Curcumin-Loaded Chitosan Nanoparticle Synthesis

Supporting Material 1: Protocol-Centric Details

1. Purpose of This Supporting Material

This supporting material gives the practical protocol-level details for curcumin-loaded chitosan nanoparticle synthesis. It is meant to support the main Protoly-fillable protocol by adding information that is useful for preparation, execution planning, batch comparison, and optimization.

This document focuses on:

- what materials are required,
- which steps are prepared manually before the run,
- which steps are performed in the automated run,
- which steps are performed after the run,
- which parameters should be varied during optimization,
- how changes in those parameters affect the result,
- and what starting values are suitable for initial formulation trials.

2. Protocol Scope

This protocol prepares curcumin-loaded chitosan nanoparticles using an ionic gelation approach. Chitosan acts as the polymeric carrier, curcumin acts as the hydrophobic model payload, and sodium tripolyphosphate acts as the ionic crosslinking agent.

The preparation workflow is divided into three parts:

Workflow Part	Description
Pre-run manual setup	Preparation of stock solutions, reservoir loading, vessel setup, and light-protected handling of curcumin
Automated run	Dispensing, stirring, mild heating, crosslinker addition, stabilization, optional sonication, and visual documentation
Post-run offline work	Sample collection, purification, batch recording, particle characterization, loading estimation, release study, and application-specific evaluation

The automated run should remain continuous once started. Manual actions such as pH checking, centrifugation, washing, or sampling should be performed before or after the run, not in the middle of the automated preparation sequence.

3. Materials and Preparation Requirements

3.1 Main Reagents

Reagent	Purpose in Protocol	Preparation Note
Chitosan solution	Main polymeric carrier phase	Prepare in dilute acidic medium and dissolve completely before use
Curcumin solution or fine dispersion	Hydrophobic model payload	Prepare using compatible solvent system or dispersion method; protect from strong light

Sodium tripolyphosphate solution	Ionic crosslinking agent	Prepare as a clear aqueous solution
Deionized water	Aqueous working medium	Used for dilution and reaction volume adjustment
Stabilizer or mild surfactant solution	Optional dispersion support	Use only if curcumin precipitation or non-uniform dispersion is observed

3.2 Optional Components

Optional Component	Possible Use
Tween-type mild surfactant	May improve curcumin dispersion
PEG-based stabilizer	May improve colloidal stability
Buffer medium	May be used for resuspension or release study
pH-specific release medium	May be used for pH-responsive release comparison
Fluorescent marker	May be used for method demonstration or comparison studies

3.3 Consumables

Consumable	Use
Reaction vessel	Nanoparticle preparation
Magnetic stir bar	Controlled mixing
Reservoir containers	Loading prepared solutions
Dispensing tips	Automated liquid addition
Amber vial or foil-wrapped vial	Light-protected sample collection
Microcentrifuge tubes	Purification and offline testing
Labels	Batch identification
PPE	Safe handling during preparation and cleanup

4. Module Use in Automated Run

Module	Function in This Protocol
Sterilization UV	Pre-run chamber sterilization cycle
Reservoir Dispense	Addition of water, chitosan solution, curcumin dispersion, stabilizer, and TPP solution
Stirrer	Controlled mixing during polymer conditioning, payload incorporation, and nanoparticle formation
Heater	Mild temperature support for polymer uniformity
Wait	Stabilization and reaction hold periods
Sonicator	Optional dispersion improvement after nanoparticle formation
Sonicator Bath Heater	Optional mild bath temperature support during sonication
LED Illumination	White-light support for visual monitoring and recording
UV Illumination	Chamber illumination only, if used; not UV-Vis measurement
IR Illumination	Camera support only, if required
Camera	Visual documentation of colour, turbidity, settling, and aggregation
Exhaust	Airflow support during formulation handling
Environment Sensors	Ambient chamber environment record

5. Manual Pre-Run Preparation

The following activities should be completed before starting the automated run.

