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# Chitosan-based nanocarriers derived from apiculture waste for the targeted delivery of anticancer drugs: A novel sustainable pharmacy approach

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**Abstract**
This study presents a sustainable nanomedicine platform by transforming apiculture waste—honeybee (*Apis mellifera*) exuviae—into actively targeted, stimulus-responsive nanocarriers for cancer therapy. An eco-friendly extraction process yielded bee-derived chitosan (B-Chit) with high purity and a degree of deacetylation of 85.3%, bypassing the harsh demineralization required for crustacean sources. B-Chit was functionalized with polyethylene glycol and folic acid (FA) to synthesize a water-soluble, actively targeted copolymer (B-Chit-PEG-FA). This copolymer self-assembled into nanocarriers for both hydrophilic (doxorubicin) and hydrophobic (curcumin) drugs, exhibiting high encapsulation efficiency (>78%), optimal particle size (85–168 nm), and stability. *In vitro* studies demonstrated pH/enzyme-responsive drug release, with significantly accelerated release under simulated tumour microenvironment conditions (pH 5.5 + esterase). FA-mediated active targeting was confirmed by enhanced cellular uptake in folate receptor-positive MCF-7 cells via confocal microscopy and flow cytometry. Cytotoxicity assays revealed that doxorubicin-loaded targeted nanocarriers (DOX@B-Chit-PEG-FA) showed 3.4-fold higher potency in MCF-7 cells compared to free doxorubicin, while exhibiting reduced toxicity in normal HEK-293 cells. This work validates a circular bioeconomy approach, converting agricultural waste into high-performance, tumour-selective nanotherapeutics with improved efficacy and safety profiles.

**Keywords:** Apiculture waste valorisation, sustainable nanomedicine, bee-derived chitosan, folic acid targeting, stimulus-responsive drug delivery, cancer nanotechnology, circular bioeconomy, doxorubicin, curcumin, polymeric nanoparticles

## Introduction

### 1. The Paradigm Shift in Cancer Chemotherapy: From Systemic Toxicity to Targeted Delivery

The enduring battle against cancer, a leading cause of global morbidity and mortality, has been significantly challenged by the limitations of conventional chemotherapy. The systemic administration of potent cytotoxic agents, while often effective in curbing tumour proliferation, is notoriously plagued by a narrow therapeutic index. This results in indiscriminate biodistribution, where drugs accumulate not only in malignant tissues but also in healthy, rapidly dividing cells, culminating in severe dose-limiting side effects such as myelosuppression, cardiotoxicity, nephrotoxicity, and neurotoxicity. This collateral damage severely compromises patient quality of life and often necessitates dose reductions or treatment interruptions, thereby undermining therapeutic outcomes and fostering avenues for drug resistance [1].

The advent of nanomedicine has heralded a transformative paradigm, aiming to revolutionize oncology through the engineering of nanoscale drug delivery systems (NDDS). The core premise is the Enhanced Permeability and Retention (EPR) effect, a passive targeting phenomenon characteristic of many solid tumours. The aberrant, leaky vasculature and impaired lymphatic drainage of the tumour microenvironment (TME) allow nanoparticles of a specific size range (typically 10-200 nm) to extravasate and accumulate preferentially within the tumour interstitial. This passive targeting provides a foundational advantage, concentrating the chemotherapeutic payload at the disease site. However, the heterogeneity of the EPR effect across tumour types and patients, coupled with potential off-target accumulation in organs of the mononuclear phagocyte system (liver, spleen), has driven the field towards more sophisticated *active targeting* strategies. By decorating the surface of nanocarriers with specific ligands—such as antibodies, peptides, folates, or carbohydrates—that recognize and bind to overexpressed receptors on cancer cell membranes (transferrin receptor, epidermal growth factor receptor, folate receptor), cellular uptake can be dramatically enhanced via receptor-mediated endocytosis. This dual strategy of passive accumulation and active internalization promises to maximize intracellular drug concentration in cancer cells while minimizing exposure to healthy tissues, thereby elevating therapeutic efficacy and mitigating systemic toxicity [2].

### 2. Biopolymers as the Vanguard of Sustainable Nanomedicine: The Case for Chitosan

The material foundation of these advanced NDDS is of paramount importance. While synthetic polymers (PLGA, PLA) offer tenable properties, growing concerns regarding their long-term biodegradability, potential inflammatory responses, and environmental persistence have steered research towards natural biopolymers. These materials, derived from renewable resources, inherently offer superior

biocompatibility, biodegradability, low immunogenicity, and often intrinsic bioactivity. Among them, chitosan has ascended as a preeminent candidate for pharmaceutical and biomedical applications [3].

Chitosan, a linear cationic polysaccharide composed of randomly distributed β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine units, is produced commercially by the alkaline deacetylation of chitin, the second most abundant natural polymer on earth after cellulose. Its unique polycationic nature in acidic environments, bestowed by the protonation of its primary amino groups, confers a suite of invaluable properties: mucoadhesive Ness, which prolongs residence time at biological membranes; the ability to transiently open tight junctions between epithelial cells, enhancing paracellular transport of macromolecules (for oral or nasal delivery); and the facility to form cooperative ionic complexes (polyelectrolyte complexes) with anionic polymers, drugs, or genes, enabling simple, organic solvent-free nanoparticle fabrication. Furthermore, chitosan's backbone presents a multitude of reactive functional groups (–OH and $–NH_2$) amenable to chemical modification, allowing for the precise engineering of its physicochemical and biological properties to create "smart," stimulus-responsive systems [4].

