showed less drastic binodal shifts compared to 400 kDa Ficoll (Figure S6). We also tested a 10 kDa DEX – 8 kDa PEG system in the presence of different molecular weight Ficoll (Figure S7). Notably, sucrose, a Ficoll monomer unit, showed negligible binodal shift even at a significantly high (20 w/w%) concentration (Figure S7A). In contrast, a binodal shift became pronounced as the Ficoll molecular weight and concentration increased (Figure S7B).

Since Ficoll also forms binary ATPS with DEX and PEG, we determined binodals for binary Ficoll – DEX and Ficoll – PEG systems (Figure S8 and Table S3 and S4). We speculate that the clear phase separation at 2.0 w/w% DEX – 2.0 w/w% PEG in presence of Ficoll may involve some Ficoll – PEG phase separation along with Ficoll crowding-facilitated DEX – PEG phase separation while 1.4 w/w% DEX – 1.4 w/w% PEG is likely predominantly DEX – PEG phase separation facilitated by background molecular crowding of Ficoll (Figure S9).

Altogether, the results indicate that Ficoll can indeed prompt DEX – PEG phase separation at concentrations below the Ficoll – PEG and Ficoll – DEX binodal concentrations and confirm the notion of ATPS binodal shift in the presence of macromolecular crowding. Local phase separation caused by local evaporation (Figure 2D and G) is not physiologically relevant, whereas local and global intracellular phase separation induced by local or global macromolecular crowding (Figure 4) is physiologically relevant and provides insights into biological LLPS and artificial cell systems under molecular crowding.

Conclusions

We exploited EWOD-enabled quantification of phase dynamics of a DEX – PEG ATPS, determined binodals and tie-lines in an automated and time-efficient manner, and verified the hypothesis of binodal shift in the presence of background macromolecules. We found the macromolecular crowding effect on binodal shift was more pronounced as the crowding molecular size and concentration increased. This technology with microanalysis capability may be particularly useful to study LLPS of cellular component solutions where various background macromolecules are present. Still, there are a few limitations and challenges. Underlying mechanisms of LLPS and molecular partitioning in the presence of background macromolecules should be elucidated by computational simulation or other analytical methods. Simple evaporation in the device is a double-edged sword in that one can take advantage of this for binodal determination, but it can also produce unwanted and slight changes in the solution concentrations even when undesired. Still we demonstrate the potential that EWOD-integrated ATPS characterization can examine combinatorial phase dynamics in the presence of phase-influencing additives.

Experimental Section

Reagents

All reagents were purchased through commercially available sources. PEG (Mw 8,000 g mol⁻¹ and 35,000 g mol⁻¹), DEX (Mw 10,000 g mol⁻¹ and 500,000 g mol⁻¹), Ficoll (Mw 70,000 g mol⁻¹ and 400,000 g mol⁻¹), and sucrose were purchased from Millipore-Sigma. 1X

PBS at pH 7.4 was prepared by dissolving a PBS tablet (Sigma, P-4417, lot 093K8202) in 200 mL of distilled deionized water. TRITC-DEX (Mw 500,000 g mol⁻¹) was purchased from TdB Consultancy.

Device Fabrication

A DMF device comprises two parallel glass plates (top and bottom plates) to sandwich and drive droplets, as shown in Scheme 1. The top glass plate held an unpatterned indium tin oxide (ITO) layer where 0.5 w/v% Teflon (AF1600, DuPont) was spun on top of the ITO layer to form a hydrophobic layer (thickness 55 nm). Driving electrodes (1 mm × 1 mm) and reservoir electrodes were fabricated on the bottom plate by photolithography and wet etching of the ITO layer in aqua regia. Subsequently, SU-8 (2002, MicroChem) was spun on the etched electrodes as a dielectric layer (thickness 2.5 μm), then UV exposed and baked. A Teflon layer (thickness 55 nm) was then spin-coated on the SU-8 to complete the bottom plate fabrication.