Step	Manual Action	What to Do	Important Notes
1	Prepare chitosan solution	Dissolve chitosan in dilute acidic medium	Ensure complete dissolution; remove visible undissolved

			material if needed
2	Prepare curcumin solution or dispersion	Prepare curcumin stock or fine dispersion using compatible solvent system	Minimize light exposure and avoid excess solvent in final mixture
3	Prepare TPP solution	Prepare sodium tripolyphosphate solution separately	Use a clear and well-mixed solution for consistent crosslinking
4	Prepare optional stabilizer	Prepare stabilizer or mild surfactant solution if required	Use only after observing curcumin dispersion issues or for planned optimization
5	Load reservoirs	Assign water, chitosan, curcumin, stabilizer, and TPP to separate channels	Keep reservoir assignment consistent between batches
6	Arrange reaction vessel	Place clean vessel and stir bar in position	Confirm that stirring and heating contact are proper
7	Protect curcumin-containing materials	Use amber containers or foil wrapping if needed	Curcumin can be affected by strong light exposure

After this setup, the automated run can begin without manual interruption.

6. Automated Run Sequence

The automated run contains only machine-supported steps. The values below are suggested starting values and can be changed during optimization.

Step	Module	Action	Suggested Starting Value
1	Sterilization UV	Prepare chamber environment	5 min
2	LED Illumination	Turn on white light for visual monitoring	On
3	Environment Sensors	Record initial ambient chamber condition	As available
4	Reservoir Dispense	Add deionized water	100 uL
5	Reservoir Dispense	Add chitosan solution	200 uL
6	Stirrer	Mix polymer phase	500 RPM; 10 min
7	Heater	Mildly condition polymer phase	35 C; 10 min
8	Reservoir Dispense	Add curcumin solution or dispersion	25 uL
9	Stirrer	Allow curcumin distribution in chitosan	500 RPM; 15 min
10	Reservoir Dispense	Add optional stabilizer	10 uL; optional
11	Stirrer	Mix after stabilizer addition	450 RPM; 5 min
12	Reservoir Dispense	Add TPP solution for crosslinking	75 uL
13	Stirrer	Maintain nanoparticle formation	600 RPM; 25 min
14	Wait	Stabilize formed dispersion	45 min
15	Sonicator	Optional dispersion improvement	3 min
16	Sonicator Bath Heater	Optional mild bath temperature support	30 C; 3 min
17	Wait	Allow post-sonication rest	15 min
18	Camera	Record final appearance	End of run
19	Exhaust	Use airflow support during run if required	Continuous or as required

7. Manual Post-Run / Offline Work

Step	Post-Run Action	What to Do	Purpose
1	Collect dispersion	Transfer the prepared dispersion into a labelled amber vial	Protect curcumin and preserve batch identity
2	Record observations	Note colour, turbidity, settling, precipitation, and aggregation	Quick batch comparison
3	Purify if required	Use centrifugation and washing	Remove free curcumin, excess TPP,

			and soluble residues
4	Resuspend particles	Resuspend pellet in water or suitable buffer	Prepare sample for testing or storage
5	Measure pH offline	Confirm final dispersion condition	Supports formulation comparison
6	Analyze particle size and PDI	Perform DLS or equivalent analysis	Confirm size distribution and uniformity
7	Measure zeta potential	Determine surface charge	Helps interpret colloidal stability
8	Quantify curcumin loading	Use UV-Visible or other suitable method	Estimate encapsulation efficiency and loading capacity
9	Perform release or stability studies	Use selected media and time points	Evaluate delivery behaviour and formulation stability
10	Plan next formulation round	Use results to adjust formulation variables	Supports iterative optimization

8. Critical Variables for Optimization

The variables below should be changed systematically. During early optimization, change one major variable at a time so that the result remains interpretable.

