CHAPTER 2

MATERIALS AND METHODS

This chapter describes the experimental design and methodologies employed to formulate and characterise biopolymeric complexes for delivering α-lipoic acid and curcumin. It outlines the materials, cross-linking techniques, analytical procedures, and instrumentation that validate the preparation and performance of these controlled-release systems.

2.1 MATERIALS

- **Polymers**
    - **Chitosan** (medium molecular weight, degree of deacetylation ~75–85%), purchased from Sigma-Aldrich (USA).
    - **Sodium Alginate**, Type I **Carrageenan**, and **Gelatin A** (bloom strength ~200–300 g) sourced from Sigma-Aldrich (USA).
    - **Soy Flour** (protein content ~40–50%) obtained from local producers or a commercial supplier (e.g., Raja Soya, Tezpur, India).
- **Filler Materials**
    - **Montmorillonite (MMT)** (K-10 grade), **Halloysite Nanotubes (HNTs)**, **Magnesium Oxide (MgO) nanoparticles**. All from Sigma-Aldrich (USA).
- **Anti-Diabetic Agents**
    - **α-Lipoic Acid (ALA)** (purity ~99%) from Sisco Research Laboratory (India).
    - **Curcumin** (≥95% curcuminoid content) from Sigma-Aldrich (USA).
- **Crosslinkers and Other Chemicals**
    - **Glutaraldehyde (GA)** (25% aqueous solution), Loba Chemie (India).
    - **Calcium Chloride (CaCl2)**, E-Merck (India).
    - **Tween 80** (surfactant) from E-Merck (India).
    - **Ethanol** (99.5%, AR grade), E-Merck (India).
    - Deionised (DI) water for all aqueous preparations.
- **Cell Culture Reagents**
    - **L6 Myotubes** (rat skeletal muscle cell line), **Dulbecco's Modified Eagle Medium (DMEM)**, Fetal Bovine Serum (FBS), **MTT reagent**, **2-NBDG** kit (Cayman, USA).
    - Antibiotics (penicillin-streptomycin), trypsin-EDTA, phosphate-buffered saline (PBS), palmitic acid and other reagents were of analytical grade.

2.2. METHODS

2.2.1. Calibration curve of α-lipoic acid

A calibration curve is essential for determining the drug's loading, encapsulation efficiency, and release rate from the complex within an appropriate solvent medium (ethanol-water), and it is plotted following the established protocol [1].

A series of known concentrations of α-Lipoic acid (prepared in double-distilled water) were analysed using a UV-Vis spectrophotometer (UV-2001, Hitachi, Tokyo, Japan) across the 200–600 nm wavelength range. A characteristic absorbance peak was observed between 227 and 334 nm, corresponding to α-Lipoic acid concentrations ranging from 0.001 to 0.005 g per 100 mL.

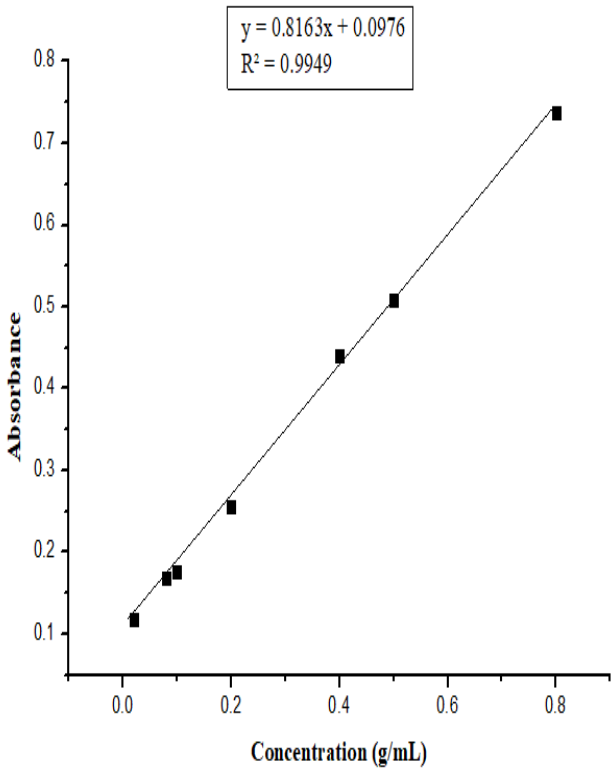

Figure 2.1. Calibration curve of Lipoic Acid

The absorbance values at 227–334 nm for each concentration were recorded and plotted, yielding a calibration curve that showed a linear relationship between absorbance and concentration. This calibration curve was subsequently used to estimate the unknown concentrations of α-Lipoic acid in release studies based on the measured absorbance values at the corresponding wavelength.

