Biological Chemistry & Chemical Biology

Active Janus Droplet as a Micro-Reactor for Automatic DNA/RNA Precipitation and Extraction

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We explore the possibility to precipitate and to extract DNA, or RNA using an active water/ethanol Janus droplet. The active Janus droplet is initially formed by a single droplet made of a water/ethanol mixture in an oil/surfactant solution. This active droplet self-propels and absorbs surfactant molecules, by advection, while moving in the oily phase. The surfactant absorption leads to a phase separation of the water/ethanol mixture and the formation of an active Janus droplet. This active Janus droplet is made of a water-rich leading droplet and an ethanol-rich trailing droplet. We employ this phase separation method to precipitate and to extract DNA, RNA, or DNA/RNA mixtures independently, in an automatic manner. This automatic precipitation and extraction of DNA, or RNA, is achieved by tuning their water solubility with the addition of salt and then it is demonstrated by fluorescence.

1. Introduction

In recent years, lab-on-chip technologies have allowed the miniaturization of laboratory devices in microchannels with dimensions of a few hundreds of micrometers.[1] These technological developments allow to reproduce a large variety of laboratory functions at the intersection of physics, chemistry and biology.[2–6] One of the most successful lab-on-chip techniques is droplet-based microfluidics, which enables the production of individual, micrometer sized, droplets with a precise control of their volumes and their reliable manipulation using microfluidic micro-pumps.[7] In particular, it was demonstrated that amplification and sequence analysis of DNA molecules could be performed in a quasi-automated manner, using droplet-based microfluidics.[8,9] These techniques can be consider to be passive lab-on-chip techniques, as the fluid manipulation in these chips is achieved via external pressure or volume-driven flow.[10,11,6] Very recently, the concept of active lab-on-chip has emerged.[10] Interestingly, active lab-on-chip does not use any external pressure and/or volume driven-flow to manipulate the different fluid phases present into the microfluidics chip.[10] The idea of active microfluidic is to employ self-propelling droplets[11] as mobile micro-robots or micro-reactors to perform standard laboratory operations in a microfluidic chip.[12–14] For example, the extraction of active biomolecules such as DNA, RNA, and proteins, is one of the most crucial and commonly used methods in molecular biology.[15] This extraction leads to downstream processes and industrial production of many consumables, including diagnostic kits.[16] DNA and/or RNA can be isolated from cells for analytical purposes.[15,16] DNA can be extracted from cells and, as DNA is generally stable under certain conditions, it can be prepared and stored in batches. DNA extraction and purification force to dissolve the cell membrane by the usage of an appropriate detergent and, then precipitate the DNA with a detergent like alcohol.[15,17] In some case, proteins are re-moved by adding an appropriate protease and RNA is removed with the use of an RNase.[15,18] DNA purification is also necessary to minimize the disturbance from non-target DNA and other contaminants during long-term storage.[15,18] RNA extraction and purification, on the other hand, also involves also some basic steps: from cell lysis to RNA precipitation and purification. These techniques are commonly used in bulk techniques,[19,15] while in this manuscript we explore the possibility of performing these techniques automatically at the micro-scale. We employ an active Janus droplet with a sub-milliter size, as a micro-robot to precipitate and extract DNA, RNA and DNA/RNA independently and automatically in a microfluidic chip. The results are that the presented active Janus droplet is comparable to an active micro-reactor.

2. Results and Discussion

In this part, we briefly describe the general properties of our active Janus droplets.

2.1. General Properties of the Water/Ethanol Active Janus Droplets

Our active Janus droplet is initially produced as a water/ethanol droplet in an oily phase (squalane oil) that contains a surfactant (Monoolein), see Scheme 1. Just after droplet production, the

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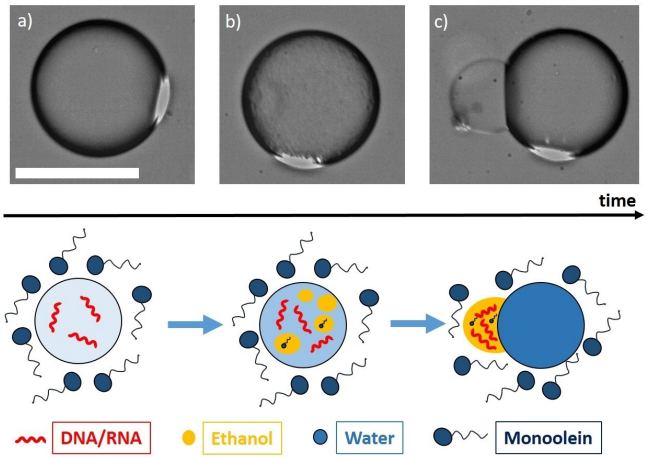
Supporting information for this article is available on the WWW under https://doi.org/10.1002/slct.202003940