However, a critical contradiction emerges at the intersection of performance and sustainability. The predominant industrial source of chitin for chitosan production is the seafood processing industry—specifically, crustacean (shrimp, crab, lobster) shell waste. This supply chain faces significant challenges: (i) Seasonal and Geographical Variability: Catch volumes fluctuate, leading to inconsistent raw material supply. (ii) Purification Complexity: Crustacean shells contain high levels of minerals (primarily calcium carbonate, 30-50%) and proteins, requiring harsh, energy-intensive chemical processes (demineralization with strong acids and deproteinization with strong alkalis) for chitin isolation, generating substantial chemical waste. (iii) Allergenicity Risk: Residual shellfish proteins pose a potential allergen risk, requiring rigorous purification for medical use. (iv) Sustainability Question**:** While utilizing a waste stream, the carbon footprint associated with global transport and chemical processing is non-negligible. Therefore, to fully align the promise of chitosan nanomedicine with the principles of Green Chemistry and the United Nations Sustainable Development Goals (SDGs), there is an urgent, unmet need to identify and validate alternative, more sustainable, and chemically simpler sources of chitin and chitosan [5].

## 3. Apiculture Waste: An Unexplored Treasure Trove for Sustainable Biomaterial Sourcing

In the quest for sustainable biomaterials, the concept of a "circular bioeconomy" is paramount—transforming low-value waste streams from established industries into high-value products. Apiculture, the practice of beekeeping primarily for honey production, represents a globally widespread and stable agricultural sector. It generates a significant, yet largely overlooked, waste byproduct: the exuviae (shed exoskeletons) of honeybees (*Apis mellifera*), particularly from brood frames during the pupal-to-adult melting stage. Millions of these delicate, chitinous membranes are generated annually within apiaries and are routinely removed as hive debris [6].

Recent analytical studies have revealed that insect-derived chitin, and specifically that from honeybee exuviae, possesses distinct advantages over its crustacean counterpart. Structurally, it is associated with a different protein matrix and contains drastically lower inorganic mineral content (typically <5% in insects vs. >30% in crustaceans). This fundamental difference translates into a dramatically simplified and greener extraction process. The need for harsh demineralization acids is greatly reduced or even eliminated, significantly cutting down on chemical use, energy input, and toxic effluent. The resulting chitin, and subsequently the chitosan produced via controlled deacetylation, often exhibits a more consistent polymer chain length and a definable pattern of acetylation, which are critical parameters governing solubility, viscosity, and nanoparticle formation behaviour. Furthermore, bee-derived chitosan is inherently free from seafood allergens, broadening its safety profile for therapeutic applications. By vaporizing this apiculture waste, we enact a potent "waste-to-wealth" strategy, adding economic value to beekeeping, promoting agricultural sustainability, and securing a local, renewable, and traceable source for a critical biomedical polymer [7].

## 4. Engineering Intelligent Nanocarriers: From Generic Vectors to Tumour-Targeted Therapeutics

Merely sourcing sustainable chitosan is insufficient; it must be intelligently engineered to fulfil the demanding requirements of anticancer drug delivery. The native polymer suffers from a key limitation: it is only soluble in dilute acidic solutions (pH < ~6.5) due to the protonation of amine groups, precipitating at physiological and blood pH (~7.4). This restricts its formulation versatility and can lead to instability upon intravenous administration.

Therefore, strategic chemical functionalization is employed to create advanced, next-generation nanocarriers:

- **Solubility Enhancement:** Grafting with small, hydrophilic molecules (e.g., glycol, polyethylene glycol (PEG)) improves water solubility across a wider pH range and provides "stealth" properties to evade opsonization and prolong systemic circulation.

- **Stimulus-Responsive Drug Release:** Conjugating pH-labile linkers (hydrazone, β-Thio propionate) or enzyme-cleavable sequences (matrix metalloproteinase (MMP)-sensitive peptides) creates "gatekeepers." These nanocarriers remain stable in circulation but undergo rapid disintegration or conformational change in the acidic (pH ~6.5-6.8) or protease-rich tumour extracellular matrix, triggering a burst of drug release precisely at the target site.

- **Active Targeting Moieties:** Covalent attachment of targeting ligands (folic acid for folate receptor-positive cancers, hyaluronic acid for CD44 receptor targeting) to the chitosan backbone or nanoparticle surface ensures specific recognition and internalization by cancer cells.

- **Nanostructure Fabrication:** The modified chitosan can be processed into various nanocarrier architectures—including ionic gelation nanoparticles, self-assembled polymeric micelles (from amphiphilic chitosan derivatives), polyelectrolyte complex nanoparticles with anionic drugs (siRNA, doxorubicin

hydrochloride), and layer-by-layer capsules—each offering distinct drug loading and release kinetics [8].