2.2.2. Calibration curve of curcumin

A calibration curve of curcumin in an appropriate solvent (ethanol-water) is plotted following a standard protocol [2, 3].

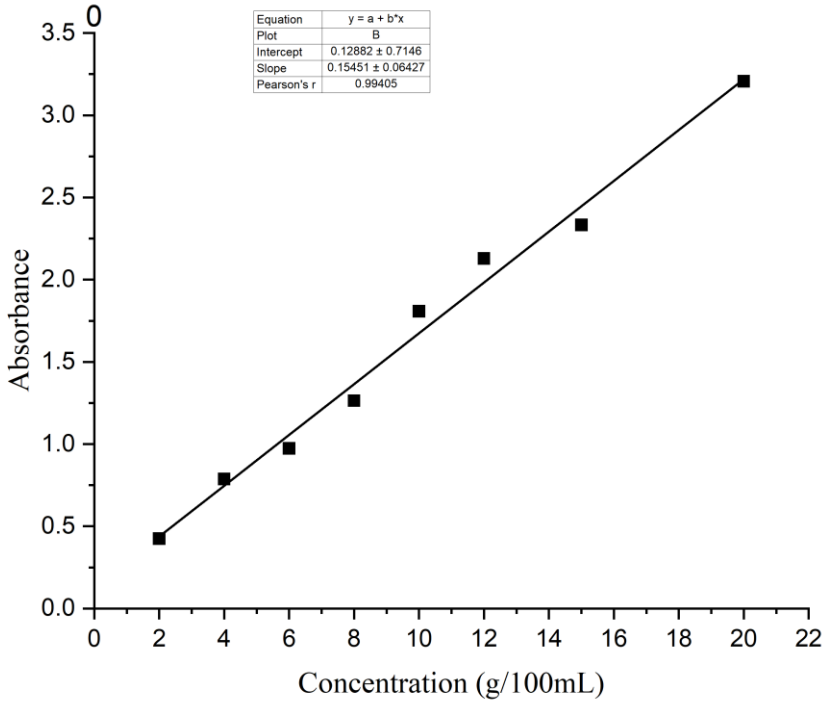

Figure 2.2. Calibration curve of Curcumin

A series of known concentrations of curcumin, prepared in a 1:1 ethanol: water mixture, were analysed via a UV–Vis spectrophotometer (UV–2001, Hitachi, Tokyo, Japan) across the 200–600 nm wavelength range. A characteristic absorbance peak was observed between 420 and 450 nm, corresponding to curcumin concentrations ranging from 0.001

to 0.005 g per 100 mL. The absorbance values at 420–450 nm for each concentration were recorded and plotted, generating a calibration curve that demonstrated a linear relationship between absorbance and concentration. This calibration curve was subsequently used to estimate the unknown concentrations of curcumin in release studies, based on the measured absorbance values at the corresponding wavelength, thereby enabling the accurate quantification of the drug release profile in subsequent experiments.