EWOD-Driven Contact Angle Measurement

A sessile droplet (volume 2 μL) of 8 w/w% DEX and 8 w/w% PEG solutions in PBS were dispensed in air on an open-surface DMF device consisting of a hydrophobic (Teflon) and dielectric (SU-8) layers-coated electrode. A Teflon-coated probe was inserted into the sessile droplet. 1 kHz AC voltage increasing from 0 to 200 V at the rate of 1 V s⁻¹ was applied between the probe and the electrode underneath the droplet. Similarly, contact angle of a DEX droplet submerged by a PEG solution was measured using pre-equilibrated ATPS solutions. Briefly, 8 w/w% DEX and 8 w/w% PEG solutions were mixed at one-to-one volume ratio in a tube followed by centrifugation at 3,000 rpm at 24 °C for 15 min. DEX-rich and PEG-rich phase solutions were collected separately and used as the dispensing solutions.

Device Manipulation

PEG and DEX solutions with appropriate concentrations were dispensed on the reservoir electrodes of the bottom plate prior to droplet manipulation. The top DMF plate was then assembled to the bottom DMF plate with a stainless steel spacer (thickness 50 μm). The unpatterned electrode on the top plate was connected to the electric ground potential. The EWOD driving and reservoir electrodes on the bottom plate were connected to relays (LU-5, Rayex Electronics) switched by a data acquisition device (USB-6509, National Instruments) and LabVIEW software. The EWOD driving signals were generated from a function generator (33210A, Agilent Technologies) and amplified via an amplifier (A-304, A.A. Lab Systems). By applying appropriate electric signals between the top electrode and proper bottom driving electrodes, EWOD could be generated for PEG, DEX, and PBS droplet manipulation. Various droplet concentrations were generated on the DMF device by merging and mixing of multiple droplets.

Determination of binodal curve and tie-lines in a DMF device

Binodal points were determined by droplet evaporation. We define the binodal state where microdroplets showing microphase separation without mixing after droplet manipulation. A

homogeneous ATPS droplet was evaporated and the droplet eventually reached the binodal state. Image analysis of the droplet provided the droplet size and volume using the channel height (50 μm) at an initial point and a binodal point. A binodal curve from total 11 binodal points was obtained using the previously reported fitting equation (ref 17) and free software R. Detail codes are described in the supporting information.

Tie-lines were determined by sequential droplet dilution and evaporation. In dilution, DEX and PEG droplets at a high concentration were generated first and PBS droplets were sequentially merged. In evaporation, DEX – PEG ATPS droplets at a sub-binodal concentration were prepared and evaporated until a clear DEX – PEG interface was visible. Each phase volume in the droplet after phase separation was estimated from a droplet image and each phase concentration was obtained using eq S1. Tie-lines were obtained from the volume ratio and initial concentration of DEX and PEG phases using Python script described in the supporting information. Each phase composition determined by dilution and evaporation is summarized in Table 2 and S1, respectively.

Binodal Characterization Under Macromolecular Crowding

400 kDa and 70 kDa Ficoll were mixed at a final concentration of 0, 1, 5, and 10 w/w% in 500 kDa DEX – 35 kDa PEG and 10 kDa DEX – 8 kDa PEG ATPSs. Binodal points were determined in the presence and absence of Ficoll. Sucrose was used as a non-crowding molecule in 10 kDa DEX – 8 kDa PEG ATPS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the NIH (GM123517 and AI116482) for funding. The Ministry of Science and Technology (Taiwan) partially supported this work under grant 106-2918-I-002 -048. TK and ST thank Ms. Michelle Menzies for collection of a part of the Ficoll data during her summer research internship.

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Figure 1.

Contact angle change of a sessile DEX (open circle) and PEG (closed circle) droplet (each 8 w/w%) on an open-surface DMF device upon applying voltage. Images show a PEG droplet at 0 V and 100 V potential applied at 1kHz AC; the droplet-substrate interfaces are indicated by white dashed lines.