Scheme 1. Upper Row: Micrograph of an active water/ethanol droplet under bright microscopy. a) Water/ethanol droplet that is self-propelling in an oily phase (total time of a few minutes). b) Water/ethanol demixes after absorbing surfactant molecules present in the oily phase. c) Picture of a long-lived, active Janus droplet, once the water/ethanol phase separation is complete. The trailing droplet is ethanol, and the leading droplet is water. Scale bar is ~100 μm.Lower Row: Sketch of the active Janus droplet formation and its usage as a micro-reactor for DNA/RNA extraction.

ethanol diffuses spontaneously in the oily phase, as it is a little more soluble in squalane oil (up to 4%).[20–22] Concurrent with this loss of ethanol, the droplet absorbs surfactant molecules dispersed into the oily phase.[12] Both effects lead to a change of the surface tension at the droplet surface and, thus, to a droplet self-propulsion via a Maragoni stress.[12,11] During self-propulsion, the active droplets are swimming at an average velocity of ≈10 μm/s, while absorbing surfactant molecules continuously. Once a critical amount of monoolein molecules is absorbed, a spontaneous demixing of the water and ethanol phases are triggered (see Scheme 1), as demonstrated by monoolein/water/Ethanol phase diagrams.[20–22] The existence of a monoolein-depleted boundary layer around the swimming active droplet can also be employed to estimate the total number of monoolein molecules adsorbed before phase separation within time t:[23,12]

$$N \approx 4\pi R^2 < j_a > t \approx 4\pi \sqrt{D\,U\,R^3 c_\infty}\, t \quad (1)$$

Considering the number of monoolein molecules N in the droplet at the onset of phase separation $N = 4\pi R3c^*/3$, where R is the droplet radius, c^* denotes the monoolein concentration in the droplet at the onset of phase separation and c_∞ is the monoolein concentration in the host phase. We arrive at the estimate of the time needed to trigger the phase separation as

$$t^* \approx \frac{1}{3}\sqrt{\frac{R^3}{DU}}\,\frac{c^*}{c_\infty} \quad (2)$$

With D being the diffusion constant of surfactant micelles and U the active droplet velocity. A more detailed calculation is available in the following Refs. [12, 23].

Once the phase separation has started, a large number of small ethanol-rich droplets are nucleated inside the mother water/ethanol droplet, see Scheme 1. These tiny droplets nucleate, growth and fuse under a vortex flow inside the active mother droplet. Within 1–2 minutes, this process leads to the creation of an active Janus droplet made of a trailing ethanol-rich droplet and a leading water-rich droplet. It is a Janus droplet because there is different chemical composition of the two opposing hemispheres.[24] It is interesting to note that the active droplet continues to swim during the timeframe of phase separation.[12] In the presence of salt, sDNA molecules become less soluble in water and precipitate in ethanol. Thus this demixing allows the precipitation of sDNA molecules, in an automatic manner and without any centrifuging steps.[12,25] And even after the active Janus droplet is formed, this active Janus droplet continues to self-propel into the oily phase.[12] After 5–10 minutes, the trailing ethanol droplet spontaneously pinches off and separates from the leading water droplet, before spreading on the substrate.[12] In general, the water-rich droplet stops moving after this separation. It is interesting to note that this ethanol-rich droplet could be programmed to be deliver at certain locations, like a pillar, using chemical programming (such process is detailed in Ref. [12]). An example of such cargo delivery is presented in Figure 1 (and supplementary movie 1). As previously described, the droplet can self-propel before the formation of Janus droplet by phase separation. Such active droplets are self-propelling at the beginning of the movie 1. Then they come in contact with the pillar and stay attached there. After a short time, a Janus droplet is eventually formed by phase separation, which produces an ethanol-rich droplet that is quickly spreading on the PDMS pillar (due to the wetting affinity between ethanol and PDMS). In fact, as the interface between the ethanol-rich droplet and the squalane oil forms a small equilibrium contact angle with a PDMS surface, the ethanol droplet spreads at the PDMS circular pillar, see

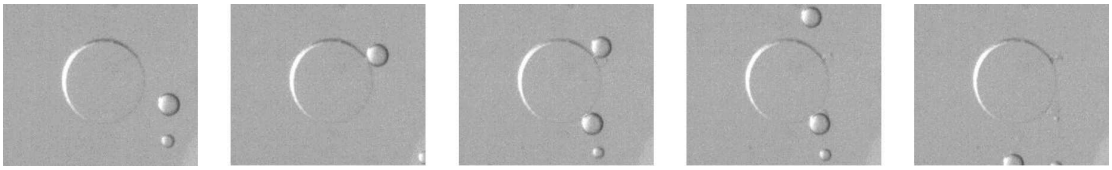


Figure 1. Time series of droplets being hydrodynamically attracted to circular PDMS pillars (1 mm in diameter) before phase separation and delivering ethanol during phase separation. After phase separation, the remaining droplet leaves the PDMS target and self-propels away from the circular pillar. The corresponding movie is available in the supplementary information.