2.2.3. Preparation of glutaraldehyde crosslinked lipoic acid loaded chitosan-MMT-alginate complex

The chitosan-alginate complex was synthesised following a previously reported protocol with slight modifications [4–6]. A sodium alginate solution, 0.3% (w/v), was prepared by dissolving sodium alginate in water, and the pH was adjusted to 5.1 by adding HCl dropwise while maintaining constant stirring at room temperature. A 20% (w/v) solution of calcium chloride ($CaCl_2$) in water was added dropwise to induce crosslinking of the alginate. A known amount of MMT (0-0.06 g) pre-dispersed in water by constant stirring for 24 h, followed by sonication, was added to the alginate under continuous stirring. Tween 80 was added to the mixture to ensure uniform dispersion. Separately, α-lipoic acid (0.01 g) dissolved in a 1:1 ethanol-water solution was added very slowly to the sodium alginate-MMT solution, and constant stirring was maintained for 2-3 h. A known amount of 0.1% (w/v) chitosan solution, prepared in 1% (v/v) acetic acid with the pH adjusted to 5.4, was gradually incorporated into the sodium alginate solution, and stirring was continued for 30 minutes. The temperature was then lowered to 0-5 $^{\circ}$C. At this point, glutaraldehyde (0-15 µL) was added dropwise to the mixture, and the temperature was raised to 45 °C. Stirring was maintained for 3-4 h to facilitate cross-linking. The resulting suspension was allowed to cool to room temperature and centrifuged. The product was freeze-dried to obtain the product. A series of samples was prepared by varying the concentrations of crosslinker and nano-clay, as shown in Table 3.1.

2.2.4. Preparation of glutaraldehyde crosslinked lipoic acid loaded gelatin-haloysite-carrageenan complex

The gelatin-carrageenan complex was created using a previously described method with minor adjustments [7–9]. A 100 mL solution of 0.3% (w/v) carrageenan in water was prepared and placed in a beaker. It was stirred vigorously using a stirrer under high

agitation at 70 °C. A known amount of HNT (0-0.06 g) pre-dispersed in water by constant stirring for 24 h, followed by sonication, was added to the carrageenan under continuous stirring. Tween 80 was added to the mixture to ensure uniform dispersion. Separately, α-lipoic acid (0.01g) dissolved in a 1:1 ethanol-water solution was added very slowly to the carrageenan-HNT solution, and constant stirring was maintained for 2-3 h. A known amount of (200 mL) gelatin A solution of 1% (w/v) was added to the beaker dropwise to attain complete phase separation. However, the weight ratio of carrageenan to gelatin was maintained at 1:2 during all the experiments. The interaction between gelatin and carrageenan occurred completely at this ratio per the coacervate % yield and viscosity measurements [7]. The pH of the mixture was then adjusted to 3.5 by adding a 2.5% (v/v) glacial acetic acid solution. The temperature was then lowered to 0-5 °C. At this point, glutaraldehyde (0-15μL) was added dropwise to the mixture, and the temperature was raised to 45 °C. Stirring was maintained for 3-4 h to facilitate cross-linking. The resulting suspension was allowed to cool to room temperature and centrifuged. The product thus obtained was freeze-dried. In this way, a series of samples were prepared by varying the concentrations of crosslinker and halloysite, as shown in Table 3.2.

2.2.5. Preparation of a glutaraldehyde-crosslinked curcumin-loaded MgO-doped chitosan–carrageenan complex

The chitosan–carrageenan complex was synthesised via a modified version of a previously reported protocol [4–6]. A 100 mL solution of 0.35% (w/v) carrageenan in water was prepared and placed in a beaker. The mixture was stirred vigorously via a mechanical stirrer under high agitation at 70 °C. The MgO nanoparticles (0.5–1.5% w/w chitosan–carrageenan) were stably suspended in 10 mL of water via a probe sonicator. Tween 80 (10 μL) was added, and the mixture was then further sonicated. This suspension was added dropwise to the carrageenan solution while stirring continuously. A curcumin solution, pre-dissolved in a 1:1 ethanol: water mixture, was gradually added to the carrageenan solution while maintaining a constant temperature throughout the process. A 0.35% (w/v) chitosan solution was slowly introduced into the system via a syringe to facilitate phase separation. The temperature of the beaker was maintained at 70 °C for an additional 15 min to ensure uniform dispersion of the polyelectrolyte nanoparticles. Next, the system was cooled to a temperature range of 5–10 °C, followed by the addition of glutaraldehyde (0–15 μL) to initiate the hardening of the capsules. Afterwards, the temperature was raised

to 45 °C, and stirring was continued for 3–4 h to complete the crosslinking process. Once the crosslinking reaction was complete, the mixture was allowed to cool to room temperature. The resulting particles were separated and washed to remove residual impurities or unreacted materials. The product was freeze-dried to obtain the particles. A series of samples was prepared by varying the crosslinker and the amount of MgO nanoparticles, as shown in Table 3.3.

