Optimizing CRISPR/Cas9 Polymeric Nanoparticle Formulations: From *In Silico* Insights to Microfluidics and Stability Strategies

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INTRODUCTION

Neurological disorders, such as stroke and neurodegenerative diseases, cause severe disability and yet treatments to replace lost neurons are lacking. REGENERAR project aims to develop non-viral polymeric nanoparticles (NPs) to deliver CRISPR/dCas9 tools for epigenetic reprogramming towards brain repair.

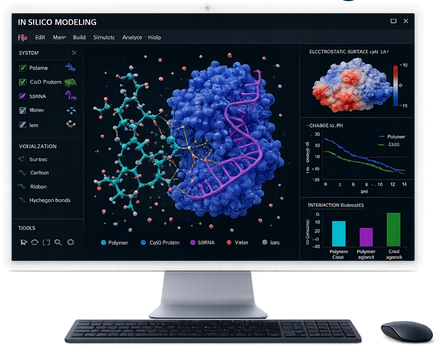
This work focuses on transitioning CRISPR/Cas9 polymeric NP formulations to pharmaceutical standards, evaluating both placebo and RNP-loaded NP and focusing on colloidal stability, size control, and reproducibility. Cas9 was used as a model for dCas9, the nuclease inactive ("dead") Cas9 variant that will be employed in later stages.

OBJECTIVES

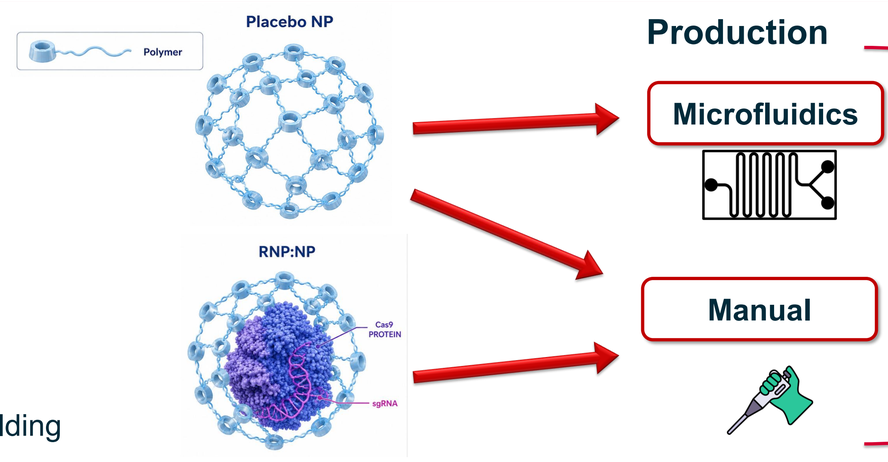
1) Understand polymer–protein interactions and stability
2) Compare microfluidic and manual nanoprecipitation (placebo NP)
3) Identify stabilizers and solvents ratio enabling meeting defined CQAs (size <300 nm; PDI <0.3);
4) Assess early RNP:NP formulations and key improvement levers

METHODS

In silico modeling

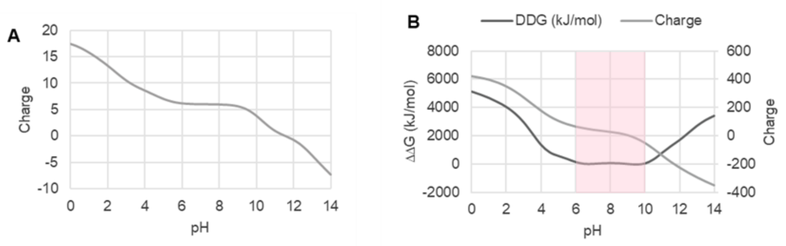


Estimation of polymer protonation, Cas9 folding free energy and charge across pH.



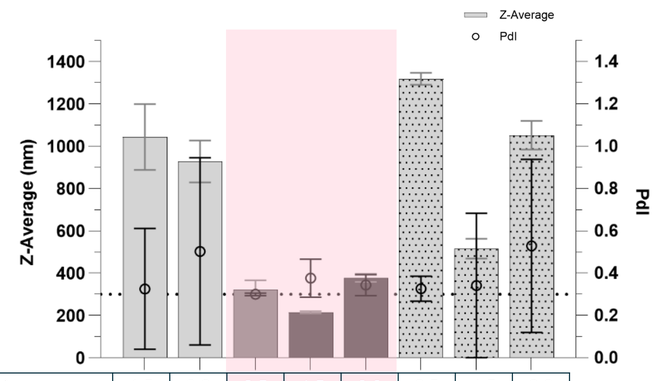
RESULTS AND DISCUSSION

1) In silico modeling



- Polymer protonation maintain mild positive charges at neutral pH, shifting only below pH 6 or above pH 9 (**A**).
- This aligns with Cas9's optimal folding range (pH 6–10) (**B**), supporting stability without pH adjustment.

