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# Novel biophysical strategy for the delivery of therapeutic microRNA molecules for Cancer and infectious diseases treatment.

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**Cancer is the most common disease worldwide and accounts for the highest number of deaths. The number of therapies available in the medical field is not yet sufficient to cure cancer. The numerous existing diagnostic methods are not cost-effective and insufficient to detect cancer in its early stages. With increasing access to tobacco and the industrialization of nations, lung cancer has become the most common cancer of the 20th century, and deaths from lung cancer have steadily increased since the early 20th century. MicroRNAs are used as therapeutics in various tumors, infections and metabolic diseases. They have been used more widely over the past decade as they can inhibit the translation process. MicroRNAs can also be used as biomarkers and help us identify early stage cancer. Current microRNA delivery systems are not 100% effective and have side effects such as inflammation etc. In this experiment we check the feasibility of microRNAs delivery using novel methods such as liquid-liquid phase separation. The genes responsible for the mutations in lung cancer are identified and the associated microRNAs target sites are identified using computational databases and software. In the case of RNA and proteins, the phase separation of the liquid droplets is observed and suggests a positive outcome.**

**Keywords:** miRNAs, Liquid-Liquid Phase separation, Protein Droplets, Lung cancer, drug delivery.

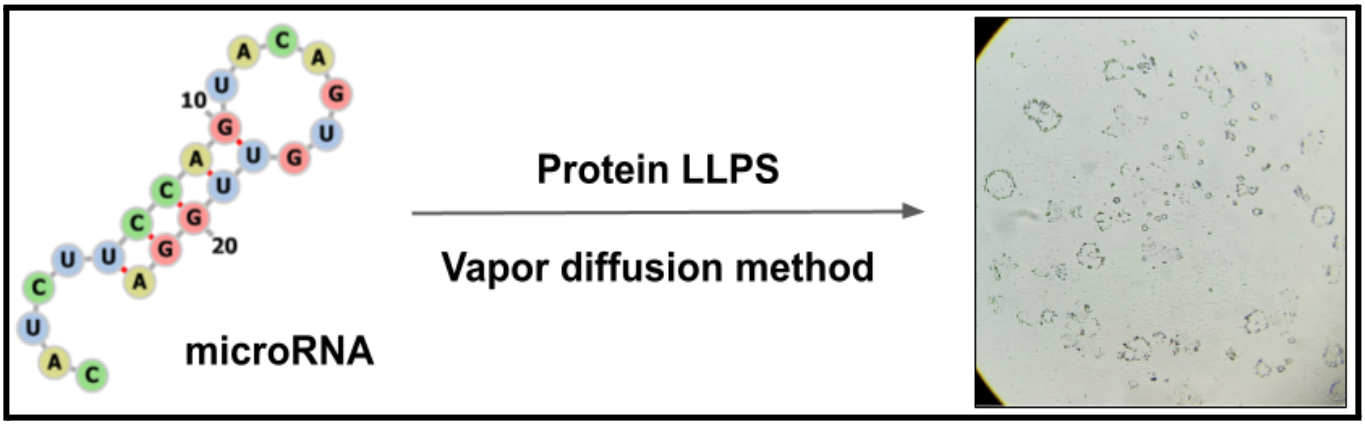


**Figure 1.** Overview of protein-RNA liquid-liquid phase separation using vapor diffusion method.

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CANCER: Cancer is a set of diseases that have afflicted living beings for more than 200 million years. Cancer is caused by human cells growing out of control and spreading to other parts of the body [1]. The causes of cancer can be varied, such as smoking, obesity, genetic changes, radiation, diet, physical activity, etc. The causes were also linked to occupations. People who worked in asbestos mines were more prone to lung cancer and radiologists were more prone to skin cancer because of the harmful X-rays [2]. The types of genes implicated in the cause of cancer are oncogenes, tumor suppressor genes, and DNA repair genes [3]. The oncogenes are the cancer-causing genes that turn a healthy cell into a cancerous cell. It is known that these changes are inherited [4]. The tumor suppressor genes are generally protective genes, but when they mutate, they grow uncontrollably and form cancer [4]. Errors in DNA repair genes can cause mutations and eventually lead to cancer [4]. There are no particular signs and symptoms in the early stages of cancer, but they appear as the mass grows [5]. Cancer originates in one