Figure 1. When the ethanol- rich droplet is eventually formed during phase separation, the droplet leaves the circular pillar via a complex mechanism of hydrodynamic interaction described in Ref. [12]. This repulsion is due to the fact that a Janus droplet produces a long-range hydrodynamic flow field (of a neutral squirmer[12]) that repels the droplet from the circular pillar. In the following, we discuss the usage of this phase separation to automatically precipitate and extract DNA, RNA or DNA/RNA mixture in an active Janus droplet.

2.2. Extraction of DNA/RNA using an Active Janus Droplet

In order to extract DNA or RNA, we follow the basic production of active Janus droplet that was described previously, whereas now a certain concentration of DNAor RNA is added to the initially formed mother droplet. Independent of the additional molecular content, the formed droplets self-propel in the surrounding oil phase and collect monoolein molecules from there and release ethanol. When achieving the concentration for the phase separation, the additional DNA or RNA molecules will collect either in the ethanol-rich or in the water-rich phase depending on details of the chemical composition of the initial mixture, i.e mainly on the salt concentration. After forming an active Janus droplet containing DNA or RNA molecules in either of the droplets, the Janus droplet will self-propel as described previously without additional molecule and can thus deliver the cargo that is contained in the ethanol-rich droplet at target locations.

The sequence of images in Figure 2 demonstrates the controlled separation of fluorescing sDNA from a water/ethanol droplet into the water-rich phase without salt and into the ethanol-rich phase in the presence of 25 mg/ml sodium acetate ($C_2H_3NaO_2$). Without salt, the sDNA molecules stay soluble in water and they are not extracted to the ethanol phase (Figure 2.a). The presence of salt reduces the sDNA solubility in water, and therefore the sDNA molecules precipitate and are extracted into the ethanol-rich droplet during the water/ethanol phase separation (Figure 2b).[12,23] In particular, the role of the salt in the protocol is to neutralize the charges on the phosphate group. A commonly used salt is sodium acetate. In solution, the positively charged of the ions neutralize the negative charges on the nucleic acid, which makes the nucleic acid apolar and, thus, less soluble in water.[26] As a result, the sDNA extraction can be controlled, chemically, by adding some salt into the initial mother water/ethanol droplet composition. However, as we will see later, the choice of the staining dye can change the amount of salt needed to trigger the extraction. It is worth emphasizing that, even in the presence of sDNA and salt, the droplets undergo the previously described phase evolution and finally form Janus droplets consisting of a water-rich leading and an ethanol-rich trailing dropletFigure .[12] At this point, it would be natural to ask if the ethanol could end up forming micelles within the droplets which could then entrap the dye and therefore result in a fluorescence signal. This seems to not be the case, as demonstrated by control experiments without DNA where no background could be observed in the ethanol droplet (for all the tested dyes).

To quantify the amount of sDNA extracted using an active Janus droplet, we will not use the Hoechst dye[27] because it cannot be used to distinguish RNA from DNA. Thus, for the rest of the manuscript, we will use Acridine Orange (AO) that can detect DNA and RNA under blue and green laser excitation respectively.[28]

We explore now the effect of pH on sDNA extraction using an active Janus droplet. In the pH range around neutral pH, from pH 5 to 9, sDNA molecules are quite stable. Low pH (<5) decreases the solubility and can cause depurination and molecular breakage.[29,30] High pH (>10) is less damaging and can be used to denature nucleic acids (but may still damage the nucleic acids).[29,30] Using AO as a fluorescent sensor to detect the

