Novel pH-Responsive Dual-Layer Chitosan-Alginate Smart Nanogels for Curcumin Delivery
with Relevance to Colorectal Cancer and Biological Validation in *Drosophila melanogaster*

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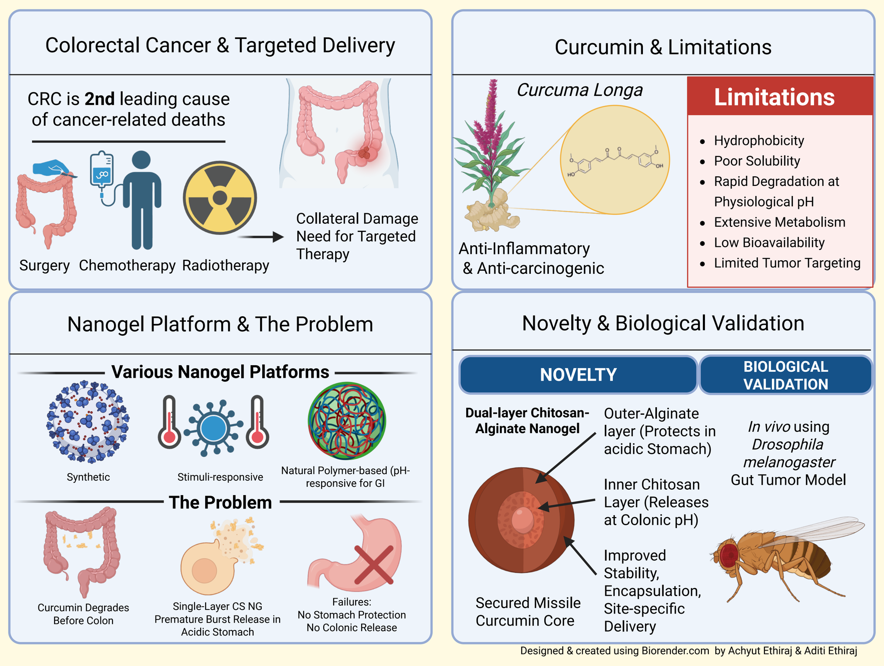
INTRODUCTION



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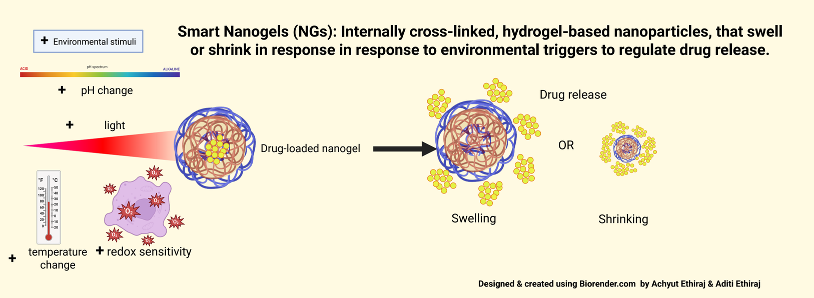
PROBLEM

- CUR delivery is limited by its rapid degradation and lack of site-specific targeting.
- Single-layer NGs fail in the stomach, as acid protonation of chitosan's amine groups triggers a substantial premature burst release at pH 1.5, which leaves CUR medically ineffective.
- Furthermore, current protocols for synthesizing dual-layer NGs are unreliable and poorly documented, key parameters scattered across chains of cross-references.
- Low encapsulation efficiency, multistep purification requirements and expensive reagents make existing methods costly and inefficient, which creates a major barrier to producing NGs for CUR delivery.

NANOGEL DESCRIPTION

Smart Nanogels (NGs): Internally cross-linked, hydrogel-based nanoparticles, that swell or shrink in response in response to environmental triggers to regulate drug release.



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RESEARCH QUESTION

- How can optimized synthesis of dual-coated chitosan-alginate nanogels enhance the targeted therapeutic potential of curcumin for the treatment of colorectal cancer (CRC)?
- What formulation parameters (pH, solvent, mixing conditions) optimize encapsulation efficiency in dual-coated NGs?
- How does chitosan & alginate coating affect NGs' surface properties, and what parameters ensure successful dual-layer formation?
- Does dual coated NGs demonstrate pH-responsive release behaviour that protects curcumin at gastric pH while releasing at colonic pH?
- How does solvent choice affect curcumin loading?
- What conditions produce a stable particle size
- Which kinetic model describes the best drug release behavior?

HYPOTHESIS

Optimized synthesis parameters will yield dual-coated chitosan-alginate nanogels with high encapsulation efficiency (>70%), spherical morphology, 200-400 nanometers, and surface charge reversal, confirming a dual-layer structure. These NGs will exhibit pH-responsive release in the colon as confirmed through in vitro drug release studies under simulated gastric and colonic conditions. *In vivo* validation in *Drosophila melanogaster* will confirm targeted midgut delivery of curcumin via dual layer NG superior to free curcumin and single-layer NGs.