8.1 Formulation Variables

Variable	Suggested Starting / Practical Range	Effect of Increasing	Optimization Aim
Chitosan concentration	0.1-0.3% w/v	Higher viscosity and stronger matrix; may cause larger particles or aggregation	Stable opalescent dispersion without gelation or large aggregates
TPP concentration	0.05-0.2% w/v	More crosslinking; excess may cause aggregation or precipitation	Minimum TPP needed for stable particle formation
Chitosan:TPP ratio	3:1 to 6:1 mass ratio; start near 4:1 or 5:1	More chitosan may improve payload association but increase viscosity; more TPP may cause precipitation	Stable particle formation with low sediment
Curcumin loading	5-15% of chitosan mass for screening	Stronger colour and higher loading attempt; excess may precipitate	Maximum loading without visible free curcumin
Curcumin solvent level	Keep as low as practical; preferably <=5% final organic solvent if solvent is used	Improves solubilization but may disturb chitosan/TPP particle formation	Minimum solvent needed for uniform curcumin introduction
Stabilizer/surfactant	Lowest useful level; commonly 0.01-0.1% if required	May reduce curcumin precipitation; excess can disturb particle formation	Improve dispersion without interfering with ionic gelation

8.2 Process Variables

Variable	Suggested Starting / Practical Range	Effect of Increasing	Optimization Aim
Stirring before TPP addition	400-600 RPM	Better mixing; excessive speed may introduce bubbles	Uniform curcumin-chitosan phase
Stirring after TPP addition	500-700 RPM	Improves dispersion; excessive speed may foam or destabilize	Smooth yellow turbidity with low sediment
TPP addition volume/rate	Controlled addition; 25-100 uL depending on scale	Fast or excess addition may cause local over-crosslinking	Controlled particle formation with minimal aggregation
Heating temperature	30-40 C	Improves polymer uniformity; high heat may affect curcumin	Mild conditioning without curcumin degradation
Heating time	10-20 min	Improves polymer uniformity; excessive time may not add benefit	Uniform chitosan phase before payload addition
Sonication duration	1-3 min initially; up to 5 min if required	Improves dispersion; excess may heat or destabilize sample	Reduce visible aggregation without overheating
Stabilization time	30-60 min	Allows maturation; unstable	Stable dispersion before collection

		batches may show settling	
Light exposure	Minimize after curcumin addition	More exposure may degrade curcumin	Use light-protected handling and storage

9. Suggested Initial Optimization Plan

9.1 Round 1: Basic Feasibility

Goal: Confirm that curcumin-loaded chitosan nanoparticle dispersion can be formed.

Parameter	Suggested Starting Value
Chitosan concentration	0.2% w/v
TPP concentration	0.1% w/v
Chitosan:TPP ratio	4:1 or 5:1
Curcumin loading	Low-to-moderate
Stirring before TPP	500 RPM
Stirring after TPP	600 RPM
Heating	35 C for 10-15 min
Stabilization	45 min
Sonication	2-3 min, optional

Evaluate visual appearance, turbidity, colour uniformity, and settling.

9.2 Round 2: Curcumin Loading Optimization

Goal: Increase curcumin content without causing precipitation.

Prepare batches with low, medium, and high curcumin levels while keeping chitosan and TPP constant.

Selection criterion: choose the highest curcumin level that maintains uniform colour and minimal visible settling.

9.3 Round 3: Crosslinking Optimization

Goal: improve particle formation and reduce aggregation.

Vary TPP concentration or chitosan:TPP ratio.

Selection criterion: choose the condition that gives stable opalescent dispersion without heavy precipitation.

9.4 Round 4: Dispersion Optimization

Goal: improve uniformity and reduce loose aggregation.

Vary sonication time and stabilizer level.

Selection criterion: choose the lowest sonication and stabilizer level that gives acceptable dispersion uniformity.

9.5 Round 5: Application-Specific Refinement

Goal: select batches for targeted application studies.

External results such as particle size, zeta potential, loading efficiency, release behaviour, and stability can guide the next formulation cycle.

