nature portfolio

Peer Review File

Diffusiophoresis promotes phase separation and transport of biomolecular condensates

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Review has equations so is submitted as pdf.

REVIEW OF DIFFUSIOPHORESIS PROMOTES PHASE SEPARATION AND TRANSPORT OF BIOMOLECULAR CONDENSATES BY DOAN ET AL.

The authors study a phase separating system in solution. There are gradients of all the relevant species, imposed using microfluidics. The inspiration is from biology (currently topical biomolecular condensates) and so the phase separating molecules are biomolecules. They have interesting results. Although I don't think they are aware of related good work, with similar behaviour, but not on biomolecules. There is a entire little field on what is sometimes called the 'ouzo'effect. In this field Hajian and Hardt [1] have done lovely work on droplets moving and dissolving. The authors should cite their work.

I think the authors study phase separation of ssDNA of length 40 monomers (DNA), and peptides of length 25 monomers (PP). They do this with DNA diffusing in from the left of their system, of length $\sim$ 1 mm, and PP diffusing from the right. And this is done with and without a salt (NaCl) gradient, also diffusing from the right. I don't know how thick their system is (presumably shallow to avoid convection) and they use conventional (not confocal) fluorescence microscopy, focusing on some focal plane in the middle of the system to image behaviour in a horizontal slice? Sorry if I have missed where the authors specify system thickness. The DNA is fluorescently tagged, Figure 3 implies that PP may be as well but the relevant part of the supplementary information mentions only the PP tagging.

They track individual condensates (hence kymograph) plots but I cannot see any condensate sizes? Are the condensates at microsope resolution? So maybe at most hundreds of nanometres and maybe smaller? In the second movie some look larger but that is just a guess.

I am not 100% sure I always understand what the authors mean when they say "diffusiophoresis", "active migration". So to be clear, in this review, I will refer to what I think are the three transport mechanisms (assuming they have eliminated any flow in their system):

1. Condensate diffusiohoresis (CDP). If the condensates are liquid and have sufficiently low viscosity then (due to Marangoni), when there are gradients, they should move with a velocity

$$\mathbf{U}_{\rm DP} = \frac{R}{(3\eta + 2\eta_C)} \sum_i \frac{1}{\Delta_i} \left(-\frac{\partial \gamma}{\partial c_i} \right) \nabla c_i \qquad (15)$$

   where the viscosity of condensate is η_C and that of the solvent-phase is η. γ is the interfacial tension of the condensate/solvent interface. $\Delta_i = (1 + (1/2)D_{Di}/D_i)$. D_i and D_{Di} are the diffusion constants for i in the solvent phase, and inside the condensate, respectively. Which is DP version of Young, Goldstein and Block's TP expression [2]. This should be sensible so long as the ratio $R/(\eta_C/\eta) > \kappa^{-1}$ for κ^{-1} the Debye length. If $\kappa^{-1} > R/(\eta_C/\eta)$ then condensate should behave more like a solid object. Then $\mathbf{U}_{\rm DP}$ is probably better given by the expressions for a solid particle, as, for example, used by Shin in their excellent PNAS with Stone and coworkers. But with additional terms from the DNA and PP gradients, see eg Anderson [3].

2. Molecular diffusion (MD):

   (a) Self diffusion (SD):

$$j_i = -D_i \nabla c_i$$

   where D_i is a (dilute) molecular diffusion constant.

   (b) Molecular diffusiophoresis (MDP)

$$j_i = -\sum_{i \neq j} M_i \nabla c_j$$

   which is diffusiophoresis in the sense of a gradient of one species pushing on another molecular species. NB When j is a salt $\nabla \ln c_j$ maybe a better approximation.

I suggest that early in the manuscript they clearly define what they think are the important transport mechanisms, give them simple names, and then use them throughout. They don't have to be the names above — it is their paper not mine — but sticking to simple names would help the reader.

As an example of where I found the text difficult to follow: on page 5 they have "We posit that the observed active migration of the droplets in the NaCl gradients is caused by diffusiophoresis, which describes a directional migration of charged colloidal particles driven by ionic solute gradients."Is "active migration"actually diffusiophoresis, in the sense of is it $\mathbf{U}_{\rm DP}$ above? If so, why the two names? Also, there are not only gradients in the salt.

In the absence of an NaCl gradient then interdiffusion of DNA and PP gives rise to condensates roughly in the middle of the bit-less-than 1 mm system. Individual condensates do not appear to move via CDP at measureable speeds, but a zone of condensates forms, which is maybe 100 µm wide and moves a few hundred µm to the right over about 1 hour.

It would be useful if the authors commented on how viscous they estimate the condensates are. This matters as the CDP scales with R for liquid condensates but has a much weaker R dependence for solid condensates. Here liquid and solid are defined by the ratio $\kappa R/(\eta_C/\eta)$ (assuming the interface of the condensate if it is solid has the thickness of around the Debye length). If this ratio is large then the driving force $\nabla\gamma$ is opposed by dissipation over a lengthscale R, while if it is small, it is opposed by dissipation over a lengthscale κ^{-1}.

Sorry I can't see any condensate size estimates but if at least some are around 100 nm, then if the condensate viscosity is a 100 times that of water, or higher, then they should behave roughly as solid objects. This would be roughly consistent with the $\sim$ 1 µm s^{-1} speeds they see. Large low viscosity liquid droplets are likely to move a lot faster.

Figures 1 and 2

I think I have worked out what the results are in Figures 1 and 2 of the manuscript. I think the results are interesting, but I am not sure I follow everything. So I will outline what I think is in these figures and ask some questions.

Given that DNA and PP should take of order tens of minutes to an hour to diffuse a mm, and phase separate together, the formation of condensates in the middle after tens of minutes makes sense.

But I am sorry I don't follow how the zone of condensates moves. The authors talk about condensates forming at the front (right-hand side) and dissolving at the back (left-hand side). But surely this is only half the story. These dissolving droplets at the back were presumably forming at the front earlier. I.e., the concentration of condensates c_{CON} at fixed x is some function of time that starts zero, increases to a maximum and then decreases back to zero — over some timescale we can call τ. Then you get a travelling zone with some function $c_{CON}(x/L_Z - t/\tau)$. This moves at some speed L_Z/τ? Here L_Z is some lengthscale, and $(x/L_Z - t/\tau)$ is (up to factors of 2π) the usual expression for argument of travelling wave functions.

The authors then add a NaCl source. As I understand it the condensates then move, to the right, i.e., uphill in NaCl concentration. This looks like clear CDP, and uphill is reasonable. The speed seems to be up to 1 µm, which if the condensates are very viscous, would be reasonable as this is a typical DP speed for a charged solid colloid in a salt gradient. Figure S2 is interesting here (this is nice work, good idea to study this). Perhaps unsurprisingly, the effective ζ potential depends on DNA/PP ratio.

As far as I can see CDP and MDP due to salt gradients does not change the behaviour qualitatively (except there maybe condensates forming directly at the far right in the first movie) but does increase the effective speed of motion of the condensate zone, by maybe 40 to 50 % (Figure 1h). This is interesting although not surprising. Just one question: there look to be two obvious timescales for a condensate here: a) time to grow/dissolve, i.e., τ above, and b) time for CDP to move condensate across condensate zone. Now both can depend on R (coarsening is often R^3). But for typical condensate sizes are they comparable? Is that why CDP just speeds up motion a bit?

In principle, it looks like there are two opposite limits. The first is where CDP is so slow that CDP does not move a condensate enough to move it during that time to a place with a significantly different environment (i.e., DNA, PP, NaCl concentrations). The opposite limit is where CDP ejects a condensate out of the zone so fast that it has no time to grow/shrink. I presume without NaCl, their system is in the first limit? And maybe it is not far from that limit even with NaCl gradients?

Figures 3 and 4

The supporting information mentions fluorescent tags for PP, but does Figure 3 imply that in a subset(?) of experiments, the DNA is also fluorescently tagged?

I am not sure I understand how n_D/n_P is measured. Do the DNA and PP have different colour fluorescent tag? Then is this the ratio of say red to blue light, if, say, DNA and PP have red and blue fluorophores? And then do they use scattering to test for condensates. I.e., they know that there is phase separation for intensity ratio $n_D/n_P = 1.2$ say because the measure (bulk fluorescence?) for both DNA and PP tags and take the ratio of light coming out, and simultaneously see scattering due to condensates? If so then of course n_D/n_P is a ratio of light intensities not molecules. This is OK, but if I missed where this was described then I apologise, if it was missed out, please add.

I think this is an important point. Back-of-the-envelope calculations suggest that if indeed the phase diagram is well approximated by a a two-phase region restricted to range of ratios of concentrations of DNA to PP that is bounded below and above, then their behaviour follows easily. You get a moving zone of condensates directly. Because over a wide range of values of x at the left of the system the DNA/PP concentration ratio should start very high (single-phase region), drop through the two-phase range, and then drop into the other single-phase region. The times at which this happens could then increase with increasing x, giving the behaviour the authors see.

Viewed from this perspective, this bounded-from-above-and-below phase separation range, is key to the authors results. So they could expand their discussion here, as it is important to be clear here as what they measure and how.

I note that it appears that zone does not penetrate much into the right-hand half of the system in the absence of salt. Is this just due to the ratio DNA/PP being always too low, i.e., always in low-ratio part of the single-phase region?

Other points:

1. The motivation is biomolecular condensates in cells, but their gradients are of NaCl. As far as I know gradients in NaCl are not common in cells; in our cells the ionic strength is roughly uniform. Now there are gradients in biomolecules, possibly even in charged species (ATP near mitochondria?). Can the authors comment if they think molecular/ionic gradients do move biomolecular condensates around, if so what species are the gradients in?

2. Writing style guides typically recommend paragraphs to be at least two sentences but usually not more than 200 words. The manuscript has a number of paragraphs significantly above 200 words. In my opinion, cutting paragraphs down to mostly < 200 words would help readability.

3. The title focuses on DP. Hajian and Hardt see more dramatic DP motion. But they don't have the moving

set of condensates plus direct mechanism in terms of the range of ratios over which condensates form. So this is a major novelty in the paper, that could be reflected in the title.

Overall, a very interesting paper, as you can tell from my questions. Because the system is complex, it is hard to write a clear paper and I think the authors may have missed some important details. I appreciate that answering the questions above will take time, but with results this interesting, I think it is worth taking this additional time to present them more clearly, and with details like what n_D/n_P is made very clear and explicit. As outlined above, I think this is a key feature of the manuscript.

They may also want to specify what is the maximum CDP speed consistent with their not observing CDP without salt, i.e., their resolution, which may be around $0.1\,\mu\mathrm{m\,s}^{-1}$. It is a little surprising that there is no apparent CDP here, despite the gradients in DNA and PP. For example, it is known that phase separating droplets induce CDP in other nearby phase separating droplets [4].

If the authors are willing to put in this work, I will be happy to read a revised manuscript and I hope to then recommend publication.
Richard Sear

[1] R. Hajian and S. Hardt, Formation and lateral migration of nanodroplets via solvent shifting in a microfluidic device, Microfluid. Nanofluid. **19**, 1281 (2015).

[2] N. O. Young, J. S. Goldstein, and M. J. Block, The motion of bubbles in a vertical temperature gradient, Journal of Fluid Mechanics **6**, 350–356 (1959).

[3] J. L. Anderson, Colloidal transport by interfacial forces, Ann. Rev. Fluid Mech. **21**, 61 (1989).

[4] N. Vladimirova, A. Malagoli, and R. Mauri, Diffusiophoresis of two-dimensional liquid droplets in a phase-separating system, Phys. Rev. E **60**, 2037 (1999).

Reviewer #2 (Remarks to the Author):

In this work, Doan and colleagues use a microfluidic platform to examine the propagation of biomolecular condensates along an ion gradient. The authors observe that an ion gradient promotes both enhanced condensate formation (between the charged peptides and the ssDNA) and quicker migration of the condensates along the gradient. The authors argue that these effects stem from the reentrant phase separation of the system combined with diffusiophoresis.

I found this work to be a very creative use of microfluidics to study the motility of condensates. The finding that an ion gradient imparts enhanced condensate formation and motility is a valuable insight to the field and one that deserves more attention. I appreciate the authors effort to parse out the mechanism of condensate propagation and am convinced that their arguments are valid. I do, however, believe that they could use some more evidence to strengthen their argument. There are several points of confusion that I believe would need to be addressed more clearly by the authors.

1. The data is clear in that the ion gradient speeds up the movement of the phase-separated particles along the wells. They make the distinction that in the wells that have no gradient, the movement of particles is stochastic, and the movement of the condensate comes purely from dissolution on the back of the wave and formation on the front, while in the presence of a gradient there is directional bias in the movement. They support this with kymographs of the 20mM salt non-gradient condition and the 1mM-20mM gradient condition. When looking at the video showing all three conditions, it seems that all three conditions look qualitatively unique. If anything, the particles are much more mobile in the 1mM salt non-gradient condition than in the 20mM salt non-gradient condition. For the rest of the paper, though, the authors focus on comparing solely the 20mM non-gradient sample and the 1mM-20mM gradient sample. To ensure that there are no more confounding effects by the difference in concentrations between the non-gradient conditions (other than those addressed by the authors already), the authors should at least include the kymograph analysis for all the samples tested.

2. The authors state in the paper “the diffusiophoretic velocity scales with the gradient of the logarithm of the salt concentration…the velocity is influenced by the absolute salt concentration where the biomolecules undergoing diffusiophoresis slow down as they migrate toward higher salt concentrations, leading to their accumulation” (pg. 6). Here they were referencing the behavior of molecules undergoing diffusiophoresis under gradient conditions. If the phase separation is in fact a result of diffusiophoresis and the diffusiophoretic velocity scales with the gradient of the logarithm of the salt concentration, then it would go a long way in supporting the authors arguments if they tested condensate motility under at least one other gradient condition. The authors do all their experiments under the 1-20mM gradient condition. There is a clear effect from non-gradient to gradient conditions, but if diffusiophoresis promoting phase separation, then there would presumably be a quantifiable difference between 1-10mM (as an example) and 1-20mM.

3. The authors chose to do a majority of their experiments at an excess ssDNA condition ([dT]40:[RGRGG]5 = 1.25:1), which confers a negative surface charge to the particles. I appreciate keeping the condition consistent for purposes of comparison, but I believe the authors should provide more justification for this choice. They do one experiment with excess positive charge (Supplementary Fig. S5), but the behavior seems to be different, and they use a different salt (KAc) than the one they use for the rest of the experiments (NaCl). The authors discuss the differences between diffusiophoretic behavior between individual positive and negative molecules but do not expand on the differences between these effects in their condensate model. I believe that expanding on these distinctions would add more clarity to their model.

4. I am curious about any differences depending on the ion of choice. The authors show clear differences in the non-equilibrium behavior in the [dT]40 and RGRGG]5 in the presence of different monovalent salts (LiCl, NaCl. KCl). I wonder how this would manifest in the combined system. Also, the authors bring up the relevance of calcium as an essential biological ion. Would these behaviors be enhanced by the addition of a divalent ion gradient?

Reviewer #3 (Remarks to the Author):

The manuscript reports the results of an experimental investigation of the impact of ion gradients on transport processes of biomolecular condensates on the mesoscale and biomolecules on the microscale. A microfluidic platform and fluorescence microscopy are used to demonstrate that ion concentration gradients can accelerate the transport of biomolecules, including nucleic acids and proteins, via diffusiophoresis. This hydrodynamic transport process is shown to facilitates localized enrichment of biomolecules, thereby promoting the location-specific formation of biomolecular condensates via phase separationR, as well as active motility of condensates, resulting in enhanced diffusion along the gradient. It is shown that the gradient-induced active motility leads to a dynamical redistribution of condensates that extends their lifetime.

The in vitro experiments of biomolecular condensates formed by RGG repeat polypeptides and ssDNA in salt gradients show several properties and behaviors, which look very intriguing to me. However, I am not familiar enough with neither biomolecular condensates nor with diffusiophoresis, to be able to judge the validity and importance of the presented results. This also includes a lack of knowledge of the relevant literature.