place, enters the bloodstream, and spreads to other parts of the body. This is known as cancer metastasis [6]. Metastases are common in the late stages of cancer [5][6]. Some cancers spread to specific parts of the body - Breast cancer tends to spread to the bones, liver, lungs, chest wall, and brain, lung cancer tends to spread to the brain, bones, liver, and adrenal glands, prostate cancer tends to spread to the bones, colon and rectal cancers tend to spread to the liver and lungs [7]. The types of cancers include carcinoma, sarcoma, leukemia, and lymphoma based on where they begin [4][5]. Estimated epidemiological trends from 2016 to 2060 predicted that liver cancer, gastric cancer, colorectal cancer, breast cancer, and lung cancer would be the most common, with lung cancer having the highest incidence worldwide [8]. These numbers could just keep rising in the absence of proper diagnostic methods and treatments. Prevention is the way to reduce the risk of cancer.

LUNG CANCER: Lung cancer is the most commonly diagnosed type of cancer worldwide. Lung cancer has the highest mortality rate in both men and women [9]. A mass grows in the lungs and grows out of control, leading to tumors. Smoking is the main cause of lung cancer although it has been found in some non-smokers due to many reasons such as environmental pollution, secondhand smoke, occupational exposure, etc [10]. The chance of getting lung cancer is very low after quitting smoking [9]. The other factors include genetics, age, gender, etc. which can cause lung cancer. Genetic mutations could be another major cause of lung cancer. The types of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [11]. NSCLC is a type of epithelial lung cancer that accounts for approximately 85% of all lung cancers [12]. The most common genetic mutations in people with NSCLC include KRAS, EGFR, ALK, MET, FGFR1, PIK3CA, BRAF, ROS1, etc. NSCLC is less aggressive than SCLC but is difficult to diagnose and has more deaths worldwide. The most common subtypes of NSCLC are adenocarcinoma (40%), squamous cell carcinoma (25%), and large cell carcinoma (10%) [12][13]. Adenocarcinoma is the most common type of cancer in non-smokers [14]. Squamous cell carcinomas begin in the squamous cells and migrate to the central parts of the lungs. The most common cause of squamous cell carcinoma is tobacco smoke [15]. Large cell carcinoma can grow in any part of the lung and tends to grow and spread rapidly [16]. Symptoms of NSCLC include cough, pleural effusion, shortness of breath, weight loss, malaise, etc. Small cell lung cancer (SCLC) accounts for about 12% of lung cancer cases, is more aggressive, and spreads easily compared to NSCLC [13]. SCLC starts in the bronchi and spreads to other parts of the lung quickly [17]. It is easy to diagnose as it spreads rapidly. SCLC is common in people who smoke. The treatment for lung cancer includes chemotherapy, immunotherapy, radiation, surgery, etc [12][17].