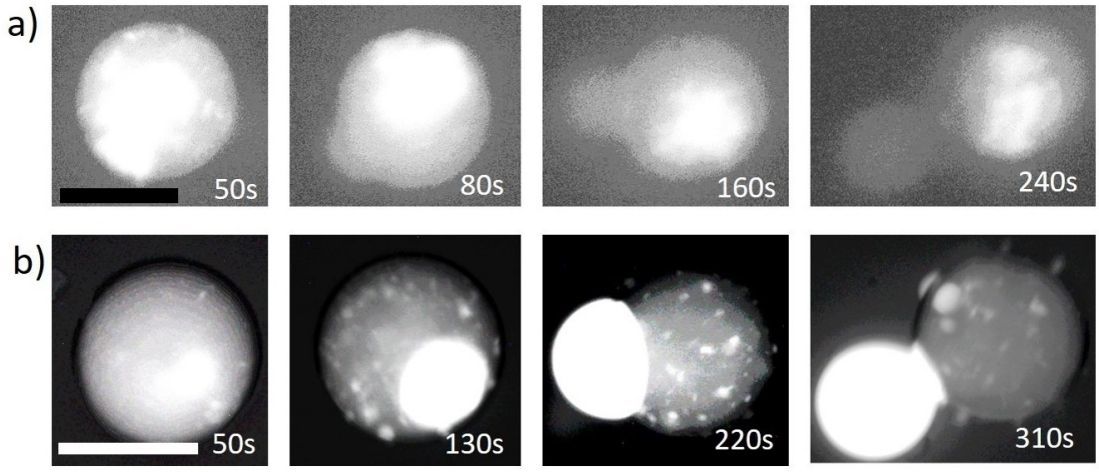


Figure 2. Extraction of sDNA molecules observed by fluorescence microscopy during the formation of an active water/ethanol Janus droplet. a) 0.2 mg/ml sDNA is stained with a dye (0.25 mg/ml Hoechst 33342) and indicates the localization of sDNA during the water/ethanol phase separation. The mother (water/ethanol) droplet does not contains any salt. As a result, the sDNA remains in the water-rich mother droplets during separation. Scale bar is approx 100 μm. b) 0.2 mg/ml sDNA is stained with a dye (0.25 mg/ml Hoechst 33342) and indicates the localization of sDNA during the water/ethanol phase separation. The mother (water/ethanol) droplet contains 25 mg/ml sodium acetate. Thus, when water/ethanol separates, sDNA molecules are spontaneously extracted from the mother droplet (water-rich) to the daughter droplet (ethanol-rich).

localization of sDNA in the active Janus droplet, we observe for pH = 7, a spontaneous partial extraction in presence of AO (Figure 3). We measure a fluorescence intensity similar in both the water-rich mother drop and the ethanol-rich trailing drop. This indicates that AO affects the solubility of sDNA. Thus, once again the extraction of sDNA molecules has been partially achieved, as sDNA is visible in both leading and trailing droplets. For pH = 10, a similar brightness distribution is visible on the formed active Janus droplet Figure 3. For pH = 4, a fluorescence signal is measurable in both droplets, but the leading water-rich droplet appears brighter than the trailing droplet (Figure 3). However, we should be cautious when comparing signal intensity between both droplets. Indeed, as the fluorescence signal changes with the droplet composition (ethanol-rich and water-rich droplets) and of the pH value, too.[28] Thus whatever the pH value, the AO dye (10 μg/ml) leads to a partial extraction of sDNA. This extraction is nevertheless able to extract a reasonable amount of sDNA molecules, as the initial sDNA concentration is rather large into the mother droplet. We also measure the kinetic of sDNA extraction as a function of the different pH-values. It results that the time of sDNA extraction is similar ≈100 s for all the tested pH values. Thus the extraction phenomena is controlled by the liquid-liquid phase separation, which is independent of the pH values.

After checking this automatic extraction method in the case of sDNA, we explore the possibility to extend t his method to RNA molecules. For this purpose, we dispersed 0.2 mg/ml of RNA into the water/ethanol mother droplet with pH buffer and only the AO dye (Figure 4). The injected RNA is the total RNA from torula yeast, thus these RNA nucleotides have a large distribution in length. We measured that RNA seems to be not extracted after the water/ethanol demixing. However, adding 25 mg/ml lithium chloride (or 25 mg/ml sodium acetate) into the initial mother water/ethanol droplet, we measure an RNA extraction into the ethanol trailing droplet. It appears that RNA molecules have been partially extracted, as RNA are visible in both leading and trailing droplet (Figure 4). This extraction is nevertheless able to extract a reasonable amount of RNA molecules, as the initial RNA concentration (0.2 mg/ml) is quite large into the mother droplet. Looking at the corresponding signal analysis (Figure 4), it appears that the total fluorescent signal is stronger when using lithium chloride. In comparison to the total signal intensity obtained with sodium acetate which indicate that lithium leads to a better RNA extraction (in particular, for large nucleotide RNA).[25] This fact is not surprising, as lithium chloride is commonly used to precipitate RNA in ethanol, in contrast to sodium acetate.[31,25] Interestingly, quasi-identical results (i.e normalized intensity measurements) can be achieved for the extraction of sDNA after adding the same salt composition (25 mg/ml lithium chloride or 25 mg/ml sodium acetate)into the mother water/ethanol droplet (Figure 4). Now we focus on the possible extraction of sDNA/RNA mixtures using our active Janus droplet. Mixtures of sDNA/RNA are complex mixtures, where both molecules may interact via specific binding.[32] First, we measured the efficiency of the extraction process using a mixture of sDNA/RNA (at 0.2 mg/ml for both) and without salt (see Figure 5). As a result, no massive extraction was visible for DNA/RNA mixtures without the presence of salt. To check the possibility of sDNA/RNA extraction, we increase gradually the amount of salt to trigger an extraction. Adding 50 μg/ml lithium chloride to a mixture of 0.2 mg/ml RNA and 0.1 mg/ml sDNA, we still do not observe a visible extraction with our fluorescent sensor (see Figure 5). Raising the amount of lithium chloride to 0.1 mg/