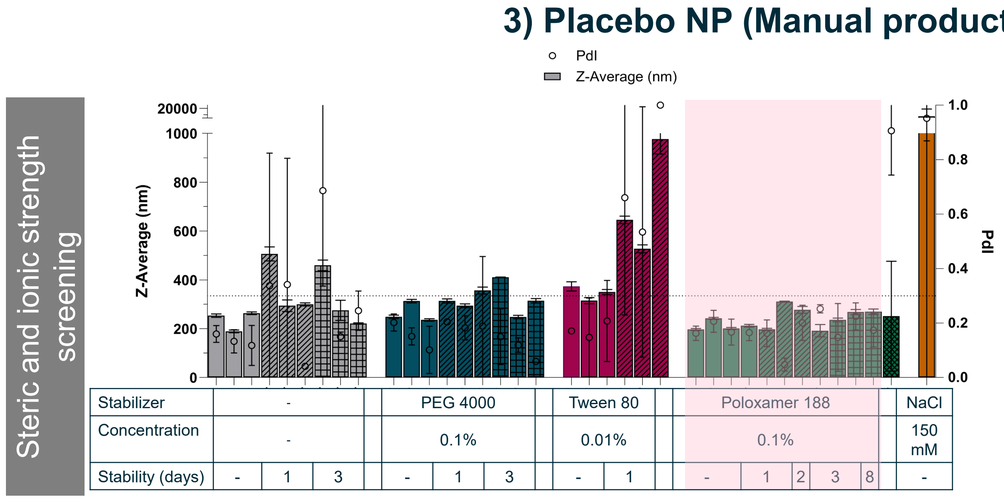
2) Placebo NP (Microfluidics production)



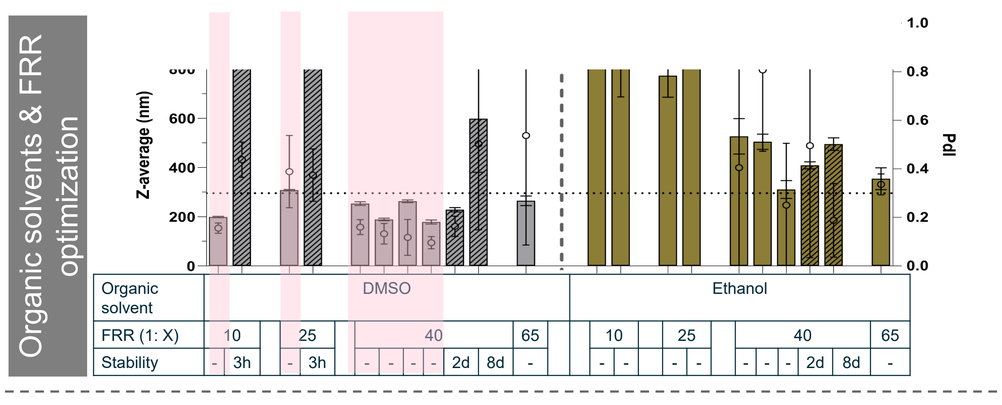
TFR (mL/min)	1.5	3.6	2.5	1.5	3.6	2.5	1.5	3.6
FRR (1: X)	10	10	25	40	40	25	40	40
Final [Polymer] (mg/mL)	0.35	0.35	**0.15**	**0.09**	**0.09**	0.35	0.35	0.35

- Polymer concentration as the main driver of NP size and PDI. Polymer concentrations above 0.3 mg/mL caused aggregation (> 900 nm).
- Total Flow Rate (TFR) and FRR (Flow Rate Ratio) are not critical determinants of particle size and PDI.

3) Placebo NP (Manual production)



Stabilizer	-			PEG 4000			Tween 80		Poloxamer 188					NaCl
Concentration	-			0.1%			0.01%		0.1%					150 mM
Stability (days)	-	1	3	-	1	3	-	1	-	1	2	3	8	-

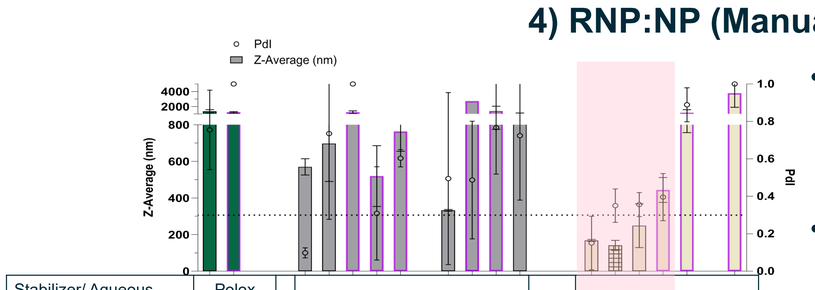


Organic solvent	DMSO								Ethanol							
FRR (1: X)	10		25		40			65	10		25		40			65
Stability	-	3h	-	3h	-	-	-	-	2d	8d	-	-	-	-	2d	8d	-

- Steric stabilization improved short-term stability, with 0.1% Poloxamer 188 and FRR 1:40 formulations maintaining CQAs for 3 days.
- PEG 4000 also improved stability but less consistently.
- Tween 80 did not improve stability.
- Ionic strength buffer (NaCl) did not improve placebo NP' size and PDI.

- DMSO as a more suitable and consistent organic solvent for placebo formulation to meet target Z-average and PDI values, specially for FRR of 1:10 and 1:40.

4) RNP:NP (Manual production)



Stabilizer/ Aqueous solvent	Polox 188		-				HEPES			
Concentration	0.1%		-				10 mM			
FRR (1:X)	40		10		40		10		40	
RNP concentration (nM)	-	32	-	120	-	116	-	120		116
Vortex during process	-		-	✓	-	✓	✓		✓	
Stability (days)	-		-		-		-	5	-	-

- In placebo NPs, combinatory effect of HEPES and vortex lead to satisfactory NPs' size and PDI in 1:10 O:A formulations.
- In RNP:NP systems, these conditions improved size and PDI and allowed identification of early leads at T0.
- Stability will be further optimized through low-temperature storage and drying approaches.

CONCLUSIONS

- Low polymer concentration and steric stabilization improved placebo NP size, PDI and short-term stability. 0.1% Poloxamer 188 emerged as the most promising stabilizer.
- RNP:NP data suggest HEPES 10 mM and vortex can improve particle size and PDI, guiding continued RNP-loaded NP optimization and characterization.
- Manual nanoprecipitation showed limited reproducibility, supporting tighter process control and transition to microfluidics production.

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