NOVELTY

- **Smart Dual-Layer Design**: Developed a core-shell structure that provides significantly greater drug protection than single-layer NGs
- **Superior NG Quality**: Achieved an optimized synthesis, with factors including particle size, stable zeta potential, and high encapsulation efficiency.
- **Precision Colon Targeting**: Utilized a pH-responsive mechanism that minimizes drug loss at gastric pH (1.5) and specifically triggers release at colon pH (7.4)
- **Simulated GI Testing**: Demonstrated efficient drug delivery using *in vitro* GI model that accurately resembled human digestion by maintaining temperature (37°C), accurate pH gradients, and relevant digestion timings
- **Biological Drug Delivery Validation**: Confirmed the targeted drug delivery using *Drosophila melanogaster* as a gut model due to its conserved digestive physiology and pH gradients that closely resemble the human digestive system.

RESEARCH DESIGN

- **Systematic Process Optimization**: Evaluated multiple synthesis variables-including solvent type, pH, mixing time, stirring RPM, composition ratios-to refine the protocol
- **Testing Protocol**: Conducted 20 experimental trials, consisting of 12 exploratory trials to optimize procedures and 8-10 confirmatory trials to ensure reproducibility
- **Statistical Modeling**: Performed Multivariate Analysis, including Pearson Correlation and Principle Component Analysis (PCA).

VARIABLES

Synthesis	Characterization	Biological Validation
Independent Variables		
Curcumin solvent (DMSO vs Ethanol vs Ethanol + Tween-80)	pH conditions during drug release	Number of nanogel layers (single-layer vs. dual-layer)
Chitosan solution pH (4.5, 5.0, 5.5)	Time points during drug release	Amount of curcumin loaded within the nanogel
Stirring speed (RPM) during ionic gelation	—	—
Dependent Variables		
Curcumin encapsulation efficiency (EE%)	UV-Vis absorbance quantification	Fluorescence imaging of curcumin distribution across gut regions (foregut, midgut, hindgut)
Particle size (SEM/TEM Feret diameter analysis)	FTIR peak shifts confirming polymer interactions	Gut region fluorescence intensity (quantified with muscle autofluorescence correction)
Particle Dispersity Index (PDI via DLS)	Fluorescence imaging of curcumin distribution	—
Constants		
Ionic gelation method used for synthesis	All instruments kept at room temperature	Drosophila larval strain and developmental stage
All instruments kept at room temperature	—	Instruments: Leica confocal microscope, FIJI/ImageJ analysis

SYNTHESIS

EXPERIMENTAL PROCEDURE

Prepare Chitosan (CS) Solution
- 100 mg CS dispersed in 39.5 ml DI water+ 0.5 mL glacial acetic acid
- Acetic acid lowers pH below CS pKa(6.3)⟶full protonation & dissolution
- stirred 24 hrs at RT for complete polymer chain hydration
- pH adjusted to 4.5-5.5 for TPP addition

Dissolving Curcumin (CUR)
- 20 mg CUR dissolved in EtOH, EtOH + Tween 80, or DMSO
- CUR is hydrophobic; EtOH acts as a co-solvent;
- Tween 80 prevents recrystallization on the aqueous contact
  DMSO and EtOH only approaches, caused precipitation and aggregation

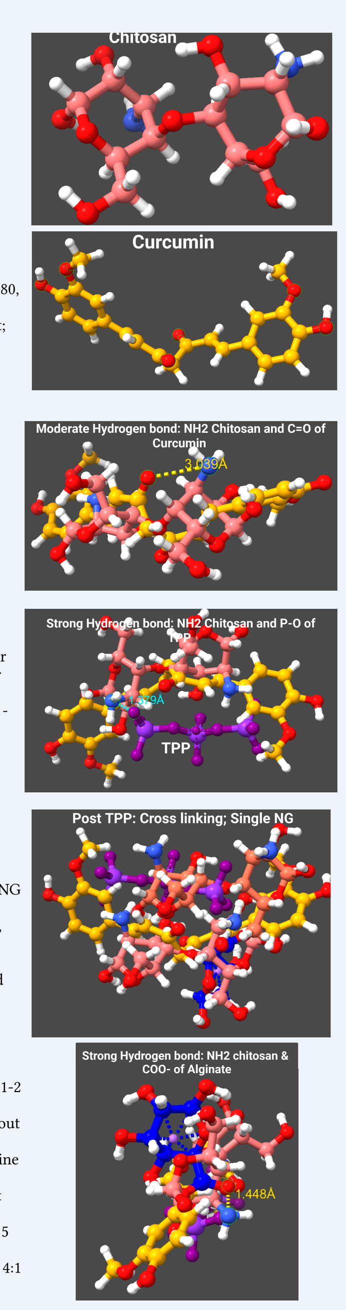
Curcumin loading
- The CUR solution added dropwise (~1 drop/ 3 sec) to CS at 400 RPM.
- 30 min of mixing +30 min of settling before TPP addition
- Controlled dropwise rate prevented curcumin precipitation at the point of contact with aqueous phase