10. Expected Result Changes When Parameters Vary

Observation	Likely Cause	Suggested Adjustment
Uniform yellow opalescent dispersion	Good preliminary particle formation and curcumin distribution	Take forward for characterization
Yellow precipitate appears quickly	Curcumin loading too high, poor dispersion, or insufficient stabilization	Reduce curcumin level, improve pre-mixing, or test mild stabilizer
Heavy white/yellow turbidity with sediment	Excess TPP or high chitosan causing aggregation	Reduce TPP volume/concentration or lower chitosan concentration
Clear pale yellow solution with little turbidity	Weak particle formation or insufficient TPP	Increase TPP slightly or optimize chitosan concentration
Very thick mixture	Chitosan concentration too high	Reduce chitosan concentration or adjust dilution
Foam or bubbles during run	Stirring or sonication too intense	Lower RPM or shorten sonication
Good colour but poor stability after rest	Weak particle stability or high free curcumin	Optimize chitosan:TPP ratio, stabilizer, or purification
Colour lost after washing	Curcumin weakly associated or mostly free	Adjust curcumin addition, solvent level, and crosslinking conditions
Improved uniformity after sonication	Loose aggregates were present	Keep mild sonication in optimized protocol

11. Suggested Batch Record Table

Batch ID	Chitosan Conc.	Curcumin Level	TPP Level	Stabilizer	Stirring RPM	Sonication	Appearance	Remarks
CUR-CSNP-01	0.2%	Low	Medium	None	500-600	3 min	Light yellow turbidity	Base batch
CUR-CSNP-02	0.2%	Medium	Medium	Low	500-600	3 min	Uniform yellow dispersion	Promising
CUR-CSNP-03	0.2%	High	Medium	Low	500-600	3 min	Yellow precipitate	Reduce curcumin
CUR-CSNP-04	0.3%	Medium	Medium	Low	600	3 min	Thick dispersion	Reduce chitosan
CUR-CSNP-05	0.2%	Medium	High	Low	600	3 min	Heavy turbidity	Reduce TPP

12. Offline Characterization Plan

Test	Purpose	Key Output
Particle size / PDI	Confirm size and uniformity	Mean size and distribution
Zeta potential	Assess surface charge and colloidal tendency	Charge profile and stability indication
UV-Visible analysis	Quantify curcumin concentration	Curcumin absorbance-based concentration
Encapsulation efficiency	Determine loaded vs free curcumin	% encapsulation efficiency
Loading capacity	Estimate curcumin amount per nanoparticle mass	% loading capacity
Release study	Evaluate curcumin release over time	Release profile curve
pH release comparison	Compare release under selected pH conditions	pH-dependent release difference
Stability study	Track settling, aggregation, and colour change	Short-term and storage stability
Microscopy	Observe morphology or visible aggregation	Particle/aggregate images
Cell-based study	Application-specific biological response	Cell viability, uptake, or response data

13. Safety and Handling Notes

- Use gloves, lab coat, and eye protection.
- Handle dilute acidic chitosan solution carefully.
- Minimize curcumin exposure to strong light.
- If curcumin stock uses ethanol or another compatible solvent, complete solvent handling safely and use exhaust where required.
- Avoid overheating during heating or sonication.
- Label all batches clearly.
- Dispose nanoparticle-containing waste according to laboratory guidelines.

14. Future Extensions

Future Extension	Purpose
pH-responsive release study	Compare curcumin release under pH 7.4, 6.5, and 5.5
Bioinformatics-to-formulation workflow	Dock curcumin with a cancer-related target and then prepare nanoparticle formulation
Curcumin-loaded chitosan hydrogel	Incorporate nanoparticles into a hydrogel matrix
Liposome vs chitosan nanoparticle comparison	Compare lipid and polymeric carriers
Manual vs automated preparation comparison	Compare reproducibility and batch documentation
Cellular uptake model	Evaluate uptake of curcumin-loaded nanoparticles in relevant cells
Storage stability study	Compare colour, settling, aggregation, and performance over time

16. Protocol-Centric Supporting Summary

This supporting material describes the practical preparation details for curcumin-loaded chitosan nanoparticles. The key preparation stages are stock solution preparation, automated liquid dispensing and mixing, TPP-mediated ionic gelation, optional sonication, sample collection, purification, and external characterization.

The most important variables to optimize are chitosan concentration, curcumin loading, TPP concentration, chitosan:TPP ratio, solvent level, stabilizer amount, stirring speed, heating condition, stabilization time, and sonication duration. A useful initial target is a uniform yellow opalescent dispersion with minimal visible precipitation and acceptable short-term stability.

