

Peer-Review Record:

Does DNA Exert an Active Role in Generating Cell-Sized Spheres in an Aqueous Solution with a Crowding Binary Polymer?

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Reviewer 1: Anonymous

Reviewer 2: Anonymous

Reviewer 3: Anonymous

Editor: Fabio Mavelli and Pasquale Stano (Guest Editor of Special Issue “Protocells-Designs for Life”)

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First Round of Evaluation

Round 1: Reviewer 1 Report and Author Response

The paper presents an interesting set of experiments on crowded dispersions of DNA that create cell-like morphologies in presence of dextran and polyethylene glycol. The DNA molecules prefer to concentrate in dextran-rich parts of the phase separated regions. The authors also demonstrate a fusion of two droplets by laser tweezers that resembles a cell division in reversed time sequence. The manuscript is very interesting and the results are novel.

Response: We appreciate the considerate comment of great importance to us for our expanding future research. Reading the following part of the comment by Reviewer 1, we have considered it is significant to explore how much DNAs entrapped in dextran microspheres could assume ordered structures.

My minor comment is that the authors present only phase contrast images of the textures. It would be of great value to also show the polarizing microscopy images as the separation of chemicals might result in formation of birefringent domains with orientational order of molecules. Such a behavior has been reported in the studies of low molecular organic compounds in presence of PEG, see Tortora *et al.*, *Soft Matter* **6**, 4157 (2010) and Park *et al.*, *Langmuir* **27**, 4164 (2011); DNA might show a similar ordering which would be clearly visible in polarizing microscopy as birefringent domains. The manuscript can be published after the authors consider this possibility; however, if additional experiments are difficult at this time, they can be performed later and form a basis of another paper.

Response: We are grateful for this impressive suggestion. Seeing the wonderful works by Prof. Lavrentovich's group, we think it is very interesting and meaningful to investigate the behavior of DNAs entrapped inside the crowded microspheres of dextran. In the present case, we simply intended to report a phenomenon of DNA entrapment, and used only a phase contrast microscope. As Reviewer 1 suggested, it would become more informative with a polarizing microscopic observation; however, we are now not ready enough to seek appropriate conditions for the formation of the birefringent domains using the microscope within a given period. We would intensely like to carry out such additional experiments in detail, and, if possible, will report them in a near future. Thank you very much.

Round 1: Reviewer 2 Report and Author Response

A very interesting paper describing an alternative use of aqueous two-phase systems to the traditional separation and purification of biological products. The paper is easy to read, easy to follow and can be of putative interest to the readers of life.

Response: We thank Reviewer 2 very much for the considerate and useful comments, and we would like to modify our original manuscript according to them as follows.

Minor comments:

Lines 32–33: What about DNA? It is also present in large concentrations?

Response: No, DNA may not be concentrated with such a range of concentration. The concentration described here (0.3–0.5 g/mL) is partly based on a total amount of biological macromolecules mostly containing RNA and protein (0.34 g/mL), whose concentrations have been mentioned by Zimmerman and Murphy (Ref. 2). On the other hand, they have also mentioned in the same report that the concentration of DNA concentrated in bacterial nucleoids are estimated to be locally 0.05–0.1 g/mL. The original sentences are somewhat ambiguous in which biological macromolecules are considered for the concentration range. Therefore, we have amended the lines by inserting a phrase, “mainly RNA and protein partly with DNA”, in the last line on the first page.

Line 40: Although Albertsson is definitely a reference in ATPS, a more recent review could also be added: e.g., “As comprehensively reviewed by Albertsson [8], and more recently by xxx [X]...”

Response: In addition to Albertsson's book, we have cited a recent review on ATPS (Molino et al., 2013) as Ref. 9, and the references that followed the added review were renumbered.

Lines 102–104: Are these the compositions described in Figure 1 as Intermediate?

Response: Yes, we mean that the composition described there can be recognized as one to be described as Intermediate in Figure 1. After very brief centrifugation, the solutions remained opaque, and did not segregated into distinct two-separated phases. To stress this point, we have added “Intermediate” to the lines.

Line 116: The line drawn in Figure 1 represents the binodal?