Therefore, I can only make a few rather general remarks:

(1) I doubt that there can be significant spatiotemporal gradients of temperature and pressure inside a cell.

(2) How can a (diffusiophoretic) mobility be viewed effectively as an enhanced diffusivity? The time dependence of the mean squared displacement should display different power laws in the two cases.

(3) The length scales in the experiment (channel width about 100 micro, channel length about 1 mm) are much larger than typical cell dimensions. Can the results be expected to hold on the cell scale?

(4) Are salt gradients in cells typically of similar magnitude as studied in the current in vitro experiment?

(5) Which mechanism in the cell would be able and sustain such gradients?

Responses to Reviewers

We thank the reviewers for their constructive comments and critical assessments of our work. The manuscript has undergone substantial revision to fully address the reviewers' critiques. For clarity, the comments of the reviewers and remarks are in plain text; our responses are in blue. With this synopsis as a prelude, we segue to point-by-point responses to each of the comments made by the reviewers.

Response to the Reviewer 1's Comments

Comment #1: The authors study a phase separating system in solution. There are gradients of all the relevant species, imposed using microfluidics. The inspiration is from biology (currently topical biomolecular condensates) and so the phase separating molecules are biomolecules. They have interesting results. Although I don't think they are aware of related good work, with similar behaviour, but not on biomolecules. There is a entire little field on what is sometimes called the 'ouzo' effect. In this field Hajian and Hardt [1] have done lovely work on droplets moving and dissolving. The authors should cite their work.

Our response: We thank the Reviewer for spending time and effort reviewing our manuscript. It was really helpful for us to receive constructive comments and suggestions. We are happy to hear from the Reviewer that our manuscript is a "very interesting paper." Following the Reviewer's comment on the Ouzo effect, we have cited the relevant work by Hajian and Hardt (citation #72 in the revised manuscript) in the Discussion section as follows:

Page 13: "*…Recent studies on diffusiophoresis with a variety of other biomolecules and biocolloids, such as membrane proteins,[69] exosomes,[48] bacteria,[46,70] and blood cells,[71] or other phase-separating systems[72] suggest the ubiquity of diffusiophoresis in biological systems where chemical gradients are ever-present, providing suitable conditions for diffusiophoresis to arise.[73] …*"

Comment #2: I think the authors study phase separation of ssDNA of length 40 monomers (DNA), and peptides of length 25 monomers (PP). They do this with DNA diffusing in from the left of their system, of length ~ 1 mm, and PP diffusing from the right. And this is done with and without a salt (NaCl) gradient, also diffusing from the right. I don't know how thick their system is (presumably shallow to avoid convection) and they use conventional (not confocal) fluorescence microscopy, focusing on some focal plane in the middle of the system to image behaviour in a horizontal slice? Sorry if I have missed where the authors specify system thickness. The DNA is fluorescently tagged, Figure 3 implies that PP may be as well but the relevant part of the supplementary information mentions only the PP tagging.

Our response: The depth of our microfluidic channel is 40 μm, which is observed under a widefield fluorescence microscope focusing on the mid-plane. Only ssDNA is tagged with Cy5 fluorescent dye in the case of phase-separated condensates (Figures 1 and 2 in the main text). In Figure 3, we reported diffusion of peptide or ssDNA alone independent of each other without any condensates. For these experiments, we used Cy5-labeled ssDNA $[dT]_{40}$ (1.2% of the total $[dT]_{40}$) and Alexa488-labeled peptide $[RGRGG]_5$ (3.7% of the total $[RGRGG]_5$). This information is now added to the main text and the Supplementary Information.

Comment #3: They track individual condensates (hence kymograph) plots but I cannot see any condensate sizes? Are the condensates at microscope resolution? So maybe at most hundreds of nanometres and maybe smaller? In the second movie some look larger but that is just a guess.

Our response: The size of our biomolecular condensates is in the range ~ 250 – 600 nm, as determined by the confocal microscopy image of condensates, which is now provided in supplementary **Figure S2a**. **Figures S2b** and **S2c** present the size distribution of $[dT]_{40}$-$[RGRGG]_5$ condensates at varying NaCl

concentrations measured via (b) confocal microscopy and (c) dynamic light scattering. Both measurements indicate that the mean diameter of the condensates is submicron, ranging from 230 nm to 660 nm. We have added this information in the main text and the Supplementary Information as follows:

Page 4: "*These condensates are polydispersed submicron-sized coacervates with mean diameters ranging between 230–660 nm depending on the buffer salt concentration (Supplementary Fig. S2).*"

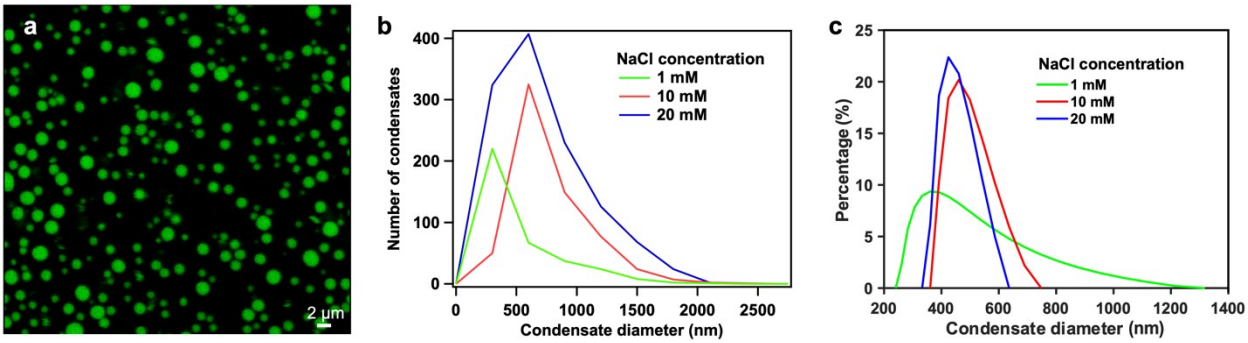

Figure S2. Size measurements of $[dT]_{40}$-$[RGRGG]_5$ condensates ($[dT]_{40}$:$[RGRGG]_5$ = 1.25:1). (**a**) Confocal image of $[dT]_{40}$-$[RGRGG]_5$ condensates in 20 mM NaCl (sample composition: 0.5 mg/ml $[RGRGG]_5$, 150 nM Alexa-488 labeled $[RGRGG]_5$, 0.625 mg/ml $[dT]_{40}$, 10 mM Tris-HCl, 20 mM DTT, and 20 mM NaCl). (**b,c**) Size distribution of $[dT]_{40}$-$[RGRGG]_5$ condensates at varying NaCl concentrations obtained from the (**b**) confocal imaging and (**c**) dynamic light scattering.

Comment #4: I am not 100% sure I always understand what the authors mean when they say "diffusiophoresis", "active migration." So to be clear, in this review, I will refer to what I think are the three transport mechanisms (assuming they have eliminated any flow in their system):
1. Condensate diffusiohoresis (CDP). If the condensates are liquid and have sufficiently low viscosity then (due to Marangoni), when there are gradients, they should move with a velocity

$$U_{DP} = \frac{R}{(3\eta + 2\eta_C)} \sum_t \frac{1}{\Delta_i} \left(-\frac{\partial \gamma}{\partial c_i}\right) \nabla c_i$$

where the viscosity of condensate is η_C and that of the solvent-phase is η. γ is the interfacial tension of the condensate/solvent interface. $\Delta_i = 1 + (1/2)D_{D_i}/D_i$. D_i and D_{D_i} are the diffusion constants for i in the solvent phase, and inside the condensate, respectively. Which is DP version of Young, Goldstein and Block's TP expression [2]. This should be sensible so long as the ratio $R/(\eta_C/\eta) > \kappa^{-1}$ for κ^{-1} the Debye length. If $\kappa^{-1} > R/(\eta_C/\eta)$ then condensate should behave more like a solid object. Then U_{DP} is probably better given by the expressions for a solid particle, as, for example, used by Shin in their excellent PNAS with Stone and coworkers. But with additional terms from the DNA and PP gradients, see eg Anderson [3].

Our response: In this study, we consider condensate diffusiophoresis as the classical diffusiophoresis of rigid spheres. Based on our previous measurements, the viscosity of the $[dT]_{40}$-$[RGRGG]_5$ is on the order of 1–10 Pa·s [*see* R1-R3]. Considering the radius of the condensates as ~240 nm, $R/(\eta_C/\eta) \sim 0.24 - 0.024\ nm \ll \kappa^{-1} \sim 1\ nm$, implying that **our condensates behave close to a rigid particle**. We have added this discussion in the main text as follows:

Page 11: "*…We note that the condensate migration velocity (~0.1 μm/s) agrees quantitatively with the theoretical estimates for the diffusiophoretic velocity of charged rigid spheres of similar zeta potential.*[60,61] *This suggests that these condensates behave close to a solid, where liquid-like phoretic transport, i.e., Marangoni propulsion is negligible. This is likely due to the viscoelastic nature of* $[dT]_{40}$-$[RGRGG]_5$ *condensates.*[30,33,62] *For highly viscous liquid drops, the viscous shear inside the drop should dominate over the viscous shear acting in the exterior, yielding the condition* $(\lambda/R) \cdot (\eta_c/\eta) \gg 1$, *where* $\lambda \sim 1\ nm$ *is the Debye layer thickness,* $R \sim 240\ nm$ *is the condensate radius,* $\eta \sim 1\ mPa \cdot s$ *is the dynamic viscosity of the*

*external solution, and η_c is the dynamic viscosity of the condensates. Based on our previous measurements of similar peptide-nucleic acid condensates,[30,33] the viscosity η_c ranges between $1-10\ Pa\cdot s$, indeed satisfying the above condition. The overall migration of the condensates being identical regardless of their size, shown by the kymographs in **Fig. 2c**, also suggests that their motion is dictated by diffusiophoresis[39] rather than the Marangoni effect, which strongly depends on the drop size.[63] ...*"

[R1] Alshareedah, I., Moosa, M. M., Pham, M., Potoyan, D. A., & Banerjee, P. R. (2021). Programmable viscoelasticity in protein-RNA condensates with disordered sticker-spacer polypeptides. *Nature Communications*, *12*(1), 6620.
[R2] Alshareedah, I., Thurston, G. M., & Banerjee, P. R. (2021). Quantifying viscosity and surface tension of multicomponent protein-nucleic acid condensates. *Biophysical Journal*, *120*(7), 1161-1169.
[R3] Alshareedah, I., Singh, A., Yang, S., Ramachandran, V., Quinn, A., Potoyan, D. A., & Banerjee, P. R. (2024). Determinants of Viscoelasticity and Flow Activation Energy in Biomolecular Condensates, *Science Advances*, DOI: 10.1126/sciadv.adi6539.

Comment #5: 2. Molecular diffusion (MD): (a) Self diffusion (SD): $j_i = -D_i \nabla c_i$, where D_i is a (dilute) molecular diffusion constant. (b) Molecular diffusiophoresis (MDP): $j_i = -\sum_{i \neq j} M_i \nabla c_j$, which is diffusiophoresis in the sense of a gradient of one species pushing on another molecular species. NB When j is a salt $\nabla \ln c_j$ maybe a better approximation. I suggest that early in the manuscript they clearly define what they think are the important transport mechanisms, give them simple names, and then use them throughout. They don't have to be the names above — it is their paper not mine sticking to simple names would help the reader. As an example of where I found the text difficult to follow: on page 5 they have "We posit that the observed active migration of the droplets in the NaCl gradients is caused by diffusiophoresis, which describes a directional migration of charged colloidal particles driven by ionic solute gradients. "Is "active migration" actually diffusiophoresis, in the sense of is it U_{DP} above? If so, why the two names? Also, there are not only gradients in the salt.

Our response: This is an excellent suggestion by the Reviewer. We have revised the main text to explicitly distinguish these two mechanisms as follows:

Page 10: *"This is attributed to the increase in the local biomolecule concentration driven by diffusiophoresis (i.e., molecular diffusiophoresis), ...", "Unlike the condensates formed without the NaCl gradient, the condensates under the non-equilibrium environments effectively move along with NaCl gradient via diffusiophoresis (i.e., condensate diffusiophoresis)."*

Our usage of "active migration" indeed refers to the diffusiophoretic motion of the condensate (CDP) in the salt gradient. To avoid any confusion, we have revised the relevant text as follows:

Page 5: "*We posit that the observed directional migration of the droplets in the NaCl gradients is caused by diffusiophoresis, which describes a spontaneous migration of charged colloidal particles driven by ionic solute gradients.[37]*"

Comment #6: In the absence of an NaCl gradient then interdiffusion of DNA and PP gives rise to condensates roughly in the middle of the bit-less-than 1 mm system. Individual condensates do not appear to move via CDP at measureable speeds, but a zone of condensates forms, which is maybe 100 μm wide and moves a few hundred μm to the right over about 1 hour. It would be useful if the authors commented on how viscous they estimate the condensates are. This matters as the CDP scales with R for liquid condensates but has a much weaker *R* dependence for solid condensates. Here liquid and solid are defined by the ratio $\kappa R/(\eta_C/\eta)$ (assuming the interface of the condensate if it is solid has the thickness of around the Debye length). If this ratio is large then the driving force $\nabla\gamma$ is opposed by dissipation over a length scale R, while if it is small, it is opposed by dissipation over a length scale κ^{-1}. Sorry I can't see any condensate size estimates but if at least some are around 100 nm, then if the condensate viscosity is a 100 times that of water, or higher, then they should behave roughly as solid objects. This would be roughly

consistent with the 1um s^{-1} speeds they see. Large low viscosity liquid droplets are likely to move a lot faster.

Our response: Thank you for your insightful suggestions and our apologies for not providing any data on the condensate size estimates. This has been addressed in the main text (Page 4) and the supplementary information (**Supplementary Fig. S2**). As discussed above in our response to Comment # 4, $\kappa R/(\eta_c/\eta) \ll 1$ suggests their motion following closer to the solid particles of similar size and surface charge. Despite the condensate size being widely dispersed, they all seem to be migrating at the same rate, which is shown in the kymograph in Fig. 2c. This size-independent motion suggests the condensate transport is mainly governed by diffusiophoresis, not Marangoni effects. As mentioned above, we have added this discussion in the main text, a reproduction of which is provided above in our response to Comment # 4.

Comment #7: Given that DNA and PP should take of order tens of minutes to an hour to diffuse a mm, and phase separate together, the formation of condensates in the middle after tens of minutes makes sense. But I am sorry I don't follow how the zone of condensates moves. The authors talk about condensates forming at the front (right-hand side) and dissolving at the back (left-hand side). But surely this is only half the story. These dissolving droplets at the back were presumably forming at the front earlier. I.e., the concentration of condensates c_{CON} at fixed x is some function of time that starts zero, increases to a maximum and then decreases back to zero over some timescale we can call τ. Then you get a travelling zone with some function $c_{CON}(x/L_Z - t/\tau)$. This moves at some speed L_Z/τ? Here L_Z is some lengthscale, and $(x/L_Z - t/\tau)$. is (up to factors of 2π) the usual expression for argument of travelling wave functions.