2.2.6. Preparation of glutaraldehyde crosslinked curcumin loaded MgO doped soy flour-MMT complex

The SF complex was prepared following a desolvation method with modifications [3, 10, 11]. Initially, 0.5 g of SF was dissolved in 50 mL of deionised water and stirred for one hour at room temperature in a beaker. Varied concentrations of montmorillonite (MMT) were swelled in 50 mL of water combined with 0.005 mL of Tween 80 (surfactant) for 24 hours. The solution was stirred vigorously using a mechanical stirrer for 48 hours and sonicated for 30 minutes. The dispersed MMT solution was added to 50 mL of a 1% (w/v) SF solution. MgO nanoparticles were stably suspended in 10 mL of water using a probe sonicator. Tween 80 (10µL) was added and further sonicated. This suspension was added to the soy flour solution dropwise while stirring continuously. A curcumin solution, pre-dissolved in a 1:1 ethanol-water mixture, was gradually added to the soy flour solution while maintaining a constant temperature throughout the process. After one hour, the system's temperature was reduced to 5–10 °C to facilitate the hardening of the nanoparticles. Glutaraldehyde (GA) was then incorporated as a cross-linker, and the temperature was increased to 45 °C. The reaction was stirred for 3 h. Rotary evaporation was conducted using a Buchi Rotavapor RII at 30 °C (Buchi Labortechnik AG, Flawil, Switzerland) to remove ethanol and replace it with an equivalent amount of deionised water. The nanoparticles were separated by centrifugation for 30 min, after which they were washed several times with water and dried.

2.2.7. Calculation of process yield

Process yield was calculated using the following equation [12].

$$Process\ \ yield(\%) = \frac{Weight\ of\ Product}{Weight\ of (Drug + MgO + Polymer)} \times 100$$

2.2.8. Calculation of drug loading and drug encapsulation efficiency

To evaluate the drug loading efficiency (LE) and encapsulation efficiency (EE) of the polyelectrolyte complexes in different formulations, the samples were subjected to ultracentrifugation at room temperature for 30 minutes. The concentration of free curcumin in the supernatant was determined by measuring the absorbance at 420–450 nm via a UV–Vis spectrophotometer. Based on the absorbance data, the encapsulation and drug loading efficiency of the nanoparticles were calculated via standard formulas from the literature [12].

$$Loading\ \ efficiency(LE)\% = \frac{(Total\ amount\ of Drug - Free\ amount\ of drug)}{Total\ amount\ of\ drug} \times 100$$

$$Encapsulation\ efficiency(EE)\% = \frac{(Total\ amount\ of Drug - Free\ amount\ of\ drug)}{Weight\ of\ dry\ polyectrolyte\ complex} \times 100$$

These calculations help determine the effectiveness of the nanoparticulate system in encapsulating and delivering curcumin. Higher efficiency values indicate better encapsulation and drug loading capacity.

2.3. CHARACTERISATION

2.3.1. Fourier Transform Infrared spectroscopy (FTIR)

Fourier Transform Infrared spectroscopy was employed to identify functional groups and intermolecular interactions in the polyelectrolyte complexes. FTIR spectra were recorded

using a Thermo Nicolet Impact-410 FTIR spectrophotometer operating in the mid-infrared range of 4000–400 cm^{-1}. Samples (~2–5 mg) were mixed with dry KBr powder (IR grade) and pressed into translucent pellets for transmission analysis. This KBr pellet method ensured a uniform matrix with minimal scattering, suitable for qualitative spectral analysis of solids. Key components and composite formulations – including chitosan, alginate, carrageenan, gelatin, montmorillonite (MMT) clay, magnesium oxide (MgO) nanoparticles, curcumin, lipoic acid, and their cross-linked polyelectrolyte complexes – were analysed. Characteristic absorption bands were identified for each functional group (e.g., –OH, –NH, –COOH, $–SO_3^-$), and shifts in peak positions or intensities were interpreted as evidence of polyelectrolyte complexation or nanoparticle interactions. All spectra were baseline-corrected, and peak assignments were compared with literature values to confirm the successful incorporation of functional moieties into the composite system.