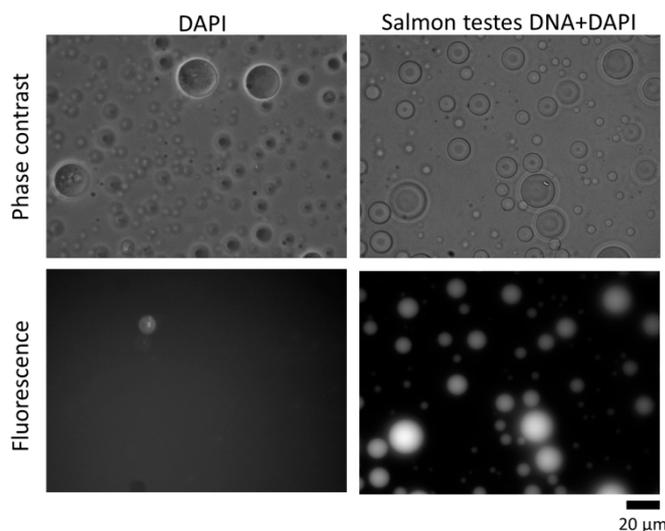
Response: The line is not consistent with the true binodal line. As described in the caption of Figure 1, the line shows only a boundary for convenience, the boundary which has been determined with the simple experiment we conducted; probably, the line could be drawn near the binodal curve. We have amended the sentence in the caption to describe this point.

Figure 1: There are only 4 Intermediate compositions in Fig 1 and these are obtained for very low concentrations of dextran. Is this a trend? Can these intermediate compositions be obtained with higher dextran concentrations? Please comment.

Response: Yes, this is a trend when one will determine which states such dextran/PEG solutions are in as we did in the present study. In an experiment with solutions of other dextran and PEG (not included in the present manuscript), we have also observed a similar trend. We consider that the reason why intermediate states might not be observed with higher dextran concentrations is because a solution containing higher-concentrated dextran is apt to be segregated into two phases relatively faster than that containing lower-concentration dextran due to difference in density between dextran and PEG. In the present experiment, after mixing dextran/PEG solutions, we made them kept stood freely or briefly centrifuged them, and then solutions with low dextran concentrations were able to remain turbid for a few hours, whereas solutions with high dextran concentration (that is, low PEG concentration) could not be stable in an intermediate state. In other words, the compositions indicated as Intermediate in Figure 1 seem to be in intermediate states stably only for some hours. With more rapid and/or longer centrifugation, of course, they could be segregated clearly into two phases. In order to make clearer such situations, in the revised version, we have added the following sentences in the end of the first paragraph of the section Results and Discussion of the amended manuscript to note the above discussed point: "The reason why intermediate states might not be observed with higher dextran concentrations is because ...could cause segregation into two phases".

Line 121: What is the partition coefficient of DNA in this system?

Response: We do not have a result with which to determine an exact value of the partition coefficient of DNA in this system, regrettably. In the present short note, our aim has focused on entrapment of DNA aggregates within dextran microspheres because we have considered the emergence of morphologies to be interesting. Of course, we also see that DNA molecules which are not included in aggregates exist in the background, though they are invisible. We have not determined a value of the partition coefficient; nevertheless, we have believed that, in some intermediate solutions, most DNA molecules can be distributed to dextran phases including dextran microspheres, judging from other experiments (microscopic observation and electrophoresis) that we conducted on samples with similar systems; we would like to show, confidentially for example, fluorescence microscopic images of DAPI-stained DNA distributed in dextran microspheres in an intermediate but different system (2.5% dextran 20,000; 8.5% PEG 6000; in the presence of ~1 mM MgCl₂ and ~10 mM Tris buffer). The images suggest DNAs are distributed to dextran microspheres almost exclusively.



Lines 121–123: A reference is missing.