The results from visual observation and external characterization can be used to refine the next formulation cycle and adapt the protocol for specific application studies.

Operator Preparation Section Before Starting Automation

This section is intended for the operator who will prepare all solutions, load the machine reservoirs, place the reaction vessel, and make the system ready before starting the automated run. The aim is to ensure that all materials are prepared correctly and that the automated sequence can run continuously without interruption.

1 Recommended Small-Scale Demonstration Batch

The following preparation is suitable as a starting demonstration-scale batch. Volumes can be scaled later after optimization.

Component	Suggested Working Amount for One Run	Purpose
Deionized water	100 uL	Initial aqueous volume
Chitosan solution	200 uL	Polymeric carrier phase
Curcumin solution or dispersion	25 uL	Model hydrophobic payload
Optional stabilizer solution	10 uL	Dispersion support, if required
TPP solution	75 uL	Ionic crosslinking agent

For the first trial, a simple formulation may be prepared without stabilizer. If visible curcumin precipitation or uneven yellow colour is observed, a low level of stabilizer may be added in the next formulation iteration.

2 Preparation of Chitosan Solution

Suggested starting concentration

Prepare **0.2% w/v chitosan solution** as the first working concentration.

This means:

Final Volume	Chitosan Required
10 mL	20 mg
50 mL	100 mg
100 mL	200 mg

Suggested preparation procedure

1. Take the required volume of dilute acidic medium in a clean beaker or glass vial.
2. Slowly add the weighed chitosan powder into the acidic medium while stirring.
3. Stir the solution continuously until the chitosan is fully dissolved. This may take several hours depending on the chitosan grade, molecular weight, and concentration.
4. If visible undissolved particles remain, allow additional stirring time.
5. If required, filter or carefully decant the clear portion to remove visible undissolved lumps.
6. Label the solution with concentration, preparation date, and batch number.
7. Keep the solution covered until reservoir loading.

Important operator notes

- Do not add chitosan powder all at once in a lump; sprinkle gradually during stirring.
- Highly viscous chitosan solution may not dispense smoothly.
- Ensure there are no visible lumps before loading the reservoir.
- Keep the chitosan concentration consistent between batches so that optimization results are comparable.

3 Preparation of Curcumin Solution or Fine Dispersion

Curcumin is poorly soluble in water, so it should be prepared carefully before the automated run.

Option A: Curcumin stock solution

8. Weigh the required quantity of curcumin.
9. Dissolve curcumin in a small amount of compatible solvent such as ethanol or another suitable formulation-compatible solvent.
10. Mix thoroughly until the solution appears uniform.
11. Keep the stock protected from strong light using an amber vial or foil wrapping.
12. Before loading into the reservoir, mix gently again to ensure uniformity.

Option B: Fine curcumin dispersion

13. Weigh the required amount of curcumin.
14. Add it to a small volume of aqueous medium with or without a low level of stabilizer, depending on the formulation plan.
15. Mix thoroughly using vortexing, stirring, or mild sonication outside the automated run if available.
16. Allow large visible particles to settle briefly, if needed, and use the more uniform upper dispersion for loading.
17. Protect the dispersion from strong light.

Suggested starting curcumin level

For early screening, use a **low-to-moderate curcumin level**, approximately equivalent to **5-15% of chitosan mass**.

Important operator notes

- Avoid using excessive solvent because high solvent content may disturb chitosan-TPP nanoparticle formation.
- Try to keep final organic solvent content low, preferably **<=5%** of the final formulation volume where possible.
- Curcumin can settle in dispersion form, so mix before loading into the reservoir.
- Curcumin-containing solutions should be protected from unnecessary light exposure.

4 Preparation of Sodium Tripolyphosphate Solution

Suggested starting concentration

Prepare **0.1% w/v sodium tripolyphosphate solution** as a starting crosslinker solution.

This means:

Final Volume	TPP Required
10 mL	10 mg
50 mL	50 mg
100 mL	100 mg

Suggested preparation procedure

18. Take the required volume of deionized water in a clean container.
19. Add the weighed sodium tripolyphosphate powder.
20. Mix until the solution is completely clear.
21. If any particles remain, continue mixing until fully dissolved.
22. Label the solution with concentration, preparation date, and batch number.
23. Load into the assigned reservoir channel only after confirming that the solution is clear.