Our response: The formation of the condensates is governed by the concentration ratio between DNA ($[dT]_{40}$) and polypeptide ($[RGRGG]_5$). Briefly, the DNA and polypeptide mixture shows stoichiometry-dependent reentrant phase separation where phase separation is only favored over a specific range of concentration ratios. We have studied this phenomenon extensively and reported it in the following paper [R4]. In the absence of diffusiophoresis, the diffusion of $[dT]_{40}$ and $[RGRGG]_5$ molecules into one another is the underlining transport mechanism leading to the phase separation (condensation and dissolution due to a reentrant phase separation), where two biomolecules encounter at a certain range of stoichiometry. Therefore, the growing/dissolving timescale is effectively the diffusion timescale of the biomolecules, which is $\tau = (L_z)^2/D$, where $L_Z = 800$ µm is the length of the channel and D is the diffusion coefficient of the biomolecules ($\sim 1\times10^{-10}$ m^2/s). In this regard, the wave speed can be expressed as $v = L_Z/\tau = D/L_Z \approx 0.1$ µm/s, which agrees with the average moving speed of the peak (≈ 0.14 µm/s) in Fig. 1h. We note that the phase separation zone, however, acts as a sink in which biomolecules are depleted in nearby region to form condensates. Therefore, the transport of biomolecules in the phase-separated zone is associated with droplet transport, which is distinct from biomolecule diffusion. This may lead to discrepancies in the estimation of the wave speed based solely on the biomolecule diffusion time scale, which is why we have decided to refrain from discussion on the wave speed.

[R4] Alshareedah, I., Kaur, T., Ngo, J., Seppala, H., Kounatse, L. A. D., Wang, W., Moosa, M. M. & Banerjee, P. R. (2019). Interplay between short-range attraction and long-range repulsion controls reentrant liquid condensation of ribonucleoprotein–RNA complexes. *Journal of the American Chemical Society*, *141*(37), 14593-14602.

Comment #8: The authors then add a NaCl source. As I understand it the condensates then move, to the right, i.e., uphill in NaCl concentration. This looks like clear CDP, and uphill is reasonable. The speed seems to be up to 1 µm/s, which if the condensates are very viscous, would be reasonable as this is a typical DP speed for a charged solid colloid in a salt gradient. Figure S2 is interesting here (this is nice work, good idea to study this). Perhaps unsurprisingly, the effective ζ potential depends on DNA/PP ratio. As far as I can see CDP and MDP due to salt gradients does not change the behaviour qualitatively (except there maybe condensates forming directly at the far right in the first movie) but does increase the effective speed of

motion of the condensate zone, by maybe 40 to 50 % (Figure 1h). This is interesting although not surprising. Just one question: there look to be two obvious timescales for a condensate here: a) time to grow/dissolve, i.e., τ above, and b) time for CDP to move condensate across condensate zone. Now both can depend on R (coarsening is often R^3). But for typical condensate sizes are they comparable? Is that why CDP just speeds up motion a bit?

Our response: In our experiments, once the condensates are formed at the left end of the channel, we did not observe any noticeable further coarsening of the condensates, likely due to the high surface charge of the condensates that prevents them from fusing [R2, R5]. Out-of-equilibrium conditions may also contribute to the reduced coarsening by suppressing Ostwald ripening [R6]. Rather, as indicated above, the condensation and dissolution of the condensates are mainly slaved by the movement of the phase-separating window, as set by the diffusion timescale of the biomolecules τ. Note that the timescale of this condensation/dissolution does not correspond to the actual formation time scale, which is much faster. As shown in Fig. 4, the range of the composition ratio n_D/n_P matches with the condensate window, which also suggests that the time scale for the formation of the condensates is much faster than the time scale at which the wave moves (τ). To clarify our points, we have added more discussions on this aspect in the main text as follows:

Page 10: "*This suggests that the formation and dissolution of the condensates occur much faster than the migration of the phase-separation region, thus indicating that the phase separation occurs in a quasi-equilibrium manner.*"

[R5] Alshareedah, I., Moosa, M. M., Raju, M., Potoyan, D. A., & Banerjee, P. R. (2020). Phase transition of RNA−protein complexes into ordered hollow condensates. *Proceedings of the National Academy of Sciences*, *117*(27), 15650-15658.
[R6] Zwicker, D., Hyman, A. A., & Jülicher, F. (2015). Suppression of Ostwald ripening in active emulsions. *Physical Review E*, *92*(1), 012317.

Comment #9: In principle, it looks like there are two opposite limits. The first is where CDP is so slow that CDP does not move a condensate enough to move it during that time to a place with a significantly different environment (i.e., DNA, PP, NaCl concentrations). The opposite limit is where CDP ejects a condensate out of the zone so fast that it has no time to grow/shrink. I presume without NaCl, their system is in the first limit? And maybe it is not far from that limit even with NaCl gradients?

Our response: In the absence of CDP, the condensates do not exhibit any directional motion, and their formation and dissolution are dictated by the reentrant phase separating window, as elaborated in the above response to Comment #9. Even in the presence of CDP, the condensates move at a rate that is only commensurate with the wave speed. Therefore, we believe that our system under NaCl gradients is still mainly governed by the equilibrium thermodynamics, as also speculated by the Reviewer.

Comment #10: The supporting information mentions fluorescent tags for PP, but does Figure 3 imply that in a subset(?) of experiments, the DNA is also fluorescently tagged?

Our response: We apologize that we did not provide enough details about the use of specific fluorescence tags for our experimental results reported in Fig. 3, this has been addressed now. For Figure 3 where we conduct experiments with individual molecules, the biomolecules, both the ssDNAs and PPs are tagged with Cy5 and Alexa488, respectively. Note that in the experiments involving a mixture of the two, only ssDNAs are tagged with Cy5. We have added the details in the main text as follows:

Page 4: "*The condensates are visualized using Cy5-labeled $[dT]_{40}$ (1.2 mol% of the total $[dT]_{40}$).*"

Page 6: "*In these experiments, not only $[dT]_{40}$ is fluorescent, but also $[RGRGG]_5$ is tagged with Alexa-488 fluorophores (3.7 mol% of the total $[RGRGG]_5$).*"

Page 7, In the caption of Fig. 3: "……*For these experiments, we used Cy5-labeled* $[dT]_{40}$ *(1.2 mol% of the total* $[dT]_{40}$*) and Alexa488-labeled* $[RGRGG]_5$ *(3.7 mol% of the total* $[RGRGG]_5$*)……*"

Comment #11: I am not sure I understand how n_D/n_P is measured. Do the DNA and PP have different colour fluorescent tag? Then is this the ratio of say red to blue light, if, say, DNA and PP have red and blue fluorophores? And then do they use scattering to test for condensates. I.e., they know that there is phase separation for intensity ratio $n_D/n_P = 1.2$ say because the measure (bulk fluorescence?) for both DNA and PP tags and take the ratio of light coming out, and simultaneously see scattering due to condensates? If so then of course n_D/n_P is a ratio of light intensities not molecules. This is OK, but if I missed where this was described then I apologise, if it was missed out, please add.

Our response: We apologize that we did not provide enough details about how the concentration ratio of ssDNA and peptide, n_D/n_P, is measured where n_D and n_P are the mass concentration of $[dT]_{40}$ and $[RGRGG]_5$, respectively. The ratio n_D/n_P is constructed from the transport measurements for individual biomolecules in the absence of condensates (Figure 3a-d), where we take the ratio of the two measurements. Here, the DNA is labeled with Cy5 (red) and the peptide is labeled with Alexa488 (green) as mentioned above. The concentration of the molecules is assumed to be linearly proportional to the fluorescence intensity, which is a valid assumption in dilute systems [R7]. The normalized intensity ratio can be used to reflect the concentration ratio of the individual biomolecule in which the maximum concentration is set by sample preparation. In the revised manuscript, we have clarified this to remove any ambiguity regarding how n_D/n_P is measured as follows:

Page 9: "*The concentrations of the biomolecules,* n_D *and* n_P*, are assumed to be linearly proportional to the fluorescence intensity, which is a valid assumption in dilute systems.*[57]"

[R7] Jung, B., Bharadwaj, R., & Santiago, J. G. (2003). Thousandfold signal increase using field-amplified sample stacking for on-chip electrophoresis. *Electrophoresis*, *24*(19-20), 3476-3483.

Comment #12: I think this is an important point. Back-of-the-envelope calculations suggest that if indeed the phase diagram is well approximated by a two-phase region restricted to range of ratios of concentrations of DNA to PP that is bounded below and above, then their behaviour follows easily. You get a moving zone of condensates directly. Because over a wide range of values of x at the left of the system the DNA/PP concentration ratio should start very high (single-phase region), drop through the two-phase range, and then drop into the other single-phase region. The times at which this happens could then increase with increasing x, giving the behaviour the authors see. Viewed from this perspective, this bounded-from-above-and-below phase separation range, is key to the authors results. So they could expand their discussion here, as it is important to be clear here as what they measure and how.

Our response: We agree with the Reviewer that the wave-like motion of the condensates due to the moving phase window is an important aspect of our work, rooted in the reentrant phase behavior of the system. We have added more discussions on this aspect as follows:

Page 9: "*Since* $[dT]_{40}$ *enters from the left while* $[RGRGG]_5$ *migrates from the right, the concentration ratio of the two biomolecules (i.e.,* n_D/n_P*) decreases along the channel. Such a distribution results in the localized zone of phase separation that is set by a finite window of* n_D/n_P*.*"

Comment #13: I note that it appears that zone does not penetrate much into the right-hand half of the system in the absence of salt. Is this just due to the ratio DNA/PP being always too low, i.e., always in low-ratio part of the single-phase region?

Our response: The reviewer is correct in making this assertion. In the absence of salt gradients, the biomolecules decay monotonically. Thus, the far-right side is not thermodynamically favorable for the phase separation to occur.

Comment – Other points: 1. The motivation is biomolecular condensates in cells, but their gradients are of NaCl. As far as I know gradients in NaCl are not common in cells; in our cells the ionic strength is roughly uniform. Now there are gradients in biomolecules, possibly even in charged species (ATP near mitochondria?). Can the authors comment if they think molecular/ionic gradients do move biomolecular condensates around, if so what species are the gradients in?

Our response: As articulated beautifully in a recent PRL paper by Sear [R8], metabolites are the primary candidates that can cause diffusiophoresis in cells. Despite the abundance of salt ions, such as Na^+, K^+, and Cl^-, their gradients are not expected to be strong due to the lack of rapid turnover of these species. Nevertheless, interdiffusion of these species into one another (while maintaining electroneutrality and osmolarity) and still provide diffusiophoresis. As a proof of concept, we have performed additional in vitro experiments with equimolar NaCl and KCl (1 mM) diffusing into each. In this scenario, the osmolarity is roughly equal across the experimental system. Yet, due to the difference in the diffusivity between ions Na^+, K^+, and Cl^-, a local electrical field is generated to drive (electro)diffusiophoresis of condensates (**Figure S8b**). While we acknowledge that this scenario is still far-fetched to be considered in living cells, these results nonetheless demonstrate the effect of multispecies diffusion on the diffusiophoresis of biomolecular condensates. We have mentioned these results in the main text and added the data in the Supplementary Information.

Page 13: "*…For instance, the interdiffusion of equimolar multicomponent solutes of Na^+ and K^+ can also induce significant diffusiophoresis and enhanced phase separation (**Supplementary Fig. S8**)…*"

[R8] Sear, R. P. (2019). Diffusiophoresis in cells: A general nonequilibrium, nonmotor mechanism for the metabolism-dependent transport of particles in cells. *Physical Review Letters*, *122*(12), 128101.

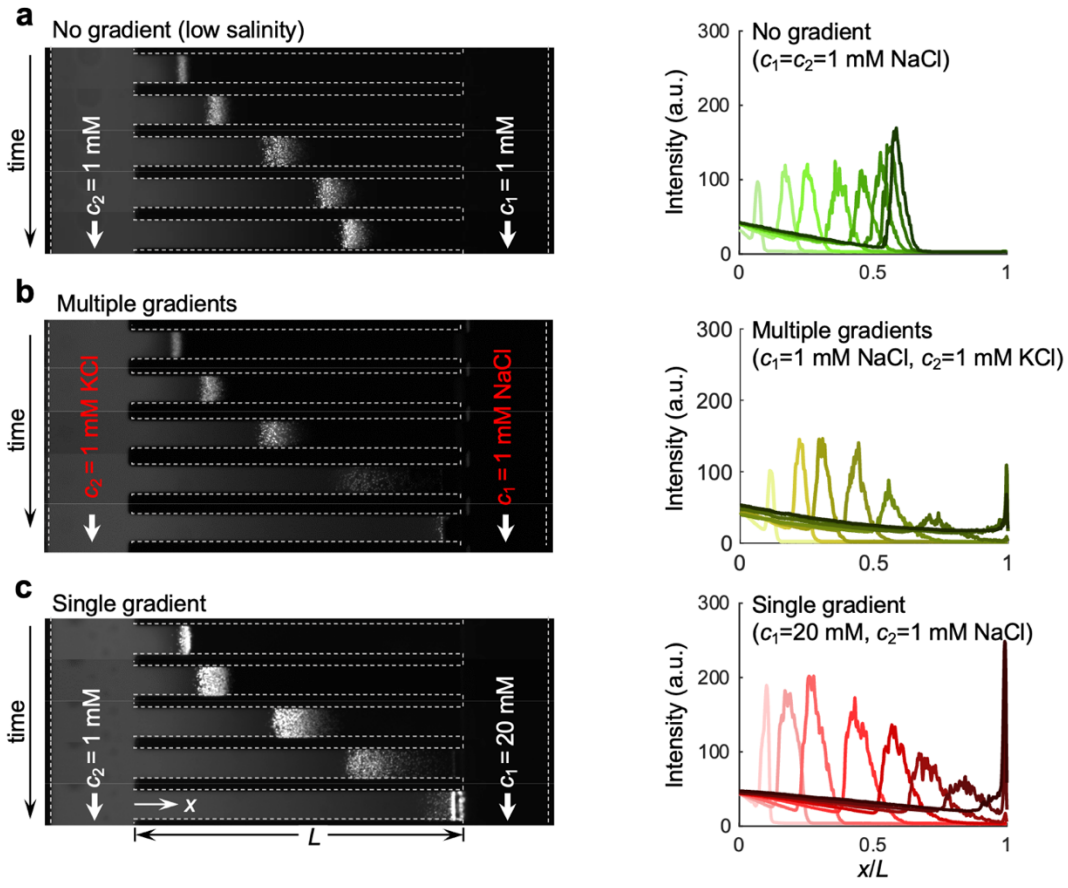

Figure S8. Formation and transport of condensates by interdiffusion of equimolar multispecies solutes. (a) The image sequences the formation and transport $[dT]_{40}$-$[RGRGG]_5$ condensates over a 60-minute period (a) without any salt gradient ($c_1 = c_2 = 10$ mM NaCl); (b) under interdiffusion of equimolar NaCl and KCl ($c_1 = 1$ mM NaCl, $c_2 = 1$ mM KCl) and (c) under NaCl gradient ($c_1 = 20$ mM, $c_2 = 1$ mM). In the case of interdiffusion of equimolar NaCl and KCl in (b), due to the difference in the diffusion coefficients of Na^+ and K^+, a local electrical field arises, causing electrophoresis. As a result, the transport and the formation of the condensate are improved compared to the case of no salt gradients (a). However, the lack of chemiphoresis in (b) due to the equimolar concentration leads to relatively weaker improvement in the transport and formation compared to the single NaCl gradient case in (c).

Comment – Other points: 2. Writing style guides typically recommend paragraphs to be at least two sentences but usually not more than 200 words. The manuscript has a number of paragraphs significantly above 200 words. In my opinion, cutting paragraphs down to mostly < 200 words would help readability.

Our response: We have edited several lengthy paragraphs according to this suggestion by the Reviewer.

Comment – Other points: 3. The title focuses on DP. Hajian and Hardt see more dramatic DP motion. But they don't have the moving set of condensates plus direct mechanism in terms of the range of ratios over which condensates form. So this is a major novelty in the paper, that could be reflected in the title.

Our response: We believe the phrase "*Diffusiophoresis promotes phase separation…*" in the title alludes to the diffusiophoresis-enhanced range of DNA/PP ratios over which condensates form, as the Reviewer mentioned. Therefore, we wish to keep the current title as is.