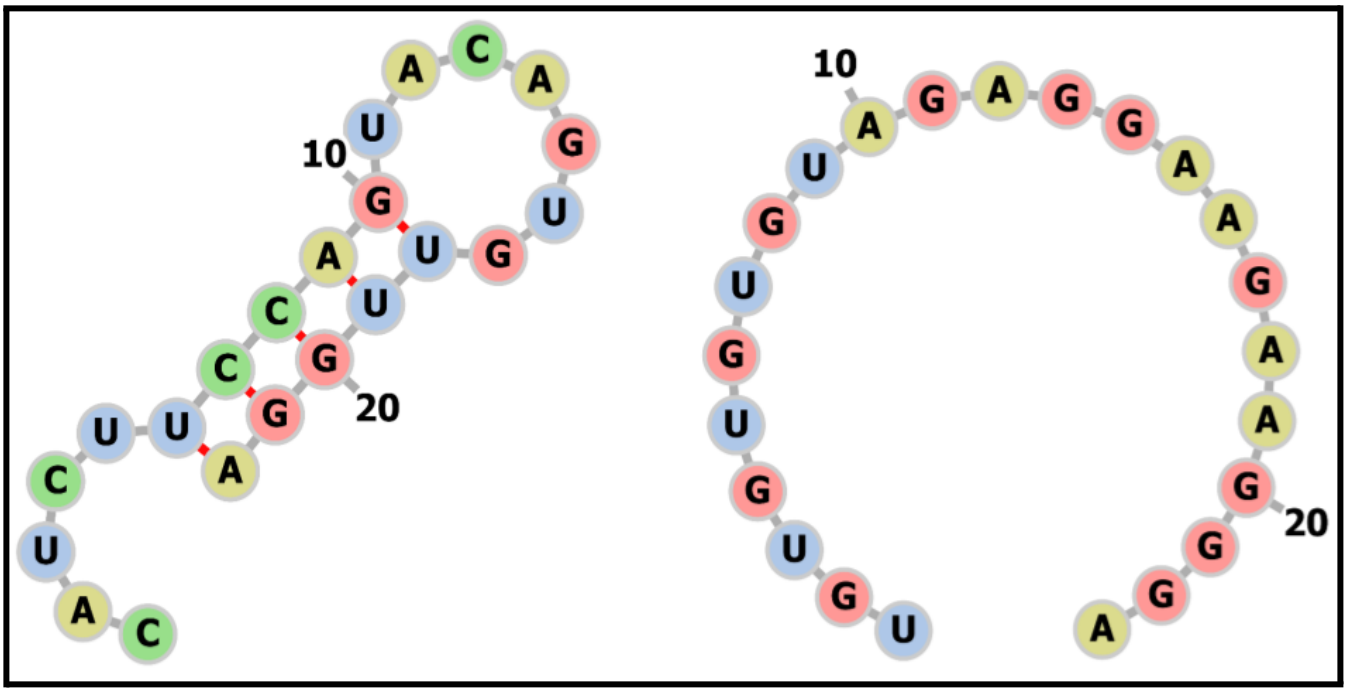


**Figure 2.** miRNAs with and without self-complementarity.

Lung cancer was a rare disease at the beginning of the 20th century, but due to reasons such as an increase in the number of smokers, environmental pollution, occupational exposure, etc., it has become a plague at the end of the 20th century [18]. About 10% to 20% of lung cancer cases are never-smokers in Asian women [19]. Despite the new technologies and advances in medicine and research, the survival rate from lung cancer does not exceed 5 years, even with the help of numerous therapies, because by the time the cancer is diagnosed, cancer has already progressed to other parts of the body [20]. The mortality rate from lung cancer is three times higher in men than from prostate cancer and twice as high in women than from breast cancer [21]. According to the International Agency for Research on Cancer (IARC), the number of lung cancer cases could increase by 45% in 2040 [22]. Lung cancer deaths are higher than colorectal, breast, prostate, and pancreatic cancers combined [23]. The survival rate varied for localized cancer (stage I-II), regional cancer (stage III), and metastatic cancer (stage IV), with localized cancer having a better survival rate compared to regional and metastatic cancer [24]. The survival rate depends on factors such as gender, age, socio economic impact, type of cancer (either NSCLC or SCLC), stage of cancer, etc [25][26]. About 40% of people survive 1 year after diagnosis, 15% survive 5 years after diagnosis and 10% survive 10 years after diagnosis [26]. Underprivileged nations are at higher risk of cigarette smoking and poor lung cancer survival rates.

NON-CODING RNAs: RNA generally undergoes translation to give corresponding proteins. But certain RNAs do not participate in the translation process and do not form a protein. These RNAs are referred to as non-coding RNAs (ncRNAs) [27]. There are two types of non-coding RNAs: housekeeping ncRNAs and regulatory ncRNAs. Housekeeping ncRNAs include ribosomal RNA (rRNA), and transfer RNA (tRNA), which normally serve essential functions of the cell such as translation, small nuclear RNA (snRNA), which is involved in splicing, and small nucleolar RNA (snoRNAs), which are involved in the modification of RNA [28][29]. The regulatory