2.3.2. X-ray Diffraction (XRD)

XRD was used to investigate the crystalline structure and phase composition of various materials and formulations. Diffraction patterns were collected on a Rigaku Miniflex X-ray diffractometer using a Cu-Kα radiation source ($\lambda \approx 0.154$ nm). Samples (powders or freeze-dried films) were packed into sample holders and scanned over a 2θ range, typically from 2° to 80° at room temperature. The instrument was operated at 30 kV and 15 mA, and scan rates were adjusted between 1° and 10° per minute depending on the sample's diffraction intensity (commonly 2°/min for routine scans). Diffraction peaks corresponding to the crystalline planes of pure lipoic acid and curcumin, as well as those of polymeric complexes and inorganic nanoparticles, were indexed by comparison with standard JCPDS data. The crystalline characteristics in polymer–nanoparticle composites were evaluated by comparing the relative intensity of sharp crystalline peaks to the broad halo of any amorphous background. For instance, neat crystalline compounds exhibited distinct, narrow peaks, whereas peaks in crosslinked polymer formulations appeared broadened or reduced, indicating partial amorphous character [12]. This analysis helped confirm the formation of nanocomposites (e.g., the exfoliation of clay layers or the dispersion of MgO nanoparticles) and any crystalline–amorphous phase transformations that occur upon cross-linking.

2.3.3. Scanning Electron Microscopy (SEM)

The surface morphology and microstructure of the polyelectrolyte complexes were examined by scanning electron microscopy. Dried gel films and cryo-fractured powder samples were mounted on metal stubs (brass holders) using conductive carbon tape. To prevent the charging of these non-conductive specimens, a thin platinum coating was applied by sputter deposition before imaging. SEM analysis was conducted on a JEOL JSM-6390LV instrument (tungsten filament electron source) at an accelerating voltage of 20 kV. The microscope was operated in high-vacuum mode using secondary electron detection to capture topographical contrast. Images were acquired at various magnifications (typically 100× to 10,000×) to observe macro to micro-scale features. The surface texture (smoothness, porosity) of the polyelectrolyte films and the distribution of embedded nanoparticles or phase-separated domains were qualitatively assessed. Fracture surface SEM images were also taken to investigate the internal morphology, revealing features such as the layered structures of polyelectrolyte complexes or the presence of inorganic fillers. All micrographs were analysed for uniformity of component dispersion (e.g., even spreading of MgO nanoparticles or clay platelets) and the absence of agglomeration. The SEM results provided insight into the efficacy of the mixing/casting process and supported correlations with mechanical or diffusion properties discussed elsewhere in the thesis.

2.3.4. Field Emission Scanning Electron Microscopy (FESEM)

In cases requiring higher-resolution imaging, field-emission scanning electron microscopy (FESEM) was conducted using a GEMINI 500 field-emission scanning electron microscope. The FESEM system features an operating voltage range of 0.2–30 kV, enabling ultra-high-resolution surface imaging. Specimens were mounted on stainless steel stubs with carbon tape and sputter-coated with gold. FESEM provided detailed insights into nanoscale features such as the size and distribution of MgO nanoparticles within polyelectrolyte complexes, the structural arrangement of MMT platelets, and the morphology of crosslinked networks. The enhanced resolution offered by FESEM facilitated a more apparent distinction between continuous polymer phases and embedded inorganic domains.

2.3.5. Energy Dispersive X-ray (EDX) spectroscopy

Energy dispersive X-ray spectroscopy (EDX) was performed in conjunction with FESEM for elemental composition analysis. The EDX detector, attached to the GEMINI 500 microscope, enabled in situ elemental analysis of specific regions on the sample. An accelerating voltage of 10–20 kV was commonly employed for EDX to excite characteristic X-rays from elements present in the sample. This technique helped confirm the distribution and relative abundance of elements such as Mg in MgO nanoparticles, Si and Al in MMT, and S in carrageenan or other sulfur-containing polymers. Elemental mapping was also carried out where appropriate, illustrating the spatial distribution of key elements across the surface or cross-sectional areas of the formulated composites.