Final Comment: Overall, a very interesting paper, as you can tell from my questions. Because the system is complex, it is hard to write a clear paper and I think the authors may have missed some important details. I appreciate that answering the questions above will take time, but with results this interesting, I think it is worth taking this additional time to present them more clearly, and with details like what n_D/n_P is made very clear and explicit. As outlined above, I think this is a key feature of the manuscript. They may also want to specify what is the maximum CDP speed consistent with their not observing CDP without salt, i.e., their resolution, which may be around 0.1 μm s^{-1}. It is a little surprising that there is no apparent CDP here, despite the gradients in DNA and PP. For example, it is known that phase separating droplets induce CDP in other nearby phase separating droplets [4]. If the authors are willing to put in this work, I will be happy to read a revised manuscript and I hope to then recommend publication.

Our response: We thank the Reviewer once again for the dedicated review of our work. The constructive feedback helped us to improve our manuscript significantly. We tried very hard to address every question that this reviewer raised and we hope the Reviewer will be persuaded by our overall efforts in revising the manuscript.

Regarding the absence of diffusiophoresis from DNA and PP gradients, this is possibly due to the biomolecules being dilute while the background solutes are concentrated. Given that the hydrodynamic radius of DNAs and PPs is around ~1−2 nm, which is comparable to the Debye layer thickness, chemiphoresis should be neglectable. Therefore, we can expect that diffusiophoresis by DNA or PP gradients is driven mainly by electrophoresis. For multi-species (electro)diffusiophoresis [R9,R10], one may express the velocity as $U_{dp} = \frac{\varepsilon kT}{e\eta}\frac{\sum_i z_i D_i \nabla n_i}{\sum_i z_i^2 D_i n_i} = 0.01\sim0.05$ nm/s, which is indeed negligible compared to the diffusiophoretic velocity driven by salt gradients. We have added this in the main text as follows:

Page 11: "*Furthermore, while one may reason that the gradients of $[dT]_{40}$ and $[RGRGG]_5$ may also induce the diffusiophoresis of the condensates, this is likely to be negligible due to the biomolecules being dilute while the background solutes are relatively concentrated. Given that the hydrodynamic radius of $[dT]_{40}$ and $[RGRGG]_5$ is around 1–2 nm, which is comparable to the Debye layer thickness, chemiphoresis should be insignificant. Therefore, we can expect that condensate diffusiophoresis by $[dT]_{40}$ or $[RGRGG]_5$ gradients*

are mainly driven by electrophoresis. For multi-species (electro)diffusiophoresis, one may express the velocity as $u_d = \frac{\varepsilon kT}{\eta e}\frac{\sum_i z_i D_i \nabla n_i}{\sum_i z_i^2 D_i n_i}$*, where ε is the permittivity,* k_B *is the Boltzmann constant, T is the temperature, e is the elementary charge, and z is the charge number. Taking the summation over all the known background solute species including the buffer, the velocity is estimates to be less than* $u_d = 0.1\, nm/s$*, which is indeed negligible compared to the condensate diffusiophoretic velocity driven by salt gradients.*"

[R9] Chiang, T. Y., & Velegol, D. (2014). Multi-ion diffusiophoresis. *Journal of Colloid and Interface Science*, *424*, 120-123.

[R10] Gupta, A., Rallabandi, B., & Stone, H. A. (2019). Diffusiophoretic and diffusioosmotic velocities for mixtures of valence-asymmetric electrolytes. *Physical Review Fluids*, *4*(4), 043702.

Response to the Reviewer 2's Comments

Comment: In this work, Doan and colleagues use a microfluidic platform to examine the propagation of biomolecular condensates along an ion gradient. The authors observe that an ion gradient promotes both enhanced condensate formation (between the charged peptides and the ssDNA) and quicker migration of the condensates along the gradient. The authors argue that these effects stem from the reentrant phase separation of the system combined with diffusiophoresis. I found this work to be a very creative use of microfluidics to study the motility of condensates. The finding that an ion gradient imparts enhanced condensate formation and motility is a valuable insight to the field and one that deserves more attention. I appreciate the authors effort to parse out the mechanism of condensate propagation and am convinced that their arguments are valid. I do, however, believe that they could use some more evidence to strengthen their argument. There are several points of confusion that I believe would need to be addressed more clearly by the authors.

Our response: We thank the reviewer for this clear and excellent summary of our manuscript. We are also grateful to the reviewer for pushing us to be more precise and clearer.

Comment #1: The data is clear in that the ion gradient speeds up the movement of the phase-separated particles along the wells. They make the distinction that in the wells that have no gradient, the movement of particles is stochastic, and the movement of the condensate comes purely from dissolution on the back of the wave and formation on the front, while in the presence of a gradient there is directional bias in the movement. They support this with kymographs of the 20mM salt non-gradient condition and the 1mM-20mM gradient condition. When looking at the video showing all three conditions, it seems that all three conditions look qualitatively unique. If anything, the particles are much more mobile in the 1mM salt non-gradient condition than in the 20mM salt non-gradient condition. For the rest of the paper, though, the authors focus on comparing solely the 20mM non-gradient sample and the 1mM-20mM gradient sample. To ensure that there are no more confounding effects by the difference in concentrations between the non-gradient conditions (other than those addressed by the authors already), the authors should at least include the kymograph analysis for all the samples tested.

Our response: Following the Reviewer's suggestion, we have added an additional kymograph for the case of condensates forming at low salinity ($c_1 = c_2 = 1$ mM NaCl) in Fig. 2b. While the condensates are more freely suspended, as shown from the jiggly trajectories, we observe no significant directionally movement of condensates, consistent with our original findings. The figure and the caption are reproduced below.

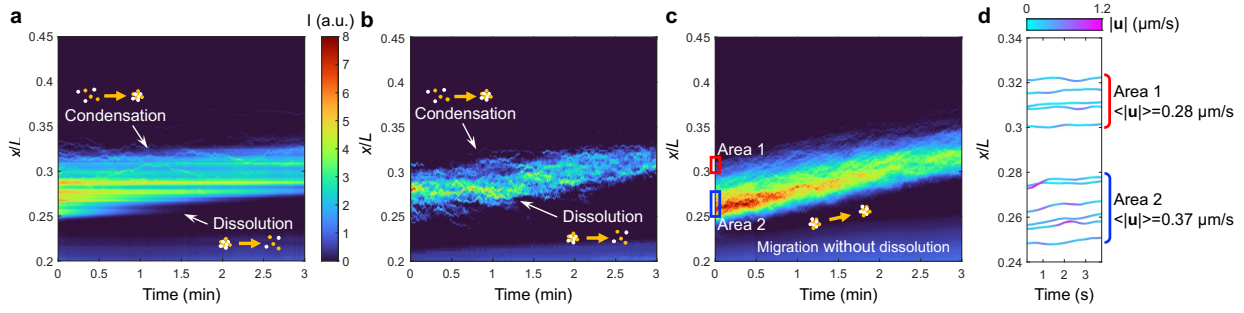

Figure 2. Spatiotemporal dynamics of biomolecular condensates reveal the influences of diffusiophoresis on their formation, transport, and dissolution. (**a**,**b**) Kymographs of the fluorescence signal of the $[dT]_{40}$-$[RGRGG]_5$ condensates without the presence of NaCl gradient ((**a**) $c_1 = c_2 = 20$ mM; (**b**) $c_1 = c_2 = 1$ mM). The waves of the condensate are formed due to simultaneous dissolution near the front and condensation near the rear. (**c**) With NaCl gradients ($c_1 = 20$ mM, $c_2 = 1$ mM), the formation of the droplets with the migration toward the higher salt concentration prevents the condensates from dissolution. The color code represents the fluorescence intensity I. (**d**) The trajectories of the individual condensates found near the front ($x/L \sim 0.31$, area 1) and rear ($x/L \sim 0.26$, area 2) of

the wave in (**c**). The color code represents the velocity magnitude $|\boldsymbol{u}|$. $\langle|\boldsymbol{u}|\rangle$ represents the mean of the time-averaged velocity magnitude of multiple condensates in each region.

Comment #2: The authors state in the paper "the diffusiophoretic velocity scales with the gradient of the logarithm of the salt concentration…the velocity is influenced by the absolute salt concentration where the biomolecules undergoing diffusiophoresis slow down as they migrate toward higher salt concentrations, leading to their accumulation" (pg. 6). Here they were referencing the behavior of molecules undergoing diffusiophoresis under gradient conditions. If the phase separation is in fact a result of diffusiophoresis and the diffusiophoretic velocity scales with the gradient of the logarithm of the salt concentration, then it would go a long way in supporting the authors arguments if they tested condensate motility under at least one other gradient condition. The authors do all their experiments under the 1-20 mM gradient condition. There is a clear effect from non-gradient to gradient conditions, but if diffusiophoresis promoting phase separation, then there would presumably be a quantifiable difference between 1-10 mM (as an example) and 1-20mM.

Our response: This is an insightful suggestion. To be fully responsive to this point, we have performed additional experiments at different gradient conditions (c_1 = 40 mM and c_2 = 20 mM NaCl). Although the magnitude of the salt gradients is roughly the same in both cases (c_1 = 40 mM, c_2 = 20 mM NaCl and c_1 = 20 mM, c_2 = 1 mM NaCl), the logarithmic dependence of diffusiophoresis leads to the stronger formation and transport of the condensates in the case of 1 mM and 20 mM NaCl. We have mentioned these results in the main text, added the plots in the Supplementary Information as Fig. S4, and reproduced below.

Page 5: "*These negatively charged condensates migrating toward the higher NaCl concentration side is consistent with previously reported diffusiophoresis of negatively charged colloids in NaCl gradients.*[37–39] *The logarithmic sensing of diffusiophoresis, i.e., the diffusiophoretic velocity dependent on the gradient of the logarithm of the salt concentration (**Supplementary Fig. S4**), further strengthens the nature of the directional migration being diffusiophoresis.*[40,41]"

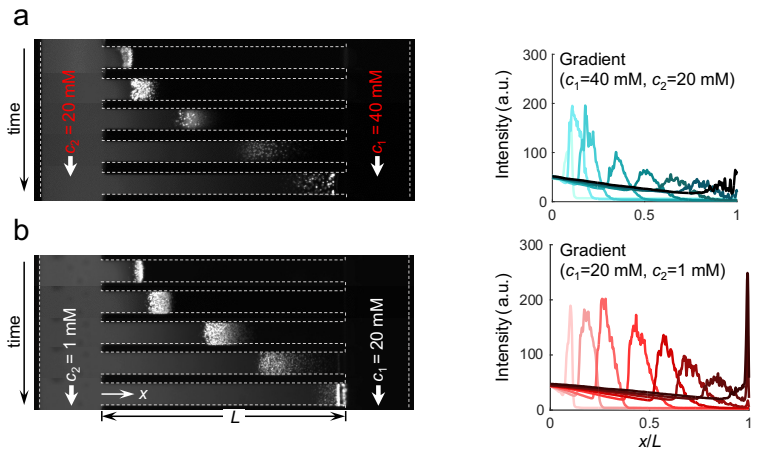

Figure S4. Formation and transport of the condensates under different salt contrasts. The image sequences and their corresponding width-averaged intensity distribution of the formation and transport $[dT]_{40}$-$[RGRGG]_5$ condensates over 60 minutes. The NaCl concentrations are (a) c_1 = 40 mM, c_2 = 20 mM, and (b) c_1 = 20 mM, c_2 = 1 mM. The concentration difference is nearly identical in both cases (~20 mM). However, the formation and transport of the condensates in (b) are stronger than in (a) due to the logarithmic dependence of diffusiophoresis.

Comment #3: The authors chose to do a majority of their experiments at an excess ssDNA condition ($[dT]_{40}$:$[RGRGG]_5$ = 1.25:1), which confers a negative surface charge to the particles. I appreciate keeping the condition consistent for purposes of comparison, but I believe the authors should provide more justification for this choice. They do one experiment with excess positive charge (Supplementary Fig. S5),

but the behavior seems to be different, and they use a different salt (KAc) than the one they use for the rest of the experiments (NaCl). The authors discuss the differences between diffusiophoretic behavior between individual positive and negative molecules but do not expand on the differences between these effects in their condensate model. I believe that expanding on these distinctions would add more clarity to their model.

Our response: The main reason for the choice of maintaining the concentration at $[dT]_{40}$:$[RGRGG]_5$ = 1.25:1 was that this condition corresponds to a highly favorable condition for condensate formation (Fig. S6). As the Reviewer correctly pointed out, this condition results in negatively charged condensates (Fig. S3), which leads to the diffusiophoresis of condensates along the NaCl gradients. The main intent of using NaCl, however, was to accelerate the motion of biomolecules, i.e., $[dT]_{40}$ and $[RGRGG]_5$, towards each other. In the same spirit, for the positively-charged peptide-ssDNA condensates, where $[RGRGG]_5$ is introduced on the left and $[dT]_{40}$ on the right side of the channel, we chose to use KAc in order to accelerate the motions of the peptide and ssDNA toward each other since KAc has a positive β factor. Diffusiophoresis of peptide-rich condensates did not show any significant motion (Fig. S7b) due to the low zeta potential caused by the asymmetric charge inversion discussed in the Supplementary Information (Fig. S3). Further discussion regarding this aspect is added in Supplementary Information as follows:

Page 4 in SI: "*Because of the opposite configuration, we choose potassium acetate (KAc) as the solute, which can drive $[RGRGG]_5$ up the gradient and $[dT]_{40}$ down the gradient due to the positive β factor (β = 0.29) such that diffusiophoresis is expected to accelerate their migration toward each other.*"

Comment #5: I am curious about any differences depending on the ion of choice. The authors show clear differences in the non-equilibrium behavior in the $[dT]_{40}$ and $[RGRGG]_5$ in the presence of different monovalent salts (LiCl, NaCl. KCl). I wonder how this would manifest in the combined system. Also, the authors bring up the relevance of calcium as an essential biological ion. Would these behaviors be enhanced by the addition of a divalent ion gradient?

Our response: Indeed, the combined system with the presence of various salts diffusing into one another can also lead to the diffusiophoresis of the condensates, as we have shown with latex particles in the past [R11]. To demonstrate this with condensates, we have now conducted additional experiments with interdiffusion of NaCl and KCl at the same concentration (1 mM). Due to the difference in the diffusivity between ions Na^+, K^+, and Cl^-, a local electrical field is generated to drive (electro)diffusiophoresis of condensates. We have mentioned these results in the main text and added the plots in the Supplementary Information as **Fig. S8**, which we have reproduced below.

Page 13: "*…For instance, the interdiffusion of equimolar multicomponent solutes of Na^+ and K^+ can also induce significant diffusiophoresis and enhanced phase separation (**Supplementary Fig. S8b**)…*"

Regarding the point of a *divalent ion gradient*: The use of divalent ions will reduce diffusiophoresis to some degree based on the classical theory, yet still expected to induce noticeable motion, as shown in the recent literature [R12]. Regarding the influence of divalent ions on phase separation, our previous studies have shown that RNA alone can phase separate in the presence of divalent ions [R13, R14, R15]. We showed that Mg^{2+} can bridge the interactions between the phosphate groups of RNA, enabling them to undergo phase separation [R15]. Moreover, the RNAs in the presence of divalent ions can exhibit temperature-dependent reentrant phase transition [R13]. In the presence of Ca^{2+} and Mg^{2+}, poly(rU) can form droplets, and an increase in the divalent ion concentration can lead to the dissolution of heterotypic polypeptide-RNA droplets and the formation of homotypic RNA alone droplets [R13]. Therefore, the presence of divalent ions will bring an additional level of complexity for the driving forces for the phase separation wherein the NAs can phase separate with divalent ions bridging the NAs, in addition to the phase separation driven solely by the obligate heterotypic interactions between the polypeptides and the NAs as observed in the case of monovalent ions. We, therefore, respectfully submit that future studies can explore such complexities in greater detail.

