Formulation of liposomes using microfluidics flow reactors fabricated using 3D printing technology

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Introduction:

Drug Delivery Technology market is expected to grow to 1.5 Tr USD by 2020, underpinning the huge need for injectible drugs. One of the key parameters to maximize for drug delivery is efficiency of delivery. Liposomes allow very efficient delivery of drugs, but currently creating liposome encapsulated drug delivery is not scalable or rapid. This is where microfluidics comes in - microfluidics allows a single step high-throughput production of liposomes encapsulating payload.

Microfluidics involves a clever choice of microreactor channel geometry, and flow rate manipulation at the microscale to create liposomes in large quantities and with a fixed size distribution. The channel geometry and dimensions are selected such that fluid flow occurs at the microscale at low Reynold's number. The uniform interface of two or more flows allow for mixing at controlled 'residence times' which allows for control on size of the liposome created. A low polydispersity index, which is a measure of the liposome size distribution, allows for more control on targeting specific tissues and organs.

Objective:

My project focused on exploring using different designs designs for microreactors and different types of lipids to deliver the CRISPR-Cas9 complex to knock down specific genes. However for the scope of my project, as I focused on the ability to formulate liposomes and exert control on its size distribution, I used GFP (green fluorescence protein) as the payload instead of CRISPR/Cas9 for my liposomes. This seemed like a cheaper alternative for a proof of concept.

The objective was to create liposome encapsulated GFP gene in a one-step process in larger quantities and with a narrower size distribution than previously produced using pipette-based mixing techniques.

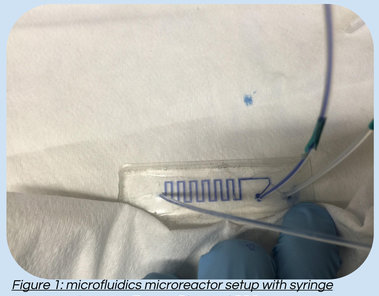

Figure 1: microfluidics microreactor setup with syringe pumps and running Trypan Blue and PBS

Materials and Methods:

Instead of using silicon wafers to create PDMS (poly dimethylsiloxane) molds for the microreactors, I used the Solus DLP 3D printer. Using 3D printing technology to achieve this is quicker, which in line with the objective of rapid processing with high throughput.

I designed the different models of chips designed using Solidworks. The model I used for the setup was a Staggered Herringbone Microreactor. I printed the .STL files using the desktop Solus printer. I baked the chip for 10 minutes in UV, poured in PDMS gel (1:10 mixing ratio using Dow Corning's Sylgard 184 Silicone Elastomer Kit), degassed it in vacuum for 30 minutes, baked it in the oven for 2 hours using oven at 110 degrees Celsius, peeled off the PDMS and then subjected it to plasma treatment to activate surfaces for 40 minutes before bonding it to glass. I punched in inlets at appropriate positions before the oxygen plasma treatment.

I mounted 1 ml syringes containing the lipid solution or the GFP DNA on separate syringe pumps (Harvard Apparatus PHD 2000 Programmable). I adjusted the flow rates as shown in the table below. Initially the syringes were loaded with Trypan Blue dye and PBS buffer solution to test the reactor.

		Flow rates (uL/min)	Duration(s)	Volume used (uL)
Run 1	Inlet 1	100	20	33.3
	Inlet 2	100	20	33.3
Run 2	Inlet 1	300	10	50.0
	Inlet 2	300	10	50.0
Run 3	Inlet 1	50	20	16.7
	Inlet 2	150	20	50.0
Run 4	Inlet 1	100	20	33.3
	Inlet 2	500	20	166.7
	Substance	Total volume needed		
Inlet 1	GFP DNA	100.0		
Inlet 2	Lipid Solution	133.3		

Table 1: Experimental setup with choice of relative flow rates and total volumetric flow rates

The lipid (D23 lipid from Xu Lab's combinatorial library) in sodium acetate solution was prepared at concentration of 100ug/mL, and the GFP DNA was prepared at 20ug/mL. Solutions were loaded onto 1mL syringes and replenished as necessary. The machine was allowed to run for the duration specified (refer to table 1) at each specific flow rate, and the fluid coming out of the outlet was collected in a 2mL eppendorf tube. Between changing flow rates, the microreactor was cleansed at the new flow rate for the specified duration.

I collected the solutions from the outlets and then tested then using Xu Lab's DLS (Dynamic Light Scattering) machine to study the liposome size and polydispersity indices.

Results:

Run Type	Effective Diameter/nm	Effective diameter Std Dev/nm	Polydispersity Index	Polydispersity Index Std Dev
Pipetting	148.74	2.5	0.229	0.01
Lipid Flow rate multiple at 200 uL/min				
1	160.28	5.57	0.257	0.014
3	181.81	2.6	0.236	0.012
Lipid flow rate multiple at 600 uL/min				
1	228.27	2.22	0.171	0.035
5	213.41	1.5	0.211	0.023

Table 2: Variation of effective diameter with relative flow rate and total volumetric flow

The experiment was repeated 5 times, however the data in table 2 is the mean of 3 experiments. The cleaning protocol was different with the first 2 experiments. In the final 3 experiments, the syringe pump was let run for the appropriate duration (refer to table 1) needed to clean out the residue in the microreactor from the last run. There is no clear trend in effective diameter variation with relative flow rates or total volumetric flow. This can be attributed to differences in cleaning techniques between experiments, leaks in the microreactor and increased residence times of residual species from previous experiments.