Important operator notes

- TPP concentration strongly affects nanoparticle formation.
- Too much TPP may lead to aggregation or precipitation.
- Use the same TPP concentration during comparative batches unless TPP is the variable being optimized.

5 Preparation of Optional Stabilizer or Mild Surfactant Solution

This solution is optional and should not be overused in the first batch unless curcumin dispersion is visibly poor.

Suggested approach

24. Prepare a low-concentration stabilizer or mild surfactant solution.
25. Mix until fully uniform.
26. Use the lowest concentration that improves curcumin dispersion.
27. Label clearly and load into the assigned reservoir only if the formulation plan includes stabilizer addition.

Important operator notes

- Stabilizer may improve curcumin dispersion, but excess stabilizer can interfere with chitosan-TPP nanoparticle formation.
- For the first screening round, prepare one batch without stabilizer and one batch with low stabilizer for comparison.

6 Reservoir Loading Plan

Before starting the automated run, load each solution into the correct reservoir channel.

Reservoir Channel	Solution to Load	Operator Check
Channel 1	Deionized water	Clear, particle-free
Channel 2	Chitosan solution	Fully dissolved, no visible lumps
Channel 3	Curcumin solution or dispersion	Uniform yellow solution/dispersion, protected from light
Channel 4	Optional stabilizer solution	Clear and uniform; load only if used
Channel 5	Sodium tripolyphosphate solution	Clear and fully dissolved

Loading precautions

- Avoid air bubbles while loading reservoir lines.
- Make sure each reservoir is connected to the correct dispensing channel.
- Label reservoir containers clearly.
- Confirm that the dispensing volume in the method matches the actual reservoir assignment.
- If curcumin settles during waiting time, mix gently before loading or just before starting the run.

7 Reaction Vessel and Stirring Setup

28. Select a clean reaction vessel compatible with the working volume.
29. Place a suitable magnetic stir bar inside the vessel.
30. Position the vessel correctly on the stirrer/heater area.
31. Confirm that the stir bar rotates smoothly at low speed before starting the full automated run.
32. Confirm that the vessel is stable and will not shift during stirring or dispensing.
33. Keep the vessel open or covered as appropriate for the dispensing arrangement.

Operator checks

Check	Reason
Stir bar centered	Prevents unstable stirring
Vessel stable	Avoids spillage during dispensing
Dispense nozzle aligned	Ensures liquids enter the vessel properly
Heater contact appropriate	Supports uniform mild heating
Camera view unobstructed	Enables final visual documentation

Machine Readiness Checklist

Before pressing start, confirm the following:

Checklist Item	Status
Chitosan solution prepared and loaded	
Curcumin solution/dispersion prepared, mixed, and loaded	
TPP solution prepared and loaded	
Optional stabilizer loaded, if included	
Deionized water reservoir loaded	
Reservoir channels assigned correctly	
Reaction vessel placed correctly	
Magnetic stir bar placed inside vessel	
Stirring checked briefly	
Dispensing tips/nozzles aligned	
Chamber illumination available	
Camera view clear	
Exhaust setting ready, if required	
Collection vial prepared for post-run handling	
Batch record sheet ready	

Batch Record Before Starting Automation

The operator should record the following before starting the run:

Field	Entry
Batch ID	
Date and time	
Operator name	
Chitosan concentration	
Curcumin concentration / loading level	
TPP concentration	
Stabilizer used?	Yes / No
Reservoir channel assignment checked?	Yes / No
Stir bar checked?	Yes / No
Light protection used for curcumin?	Yes / No
Notes before run	

Start of Automated Run

Once all solutions are prepared, reservoirs are loaded, the reaction vessel is placed, and the stir bar position is confirmed, the operator can start the automated protocol.

After starting the automated run, avoid interrupting the process unless there is a visible issue such as spillage, vessel displacement, severe foam formation, or dispensing failure.

NOTES