2.4. *IN VITRO* DRUG RELEASE STUDIES

In all formulations, dried samples of the drug-loaded complexes were accurately weighed and immersed in two different buffer media—pH 1.2 (simulated gastric conditions) and pH 7.4 (simulated intestinal conditions). Each sample was continuously stirred at 37 ± 0.5°C to simulate physiological temperature. At predetermined intervals, a 5 mL aliquot was withdrawn, filtered to remove any residual particles, and analysed with a UV-vis spectrophotometer to determine the cumulative amount of drug released at each time. For **α-lipoic acid** formulations, measurements were performed at **227–334 nm**, while for **curcumin** systems, the absorbance was recorded at **420–450 nm** [4]. After each withdrawal, an equivalent volume of fresh buffer (5 mL) at the same pH was added to maintain a constant volume and ensure sink conditions throughout the test. All experiments were conducted in triplicate, and the results are presented as mean ± standard deviation to confirm the reproducibility and accuracy of the *in vitro* release profiles.

2.5. BIOLOGICAL ASSAYS

2.5.1. Glucose uptake (2-NBDG) assay

The glucose uptake assay, which employs the fluorescent glucose analogue 2-NBDG, was conducted using a cell-based assay kit (Cayman, USA) according to previously published protocols [13, 14]. In each experiment, L6 myotubes were starved of serum overnight in Krebs' Ringer Phosphate (KRP) buffer containing 0.2% bovine serum albumin (BSA).

The cells were then treated with polymeric formulations incorporating various concentrations of glutaraldehyde (GA) and, where indicated, nanoparticles (e.g., halloysite nanotubes, montmorillonite, MgO) for 1 h. To induce an insulin-resistant state, 0.75 mM palmitic acid was added and incubated for 6 h and 30 min. Insulin (100 nM) was administered before the end of the incubation period. Subsequently, 2-NBDG was introduced 5 min before terminating the experiment. The cells were then lysed, and fluorescence intensity was measured using the Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland). All samples were prepared in triplicate.

2.5.2. Cell viability assay (MTT)

The MTT assay assessed cell viability using previously established protocols [15, 16]. The cell lines were seeded into 96-well plates and exposed to varying concentrations of the formulations for 24 h. After treatment, 10 µL of MTT reagent (5 mg/mL in phosphate-buffered saline, PBS) was added to each well and incubated at 37°C for 4 h, allowing metabolically active cells to reduce MTT to insoluble formazan crystals. The resulting crystals were dissolved in 100 µL of acidic isopropanol, followed by an additional 30 min incubation at 37°C. Absorbance was measured at 570 nm using a Multiskan GO Microplate Spectrophotometer (Thermo Scientific, Finland). Wells containing only acidic isopropanol served as blanks, while untreated cells (exposed only to medium) were designated 100% viable controls. Each experiment was carried out in triplicate, and the results are presented as the mean ± standard deviation.

2.6. STATISTICAL ANALYSIS

Statistical analysis was conducted to ensure the reliability and reproducibility of the experimental results. All experiments were typically performed in triplicate ($n = 3$) or more to confirm consistency. Data were expressed as the mean ± standard deviation (SD). The statistical significance of differences between groups was evaluated using either a one-way analysis of variance (ANOVA) or a t-test, with a significance threshold set at $p < 0.05$. For graphical representation and curve fitting, the software Origin was utilised.

2.7. REFERENCES

[1] Devi, N., Maji, T. K., Preparation and Evaluation of Gelatin/Sodium Carboxymethyl Cellulose Polyelectrolyte Complex Microparticles for Controlled Delivery of Isoniazid. *AAPS PharmSciTech,* 10:1412, 2009.