Ionic Gelation (TPP crosslinking)
- 20 mg TPP in 10 mL DI water added dropwise over 20 min; 30 min of stirring + 30 min of resting after addition.
- RPM varied by phase to match solvent properties -
- Phase 1: 200 RPM (DMSO has high solubilizing capacity)
- Phase 2: 400 RPM (EtOH- weaker solubilization)
- Phase 3: 1000 RPM (EtOH+ Tween 80⟶ higher viscosity)

Centrifugation
- 10,000 RPM intermittently, 30 min total⟶ isolate NG pellet
- Pellets split: One portion resuspended in DI water, one for freeze-drying for characterizations
- Supernatant analyzed via UV-Vis Spectroscopy at 425 nm ⟶ encapsulation efficiency was calculated from the standard curcumin curve

Sodium Alginate (SA) Coating
- 25mg of SA in 25 mL DI water (0.1% w/v), stirred 1-2 hrs, pH adjusted to 5.5 (0.1M HCl)
- pH 5.5 ensured partial carboxyl protonation without premature gelling
- CS:SA ratios tested 1:1, 2:1, 3:1, and 4:1 to determine optimal coating conditions.
- CS-CUR NGs added at 1 ml/min, stirred 20 min at 400-500 RPM, rested 10 min (90 min total).
- 5-10 ml of 10 mM $CaCl_2$ added dropwise, stirred 15 min
- Ratios 1:1 & 1:2 ⟶ bridging flocculation.; 3:1 and 4:1 successful

NG recovery with Centrifuge
- The NG suspension was centrifuged intermittently at 10,000 rpm for a total of 30 min to remove free CUR and isolate the NG pellet.
- Dual CS-Alg NG is synthesized subject to characterization



Distance (Å)	Interaction Type
< 2.0 Å	Strong electrostatic/ionic
2.0-2.5 Å	Strong H-bond
2.5-3.2 Å	Moderate H-bond
3.2-4.0 Å	Weak H-bond/ van der Waals
> 4.0 Å	Minimal interaction

Ref: Jeffrey, George A. An Introduction to Hydrogen Bonding. Oxford UP, 1997.

All pictures taken during the experimentation ; molecular interaction pictures generated using ChimeraX by Achyut Ethiraj & Aditi Ethiraj

LYOPHILIZATION/FREEZE DRYING

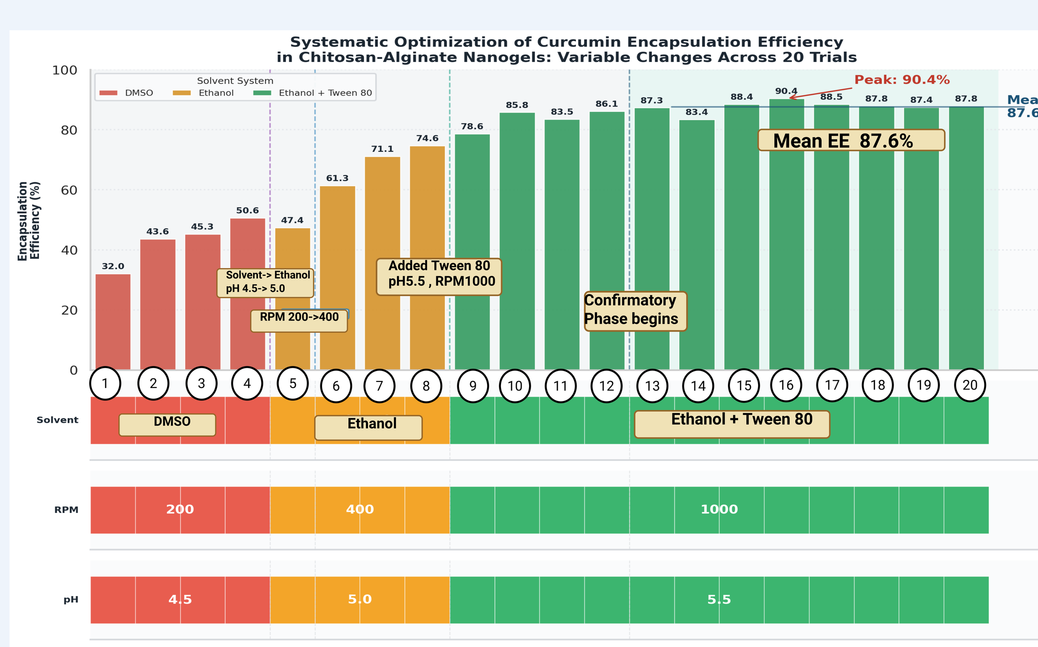
All pictures were taken during the experimentation by Achyut Ethiraj & Aditi Ethiraj