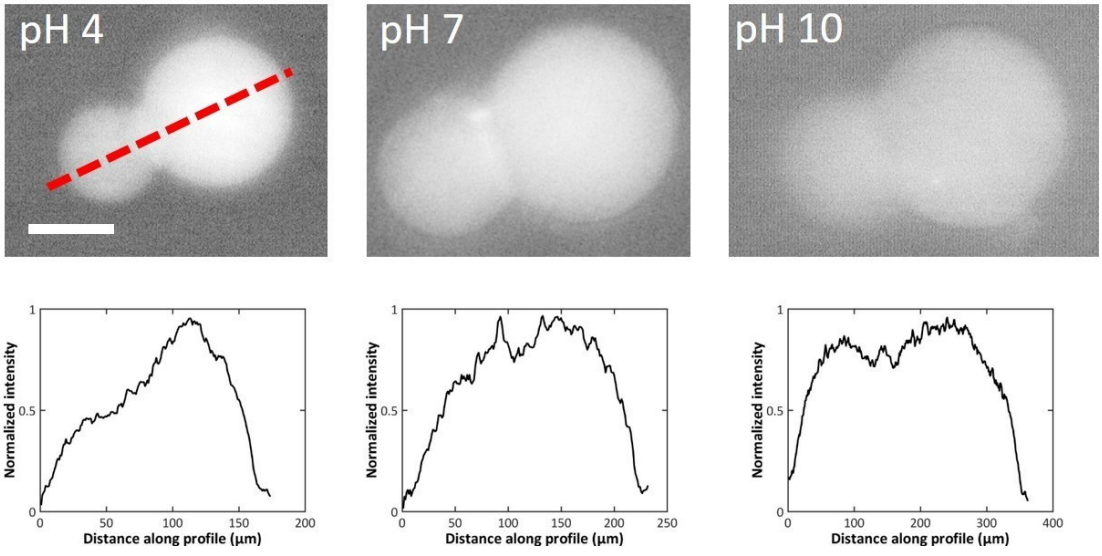


Figure 3. Upper Row: Fluorescence pictures of an active Janus droplet as function of different initial pH values. The sDNA fluorescent sensor is orange acetate (OA). The chemical composition of the mother droplet is 50%/50% ethanol/water, 0.2 mg/ml DNA, 10 μg/ml OA. pH buffers were purchased from Carl Roth with the following compositions, pH 4: Citric acid monohydrate/ Sodium hydroxid solution/Sodium chloride. pH 7: Monopotassium phosphate/ Sodium hydroxide. pH 10: Boric acid/Sodium hydroxide solution/Potassium chloride. The scale bar is ≈100 μm.Lower Row: The corresponding analysis of the relative normalized intensity from the corresponding fluorescent pictures. The signal intensity is plotted as function of the distance along profile presented by the red dashed line.