ncRNAs include short ncRNAs and long ncRNAs. The short ncRNAs are micro-RNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA), which are <200 nucleotides in length. The long ncRNAs are competitive endogenous RNA (ceRNA) and enhancer-derived RNA (eRNA) that are >200 nucleotides in length [30]. The regulatory ncRNAs are from 1% of the total genome [31]. The regulatory ncRNAs play an important role in regulating the various processes of the cell and other networks [32]. The short ncRNAs - siRNA and miRNA are similar in length and participate in the RNA Interference pathway (RNAi) [33]. The RNAi pathway, also called post-transcriptional gene silencing, is the process of sequence-specific gene suppression [34]. In the RNAi pathway, the short RNA binds to the DNA and inhibits the gene expression [35].

MICRO-RNAs: MicroRNAs (miRNA/miR) are short, non-coding RNAs approximately 22-25 nucleotides in length. The miRNAs are single-stranded RNA molecules that help in the RNAi pathway by binding to the mRNA molecule [36]. The miRNAs were first discovered in the early 1990s in the larval form of *C.elegans*. Let-7 was the first miRNA to be discovered, and let-7 was found to play an important role in the development of the later larval stage to an adult [37]. miRNA gene or intron was first transcripted into pri-miRNA with the help of RNA Pol II/III in the nucleus. The pri-miRNA is cleaved with Drosha and DGCR8 to form a pre-miRNA. The pre-miRNA is now transported to the cytoplasm with the help of a transporter molecule called Exportin-5 in combination with Ran-GTP. The pre-miRNA is cleaved with Dicer to form a miR duplex. Argonaute proteins act on the duplex and break down one of the strands and help form the RNA-Induced Silencing Complex (RISC) and they process to form a mature microRNA. The mature microRNA can now function in various cellular processes such as translational repression, mRNA deadenylation, etc [36][37][38][39]. MicroRNA functions include a) post-initiation mechanisms - miRNAs block translation elongation, b) co-translational protein degradation - polypeptide chain is degraded, c) miR-mediated mRNA decay - deadenylation and decapping, d) inhibition mechanisms - inhibition of ribosomal subunit joining, competition for cap structure and inhibition of mRNA circularization by deadenylation [40].

There are two types of cancer-associated microRNAs - OncomiRs and tumor-suppressing miRs. The dysregulation of certain miRs leads to the formation of OncomiRs [41]. The tumor suppressor miRs generally help down-regulate the oncogenes. But in some cases, when the number of tumor-suppressing miRs is reduced, oncogenes leading to cancer are rapidly produced [42]. Tumor suppressor miRNAs are used in gene therapies to inhibit cancer progression. miRNA masking, small molecule inhibitors, miRNA sponges, etc are used to downregulate OncomiRs [42]. The RNAi pathway has gained a lot of favor because of its significance as an experimental tool [43]. Various therapeutics are currently available for cancer, such as small molecules, antibodies, etc. Limitations include that the therapy only works when the tumor has a specific target and cannot lead to long-term efficacy [44]. MicroRNAs can act on multiple targets simultaneously and can efficiently inhibit the gene expression of the cancer-causing genes [45]. MicroRNAs also act as biomarkers for various diseases as they circulate in the bloodstream [46]. The imaging and invasive techniques currently used to diagnose cancer can be very costly and uncomfortable for patients [47]. MiRNAs can be used for prediction and prognosis purposes [46]. Only the blood sample is required to identify the miRNAs [47].

Various delivery systems are in practice to efficiently deliver the miRNAs to the tumor microenvironment. The vectors are viral vectors and non-viral vectors [48]. The viral vectors are the genetically engineered viruses that can carry oligonucleotides. The types of viral vectors include

retroviral vectors, adenoviral vectors, lentivirus vectors, bacteriophage associated vectors [49]. The viral vectors are useful because of their low immunogenicity and stable gene expression, but also have disadvantages such as low packaging capacity, viruses that do not replicate in the host cell leading to infections, and high carcinogenic potential due to insertional mutagenesis [49][50]. Non-viral vectors include inorganic materials, lipid-based nanocarriers, polymeric vectors, etc. [48]. The inorganic particles like gold and silicon are used in nanotechnologies to deliver miRNAs. The lipid-based nanocarriers include cationic NLCs, neutral NLCs, and target-modified NLCs. The disadvantages include lack of targeting efficiency, low packing efficiency, etc. [51].