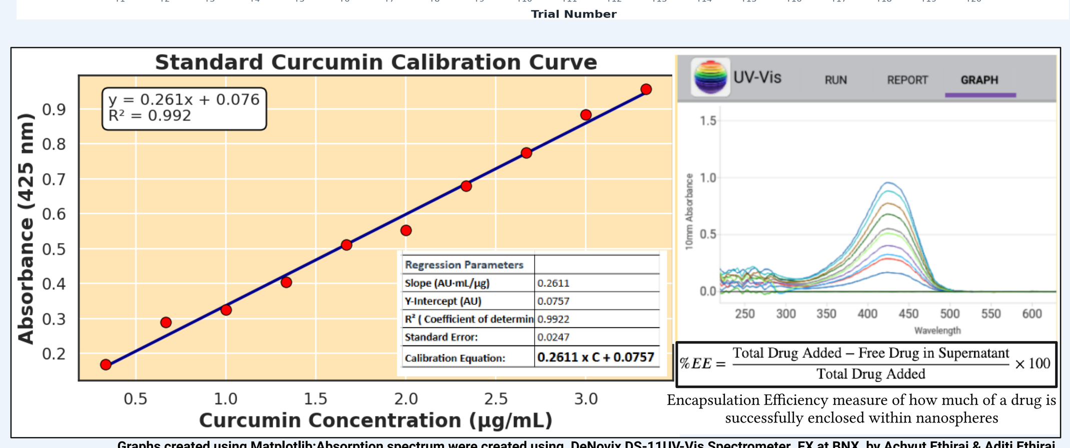
Sample Preparation: Placed liquid NG sample in a petri dish with trehalose 5% w/v as a cryoprotectant

Cold Trap: Homemade cooling system using dry ice (solid CO_2) in 91% isopropyl alcohol maintained the temperature at -78°C to sustain a high-vacuum environment

Sublimation: A vacuum pump was utilized to reduce the internal pressure, which enabled evaporation of liquid/solvent

UV-VIS SPECTROSCOPY

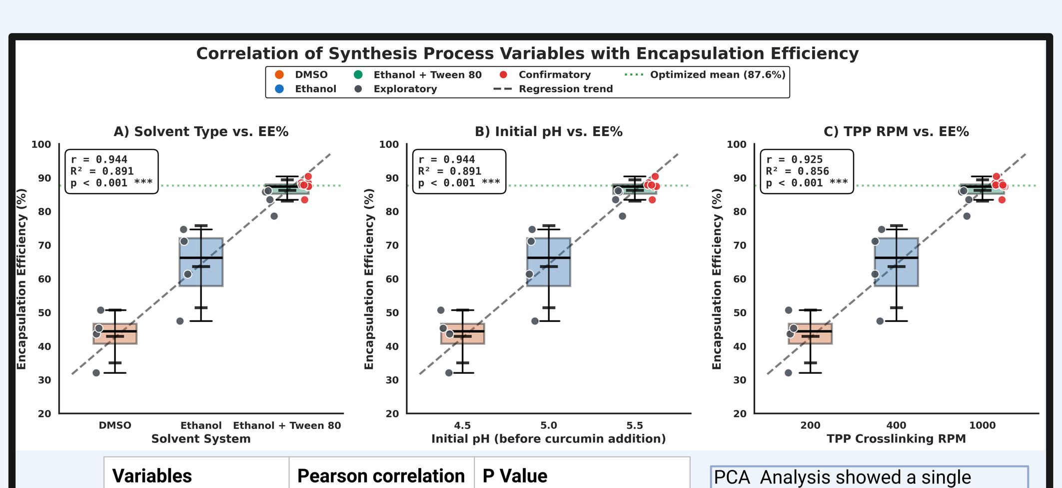




$$\%EE = \frac{\text{Total Drug Added} - \text{Free Drug in Supernatant}}{\text{Total Drug Added}} \times 100$$

Encapsulation Efficiency measure of how much of a drug is successfully enclosed within nanospheres

Graphs created using Matplotlib;Absorption spectrum were created using DeNovix DS-11UV-Vis Spectrometer FX at BNX by Achyut Ethiraj & Aditi Ethiraj



Variables	Pearson correlation	P Value
Solvent type	94.4%	<0.001
pH before adding Curcumin	94.4%	<0.001
RPM for TPP for crosslinking	92.5%	<0.001

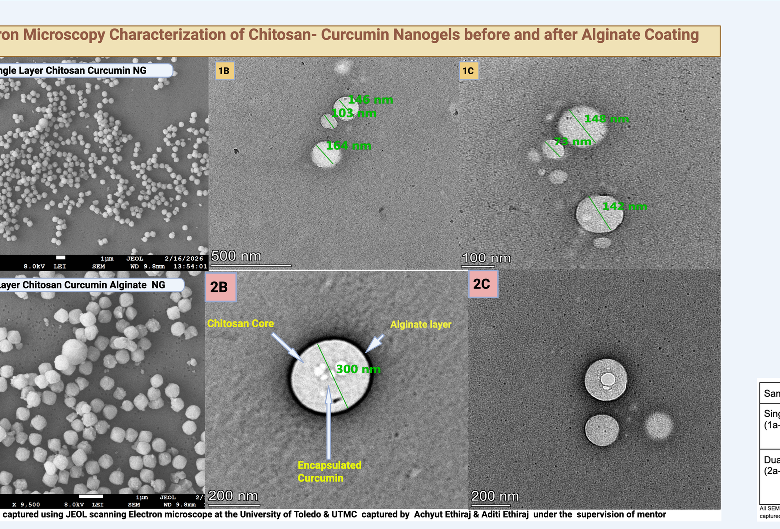
PCA Analysis showed a single dominant component (PC1=97.5%) driven equally by the solvent type, pH and RPM for crosslinking, confirming these variables collectively drove the improvement in encapsulation efficiency across 20 trials.