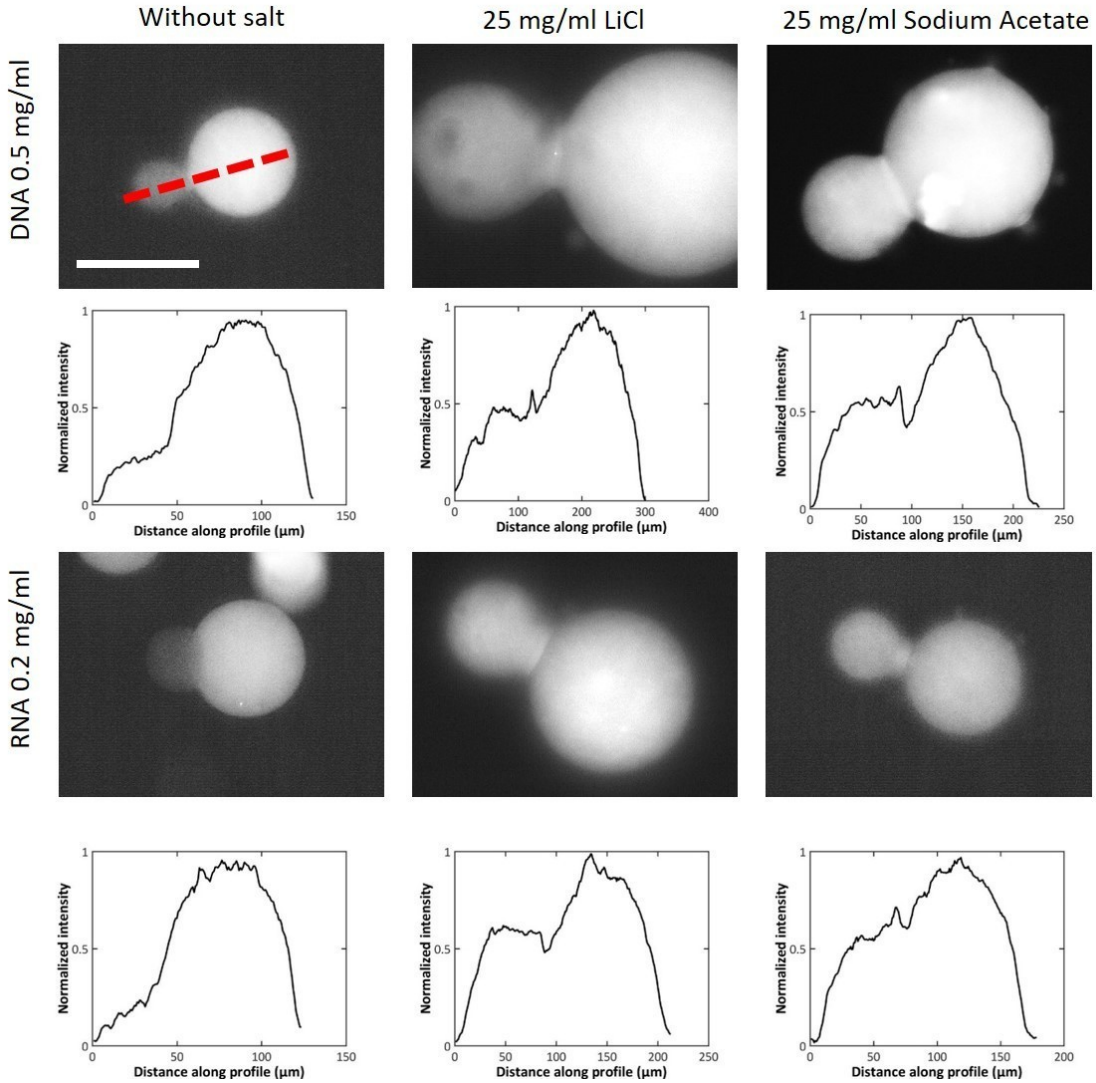


Figure 4. Upper Rows :Fluorescence pictures of a Janus active droplet with sDNA, and as a function of different salt compositions and concentrations. The sDNA fluorescent sensor is acridine orange (AO, 10 µg/ml). Below, the corresponding analysis of the normalized intensity from the corresponding fluorescent images. The signal intensity is plot as a function of the distance along the profile presented by the red dashed line. Lower Rows: Fluorescence images of a Janus active droplet with RNA, and as function of different salt composition and concentration. The RNA fluorescent sensor is orange acetate (OA, 10 µg/ml). Below, the corresponding analysis of the normalized intensity from the corresponding fluorescent images. The signal intensity is plotted as a function of the distance along the profile presented by the red dashed line.

ml, a notable extraction of RNA is visible under blue illumination of our sensor (AO, 10 µg/ml), and a slightly weaker signal of sDNA extraction could be visible under green laser illumination. Similar results were obtaining with 20% isopropanol into the composition of the water/ethanol mother droplet. As isopropanol is a solvent commonly used to precipitate DNA, or RNA, we try to employ it and observe that it works but the total fluorescent signal intensity looks even weaker,[33,34] see Figure 5. The results show that sDNA and RNA molecules can be extracted simultaneously using the phase separation of our water/ethanol active droplet. The amount of extracted sDNA/RNA molecules can be controlled by the amount of salt into the water/ethanol mother droplet. Raising the sodium acetate concentration is increasing the amount of extracted material (see Table 1), until a threshold concentration where it seems to have no more a significant contribution. The fluorescence dye signal appears to be weaker for sDNA/RNA mixtures. This is probably due to specific binding

Table 1. Efficiency of extraction based on fluorescence measurements and expressed in %.. **sDNA** corresponds to the amount of sDNA extracted in a droplet containing sDNA and RNA. **RNA** is corresponding to the amount of RNA extracted in a droplet containing sDNA and RNA. They were measured at 0.2 mg/ml sDNA (or RNA) concentration.