LIQUID-LIQUID PHASE SEPARATION (LLPS): Phase separation is a process in which an immiscible liquid is separated from the mixture into two distinct components. LLPS is a reversible physicochemical phenomenon that consists in the mixing of two distinct liquid phases with different solute concentrations [52]. It is a biological procedure in the cells where the condensates appear like oil droplets in water under various interacting forces[53]. Macromolecules condense with the liquid droplets, forming condensates that contribute to many biological processes and regulatory mechanisms [52][54]. The molecules condense into a dense phase and the dense phase coexists with the aqueous phase. LLPS is a relatively new biophysical method first described by Brangwynne *et. al* as a phase-separated condensate of RNA and proteins [55]. Eukaryotic cells have membrane-bound organe -lles (Endoplasmic Reticulum) and membrane -less organelles (Stress granules) [56]. LLPS is the underlying mechanism for the formation of membrane-less organelles and their functions [56][57]. The driving forces of LLPS include assembly, sequestration, reaction crucible, packaging transports, protein-protein and protein-RNA interactions, weak interactions between intrinsically disordered regions [56]. Databases like (LLPSDB, PhaSePro, PhaSepDB, DrLLPS, RNAgranule -DB, and HUMAN CELLMAP) are developed to identify the membrane-less organelles based on LLPS [58]. Interactions between droplets and enzymatic solutes will achieve functions such as protocell formation, applications such as drug delivery vehicles, and so on [59]. In this study, the total cellular RNA was mixed with proteins to set up the hanging drop vapor diffusion droplets in order to evaluate the formation of LLPS. This will serve as a prototype for future studies.

**Materials & Methods:**

*NCBI search:* Five genes were identified as the most mutating genes in lung cancer. The five genes include KRAS, EGFR, FGFR1, ALK, MET. KRAS is the highly mutated gene and MET is the gene that mutates about 1% in the cases of lung cancer. EGFR has the most number of mutations after KRAS. In this study, we focus on EGFR which accounts for 10%-25% of the cases. The gene EGFR was searched in the gene database of NCBI, and 9 transcript variants were found. Using mirdb.org, microRNAs related to EGFR were listed based on their target scores and target ranks. 118 microRNAs were found to have more than 60 target scores. Of the 118 microRNAs, they were further filtered out based on their self-complementarities. The microRNAs with no self-complementarities are preferred.

*Computing the RNA secondary structure:* The secondary structures of the 118 microRNAs were predicted using the RNAfold web server. The sequences of the microRNAs were pasted into the webserver and the secondary structures were analyzed. The microRNAs with no self-complementarities were preferred as they can bind perfectly to the gene sequence without binding to themselves. Fourteen out of 118 microRNAs were found to be without any self-complementarities whicher were chosen for further study to avoid any self-complementarity bias.

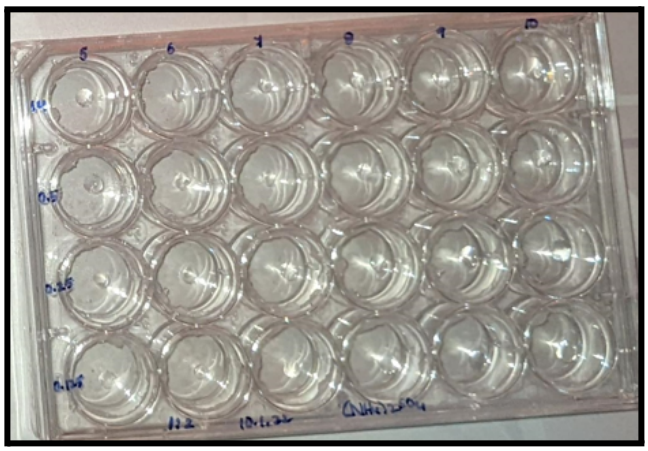

**Figure 3.** A 24-well plate showing hanging drops.

The microRNAs of the EGFR gene till the 92 target score were taken and aligned using ClustalO, to check if all the microRNAs have the same 3'-UTR sequence. The microRNAs are aligned in a perfect way which indicates that all the microRNAs have the same 3'-UTR sequence. The 3'-UTR sequences of the microRNAs are now aligned with the EGFR transcript variants with the help of the Global Align of BLAST database from NCBI. The Global Align results helped in the identification of the CDS and the 3'-UTR sequence.