All graphs generated using google sheets & Matplotlib by Achyut Ethiraj & Aditi Ethiraj

CHARACTERIZATION

SEM/TEM (ELECTRON MICROSCOPY)

Electron Microscopy Characterization of Chitosan- Curcumin Nanogels before and after Alginate Coating



All SEM images captured using JEOL scanning Electron microscope at the University of Toledo & UTMC captured by Achyut Ethiraj & Aditi Ethiraj under the supervision of mentor

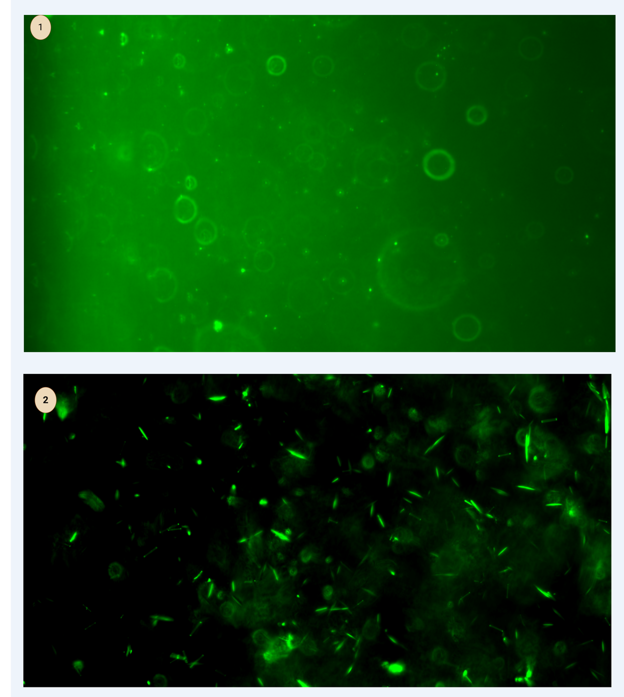
TEM SIZE CHARACTERIZATION
Feret Diameter measurements (n=12)

Samples	Single NG (nm)	Dual NG (nm)
1	198	379
2	153	395
3	149	378
4	65	419
5	137	382
6	168	323
7	145	413
8	161	419
9	165	233
10	101	223
11	183	229
12	146	313
SD	37.20	79.41
Average (nm)	147.58	342.17
Min	65	233
Max	198	419
PDI	0.064	0.054

Sample	SEM Analysis	TEM Analysis
Single-Layer NG (1a-1c)	- Uniform - Spherical - Narrow size distribution	• Dense matrix • Smooth internal structure • Monodispersity Index, with (PDI<0.3)
Dual-Layer NG (2a-2C)	- Textured - Larger - Diameter	• The observed electron-dense region (resulted by uranyl acetate) validates the presence of the alginate coating. • Highly uniform size, with PDI<0.3

All SEM images captured using JEOL Scanning Electron Microscope at the University of Toledo & TEM images UTMC captured by Achyut Ethiraj & Aditi Ethiraj under the supervision of mentor

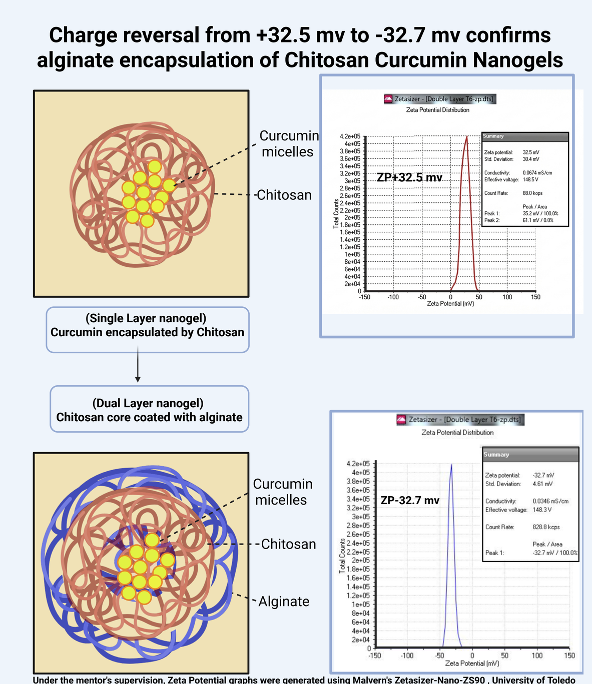
FLUORESCENCE IMAGING



1. **Solvent: EtOH & Tween 80:** Distinct green spheres with uniform curcumin distribution in chitosan core and visible alginate shell (no aggregation).Ring-like" alginate shell.
2. **DMSO as solvent:** Bright green rod-shaped structures indicating curcumin crystallization outside the nanogel matrix.