Salt Concentration (mg/ml)	0.1	1	10	25	30
sDNA and LiCl	40%	40%	50%	50%	50%
sDNA and $C_2H_3NaO_2$	30%	50%	60%	60%	60%
RNA and LiCl	40%	40%	50%	50%	50%
RNA and $C_2H_3NaO_2$	30%	30%	30%	40%	40%
sDNA and LiCl	40%	40%	40%	40%	40%
RNA and LiCl	20%	20%	20%	20%	20%

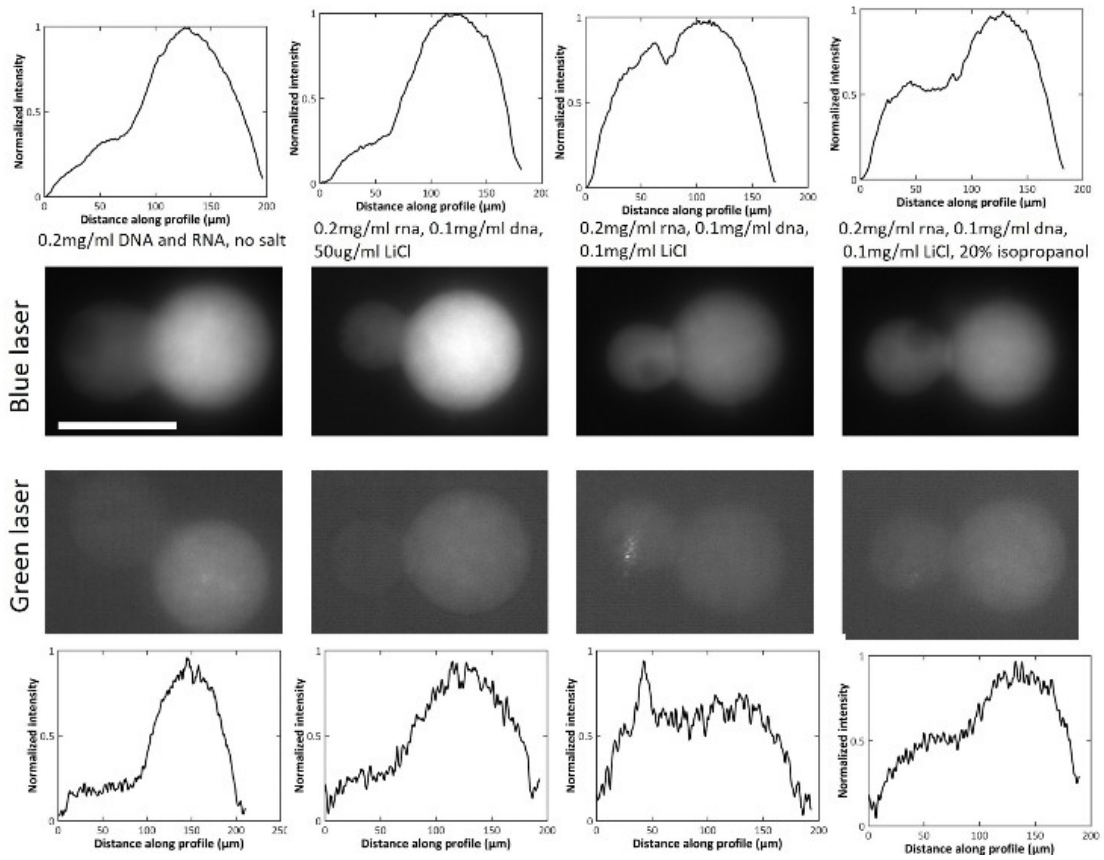


Figure 5. Fluorescence pictures of a active Janus active droplet with sDNA and RNA and as a function of different salt composition and concentration. The sDNA and RNA fluorescent sensor is acridine orange (AO, 10 μg/ml). The upper row shows the fluorescent images under blue laser illumination (473 nm), which detect RNA. The upper row shows the fluorescent pictures under green laser illumination (532 nm), which detect DNA

between sDNA and RNA nucleotide which may hinder the staining of our AO dye. Still, we try to measure quantitatively the amount of extracted sDNA/RNA using two different photometric measurements devices like the *NanoDrop One* and the *Nanodrop 8000* from ThermoFisher without any satisfying results. We think that the tiny liquid volume that we employed was the problem which prevented the quantification with these photometric devices. We could also have performed fluorometric measurements on our system. However, it would require a large volume of liquid, that are much bigger than the droplet employed here. It would require a biphase (oil and water/ethanol) in a falcon, which would be a different system than the one that we used (and a passive system). Thus, it would be questionable if these results could be used for our tiny active droplets. It results that our active droplet method enables the automatic precipitation and extraction of sDNA, RNA and sDNA/RNA mixture. This method does not require a centrifuging step and could be performed on liquid quantities at the microscale.[31] The efficiency of this method could only be estimated qualitatively. However, the large concentration of initial molecules guarantees ($\approx$0.1–1 mg/ml) that a large number of sDNA, RNA, or sDNA/RNA molecules is extracted.