*Hanging drop vapor diffusion plates:* First, pilot studies were performed with protein and buffer to verify phase separation. To carry out the liquid-liquid phase separation experiments, two buffers with different pH values and different molarities were prepared. The two buffers are sodium chloride (NaCl) and ammonium sulfate ($(NH_4)_2SO_4$) with different molarities of 1 M, 0.5 M, 0.25 M, and 0.125 M are prepared. The different molar buffers are now adjusted to different pH values with 1N HCl or 1N NaCl as acid or base. The different pH values are 5,6,7,8,9,10. The various tubes of the pH are labeled accordingly. The 24-well tissue culture plates were taken to fix the protein droplets. The 24-well plate is now filled with 1 ml of the buffer of different molarities and pH values. The four rows of the plate have four different molarities and the six columns each have different pH values of the buffer. The pilot experiments are carried out under four conditions. Protein and buffer are mixed in ratios of 1:2 and 2:2 with NaCl and (NH4)2SO4. Four plates were fixed with the ratios 1:2 protein:NaCl, 2:2 protein:NaCl, 1:2 protein:$(NH_4)_2SO_4$ and 2:2 protein:$(NH_4)_2SO_4$. A total of 2$\mu$l drops were placed on the coverslip and mixed with the micropipette. The coverslip was carefully placed on the wells of the tissue culture plate. The plates were left undisturbed for 2 weeks to allow the droplets to condense. The plates are later viewed under the microscope to observe phase separation. The same experiments are now conducted with protein, buffer, and whole-cell RNA molecules. These experiments are carried out under two conditions. Protein, RNA, and buffer are mixed in ratios of 0.5:0.5:1 with NaCl and $(NH_4)_2SO_4$. A total of 2$\mu$l drop was placed on the coverslip and mixed with the micropipette. The coverslip was carefully placed on the wells of the tissue culture plate. The plates were left undisturbed for 2 weeks to allow the droplets to condense. The plates are later viewed under the microscope to observe phase separation.

**Results and Discussion:**

*LLPS were obtained in multiple conditions:* For the experiments conducted with protein, RNA and buffer, phase separation is observed in the following five conditions of which three wee prominently visible as shown in Figure 4:

(i.) [0.5:0.5:1 protein:RNA:NaCl] ; buffer concentration - 0.5M, pH - 10.
(ii.) [0.5:0.5:1 protein:RNA:NaCl] ; buffer concentration - 0.25M, pH - 6.
(iii.) [0.5:0.5:1 protein:RNA:NaCl] ; buffer concentration - 0.25M, pH - 8.
(iv.) [0.5:0.5:1 protein:RNA:$(NH_4)_2SO_4$]; buffer concentration - 0.5M, pH - 10.
(v.) [0.5:0.5:1 protein:RNA:$(NH_4)_2SO_4$]; buffer concentration - 0.125M, pH - 8.