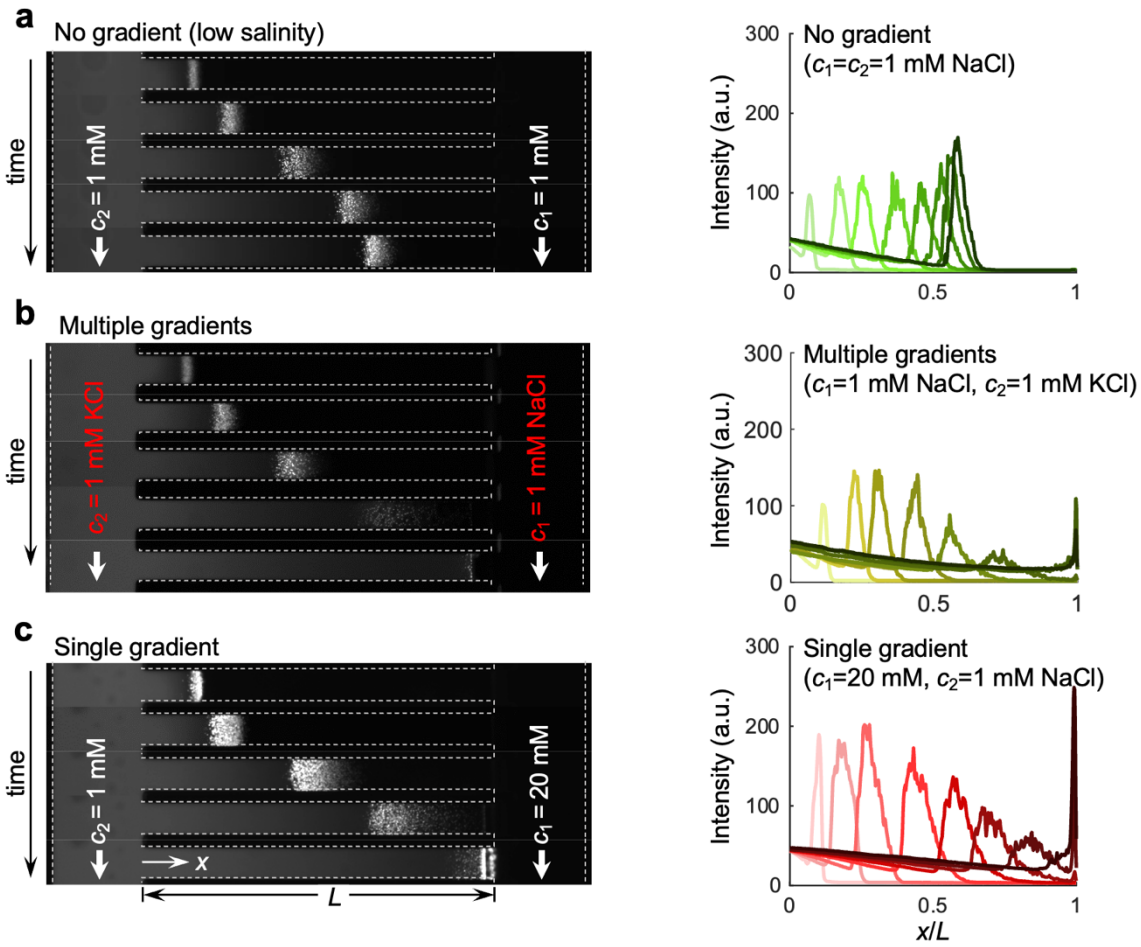

Figure S8. Formation and transport of condensates by interdiffusion of equimolar multispecies solutes. (a) The image sequences the formation and transport $[dT]_{40}$-$[RGRGG]_5$ condensates over a 60-minute period (a) without any salt gradient ($c_1 = c_2 = 10$ mM NaCl); (b) under interdiffusion of equimolar NaCl and KCl ($c_1 = 1$ mM NaCl, $c_2 = 1$ mM KCl) and (c) under NaCl gradient ($c_1 = 20$ mM, $c_2 = 1$ mM). In the case of interdiffusion of equimolar NaCl and KCl in (b), due to the difference in the diffusion coefficients of Na^+ and K^+, a local electrical field arises, causing electrophoresis. As a result, the transport and the formation of the condensate are improved compared to the case of no salt gradients (a). However, the lack of chemiphoresis in (b) due to the equimolar concentration leads to relatively weaker improvement in the transport and formation compared to the single NaCl gradient case in (c).

[R11] Shin, S., Um, E., Sabass, B., Ault, J. T., Rahimi, M., Warren, P. B., & Stone, H. A. (2016). Size-dependent control of colloid transport via solute gradients in dead-end channels. *Proceedings of the National Academy of Sciences*, *113*(2), 257-261.

[R12] Wilson, J. L., Shim, S., Yu, Y. E., Gupta, A., & Stone, H. A. (2020). Diffusiophoresis in multivalent electrolytes. *Langmuir*, *36*(25), 7014-7020.

[R13] Onuchic, Paulo L., et al. "Divalent cations can control a switch-like behavior in heterotypic and homotypic RNA coacervates." *Scientific Reports* 9.1 (2019): 12161.

[R14] Pullara, Paul, Ibraheem Alshareedah, and Priya R. Banerjee. "Temperature-dependent reentrant phase transition of RNA–polycation mixtures." *Soft Matter* 18.7 (2022): 1342-1349.

[R15] Wadsworth, Gable M., et al. "RNAs undergo phase transitions with lower critical solution temperatures." *Nature Chemistry* (2023): 1-12.

Response to the Reviewer 3's Comments

Comment: The manuscript reports the results of an experimental investigation of the impact of ion gradients on transport processes of biomolecular condensates on the mesoscale and biomolecules on the microscale. A microfluidic platform and fluorescence microscopy are used to demonstrate that ion concentration gradients can accelerate the transport of biomolecules, including nucleic acids and proteins, via diffusiophoresis. This hydrodynamic transport process is shown to facilitate localized enrichment of biomolecules, thereby promoting the location-specific formation of biomolecular condensates via phase separation, as well as active motility of condensates, resulting in enhanced diffusion along the gradient. It is shown that the gradient-induced active motility leads to a dynamical redistribution of condensates that extends their lifetime. The in vitro experiments of biomolecular condensates formed by RGG repeat polypeptides and ssDNA in salt gradients show several properties and behaviors, which look very intriguing to me. However, I am not familiar enough with neither biomolecular condensates nor with diffusiophoresis, to be able to judge the validity and importance of the presented results. This also includes a lack of knowledge of the relevant literature. Therefore, I can only make a few rather general remarks:

Our response: We thank the Reviewer for reviewing our manuscript. We are happy to hear from the Reviewer that our manuscript is "very intriguing."

Comment #1: I doubt that there can be significant spatiotemporal gradients of temperature and pressure inside a cell.

Our response: We appreciate this point raised by the reviewer. The alteration in the environment can induce a spatiotemporal temperature gradient within the cell. For instance, changes in temperature gradients have been observed in murine fibroblast cells in response to abiotic stress [R16]. In another case, the temperature of HeLa cells fluctuates during cell metabolism [R17]. Recent thermometry studies have shown that the nucleus and the other organelles can lead to local variations in intracellular temperatures due to their behaviors as local heat sources [R18]. Moreover, using quantum dot-based fluorescence imaging, it was shown that the neurites can have different temperatures from the cell body [R19]. These studies signify the presence of a temperature gradient inside the cell. Similarly, pressure gradients are also evident within cells. For example, variations in salt concentration can lead to both osmotic and hydraulic pressures across the cell [R20]. During the cell protrusion of *Entamoeba histolytica*, the pressure gradient also dynamically changes over time [R21]. We have added some of these discussions in the main text as follows:

Page 2: "*For instance, thermometry studies have shown that the nucleus and other intracellular organelles can lead to local variations in intracellular temperatures due to their behaviors as local heat sources.*[14] *Pressure gradients are also evident within the living cells where the variations in the ion concentration lead to both osmotic and hydraulic pressures across the cell.*[15]"

[R16] Yang, J. M., Yang, H., & Lin, L. (2011). Quantum dot nano thermometers reveal heterogeneous local thermogenesis in living cells. *ACS Nano*, *5*(6), 5067-5071.
[R17] Donner, J. S., Thompson, S. A., Kreuzer, M. P., Baffou, G., & Quidant, R. (2012). Mapping intracellular temperature using green fluorescent protein. *Nano Letters*, *12*(4), 2107-2111.
[R18] Okabe, Kohki, et al. "Intracellular temperature mapping with a fluorescent polymeric thermometer and fluorescence lifetime imaging microscopy." *Nature Communications* 3.1 (2012): 705.
[R19] Tanimoto, Ryuichi, et al. "Detection of temperature difference in neuronal cells." *Scientific Reports* 6.1 (2016): 22071.
[R20] Li, Y., Konstantopoulos, K., Zhao, R., Mori, Y., & Sun, S. X. (2020). The importance of water and hydraulic pressure in cell dynamics. *Journal of Cell Science*, *133*(20), jcs240341.
[R21] Boquet-Pujadas, A., Lecomte, T., Manich, M., Thibeaux, R., Labruyère, E., Guillén, N., ... & Dufour, A. C. (2017). BioFlow: a non-invasive, image-based method to measure speed, pressure and forces inside living cells. *Scientific Reports*, *7*(1), 9178.

Comment #2: How can a (diffusiophoretic) mobility be viewed effectively as an enhanced diffusivity? The time dependence of the mean squared displacement should display different power laws in the two cases.

Our response: In essence, solute gradients drive diffusiophoresis where the particle motion is slaved by the diffusion of solutes. Therefore, diffusiophoresis should also be diffusive [R22]. This allows us to treat diffusiophoresis as an additional drift term in the flux equation with the driving force being solute gradients, rendering diffusion. This diffusive aspect of diffusiophoresis is also demonstrated in detail in previous publications [R23, R24].

[R22] Velegol, D., Garg, A., Guha, R., Kar, A., & Kumar, M. (2016). Origins of concentration gradients for diffusiophoresis. *Soft Matter*, *12*(21), 4686-4703.
[R23] Ault, J. T., Warren, P. B., Shin, S., & Stone, H. A. (2017). Diffusiophoresis in one-dimensional solute gradients. *Soft Matter*, *13*(47), 9015-9023.
[R24] Ault, J. T., Shin, S., & Stone, H. A. (2018). Diffusiophoresis in narrow channel flows. *Journal of Fluid Mechanics*, 854, 420-448.

Comment # 3: The length scales in the experiment (channel width about 100 microns, channel length about 1 mm) are much larger than typical cell dimensions. Can the results be expected to hold on the cell scale?

Our response: As mentioned above, diffusiophoresis is diffusive. Therefore, the transport dynamics should be self-similar such that the overall dynamics are identical regardless of length/time scales when rescaled by diffusion length and time scales. In this regard, we expect our results to hold in cellular scales. It was argued previously that inside metabolically active cells, diffusiophoresis can significantly contribute to the transport of cellular species if particle sizes are tens of nanometers or more [R8].

Comment #4: Are salt gradients in cells typically of similar magnitude as studied in the current in vitro experiment? Which mechanism in the cell would be able and sustain such gradients?

Our response: Living cells are complex entities, and the size of gradients of molecular solutes in the cytoplasm remains largely unknown due to the experimental limitations as well as the complexity and heterogeneity of the cell, the magnitude of the gradients is expected to be much smaller than in the current experiments. Nevertheless, small chemical gradients are still expected to induce considerable diffusiophoresis in cells. For instance, a recent estimation of ATP gradients in *E. coli* is predicted to be around $10^5 - 10^6$ μm^{-4} owing to the fast turnaround rate of ATPs in the cytoplasm [R8]. These gradients are expected to induce the motion of nanoscale colloids with a speed of ~0.1 μm/s, which happens to coincide with the speed of condensates we observed in our study. This speed is also consistent with the experimental observation by Parry et al. where they measured the metabolic-dependent motion of nanoscale biocolloids (enzyme granules, plasmid, and viral proteins) in *E. coli* [R25]. In their study, the distribution of the displacements was shown to be far from Gaussian, implying the non-uniform nature of the cytoplasm. The complexity of the cytoplasm, however, implies that the directional migration is difficult to unambiguously show that the motion is solely due to one specific mechanism. In this regard, our investigation provides a clear understanding of how diffusiophoresis alone can impact the transport of DNA/polypeptide condensate systems without other mechanisms involved.

[R25] Parry, B. R., Surovtsev, I. V., Cabeen, M. T., O'Hern, C. S., Dufresne, E. R., & Jacobs-Wagner, C. (2014). The bacterial cytoplasm has glass-like properties and is fluidized by metabolic activity. *Cell*, *156*(1), 183-194.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The results are impressive and interesting, as I noted in my first review. The authors have fixed a number of omissions - eg saying DNA also has fluorescent tags in some experiments. I thank them for that. The reader can now understand much of what the authors do. Thanks also for shrinking paragraphs to a more manageable length. I think the results are worthy of publication and would like to see them published. I would not write the paper as the authors do but it is their paper not mine, and endless rounds of revision are not useful. I do have some comments below that I think need addressing, so a third round is needed but I hope this can be kept small then Nature Comms should decide to publish/not publish.

Response to the revisions and authors' response: The authors have put a lot of work in to improve the manuscript. Thank you. I agree with their replies, with a couple of exceptions.

Comment #7 I think the authors are implying that nucleation and growth is much faster than the time for the biomolecules to diffuse the approx 1 mm length of their system. Other than that the timescale they observe is consistent with this, I don't see any direct evidence of this. If they have direct evidence, this would strengthen the paper, as it is important to understand what are the characteristic timescales in a system. This evidence may be in condensate trajectories which I cannot resolve in Fig 2, if that is what Fig. 2 shows.

Comment #8 [R6] has chemical reactions in them, so I am sorry I do not see the relevance. The authors do not mention any irreversible chemical reactions in their system.

Comment - Other points: I am not sure I find either the title or abstract very clear but it is the authors' paper.

Final comment: I am sorry I do not see any direct evidence in the manuscript that chemiphoresis is dominated by electrophoresis. I understand the direction of diffusiophoresis is consistent with electrophoresis but this is not, I think, strong evidence.

In terms of the other two reviewers' comments: I agree with them, and they make good points. In particular, reviewer 3 is correct to doubt that there are temperature and pressure gradients in cells. I return to temperature gradients below. Regarding pressure gradients, I note that the examples given by the authors in their reply are very special cases.

I think I can illustrate the differences between how I would approach paper writing and the authors by discussing the section “The promotion of phase separation is a ….”. When I give students feedback on report writing I tell them to try and avoid vague words like “promote”. Also, they see phase separation with and without salt gradients so I am sorry I am not sure that they have evidence that diffusiophoresis driven by salt gradients increases the amount of phase separation in any way they have quantified in the manuscript. However, the word “promote” is so vague that if the authors want to use it is hard for me to say they are wrong. Maybe it is justified by some intensity peaks being of order 10% higher with a salt gradient than without, but I am not sure that their most interesting result is an increase of roughly 20% in a peak intensity?

In this section they have some new and interesting results in Fig 3. These show the oppositely charged DNA and peptide molecules being pushed in opposite directions by salt gradients. This is what we should expect but it is very nice to see this data. It would be good to publish it.

However I think they are using Fig 3 to help them claim “This local enrichment of both of these oppositely charged biomacromolecules directly promotes the phase separation …”. I am sorry I can see no direct evidence of this in the manuscript. Phase separation is a complex, extremely non-linear, phenomena that is tough to understand and model. For example, I would guess that phase separation tends to flatten out gradients in the DNA and peptide, because the system tends towards the equilibrium coexistence, which is gradient free. This may or may not be why they see, if I understand correctly, little diffusiophoresis with gradients in DNA and the peptide.

The complexity of phase separation affects our ability to interpret the authors’ results. For example they have a number of plots of intensity along the channel, and I think the idea is that the peaks in fluorescence intensity are where phase separation occurs. But phase separation should not directly affect intensity (I think this is what the authors assume). Presumably what happens is that phase separation dramatically reduces mobility of the phase separating molecules (as they form slow moving condensates), and so phase separated regions act as sinks for the fluorescent molecules and hence become brighter. If this is correct then the peaks in intensity observed by the authors are slightly indirect consequences of phase separation, which makes it hard to prove or even disprove statements like “promotes phase separation”.