[2] Banik, N., Ramteke, A., Maji, T. K., Carboxymethyl chitosan-montmorillonite nanoparticles for controlled delivery of isoniazid: evaluation of the effect of the glutaraldehyde and montmorillonite. *Polym Adv Technol,* 25:1580–1589, 2014.

[3] Gogoi, P., Das, M. K., Ramteke, A., Maji, T. K., Soy flour–ZnO nanoparticles for controlled release of silibinin: Effect of ZnO nanoparticle, surfactant, and cross-linker. *International Journal of Polymeric Materials and Polymeric Biomaterials,* 67:543–552, 2018.

[4] Gogoi, P., Dutta, A., Ramteke, A., Maji, T. K., Preparation, characterization and cytotoxic applications of curcumin-(±) α-lipoic acid coloaded phosphorylated chitosan nanoparticles in MDA MB 231 breast cancer cell line. *Polym Adv Technol,* 31:2827–2841, 2020.

[5] Banik, N., Hussain, A., Ramteke, A., Sharma, H. K., Maji, T. K., Preparation and evaluation of the effect of particle size on the properties of chitosan-montmorillonite nanoparticles loaded with isoniazid. *RSC Adv,* 2:10519, 2012.

[6] Devi, N., Maji, T. K., Microencapsulation of isoniazid in genipin-crosslinked gelatin-A–κ-carrageenan polyelectrolyte complex. *Drug Dev Ind Pharm,* 36:56–63, 2010.

[7] Devi, N., Maji, T. K., Genipin crosslinked microcapsules of gelatin A and κ-carrageenan polyelectrolyte complex for encapsulation of Neem (Azadirachta Indica A.Juss.) seed oil. *Polymer Bulletin,* 65:347–362, 2010.

[8] Al-Zebari, N., Best, S. M., Cameron, R. E., Effects of reaction pH on self-crosslinked chitosan-carrageenan polyelectrolyte complex gels and sponges. *Journal of Physics: Materials,* 2:015003, 2018.

[9] Sonawane, R. O., Patil, S. D., Gelatin–κ-carrageenan polyelectrolyte complex hydrogel compositions for the design and development of extended-release pellets. *International Journal of Polymeric Materials and Polymeric Biomaterials,* 66:812–823, 2017.

[10] Teng, Z., Luo, Y., Wang, Q., Nanoparticles Synthesized from Soy Protein: Preparation, Characterization, and Application for Nutraceutical Encapsulation. *J Agric Food Chem,* 60:2712–2720, 2012.

[11] Banik, N., Hussain, A., Ramteke, A., Sharma, H. K., Maji, T. K., Preparation and evaluation of the effect of particle size on the properties of chitosan-montmorillonite nanoparticles loaded with isoniazid. *RSC Adv,* 2:10519, 2012.

[12] Sharma, D., Maji, T. K., Chitosan-alginate complex for the controlled delivery of α-lipoic acid in diabetes therapy: Modulation by montmorillonite and glutaraldehyde. *Indian Journal of Chemical Technology,* 2025. https://doi.org/10.56042/ijct.v32i1.14810

[13] Khatun, B., Rohilla. S., Rather, M. A., Sinha, A., Dasgupta, S., Mandal, M., Maji, T. K., Improved bioactivities of curcumin pyrazole and its HPβCD inclusion complex compared to curcumin. *Journal of Chemical Sciences,* 135:16, 2023.

[14] Pal, D., Dasgupta, S., Kundu, R., Maitra, S., Das, G., Mukhopadhyay, S., Ray, S., Majumdar, S. S., Bhattacharya, S., Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat Med,* 18:1279–1285, 2012.

[15] Mazumder, S., Sinha, A., Ghosh, S., Sharma, G. C., Prusty, B. M., Manna, D., Pal, D., Pal, C., Dasgupta, S., Leishmania LPG interacts with LRR5/LRR6 of macrophage TLR4 for parasite invasion and impairs the macrophage functions. *Pathog Dis*, 2023. https://doi.org/10.1093/femspd/ftad019

[16] Kumar, P., Nagarajan, A., Uchil, P. D., Analysis of Cell Viability by the MTT Assay. *Cold Spring Harb Protoc,* 6, 2018.