Response: We thank the reviewer very much, and would like to amend the lines to avoid misconducting readers. On the lines of the original manuscript, we said DNA molecules were distributed usually to dextran phases as if DNA should preferred to dextran-rich phases in any compositions. However, as mentioned in the book by Albertsson (Reference 8 in the manuscript), of course, the partition coefficient, that is, the trend to which phase of a dextran/PEG system DNA molecules are distributed, may very sensitively depend on the kind and concentration of dextran, PEG and salt, and the base content, length and helix structure of DNA. What we intended to say with these lines in the original manuscript is that most DNA molecules were distributed to a dextran phase in the composition we chose here. As Albertsson has mentioned therein, in the ATPS, higher molecular weight nucleic acids partition completely in favor of either the lower or the upper phase. This implies that once a condition of a dextran/PEG solution containing large DNAs is given, almost all DNA molecules will be partitioned into either phase decisively. And, in the conditions we used here, we observed DNA molecules were almost completely entrapped inside dextran microspheres. To mention this point accurately, we have removed the lines from the original manuscript (“Since large DNA molecules ...contains very little DNA.”) and inserted the following new sentences: “DNA molecules are generally partitioned...from the PEG phase macroscopically.”

Figures 2–4: The composition of the ATPS used should be referred in the figures legends.

Response: We used the identical composition (indicated with an arrow in Figure 1) in the laser experiment. We have also inserted the composition used there in the figure legends.

Line 135: Figure 2b, represents a sample form the upper region of the vessel and Figure 2c from the lower region?

Response: We are grateful for the comment. As the reviewer has commented, Figure 2b and Figure 2c represent samples from the upper region and the lower region, respectively. In the original description this issue was not clear, as indicated by the reviewer. We have thus inserted “upper” and “lower” to make it clear from which region the sample was taken.

Line 148: Please replace “finally” by “were successfully”

Response: We have replaced it as the reviewer commented.

Figure 4: No laser used in Figure 4c?

Response: No, we did not use laser for induction of fusion between the two juxtaposed microspheres. We amend the caption to make clear this point. We have also made a small change in the last line of the section Introduction.

Lines 168–176: It is somehow possible to observe the formation and encapsulation of the DNA under the microscope?

Response: We think that it is possible now under a certain condition. Although a chamber which we used in the present experiment was simple, it was difficult to regulate a sample condition for direct observation on encapsulation of DNA inside dextran microspheres after mixing polymers and DNAs. Instead, as we wrote in the original manuscript (in the second paragraph from the last one of the section Results and Discussion), we occasionally observed that dextran apparently gathered to form a spherical shape around DNA molecules that were trapped under laser radiation in PEG solutions. We did not show the microscopic image in the original manuscript, but we would like to insert it as Figure 5 to mention the potential that DNA aggregates may serve as a core for formation of dextran microspheres under laser radiation.

Round 1: Reviewer 3 Report and Author Resposne

In this article, the authors show the role of DNA in generating cell-like morphology in an aqueous solution crowded with the polymers dextran and PEG. Although the potential of DNA aggregate as a core of microdroplet is interesting, the experimental results presented in this article (Figure 2) are insufficient to show the active role of DNA. The authors should carefully compare the results in the presence of DNA with those in the absence of DNA, and show how DNA-entrapping dextran microdroplets emerge. Second Round of Evaluation

Response: We are very grateful for the significant comment. We have agreed with the reviewer in that we were not able to show the clear evidence that a sufficient result in the original manuscript to argue DNA had an active role in generating microspheres of cell-like morphology. Formation of dextran microdroplets and encapsulation of DNA aggregates occur rapidly and simultaneously, and therefore we consider that it is now difficult to conduct the experiment to manifest how actively DNAs influence emergence of dextran microspheres by quantifying the extent to which, for example, size, stability and growth rate of the microspheres are affected by the presence of DNAs. We think that the aim of the present work is to experimentally show the cell-like structure consisted of DNA aggregates and dextran crowded by PEG. We have been studying the system, and we will like to report results from a quantitative standpoint in future. We have inserted a new figure (Figure 5) to the end of the revised manuscript, the figure which shows dextran gathered around DNA aggregates that were trapped by laser and grew to be a larger microsphere, and we are expecting that the figure 5 serves an additional illustrative example to imply the possibility that DNA potentially functions as a core for emergence of cell-like microspheres under some conditions.

Second Round of Evaluation

Round 2: Reviewer 3 Report

The revised manuscript has been significantly improved.

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