Leveraging the protein liquid-liquid phase separation droplets as potential drug delivery vehicles.

[1, 2]Manikanta Sodasani and [2]Ravikiran S. Yedidi*
[1]GIET School of Pharmacy, Rajahmundry, AP, India; [2]The Center for Advanced-Applied Biological Sciences & Entrepreneurship (TCABS-E), Rajahmundry, AP, India.
(*Correspondence to RSY: tcabse.india@gmail.com)

Protein liquid-liquid phase separation (LLPS) has been a relatively new field of study that revealed multiple biological processes that were otherwise mysterious previously. During stress, certain proteins are known to form LLPS droplets in the cell (both cytoplasm and nucleus) in order to protect the critical biological functions to jump start the cellular physiology while recovering from the stress conditions. In this study, we propose the usage of such LLPS droplets for targeted delivery of micro-RNA (miRNA) molecules for various therapeutic purposes. This *in vitro* strategy will test dehydrin protein (ERD10) extracted from *Arabidopsis thaliana* seeds which will then be mixed with miRNA in various dosages to test for the LLPS characteristics. These LLPS droplets containing EDR10 and miRNA will then be used for targeted delivery *in vivo*. We are going to extend this LLPS droplet-based targeted delivery strategy further to small molecules and biologics besides nucleic acids in the future.

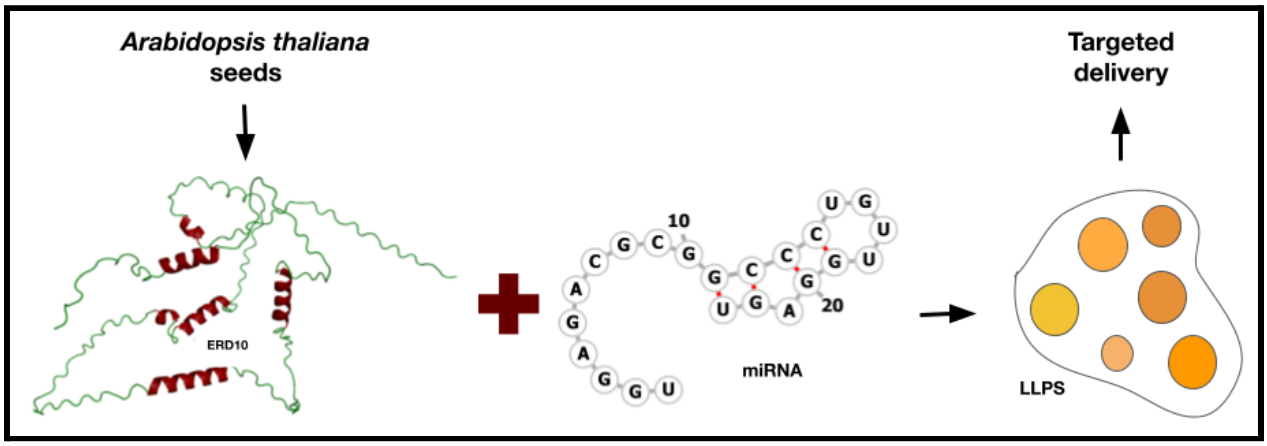

Figure 1. Overview of the proposed miRNA targeted delivery strategy using ERD10-based LLPS strategy.

The intrinsic structural disorderness of certain proteins leads to a meaningful aggregation of such proteins during the stress conditions such that the cellular biochemical complexes stay hydrated instead of precipitating in the cytoplasm. These intrinsically disordered proteins (IDPs) are often targets of cellular proteasomes. However, the proteasomes (multi-protein and megadalton complexes) themselves were known to form protein liquid-liquid phase separated droplets (LLPS) in the cytoplasm (1, 2). In this study, we propose to take advantage of such naturally occuring LLPS phenomenon for targeted drug delivery, primarily to the delivery of micro-RNAs (miRNA). The concept of using miRNAs as therapeutic agents has long been utilized with little to no success due to lack of proper delivery platforms. We believe that by using the LLPS platform one can successfully deliver the versatile and unstable molecules such as

miRNAs. One can question the capability of these membraneless LLPS droplets as carriers of miRNA but it has been recently shown that the LLPS droplets may act as membraneless organelles in response to the environmental factors (3).

We have previously evaluated various proteins for their LLPS capabilities using different approaches. In this study we propose using an IDP, dehydrin (ERD10) extracted from the seeds of *Arabidopsis thaliana* (4, 5). Owing to its IDP nature, ERD10 (6) has been previously shown to exhibit LLPS capabilities and is highly economical even for large scale preparations in the future once a prototype is established. The miRNA-139-3p which is involved in many types of cancers will be evaluated for delivery using the ERD10-based LLPS droplets. As shown in Figure 1, the purified ERD10 protein will be mixed with miRNA-139 in different proportions to obtain at least 5-10 formulations. These formulations will then be used to evaluate the targeted delivery of miRNA both *in vitro* and *in vivo*. Beginning with the small miRNA molecules, we are going to further extend this study to the delivery of large RNA molecules such as the complementary endogenous RNA (ceRNA) molecules which are also known as long intergenic non-coding RNA molecules. It has been previously shown that ceRNA molecules act as molecular sponges towards the miRNA molecules which control the gene expression in the cells (7). For example, p53 (the guardian of the cell) is often downregulated in many cancers. In such cases, one can deliver the corresponding ceRNA that can sponge out the miRNA molecules which are responsible for the downregulation of p53.

In addition to the RNA molecules, we are also interested in using this LLPS for small molecule delivery. However, the challenge is in preventing these small molecules from binding the ERD10 with high binding affinity within the LLPS droplet that would hinder the desired delivery concentrations. In such cases, one can overload the ERD10-based LLPS droplets with small molecules in order to saturate all the binding sites within ERD10. Typically a very common question about protein-based delivery systems is how one can protect these delivery systems from the human immune system without triggering any immune responses. Our current study using genetic engineering has answered a few preliminary questions regarding this concern. However, the details will be published separately as they are beyond the scope of this applications report.

Currently, we are in the process of evaluating different formulations of the ERD10 and miRNA to test the feasibility of obtaining the LLPS droplets of the mixture. These mixture-based LLPS droplets will then be evaluated for the miRNA delivery efficiency. The data obtained from the *in vitro* and *in vivo* studies will be published in the future issues of TCABSE-J.

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Conflict of interest: The strategic communication presented here is an ongoing project currently at TCABS-E, Rajahmun--dry, India. The authors invite collaborations without any conflict of interest.