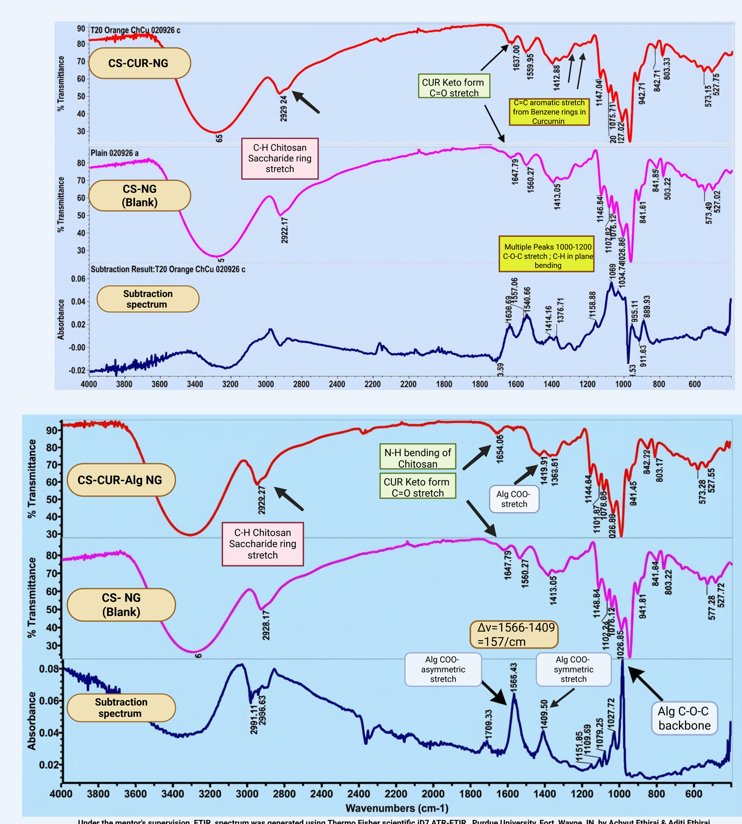
ZETA POTENTIAL DISTRIBUTION

Charge reversal from +32.5 mv to -32.7 mv confirms alginate encapsulation of Chitosan Curcumin Nanogels



Under the mentor's supervision, Zeta Potential graphs were generated using Malvern's Zetasizer-Nano-ZS90 , University of Toledo by Achyut Ethiraj & Aditi Ethiraj

ATR-FTIR SPECTROSCOPY



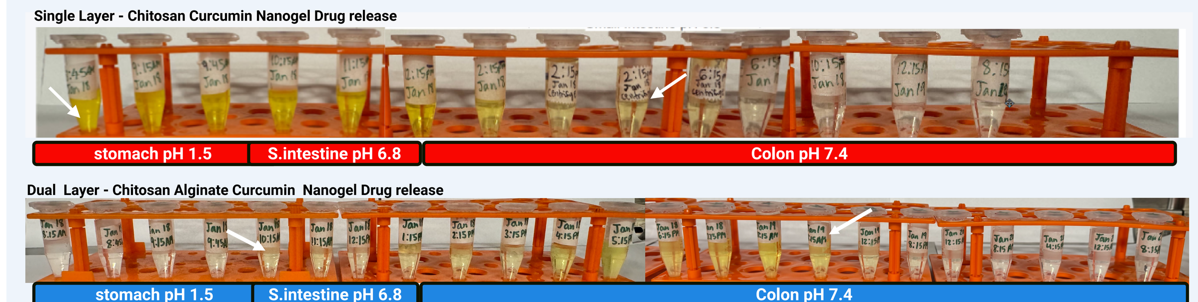
Under the mentor's supervision, FTIR spectrum was generated using Thermo Fisher scientific iD7 ATR-FTIR , Purdue University, Fort Wayne, IN by Achyut Ethiraj & Aditi Ethiraj