The complexity is impressive in simply phase separating oil and water, and it is doubly so with their system of larger charged molecules where the species they are using to drive diffusiophoresis (salt) also affects phase separation.This combination of an intrinsically complex phenomena and a rather complex system, makes interpreting results difficult. I do not understand much of what is going on, and after reading two drafts very carefully, I think the authors do not understand much of what is going on, either.

But then I think it is unhelpful to use phrases such as “promote the phase separation”. A clearer paper would present results and be clearer on what can and cannot be inferred from these results. But as noted above it is their paper not mine and I cannot write it for them. Statements which I think are more definitive than I would write occur throughout the manuscript.

Could I also comment on Fig 2. Is what we are seeing in Fig 2a, b and c, many individual trajectories of individually tracked condensates - as the bottom paragraph of page 5 seems to imply - or is an average intensity - as the intensity scale bar for Fig 2a seems to imply. Also, in this bottom paragraph the authors state “… delays their dissolution” while in the caption of Fig 2 they say “prevents the condensates from dissolution”. Is it “delay” or “prevent”?

Also if indeed they track individual condensates then these trajectories must contain huge amounts of information that is missing from Fig 2 but would help understand the system. For example, if individual condensates are tracked then presumably they could determine how long they live for, and whether they are present at the end of the experiment. This would allow the authors to show readers if it is delay or prevent. At the top of page 10 the authors say “condensate streaks that fade in and out..” which again suggests that they are tracking individual condensates. But I am sorry I cannot resolve individual streaks in Fig 2. If they can track individual condensates they will have to replot.

Fig 2 also has Fig 2d which has estimated speeds. I am sorry I do not see how these speeds are estimated. It would help if the authors explain this.

I am sorry I also need to highlight the aspect in which unfortunately the manuscript has been made worse. This is the citing of reference 14 (Okabe et al, Nat Comm (2012)) to support the idea of temperature gradients. This paper is clearly wrong, as has been shown for example by Buffou et al (https://www.nature.com/articles/nmeth.3073) in 2014. Temperature differences of order 0.1C across cells, as Okabe et al claim, are unsustainable. As I am someone who understands the basics of thermal diffusion - unlike Okabe et al. and the reviewers Nat Comm employed in 2012 - the continued credence given to these claims of temperature gradients is irritating.

See Buffou et al (2014) for details but the argument is basically trivial. Consider a cell 10 micrometres across, then if there is a temperature difference of 0.1 C across it, there is a flux of thermal energy across the cell - due to the inevitable thermal diffusion that occurs whenever there is a temperature gradient. The thermal conductivity of tissue is roughly similar to that of water which is order k = 1 W/(K m). So the power (W) of the thermal transport across the cell is just the product of the thermal conductivity, the T gradient which is of order 10,000 K/m, and the cross-

sectional area of the cell which is 100 micrometres squared. I.e., thermal flux (1D x axis for simplicity) $j = - k \, d T/ d x$ with power just flux times area A so power $P = kA \, d T/ d x$.

This is a power P of order 1 microWatt. The human body has roughly 10 trillion cells, so if each has a power consumption of a microWatt that implies that humans have a power consumption of order 10 million W. The actual figure for humans is 100 W (approximately equivalent to the 2000 kilocalories per day we see on the back of food packets). The two figures differ by a factor of 100,000! Our bodies cannot of course sustain 10 million W as there is no chemical source of power for it, and even if there was, the dissipated heat would cause us to boil.

My estimate of 1 microW is of course just a rough estimate, for example the power consumption of one of our cells almost certainly can peak at larger values than a 100 W/(10 trillion) = 10 pW. For example Buffou et al. quote 100 pW. However, the cell power consumptions needed to sustain 0.1 C temperature differences across cells 10 micrometres across (for any sensible length of time) are clearly ridiculous. And for 100 nm mitochondria the numbers are even more ridiculous as the gradients scale inversely with lengthscale.

In short either our understanding of thermal transport - well tested over perhaps 200 years now - is wrong by orders of magnitude for cells. Or Okabe et al, Nat Comm (2012) are wrong. I would bet a lot of my money on our understanding of thermal transport applying - to within an order of magnitude or two - to cells.

In an ideal world, Nat Comm would put something like an expression of concern on articles like that of Okabe et al.. In the absence of such markers, authors like Doan et al., should do adequate literature reviews on temperature gradients in cells and see that claims such as those of Okabe et al. are very unlikely to be correct. I cannot recommend publication of a manuscript that uncritically cites such a misleading paper. If the authors wish to mention temperature gradients in cells - and they don't have to mention these gradients as they don't exist - then they should cite papers that are correct, such as that of Buffou et al..

Data availability: The authors state that "all relevant data are included". This is a bit inaccurate. They don't include any data, such as tracked condensate positions, sizes etc. I don't know what NSF requirements are, but some funders are encouraging authors to share the data, eg the data which underlies or is plotted in figures. The authors do not do this. Including some data would improve the paper's contribution to the field.

Richard Sear, 29th Feb 2024

Reviewer #2 (Remarks to the Author):

The authors have addressed all issues raised by this reviewer. I therefore endorse this paper for immediate publication in Nature Communications.

Reviewer #4 (Remarks to the Author):

My report is attached as a pdf file.

The manuscript by Doan et al. reports the use of a cleverly designed microfluidic device to investigate the effect of salt concentration gradients on the transport of nucleic acids, proteins and their condensate. The experimental findings clearly show that diffusiophoresis can (i) enhance the formation of biomolecular condensates by increasing the transport rates of their molecular constituents and (ii) promote the directed motion of condensates along the gradients. These results are not only exciting, but also very intriguing to an interdisciplinary audience, improving our understanding of the potential role of diffusiophoresis in biological systems.
Overall, the manuscript is very well written. The proposed interpretations of the underlying physical mechanisms are well supported by the experimental observations. However, there a few important points that deserve further clarifications and discussions.

1) In their response, Authors have addressed all points raised by Reviewer 3. However, I disagree with their answer to Comment #2. I would say that diffusiophoresis per se is not a diffusive process. Diffusiophoresis transport may appear as a diffusive process under certain conditions. More specifically, for colloids exposed to a time-dependent inhomogeneous salt concentration field, c, that is relaxing by diffusion, the gradient of $\log c$ scales as

$$\nabla \log c \simeq \frac{\Delta c}{c\ \delta_c} \simeq \frac{1}{\sqrt{D_s\ t}}$$

where δ_c is the salt diffusion length scale, D_S the salt diffusivity and t the time. Therefore, the diffusiophoresis velocity of the particle can be written as

$$u_{DP} = \frac{\mathrm{d}x_p}{\mathrm{d}t} = M_{DP} \nabla \log c \simeq \frac{M_{DP}}{\sqrt{D_s\ t}}$$

where M_{DP} is the diffusiophoresis mobility and x_p the position of the particle in a 1D domain. By integrating over time, one gets

$$x_p(t) - x_p(0) \simeq \sqrt{D_{eff}\ \ t}$$

where $D_{eff} \propto \frac{M_{DP}^2}{D_s}$ is an effective diffusion coefficient. Hence, in this case diffusiophoresis can be viewed effectively as an enhanced diffusivity, and the mean square displacement of the particle scales as t. These are similar conditions to the ones examined in the references R22, R23, R24 cited by the authors in their response to Reviewer #3. However, for instance, if the salt concentration field is stationary, as it is between a low and high salt concentration reservoir after the initial relaxation period, the term $\nabla \log c$ is time-independent and it follows

$$x_p(t) - x_p(0) \simeq\ D_{DP} \nabla \log c\ \ t$$

Therefore, in this case the diffusiophoresis transport is not diffusive and, as remarked by Reviewer #3, the mean square displacement of the particle scales with a different power of time, namely it scales as t^2.

Consequently, the following sentence at page 8 of the manuscript

"*Given the units for mobility being identical to the diffusivity, diffusiophoretic mobility can alternatively be viewed effectively as an enhanced diffusivity*."

should be revised by highlighting under what conditions the diffusiophoretic transport has a diffusive character.

2) At pH~7, Alexa 488 dye carries two negative charges[1] whereas cy5 dye carries one positive charge[2]. Now in experiments in Fig.1 and 2, the ssDNA is tagged with Cy5 whereas $[RGRGG]_5$ is not fluorescently labelled. Conversely, for the experiments in Fig.3 both ssDNA and $[RGRGG]_5$

are tagged with Cy5 and Alexa 488, respectively. Finally, for zeta potential and turbidity measurements, neither the peptide or ssDNA is tagged with a fluorescent dye. It is known that fluorescent tags can alter the charge and, consequently, the behaviour of biomolecules[3-5]. How do these additional charges affect the diffusiophoresis mobility of fluorescently-labelled ssDNA and $[RGRGG]_5$ and the phase separation processes? What are the experimental limitations introduced by the necessary use of fluorescent tags for the investigation of the molecule/condensate dynamics? Should have the zeta-potential and turbidity measurements been performed with tagged-molecules to replicate the experimental conditions within the microfluidic systems?

4) The diffusiophoresis process, described in the paper, is not an active transport mechanism (like self-diffusiophoresis). Hence, the terms “active motility”, “active migration”, “active transport”, etc. should not be used to describe this process.

5) In Section “Salt-dependent diffusiophoretic mobility of biomolecules” in the Supplementary Material, it is correctly stated that the zeta potential scales with the local salt concentration c as $\zeta \sim 1/\sqrt{c}$. However, it appears that zeta potential measurements have been performed in absence of NaCl salt. Why were not ζ measurements taken at 1mM and 20mM salt concentrations? Mayne negligible effects are expected at these salt concentrations?

6) It appears that the role of diffusioosmosis in the microfluidic channel was not mentioned in the manuscript. Could the Authors comment in the manuscript on whether they expect negligible effects of diffusioosmosis driven flows within the dead-end channel?

7) I cannot find in the revised manuscript information regarding the channel depth. I advise to include all key dimensions of the devices in the Supplementary Material Section “Microfluidic Device Fabrication”.

8) Unless I am mistaken, it appears that Fig. 2d is not mentioned and discussed in the main text of the manuscript. If so, it is recommended to include a description of Fig. 2d within the main text.

To conclude, this is an excellent manuscript, and I recommend the publication, provided the points above are addressed.

PS: Reviewing the manuscript, I noticed a couple of typos

- Main manuscript: Pg 8, line 6 of the first paragraph, “Both The […]”

- Supplementary Material: Figure S7, caption, I think it should read “[…] or (d) with NaCl gradients […]” instead of “[…] or (d) with NaCl gradients […]”

References

[1] Yin, L., Wang, W., Wang, S., Zhang, F., Zhang, S., & Tao, N. (2015). How does fluorescent labeling affect the binding kinetics of proteins with intact cells?. *Biosensors and Bioelectronics*, *66*, 412-416.

[2] https://www.sigmaaldrich.com/GB/en/technical-documents/technical-article/genomics/gene-expression-and-silencing/analyzing-fluorescent-dye-dna-labeling

[3] Quinn, M. K., Gnan, N., James, S., Ninarello, A., Sciortino, F., Zaccarelli, E., & McManus, J. J. (2015). How fluorescent labelling alters the solution behaviour of proteins. Physical Chemistry Chemical Physics, 17(46), 31177-31187.

[4] Gajraj, A.; Ofoli, R. Y. Effect of extrinsic fluorescent labels on diffusion and adsorption kinetics of proteins at the liquid-liquid interface. Langmuir 2000, 16, 8085– 8094, DOI: 10.1021/la000305r
View

[5] Bingaman, S.; Huxley, V. H.; Rumbaut, R. E. Fluorescent dyes modify properties of proteins used in microvascular research. *Microcirculation* **2003**, *10*, 221– 231, DOI: 10.1080/713773616

Response to the Reviewer 1's Comments

Comment #1: The results are impressive and interesting, as I noted in my first review. The authors have fixed a number of omissions - eg saying DNA also has fluorescent tags in some experiments. I thank them for that. The reader can now understand much of what the authors do. Thanks also for shrinking paragraphs to a more manageable length. I think the results are worthy of publication and would like to see them published. I would not write the paper as the authors do but it is their paper not mine, and endless rounds of revision are not useful. I do have some comments below that I think need addressing, so a third round is needed but I hope this can be kept small then Nature Comms should decide to publish/not publish.

Response to the revisions and authors' response: The authors have put a lot of work in to improve the manuscript. Thank you. I agree with their replies, with a couple of exceptions.

Our response: We thank the Reviewer for taking the time once again to provide constructive feedback and a critical assessment of our work. We hope our response, which includes new experimental results and further clarifications in texts, fully addresses the Reviewer's comments and concerns.

Comment #2: I think the authors are implying that nucleation and growth is much faster than the time for the biomolecules to diffuse the approx 1 mm length of their system. Other than that the timescale they observe is consistent with this, I don't see any direct evidence of this. If they have direct evidence, this would strengthen the paper, as it is important to understand what are the characteristic timescales in a system. This evidence may be in condensate trajectories which I cannot resolve in Fig 2, if that is what Fig. 2 shows.

Our response: In our experimental system to probe diffusiophoresis, it is difficult to probe the nucleation and growth dynamics of the condensates over an extended period of time (> 10 minutes) due to the limitation in the continuous imaging of Brownian condensates and the continuous shift of the phase-separating window, causing the visualization of the condensates short-lived. Figure R1 shows the condensate images around the wave over time, which is reconstructed from Figure 1c,d in the manuscript (Movie S1). When the biomolecules are initially mixed, the condensates are nucleated immediately within the first few seconds. Since the phase-separating window (i.e., condensate wave) moves relatively fast in the early stage and the window is narrow (due to the steep biomolecular gradients), this condition indicates that the nucleated condensates will only stay within the window for a short period of time (< 1 minute). Toward the later stage (> 10 minutes), where the wave slows down and the window broadens, the condensates tend to stay within the window for a much longer period (~10 minutes). Nonetheless, the images taken after 10 minutes do not exhibit any further noticeable difference.

For the case of NaCl gradients (Figure R1, bottom panel), the condensates as early as 1 minute appear to be already similar to the ones after 10 minutes. This is likely due to the diffusiophoretic advection by NaCl gradients, forcing the condensates to move along with the wave and stay within the moving phase-separating window, which aligns with the main claim of this study. This feature allows the condensates to grow over time. The visual similarity between the 1-minute and 10-minute condensates indicates that the nucleation and growth occur within a minute.

We note that the phase-separating window is associated with a spatially varying DNA-to-peptide ratio, which will affect the local growth rate due to the varying interfacial conditions. This has been published by the Banerjee group [R1; Ref. 29 in the main text]. This spatiotemporal inhomogeneity and variations in interfacial charge as a function of mixture stoichiometry add an extra layer of complexity to quantifying the coarsening dynamics. Nevertheless, Figure R1 shows that the condensates form and grow immediately upon mixing, so their condensation/dissolution occurring through the moving window is limited by the transport of the biomolecules.

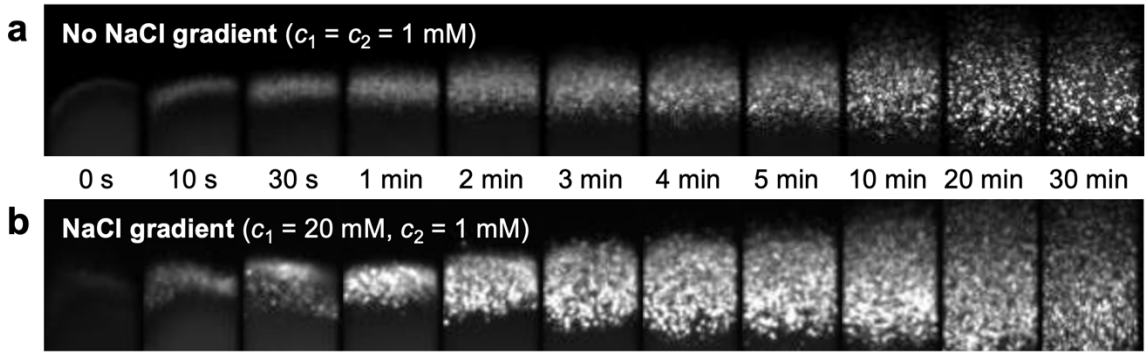

Figure R1. Close-up images of the condensates that were taken from Movie S1 (Figure 1c,d in the manuscript). **(a)** No gradient ($c_1 = c_2 = 1$ mM). **(b)** NaCl gradient ($c_1 = 20$ mM, $c_2 = 1$ mM).