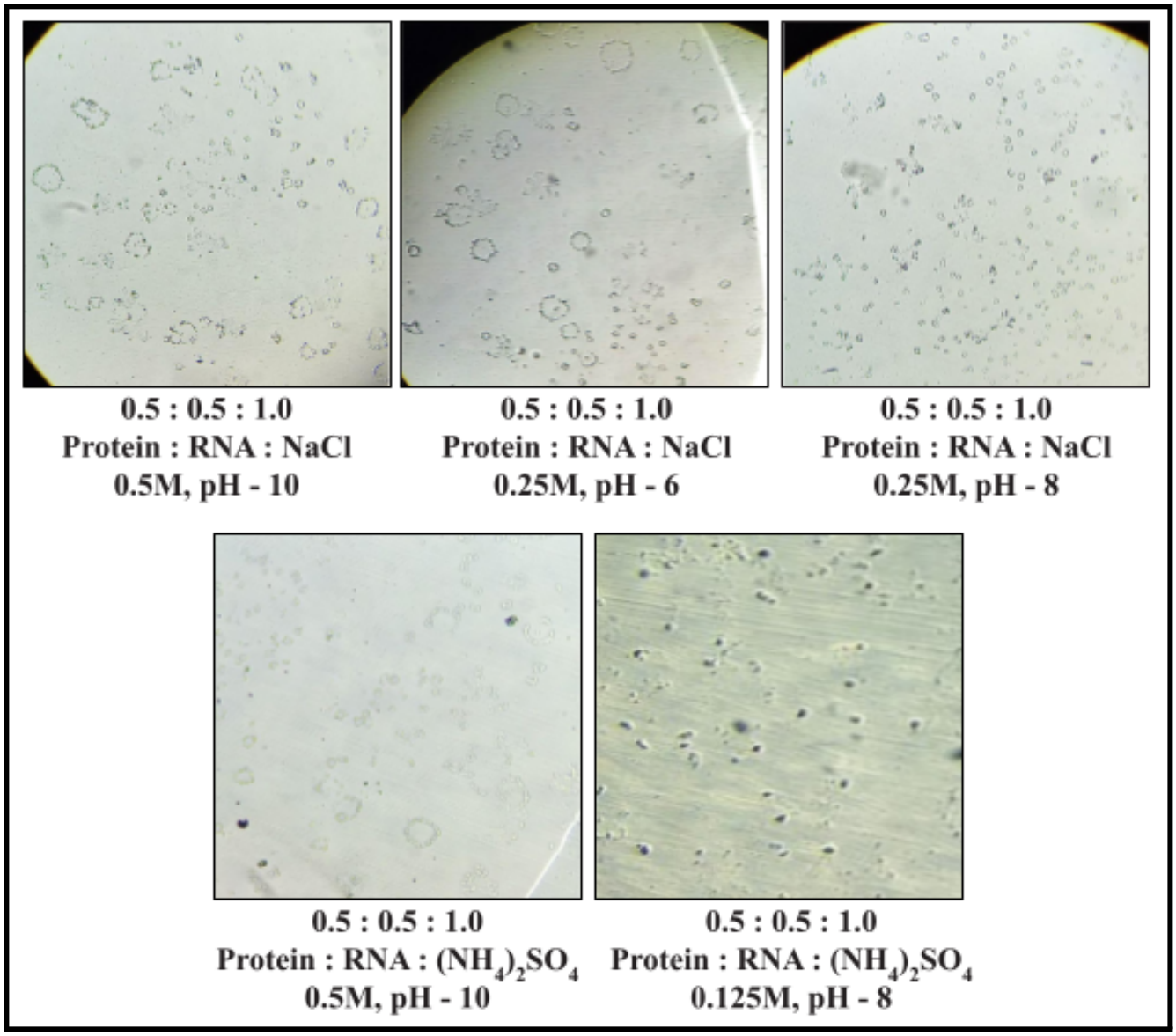


**Figure 4.** Hit conditions that showed LLPS of protein-RNA.

The present study clearly demonstrates that it is indeed possible to obtain liquid-liquid phase separations (LLPS) using a mixture of protein and microRNA. The hit conditions will be further expanded to fine tune the exact conditions of the LLPS. Based on the results in this study we conclude that it is possible to obtain LLPS using protein-RNA mixtures and in future, these hit conditions will be used as novel drug delivery systems. We will check the phase separation with different proteins to find out which protein is the best. We will examine the different sizes and concentrations of microRNAs to test the concentration at which phase separation can be performed with RNAs. The current study has tested and proved that it is indeed possible to obtain LLPS droplets not only with proteins but also with a mixture of protein-RNA. These results suggest that the protein-RNA mixed LLPS droplets can be evaluated further for the feasibility of microRNA delivery as a therapeutic approach.

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