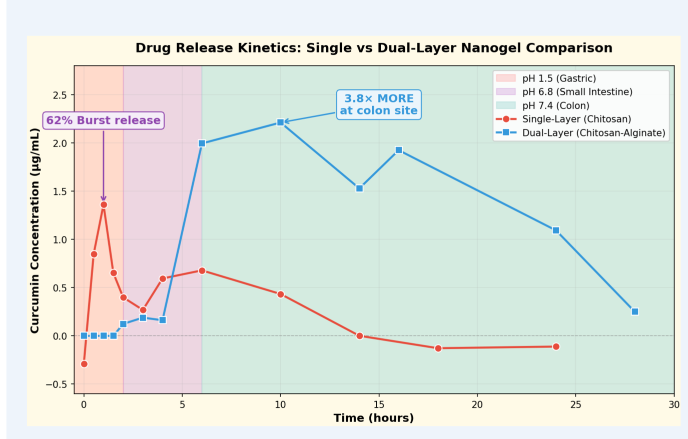
- Chitosan Curcumin H bonding: C=O 1647->1637 cm^{-1}shift
- Curcumin aromatic C=C (1500-1600 cm^{-1})
- Keto-form C=O stretch (1637 cm^{-1}) isolated in Single NG indicate successful loading of curcumin
- NH bending of Chitosan retained in Dual layer NG, confirms the core integrity after alginate coating.
- TPP characteristic peak between 800-1200

- Alginate shell confirmed in Dual NG: symmetric COO- stretch (1566 cm^{-1}) asymmetric stretch (1409 cm^{-1}) and C-O-C backbone (1027 cm^{-1}) appear in subtraction spectrum .
- The observed separation of 157 between symmetric (1409 cm^{-1}) & asymmetric (1566 cm^{-1}) stretches in subtraction spectrum indicate strong Polyelectrolyte Complex (PEC)

IN VITRO: DRUG RELEASE KINETICS

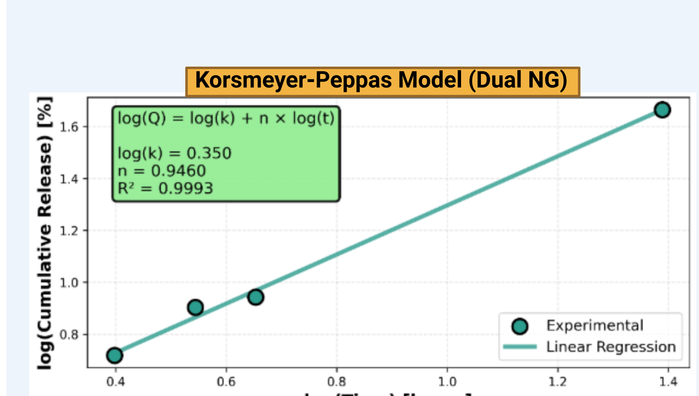
pH responsive drug release kinetics across simulated Gastrointestinal environment





Drug release behavior of Single & Dual NG was evaluated under gastrointestinal tract: Stomach (pH1.5), small intestine (pH6.8), and colon (pH 7.4).

- At each time point, supernatant samples were collected and curcumin concentration was quantified by UV - Vis spectroscopy (λ = 425nm) against a pre generated standard calibration curve.
- Single layer Nanogels exhibited premature drug release beginning at gastric pH, indicating limited protection in the upper GI tract.
- Dual layer Nanogels demonstrated significantly suppressed release at stomach and small intestinal pH, with sustained curcumin release occurring preferentially at colonic pH 7.4.

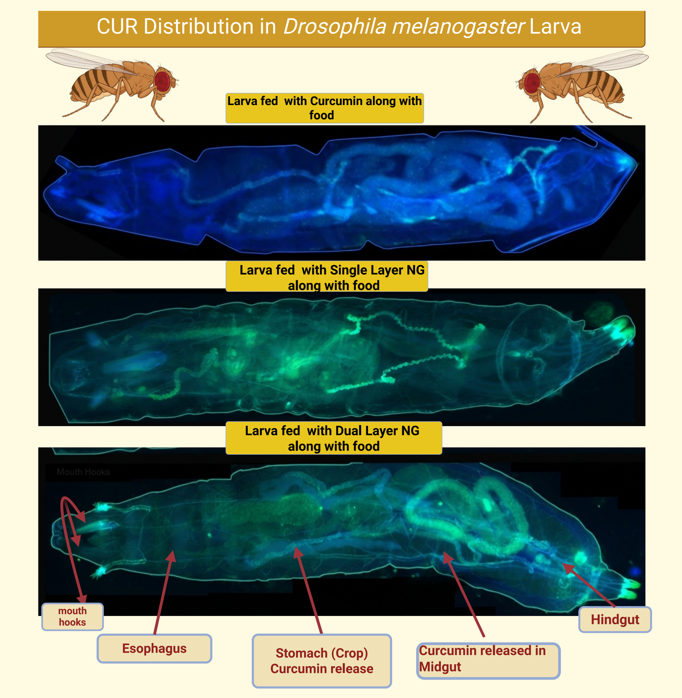


All graphs were generated using Matplotlib and google sheet by Achyut Ethiraj & Aditi Ethiraj