[R1; Ref. 29 in the main text] Alshareedah, I., Moosa, M. M., Raju, M., Potoyan, D. A., & Banerjee, P. R. (2020). Phase transition of RNA− protein complexes into ordered hollow condensates. *Proceedings of the National Academy of Sciences*, *117*(27), 15650-15658.

Comment #3: [R6] has chemical reactions in them, so I am sorry I do not see the relevance. The authors do not mention any irreversible chemical reactions in their system.

Our response: We would like to point out other recent studies that have shown that purely advective processes could suppress the growth of condensates formed by liquid-liquid phase separation including biomolecular condensates [R2-R4]. Apart from the (weakly) advective processes occurring in our system, the strongly charged surface of our condensates is likely the reason for any lack of coalescence, thus suppressing the late-stage coarsening, as shown in Figure R1.

[R2] Adkins, R., Kolvin, I., You, Z., Witthaus, S., Marchetti, M. C., & Dogic, Z. (2022). Dynamics of active liquid interfaces. *Science*, *377*(6607), 768-772.
[R3] Tayar, A. M., Caballero, F., Anderberg, T., Saleh, O. A., Cristina Marchetti, M., & Dogic, Z. (2023). Controlling liquid–liquid phase behaviour with an active fluid. *Nature Materials*, *22*(11), 1401-1408.
[R4] Arnold, D. P., Gubbala, A., & Takatori, S. C. (2023). Active surface flows accelerate the coarsening of lipid membrane domains. *Physical Review Letters*, *131*(12), 128402.

Comment #4: I am sorry I do not see any direct evidence in the manuscript that chemiphoresis is dominated by electrophoresis. I understand the direction of diffusiophoresis is consistent with electrophoresis but this is not, I think, strong evidence.

Our response: Our apologies for the confusion. This was our mistake. We have now included chemiphoresis in estimating the condensate velocity driven by biomolecule gradients. The velocity is estimated to be around 0.4 nm/s. We have revised this part in the manuscript as follows:

Page 11-12, Paragraph 1: "…For multi-species diffusiophoresis, one may express the particle velocity as $u_d = \frac{\varepsilon kT}{\eta e}\frac{\sum_i z_i D_i \nabla n_i}{\sum_i z_i^2 D_i n_i}\zeta + \frac{\varepsilon}{8\eta}\frac{\sum_i z_i^2 \nabla n_i}{\sum_i z_i^2 n_i}\zeta^2$, where ε is the permittivity, k_B is the Boltzmann constant, T is the temperature, e is the elementary charge, and z is the charge number.[60,61] Taking the summation over all the known background solute species, including the buffer, the velocity is estimated to be about $u_d = 0.4$ nm/s, which is indeed negligible compared to the condensate diffusiophoretic velocity driven by salt gradients."

Comment #5: Other points: I am not sure I find either the title or abstract very clear but it is the authors' paper. I think I can illustrate the differences between how I would approach paper writing and the authors by discussing the section "The promotion of phase separation is a ….". When I give students feedback on report writing I tell them to try and avoid vague words like "promote". Also, they see phase separation with and without salt gradients so I am sorry I am not sure that they have evidence that diffusiophoresis driven by salt gradients increases the amount of phase separation in any way they have quantified in the manuscript. However, the word "promote" is so vague that if the authors want to use it is hard for me to say they are wrong. Maybe it is justified by some intensity peaks being of order 10% higher with a salt gradient than without, but I am not sure that their most interesting result is an increase of roughly 20% in a peak intensity?

In this section they have some new and interesting results in Fig 3. These show the oppositely charged DNA and peptide molecules being pushed in opposite directions by salt gradients. This is what we should expect but it is very nice to see this data. It would be good to publish it. However I think they are using Fig 3 to help them claim "This local enrichment of both of these oppositely charged biomacromolecules directly promotes the phase separation …". I am sorry I can see no direct evidence of this in the manuscript. Phase separation is a complex, extremely non-linear, phenomena that is tough to understand and model. For example, I would guess that phase separation tends to flatten out gradients in the DNA and peptide, because the system tends towards the equilibrium coexistence, which is gradient free. This may or may not be why they see, if I understand correctly, little diffusiophoresis with gradients in DNA and the peptide.

The complexity of phase separation affects our ability to interpret the authors' results. For example they have a number of plots of intensity along the channel, and I think the idea is that the peaks in fluorescence intensity are where phase separation occurs. But phase separation should not directly affect intensity (I think this is what the authors assume). Presumably what happens is that phase separation dramatically reduces mobility of the phase separating molecules (as they form slow moving condensates), and so phase separated regions act as sinks for the fluorescent molecules and hence become brighter. If this is correct then the peaks in intensity observed by the authors are slightly indirect consequences of phase separation, which makes it hard to prove or even disprove statements like "promotes phase separation".

The complexity is impressive in simply phase separating oil and water, and it is doubly so with their system of larger charged molecules where the species they are using to drive diffusiophoresis (salt) also affects phase separation.This combination of an intrinsically complex phenomena and a rather complex system, makes interpreting results difficult. I do not understand much of what is going on, and after reading two drafts very carefully, I think the authors do not understand much of what is going on, either. But then I think it is unhelpful to use phrases such as "promote the phase separation". A clearer paper would present results and be clearer on what can and cannot be inferred from these results. But as noted above it is their paper not mine and I cannot write it for them. Statements which I think are more definitive than I would write occur throughout the manuscript.

Our response: We appreciate the Reviewer for providing a lengthy concern regarding our claim on diffusiophoresis enhancing phase separation of the biomolecules. While we understand the Reviewer's concern, we respectfully disagree with the Reviewer, particularly their points regarding whether phase separation is being "promoted" by diffusiophoresis. However, it is the reviewer's prerogative to make such assertions. The same argument holds for the lengthy discussions on how to write a paper. Below, we try our best to answer the question raised on its merit.

First of all, we note that the peak intensity provided in Figure 1e-g is not an ideal quantitative measure of phase separation since these are width-averaged intensities so that the locally enhanced fluorescence signal of the condensates is averaged out with the darker background. Moreover, the local intensity of the condensates under diffusiophoresis has already been saturated to the maximum value (intensity = 255; we

use 8-bit imaging), while the fluorescence intensities of the condensates in the absence of salt gradients were far below the saturation (Figure R2). We intentionally raised the light intensity in all cases to visualize the condensates in the no-gradient cases to match the baseline intensity consistently across all the cases, which caused the local pixel intensity of the condensates under diffusiophoresis to saturate. As shown in Figure R2, the local intensity enhancement by diffusiophoresis is far greater than 20% (exceeding 100% before saturation).

Furthermore, to further strengthen our claim on diffusiophoresis affecting biomolecular phase separation, we conducted a counter-experiment where we imposed a negative (reverse) NaCl gradient in the same system. As shown in Figure R3, after the initial condensate formation, the subsequent dynamics of the condensates are dramatically altered, where the condensates not only slow down but dissolve faster. This additional data, along with positive NaCl gradients, undoubtedly shows that diffusiophoresis impacts the phase separation dynamics depending on the direction of the NaCl gradients. We have now added the local maximum intensity (Figure S2) and the negative gradient data (Figure S5) in the Supplementary Information. We believe this additional data clarifies the effect of diffusiophoresis on phase separation.

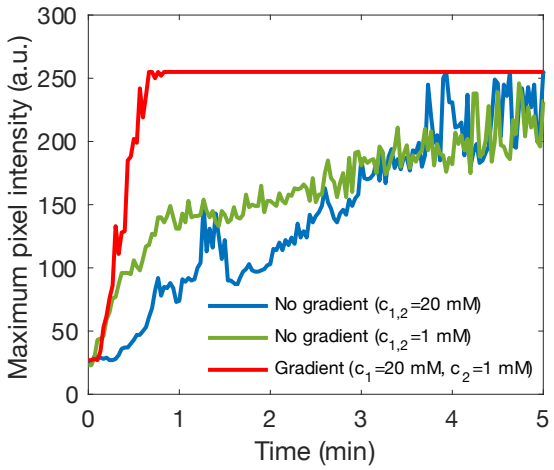

Figure R2 (Fig. S2). Local maximum fluorescence intensity of the condensates over time under different salt conditions. Data were processed from Movie S1 (Figure 1b-d). Blue, green, and red curves indicate, respectively, $c_1 = c_2 = 20$ mM (high salinity), $c_1 = c_2 = 1$ mM (low salinity), and $c_1 = 20$ mM, $c_2 = 1$ mM (NaCl gradient).

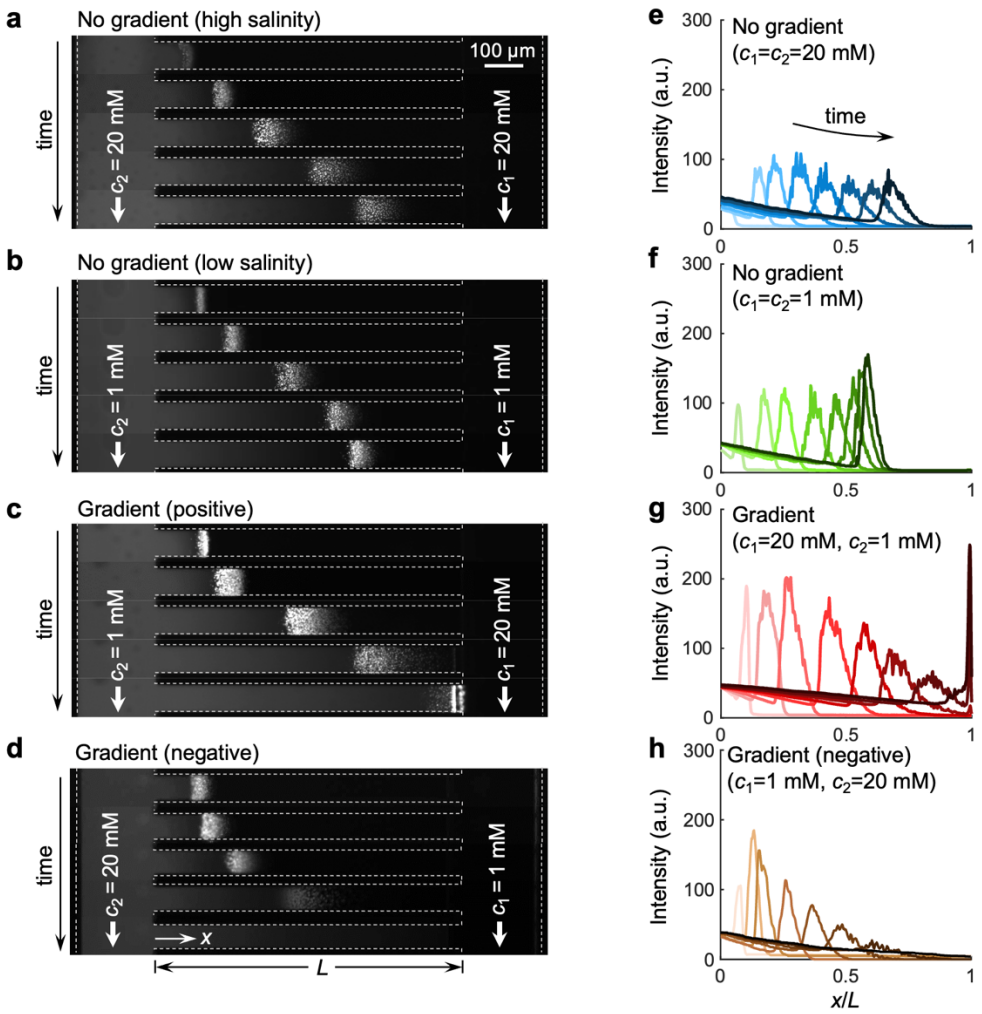

Figure R3. (**a-d**) Fluorescence image sequences and (**e-h**) their corresponding width-averaged intensity distribution of the $[dT]_{40}$-$[RGRGG]_5$ condensates over a period of 60 min. The NaCl concentrations are (**a,e**) $c_1 = c_2 = 20$ mM, (**b,f**) $c_1 = c_2 = 1$ mM, (**c,g**) $c_1 = 20$ mM, $c_2 = 1$ mM, and (**d,h**) $c_1 = 1$ mM, $c_2 = 20$ mM. The timestamps for the images in (**a-d**) are 1, 5, 20, 40, and 60 min, and for the plots in (**e-h**) are 1, 5, 10, 20, 30, 40, 50, and 60 min.

Comment #6: Could I also comment on Fig 2. Is what we are seeing in Fig 2a, b and c, many individual trajectories of individually tracked condensates - as the bottom paragraph of page 5 seems to imply - or is an average intensity - as the intensity scale bar for Fig 2a seems to imply. Also, in this bottom paragraph the authors state "... delays their dissolution" while in the caption of Fig 2 they say "prevents the condensates from dissolution". Is it "delay" or "prevent"?

Also if indeed they track individual condensates then these trajectories must contain huge amounts of information that is missing from Fig 2 but would help understand the system. For example, if individual condensates are tracked then presumably they could determine how long they live for, and whether they are present at the end of the experiment. This would allow the authors to show readers if it is delay or prevent. At the top of page 10 the authors say "condensate streaks that fade in and out.." which again suggests that they are tracking individual condensates. But I am sorry I cannot resolve individual streaks in Fig 2. If they can track individual condensates they will have to replot.

Fig 2 also has Fig 2d which has estimated speeds. I am sorry I do not see how these speeds are estimated. It would help if the authors explain this.

Our response: The kymographs in Figure 2a-c are constructed by plotting the intensity distribution within a region of interest (ROI) that spans across the centerline of the channel. The width of the ROI is about 10% of the channel width. In this way, we effectively obtain condensate pathlines without smoothing out too much, while keeping the migrating condensates within the ROI. Therefore, the kymograph shows an ensemble of many short-lived trajectories

Also, the wording in Figure 2 caption should be "delay", as the condensates will eventually dissolve in the long run. We have changed this accordingly in the revised main text.

It is difficult to extract a continuous streak of a particular condensate due to their strong Brownian fluctuation, making them easily disappear from the focal point, as shown in Movie S2. Regarding the velocity calculations in Figure 2d, we obtained several continuous streaks from individual condensates that lasted for at least a few seconds and took the time- and ensemble average of them, such that $\langle |\boldsymbol{u}| \rangle = \sum_{i=1}^{N} \int |\boldsymbol{u_i}(t)| dt \,/ N$. This information is added in the caption of Figure 2.

Comment #7: I am sorry I also need to highlight the aspect in which unfortunately the manuscript has been made worse. This is the citing of reference 14 (Okabe et al, Nat Comm (2012)) to support the idea of temperature gradients. This paper is clearly wrong, as has been shown for example by Buffou et al (https://www.nature.com/articles/nmeth.3073) in 2014. Temperature differences of order 0.1C across cells, as Okabe et al claim, are unsustainable. As I am someone who understands the basics of thermal diffusion - unlike Okabe et al. and the reviewers Nat Comm employed in 2012 - the continued credence given to these claims of temperature gradients is irritating.