3. Conclusion

We explored the possibility of using an active Janus droplet to precipitate and extract sDNA, RNA, or sDNA/RNA mixtures in a quasi-automatic manner. Our active Janus droplet is, initially, made of a water/ethanol droplet that spontaneously self-propels into an oily phase containing surfactant molecules. Upon self-propulsion, this active droplet absorbs surfactant molecules which, after a certain concentration threshold, triggers a phase separation between the water and the ethanol phase. This leads to the formation of a long-living active Janus droplet. We demonstrated that the water/ethanol demixing can be used to precipitate and automatically extract sDNA, RNA, or sDNA/RNA molecules from the water-rich leading droplet to the trailing ethanol. If the initial water/ethanol droplet does not contain any salt, we observed no precipitation of sDNA, RNA, or sDNA/RNA and thus no extraction is observed during the demixing process. However, if a sufficient amount of salt is present inside the initial water/ethanol, we measured a successful sDNA, RNA, or sDNA/RNA precipitation and, thus, extraction during the demixing process. The efficiency of the precipitation/extraction is measured using fluorescence via a staining dye. If this technique is satisfying from the qualitative point-of-view, this method is not yet able be extended to a

quantitative level. However, in contrast to the laboratory method, our microreactor does not need any additional centrifuging step, the precipitation and extraction is performed in minutes (compare to hours in lab), and we manipulate liquid volume below the nanoliter. Thus, we hope this automatic, fast, method will attract many researchers willing to use active droplets as micro-reactor, or micro-robots, for biophysical and chemical applications.

Experimental Section

Observation chambers were fabricated from glass slides cut into squares of about (2.5×2.5) cm^2. These glass squares were pre-cleaned (detailed procedure available in[12]), before being coated with octadecyl-trichlorosilane (OTS). To do this coating, the glass slides were immersed for 12 min in a solution consisting of 50 ml bicyclohexane, 20–40 drops of carbon tetrachloride, 5–20 drops of OTS, and were afterwards rinsed with chloroform. The chip is finalized by overlapping the two glass slides with a spacer (150 μm) and glued with epoxy glue. All active droplets were produced at room temperature. To produce these active droplets, we fill the chip with an oil/surfactant mixture (squalene oil with 15 mM monoolein). Then we bring, manually, a glass capillary (20 μminner diameter that is connected to a syringe pump) filled with a water/ethanol mixture (1:1). An homebuild seryngue pump controls the injected flow, that will produce the active droplets. The typical radius of the produced droplet is between 30 and 80 μm depending on the flow and the exact capillary dimensions. A detailed protocol is also reported in the following References [12,23]. All non-fluorescent chemicals were purchased from Sigma-Aldrich. Acridine Orange (from Sigma-Aldrich, 235474) that stains to sDNA and is excitable with green laser (500/526 nm), and stains to RNA and is excitable with blue laser(460/650 nm). Total ribonucleic acid (RNA) from torula yeast was purchasedfrom Sigma-Aldrich (R6625). Single-strand deoxyribonucleic acid (sDNA) from herring sperm was purchased from Sigma-Aldrich (D7290), like Hoechst 33342 (ThermoFisher). pH buffers were purchased from Carl Roth with the following compositions, pH 4: Citric acid monohydrate/ Sodium hydroxid solution/Sodium chloride. pH 7: Monopotassium phosphate / Sodium hydroxide. pH 10Boric acid/Sodium hydroxide solution/ Potassium chloride. Extraction efficiency is measured using fluorescence measurements by comparing the difference in absolute signal intensity between the signal of the mother droplet without salt and the signal of the mother droplet with salt. The two different mother droplets are compared if their size is similar. However, it remains difficult to compare a fluorescence signal between a droplet made of mainly water (mother droplet) and mainly ethanol (daughter droplet).

Acknowledgements

M.L and R.S acknowledge funding from SPP1726 (DFG). Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: Saarland University · Experimental physics and Center for Biophysics · Saarbruecken · Germany

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Submitted: October 13, 2020
Accepted: April 7, 2021