Figure 2.

ATPS droplet dilution and evaporation. (A) Droplets of 4 w/w% PEG (left), 4 w/w% DEX (top), and PBS (right) solutions were generated. (B) After merging the PEG and DEX droplets, a clear DEX – PEG interface appeared in the droplet. A region (dashed rectangle) showing the liquid-liquid interface (white dashed line) is enlarged in (E). (C) The DEX – PEG interface disappeared after merging the PBS droplet. A region (dashed rectangle) is magnified in (F). (D) Tiny DEX droplets emerged near the droplet boundary after evaporation. A region (dashed rectangle) is zoomed in (G). The DEX – PEG interface is highlighted (white dashed line). (A-D) Scale bar 1 mm and (E-G) scale bar 500 μm .

Figure 3.

Binodal curves and tie-lines determined by EWOD-assisted droplet manipulation. (A) Binodal points were obtained by evaporation of homogeneous ATPS droplets from below the binodal concentration until it reached the critical point. A binodal curve was plotted using a fitting equation (black dashed line) and compared with the one determined by conventional bulk dilution (grey solid line). (B) Tie-lines were obtained based on the volume ratio and bulk concentration of ATPS droplets upon serial dilution (see the upper right inset in B). Phase compositions of the DEX-rich and PEG-rich phases were obtained using Python

scripts (supporting information). The phase composition of each data (point i - v) was summarized in Table 2.

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Figure 4.

Binodal shifts in the presence of 400 kDa Ficoll in a 500 kDa DEX – 35 kDa PEG system. (A) Sub-binodal droplets containing DEX – PEG solutions (approximately 1.4 w/w% DEX – 1.4 w/w% PEG in the top row and 2.0 w/w% DEX – 2.0 w/w% PEG in the bottom row, respectively) were prepared in the presence (left column) and absence (right column) of 10 w/w% Ficoll. A liquid-liquid interface is indicated by a white dashed line. Scale bar 1 mm. (B) Binodal points of DEX – PEG droplets in the presence of Ficoll were plotted and fitted: 1 w/w% (open diamond / light grey dashed line), 5 w/w% (open square / grey dashed line),

and 10 w/w% (open circle / black dashed line). The DEX – PEG concentrations used in (A) is highlighted with open and closed black stars. A binodal curve in the absence of Ficoll is also plotted (grey solid line).

Scheme 1.

Droplet manipulation by EWOD in a DMF device. (A) Cross section of a DMF device. DEX and PEG droplets generated from dedicated reservoirs are merged to form a DEX – PEG ATPS. The droplets are sandwiched between two glass plates coated with proper electrodes, a dielectric layer, and hydrophobic coatings. (B) Top-view of the DMF device for droplet manipulation: splitting, moving, mixing, and evaporating the droplets. PEG, DEX, and PBS droplets generated from the reservoirs (not shown) are depicted as an example.

Table 1.

Bulk concentrations in the DEX – PEG ATPS as shown in Figure 2.

ATPS Droplets	(A) 1-phase	(B) 2-phase	(C) 1-phase	(D) 2-phase
DEX Size / mm ²	1.1	1.3	3.2	2.1
PEG Size / mm ²	1.2	0.94	3.2	2.1
[DEX] / w/w%	4.0	1.9	1.3	2.0
[PEG] / w/w%	4.0	2.1	1.5	2.3

Table 2.

Phase composition determined by tie-lines as shown in Figure 3.

[DEX] / w/w% [PEG] / w/w%	Point 1	Point 2	Point 3	Point 4	Point 5
Bulk Concentration	10 5.8	6.5 3.4	5.4 2.6	4.6 2.1	7.3 4.7
DEX-rich Phase	24 0.34	16 0.34	15 0.34	12 0.34	19 0.34
PEG-rich Phase	0.017 9.6	0.017 5.4	0.017 3.9	0.69 3.1	0.017 7.5