See Buffou et al (2014) for details but the argument is basically trivial. Consider a cell 10 micrometres across, then if there is a temperature difference of 0.1 C across it, there is a flux of thermal energy across the cell - due to the inevitable thermal diffusion that occurs whenever there is a temperature gradient. The thermal conductivity of tissue is roughly similar to that of water which is order $k = 1$ W/(K m). So the power (W) of the thermal transport across the cell is just the product of the thermal conductivity, the T gradient which is of order 10,000 K/m, and the cross-sectional area of the cell which is 100 micrometres squared. I.e., thermal flux (1D x axis for simplicity) $j = -k\, \mathrm{d}T/\mathrm{d}x$ with power just flux times area A so power $P = kA\, \mathrm{d}T/\mathrm{d}x$.

This is a power P of order 1 microWatt. The human body has roughly 10 trillion cells, so if each has a power consumption of a microWatt that implies that humans have a power consumption of order 10 million W. The actual figure for humans is 100 W (approximately equivalent to the 2000 kilocalories per day we see on the back of food packets). The two figures differ by a factor of 100,000! Our bodies cannot of course sustain 10 million W as there is no chemical source of power for it, and even if there was, the dissipated heat would cause us to boil.

My estimate of 1 microW is of course just a rough estimate, for example the power consumption of one of our cells almost certainly can peak at larger values than a 100 W/(10 trillion) = 10 pW. For example Buffou et al. quote 100 pW. However, the cell power consumptions needed to sustain 0.1 C temperature differences across cells 10 micrometres across (for any sensible length of time) are clearly ridiculous. And for 100 nm mitochondria the numbers are even more ridiculous as the gradients scale inversely with lengthscale.

In short either our understanding of thermal transport - well tested over perhaps 200 years now - is wrong by orders of magnitude for cells. Or Okabe et al, Nat Comm (2012) are wrong. I would bet a lot of my money on our understanding of thermal transport applying - to within an order of magnitude or two - to cells.

In an ideal world, Nat Comm would put something like an expression of concern on articles like that of Okabe et al.. In the absence of such markers, authors like Doan et al., should do adequate literature reviews on temperature gradients in cells and see that claims such as those of Okabe et al. are very unlikely to be correct. I cannot recommend publication of a manuscript that uncritically cites such a misleading paper. If the authors wish to mention temperature gradients in cells - and they don't have to mention these gradients as they don't exist - then they should cite papers that are correct, such as that of Buffou et al.

Our response: We thank the Reviewer for pointing out the critique of Okabe et al (2012), which we were unaware of. We have removed this reference and the associated discussion from the manuscript accordingly.

In addition, we have now discussed at the bottom paragraph of Page 6 and cited a landmark recent study (King et al, 2024, Cell, PMID: 38503281) showing almost a unit of pH change across the 3 subphases of the nucleolus, which may drive the advective inside-out migration of the ribosomal RNAs might be due to the diffusiophoretic transport induced by H^+ ion gradients.

Comment #8: Data availability: The authors state that "all relevant data are included". This is a bit inaccurate. They don't include any data, such as tracked condensate positions, sizes etc. I don't know what NSF requirements are, but some funders are encouraging authors to share the data, eg the data which underlies or is plotted in figures. The authors do not do this. Including some data would improve the paper's contribution to the field.

Our response: The raw data were already uploaded on the journal submission website during the initial revision on Feb. 9, 2024. We believe they are made available to the public only if the manuscript is published.

Response to the Reviewer 4's Comments

Comment #1: The manuscript by Doan et al. reports the use of a cleverly designed microfluidic device to investigate the effect of salt concentration gradients on the transport of nucleic acids, proteins and their condensate. The experimental findings clearly show that diffusiophoresis can (i) enhance the formation of biomolecular condensates by increasing the transport rates of their molecular constituents and (ii) promote the directed motion of condensates along the gradients. These results are not only exciting, but also very intriguing to an interdisciplinary audience, improving our understanding of the potential role of diffusiophoresis in biological systems. Overall, the manuscript is very well written. The proposed interpretations of the underlying physical mechanisms are well supported by the experimental observations. However, there a few important points that deserve further clarifications and discussions.

Our response: We thank the Reviewer for taking the time to review our manuscript and provide constructive feedback. We were pleased to hear that our manuscript is "exciting and intriguing" and "very well written." The comments and questions helped improve our manuscript significantly. In what follows, we provide a point-by-point response to the Reviewer's comments.

Comment #2: In their response, Authors have addressed all points raised by Reviewer 3. However, I disagree with their answer to Comment #2. I would say that diffusiophoresis per se is not a diffusive process. Diffusiophoresis transport may appear as a diffusive process under **certain conditions.** More specifically, for colloids exposed to a time-dependent inhomogeneous salt concentration field, c, that is relaxing by diffusion, the gradient of $\log c$ scales as

$$\nabla \log c \simeq \frac{\Delta c}{c\delta_c} \simeq \frac{1}{\sqrt{D_S t}}$$

where δ_c is the salt diffusion length scale, D_S the salt diffusivity and t the time. Therefore, the diffusiophoresis velocity of the particle can be written as

$$u_{DP} = \frac{dx_p}{dt} = M_{DP}\nabla \log c \simeq \frac{M_{DP}}{\sqrt{D_S t}}$$

where M_{DP} is the diffusiophoresis mobility and x_p the position of the particle in a 1D domain. By integrating over time, one gets

$$x_p(t) - x_p(0) \simeq \sqrt{D_{eff} t}$$

where $D_{eff} \propto \frac{M_{DP}^2}{D_S}$ is an effective diffusion coefficient. Hence, in this case diffusiophoresis can be viewed effectively as an enhanced diffusivity, and the mean square displacement of the particle scales as t. These are similar conditions to the ones examined in the references R22, R23, R24 cited by the authors in their response to Reviewer #3. However, for instance, if the salt concentration field is stationary, as it is between a low and high salt concentration reservoir after the initial relaxation period, the term $\nabla \log c$ is time-independent and it follows

$$x_p(t) - x_p(0) \simeq D_{DP}\nabla \log c\, t$$

Therefore, in this case the diffusiophoresis transport is not diffusive and, as remarked by Reviewer #3, the mean square displacement of the particle scales with a different power of time, namely it scales as t^2. Consequently, the following sentence at page 8 of the manuscript "Given the units for mobility being

identical to the diffusivity, diffusiophoretic mobility can alternatively be viewed effectively as an enhanced diffusivity." should be revised by highlighting under what conditions the diffusiophoretic transport has a diffusive character.

Our response: We regret for causing some confusion to the previous Reviewer 3. There is a clear difference between a process *being diffusion* and a process *behaving diffusively*, where the mean square displacement characterizes the latter, as the Reviewer correctly pointed out. For a linear, steady-state solute concentration gradient, the macroscopic transport of the solutes will *appear advective* following Fick's law (i.e., constant solute flux), but the process is undoubtedly governed by diffusion. What we meant to say in this context was that diffusiophoresis is governed by the *diffusion* of solutes, not necessarily that diffusiophoresis is *diffusive*. To avoid any further confusion, we have added this discussion in the main text as follows:

Page 8, Paragraph 1: "*Given the units for mobility being identical to the diffusivity, diffusiophoretic mobility can alternatively be viewed effectively as an enhanced diffusivity since diffusiophoresis of the condensates is ultimately dictated by the diffusion of solutes.*"

Comment #3: At pH~7, Alexa 488 dye carries two negative charges whereas Cy5 dye carries one positive charge. Now in experiments in Fig. 1 and 2, the ssDNA is tagged with Cy5 whereas $[RGRGG]_5$ is not fluorescently labelled. Conversely, for the experiments in Fig. 3 both ssDNA and $[RGRGG]_5$ are tagged with Cy5 and Alexa 488, respectively. Finally, for zeta potential and turbidity measurements, neither the peptide or ssDNA is tagged with a fluorescent dye. It is known that fluorescent tags can alter the charge and, consequently, the behaviour of biomolecules3-5. How do these additional charges affect the diffusiophoresis mobility of fluorescently-labelled ssDNA and $[RGRGG]_5$ and the phase separation processes? What are the experimental limitations introduced by the necessary use of fluorescent tags for the investigation of the molecule/condensate dynamics? Should have the zeta-potential and turbidity measurements been performed with tagged-molecules to replicate the experimental conditions within the microfluidic systems?

Our response: In our experiments, the fraction of the tagged DNAs and polypeptides is small. For example, Cy5-labeled $[dT]_{40}$ and Alexa 488-labeled $[RGRGG]_5$ constitute 1.2 mol% and 3.7 mol% of the total $[dT]_{40}$ and $[RGRGG]_5$ samples, respectively. Moreover, $[dT]_{40}$ consists of 40 negatively charged thymine, giving the ssDNA a net negative charge of 40 per strand. On the other hand, $[RGRGG]_5$ contains positively charged arginine residues and neutral glycine residues, resulting in a total of 10 positive charges per peptide. Since Cy5 and Alexa 488 carry 1 negative and 2 positive charges, adding the dye increases the total charge of the tagged $[dT]_{40}$ and $[RGRGG]_5$ by 2.5% and 20%, respectively. Taking into account the molar fractions of the tagged species, the total charge increase for $[dT]_{40}$ and $[RGRGG]_5$ samples is estimated as 0.03% and 0.7%, respectively. Therefore, we expect negligible influence of tagged species on the measurements of zeta potential, turbidity, diffusiophoretic mobility, and phase separation.

Comment #4: The diffusiophoresis process, described in the paper, is not an active transport mechanism (like self-diffusiophoresis). Hence, the terms "active motility", "active migration", "active transport", etc. should not be used to describe this process.

Our response: We have removed the phrase "active" from the manuscript accordingly.

Comment #5: In Section "Salt-dependent diffusiophoretic mobility of biomolecules" in the Supplementary Material, it is correctly stated that the zeta potential scales with the local salt concentration c as $\zeta \sim 1/\sqrt{c}$. However, it appears that zeta potential measurements have been performed in absence of NaCl salt. Why were not ζ measurements taken at 1mM and 20mM salt concentrations? Maybe negligible effects are expected at these salt concentrations?

Our response: The measurement of the zeta potential was indeed conducted in the presence of 10 mM NaCl, which is roughly the mean of 1 mM and 20 mM NaCl. We apologize for missing this information. This information is now added to the Supplementary Information as follows:

SI Page 3, Paragraph 3: "…containing 10 mM Tris-HCl and 10 mM NaCl…"

SI Page 8, Figure S8 caption: "…ratios (ssDNA/RNA-to-protein; $n_{\{D,R\}}/n_P$) in 10 mM Tris-HCl and 10 mM NaCl…"

Comment #6: It appears that the role of diffusioosmosis in the microfluidic channel was not mentioned in the manuscript. Could the Authors comment in the manuscript on whether they expect negligible effects of diffusioosmosis driven flows within the dead-end channel?

Our response: We expect negligible diffusioosmosis in NOA-81 microfluidic channels due to the low surface charge. We measured the zeta potential of NOA-81 microparticulates (diameter = 1~2 μm), which we obtained by grinding a fully cured NOA-81 film. The results showed a slightly negative potential of about –12.3 mV. We also conducted an additional experiment to demonstrate the minor effect of diffusioosmosis by comparing the particle distribution in a PDMS channel (Figure R4a), which experiences a strong diffusioosmosis due to the large surface charge (wall zeta potential ~ –50 mV [R5]), to NOA-81 channel (Figure R4b). As the particles undergo diffusiophoresis in the PDMS channel, the strong diffusioosmosis results in the curved particle front, which has also been discussed in a number of previous studies [R6-R8]. However, the particles migrating via diffusiophoresis in the NOA-81 channel do not show any curved front, but rather exhibit nearly a flat particle front, suggesting negligible diffusioosmotic flow. We have added this aspect in the manuscript and the SI as follows:

Page 3, Paragraph 4: "…The microfluidic channel is made out of a UV-curable epoxy (NOA-81) due to its low surface charge (zeta potential = –12.3 mV; Supplementary Information) and excellent compatibility with in situ photopolymerization of polyethylene glycol diacrylate (PEGDA) hydrogels…"

SI Page 3, Paragraph 4: "…We also measured the zeta potential of NOA-81 microparticulates (diameter = 1~2 μm), which we obtained by grinding a fully cured NOA-81 film. The zeta potential of the NOA-81 particles in 10 mM NaCl solution is measured to be -12.3 ± 0.4 mV, suggesting negligible diffusioosmotic flow."

[R5] Ocvirk, G., Munroe, M., Tang, T., Oleschuk, R., Westra, K., & Harrison, D. J. (2000). Electrokinetic control of fluid flow in native poly (dimethylsiloxane) capillary electrophoresis devices. *Electrophoresis*, *21*(1), 107-115.

[R6] Shin, S., Um, E., Sabass, B., Ault, J. T., Rahimi, M., Warren, P. B., & Stone, H. A. (2016). Size-dependent control of colloid transport via solute gradients in dead-end channels. *Proceedings of the National Academy of Sciences*, *113*(2), 257-261.

[R7] Shin, S., Ault, J. T., Feng, J., Warren, P. B., & Stone, H. A. (2017). Low-Cost Zeta Potentiometry Using Solute Gradients. *Advanced Materials*, *29*(30), 1701516.
[R8] Alessio, B. M., Shim, S., Gupta, A., & Stone, H. A. (2022). Diffusioosmosis-driven dispersion of colloids: a Taylor dispersion analysis with experimental validation. *Journal of Fluid Mechanics*, *942*, A23.

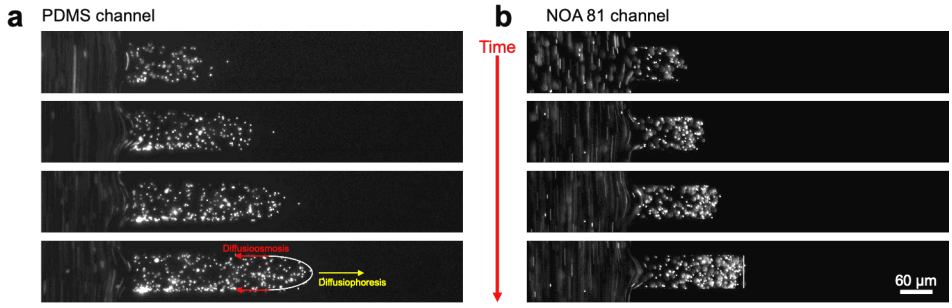

Figure R4. Comparison between particle diffusiophoresis in (a) PDMS and (b) NOA-81 channels (polystyrene, diameter = 1 μm) induced by NaCl gradients (left side = 1 mM, right side = 30 mM).

Comment #7: I cannot find in the revised manuscript information regarding the channel depth. I advise to include all key dimensions of the devices in the Supplementary Material Section "Microfluidic Device Fabrication".

Our response: The depth of the channel is 40 μm. We have added this detail in the manuscript and the SI as follows:

Page 4, Figure 1 caption: "…horizontal channel (length L = 800 μm; width = 60 μm; depth = 40 μm) that allows…"

SI Page 1, Paragraph 1: "Subsequently, the NOA-81 channel is molded from the PDMS mold (depth = 40 μm) and partially cured under UV light (IntelliRay 400, Uvitron)."

Comment #8: Unless I am mistaken, it appears that Fig. 2d is not mentioned and discussed in the main text of the manuscript. If so, it is recommended to include a description of Fig. 2d within the main text.

Our response: We thank the reviewer for spotting the mistake. We have discussed Fig. 2d in the main text but mistakenly labeled it as Fig. 2c. We have corrected the error accordingly.

Comment #9: To conclude, this is an excellent manuscript, and I recommend the publication, provided the points above are addressed.

Our response: We thank the Reviewer once again for providing constructive comments and for supporting our work.

Comment #10: PS: Reviewing the manuscript, I noticed a couple of typos - Main manuscript: Pg 8, line 6 of the first paragraph, “Both The […]” - Supplementary Material: Figure S7, caption, I think it should read “[…] or (d) with NaCl gradients […]” instead of “[…] or (d) with NaCl gradients […]”

Our response: We have revised the typos accordingly.