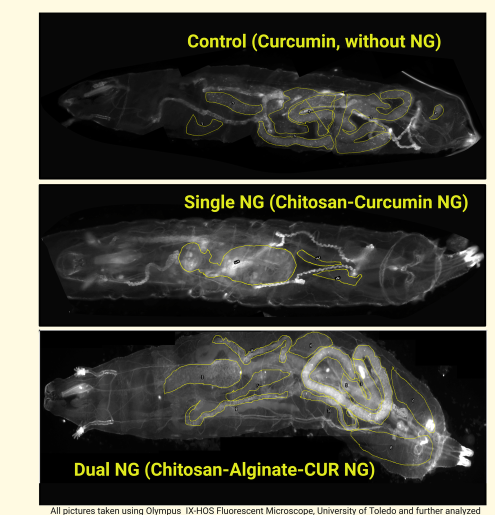
Mechanism	Exponent (n)	Physical Response
Fickian Diffusion	n ≤ 0.43	Concentration Gradient: Passive migration through a static polymer matrix
Anomalous Transport	0.43 < n < 0.85	Synchronous Dynamics: Simultaneous drug diffusion and polymer relaxation
Case II Transport	n = 0.85	Swelling Front: The environment moves into the gel at a constant speed, stretching it at a steady rate
Super Case II Transport	n > 0.85	Matrix Expansion: Rapid polymer stretching that actively pumps the drug out

VALIDATION

IN VIVO DROSOPHILA GUT MODEL



FLUORESCENCE QUANTIFICATION



All pictures taken using Olympus IX-HOS Fluorescent Microscope, University of Toledo and further analyzed using imageJ(Fiji) software by Achyut Ethiraj & Aditi Ethiraj

NG Type	Location	Mean gray Value	Integrated Density	Curcumin Delivery
Control (CUR, No NG)	Crop/Stomach (pH 2.0)	72.16	21384470	31.86%
Control (CUR, No NG)	Prox Midgut (pH 6-7.0)	81.6	24183379	36.03%
Control (CUR, No NG)	Distal Midgut (pH 7-9.5)	72.7	21545558	32.10%
Single NG (CS-CUR)	Crop/Stomach (pH 2.0)	255	38956037	92.00%
Single NG (CS-CUR)	Prox Midgut (pH 6-7.0)	76	1211764	3%
Single NG (CS-CUR)	Distal Midgut (pH 7-9.5)	110	2132100	5%
Dual NG (CH-ALG-CUR)	Crop/Stomach (pH 2.0)	92.614	21055366	12.40%
Dual NG (CH-ALG-CUR)	Prox Midgut (pH 6-7.0)	192.322	17571184	10.40%
Dual NG (CH-ALG-CUR)	Distal Midgut (pH 7-9.5)	758.561	130993251	77.20%

RESULTS

Measured Variables	Single-Layer NG	Dual-Layer NG
Morphology	Spherical and Uniform	Spherical, dense core shell structure
Mean Size	147 nm	342 nm
Polydispersity Index (PDI)	0.064	0.054
Zeta Potential	+32 mv	-32 mv
Encapsulation Efficiency	87.6%	87.6 %
Fluorescence imaging	Uniform CUR distribution in NG matrix	Uniform CUR distribution in NG matrix
FTIR (Salient Findings)	CS: Saccharide ring stretching (2929) CUR: • Multiple peaks C=C aromatic ring stretching (1500- 1600) • Peak shifts at 1637-1647: interaction between CUR C=O and NH of CS	Alginate: • COO- stretch (symmetric: 1566 and asymmetric: 1409) • C-O-C stretch (1027) TPP: • O-P-O stretching at 800-1200
In vitro CUR release Stomach	63%	<1%
In vitro CUR release in S. intestine	29.5%	26%
In vitro CUR release in Colon	8.3%	74%
Drosophila Gut Model CUR release in stomach	92%	22%
Drosophila Gut Model CUR release in Colon	8%	78%

CONCLUSION

- To attain high encapsulation efficiency, optimization of three key parameters include: ethanol & tween 80 as the solvent for curcumin, initial pH of 5.5 in the chitosan solution, and a higher crosslinking RPM, all played a major role in achieving our synthesis of smart nanogels.
- Compared to the single-layer nanogel, the dual-layer system eliminated premature gastric release, and increased the colonic curcumin delivery in vitro. In vivo Drosophila validation confirmed this trend.
- Drug release kinetics analyzed using the Korsmeyer-Peppas model showed a strong fit with super case II transport behavior, indicating release was mainly controlled by polymer swelling and relaxation rather than simple diffusion, which supports the idea of controllable and predictable pH responsive release.
- The dual-layer chitosan-alginate smart nanogel successfully enhanced the targeted delivery of curcumin to the colon. The alginate outer layer protected the payload under acidic stomach conditions, while the chitosan core enabled controlled swelling and release at colonic pH.

FUTURE DIRECTIONS

- Implement microfluidic assisted synthesis to reduce the NG particle size
- Conduct NG cytotoxicity tests on HCT-116 CRC cell lines (MTT and Clonogenic Assays)
- Extend the research to include in vivo mouse studies Investigate cellular uptake efficiency of NG by HCT-116 cells using flow cytometry
- Understand internalization mechanisms using specific endocytosis inhibitors to identify the biological pathways of NG entry.

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