The first 2 experiments used water as a cleaning medium instead of the experimental solutions. Moreover the microreactor used has a small leak which had to be secured. Ideally, a new microreactor should have been used for each experiment to avoid higher residence times for particles from previous runs lingering in reactor. Additionally, it would be better to use run the syringe pumps for the same duration of time for each flow rate. However the size distribution graphs from DLS studies indicated that the monodispersity was improved for the microfluidics case.

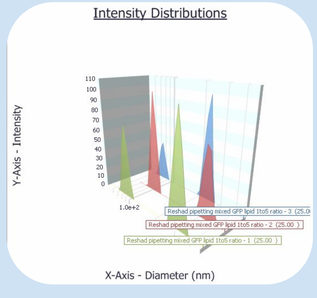

Figure 2: Size distribution graph for pipetting technique

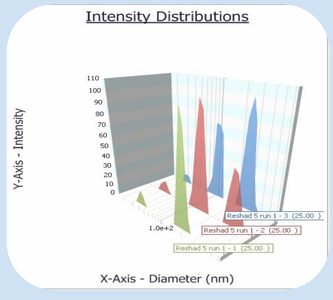

Figure 3: Size distribution graph for 200uL/min 1 RFR

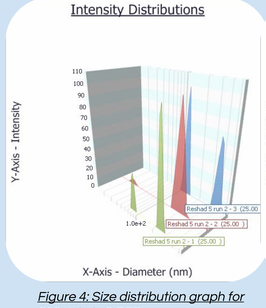

Figure 4: Size distribution graph for 200uL/min 3 RFR

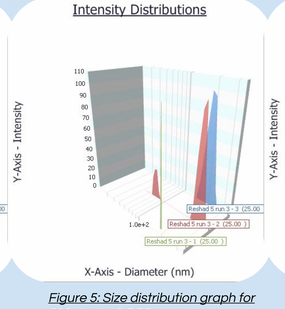

Figure 5: Size distribution graph for 600uL/min 1 RFR

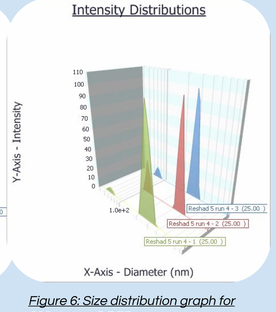

Figure 6: Size distribution graph for 600uL/min 5 RFR

Note: All the graphs above are from the last experiment in the series of repeats

Conclusions:

Further experimenting is necessary to establish a relationship between effective diameter of liposomes before moving on to delivery of CRISPR/Cas9 complex for gene editing. From DLS studies, we are able to see a narrower distribution of particle sizes, and a smaller difference in diameters causing peak intensity. However, I have demonstrated that microreactors can be fabricated wherein flow rates can be controlled using syringe pumps using the fast processing capabilities of 3D printing, and the fine imprinting abilities of PDMS gel.

Figure 7: 3D printed chip and PDMS mold for microreactor

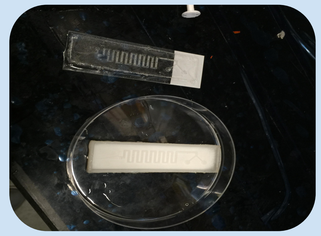

Figure 8: 3D printed chip and microreactor fabricated from glass slide and PDMS mold

Future Works:

There were issues with the oxygen plasma treatment apparatus, which limited the number of reactors that could be fabricated. In the future, there should be more repeats at each flow rate. Additionally, it would be worthwhile to explore more flow rate ratios at a fixed total volume of flow. Previous experiments (Delai Chen) have found reproducible results for effective diameters which leveled off at ~70nm at 200uL/min. Consistent cleaning protocol should be used.

For other avenues of research that could stem from this, we could introduce 3D print other chip designs. We could also try other techniques of microfluidics such as hydrodynamic fluid focussing, with different kinds of junctions. Using multiples inlets (i.e. more syringe pumps), we can incorporate more of the process into the microreactor, e.g. formulation of the lipid in sodium acetate solution.

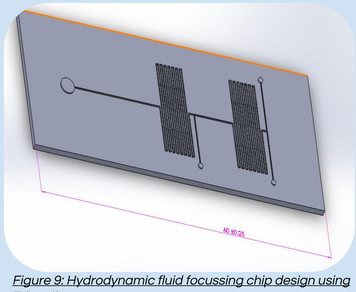

Figure 9: Hydrodynamic fluid focussing chip design using T-junctions

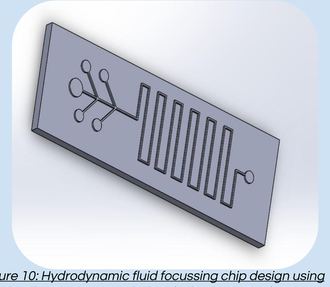

Figure 10: Hydrodynamic fluid focussing chip design using chicken leg junctions and inlets for lipid solution formation

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