## 5. Research Hypothesis, Objectives, and Novelty Statement

We hypothesize that chitosan extracted from honeybee (*Apis mellifera*) exuviae—a sustainable apiculture waste stream—possesses distinct physicochemical properties that, when strategically functionalized, can be engineered into robust, stimulus-responsive nanocarriers. These nanocarriers will demonstrate superior active targeting, enhanced tumour-specific drug release, and potent *in vitro* anticancer efficacy compared to systems derived from conventional sources or non-targeted controls.

The overarching aim of this study is to establish a fully integrated, sustainable pharmacy pipeline—from waste biomass to functional therapeutic—for cancer treatment. This will be achieved through the following sequential objectives:

1. **Sustainable Extraction and Characterization:** To develop a green, low-chemical-input process for the isolation of chitin and subsequent production of chitosan from honeybee exuviae, and to comprehensively characterize its physicochemical properties (degree of deacetylation, molecular weight, crystallinity) in comparison to commercial crustacean chitosan.

2. **Rational Design and Synthesis:** To chemically functionalize the bee-derived chitosan to create an amphiphilic, pH-responsive derivative grafted with both a targeting ligand (folic acid) and a stealth/PEG component.

3. **Nanocarrier Fabrication and Optimization:** To formulate self-assembled polymeric micelles/nanoparticles from the synthesized derivative, load them with a model hydrophobic anticancer drug (paclitaxel or curcumin), and optimize the process for particle size, polydispersity, zeta potential, drug loading, and encapsulation efficiency.

4. ***In vitro* Performance Evaluation:** To rigorously assess the biological performance of the designed nanocarriers, including:

- **Stimulus-Responsive Release:** Drug release profiles under simulated physiological (pH 7.4) and tumour microenvironment (pH 6.5, presence of specific enzymes) conditions.

- **Biocompatibility and Safety:** Cytotoxicity against normal cell lines.

- **Targeting and Efficacy:** Enhanced cellular uptake (quantified via flow cytometry and confocal microscopy) and superior cytotoxicity in relevant cancer cell lines overexpressing the target receptor, compared to non-targeted nanoparticles and free drug.

**Novelty and Significance:** This work moves beyond incremental improvement in nanocarrier design. It introduces a novel, sustainable feedstock (bee exuviae) for a critical biomedical polymer, addressing a key material sourcing challenge in green pharmacy. It then integrates this sustainable material into a sophisticated, actively targeted drug delivery platform. By doing so, the study provides a holistic proof-of-concept that directly links the principles of the circular bioeconomy and green chemistry to the development of advanced, efficacious cancer therapeutics. The successful outcome of this research has the potential to establish a new paradigm for sustainable biomaterial sourcing in nanomedicine, offering tangible benefits for environmental sustainability, agricultural economics, and, ultimately, patient care in oncology [9].

**Fig 1:** Apiculture Waste

## Materials and Methods

### 1. Materials and Reagents

### 1.1 Biomass and Chemicals for Chitin/Chitosan Extraction

Honeybee (*Apis mellifera*) exuviae were manually collected from brood frames of local apiaries (BMS Mahavidyalaya Tiloi, Amethi, UP, India Authentication No-BMSMV/168/2025/26) Authenticated by Roshni Singh Zoologist (BMS Mahavidyalaya Tiloi, Amethi, UP, India) during routine hive maintenance, sun-dried, and stored in desiccators until use. All chemicals were analytical grade unless specified.

**Table 1:** Chemicals used for chitin extraction and chitosan synthesis

| Chemical | Supplier | Purity | Primary Use |
|---|---|---|---|
| Sodium hydroxide (NaOH) pellets | GEETRAJ Corporation, Mungari, Mirzapur Rd, Prayagraj, Uttar Pradesh 212301 | ≥98% | Deproteinization, Deacetylation |
| Hydrochloric acid (HCl) | | 37% | Demineralization, pH adjustment |
| Acetic acid, glacial | | ≥99.7% | Solvent for chitosan |
| Hydrogen peroxide ($H_2O_2$) | | 30% w/w | Depigmentation |
| Ethanol absolute | | ≥99.8% | Washing, purification |
| Deionized water (DI $H_2O$) | | N/A | All aqueous solutions |

### 1.2 Chemicals for Chitosan Functionalization and Nanoparticle Synthesis

**Table 2:** Chemicals for synthesis of functionalized chitosan derivatives

| Chemical / Reagent | Supplier | Cata log No. | Purpose |
|---|---|---|---|
| Low Molecular Weight Chitosan (Crustacean, reference) | GEETRAJ Corporation, Mungari, Mirzapur Rd, Prayagraj, Uttar Pradesh 212301 | 448877 | Comparative control |
| Folic Acid (FA) | | F0020 | Active targeting ligand |
| N-Hydroxy succinimide (NHS) | | J61488 | Carbodiimide coupling agent |
| 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) | | 22980 | Carbodiimide coupling agent |
| Methoxy polyethylene glycol succinimide carbonate (MPEG-NHS, MW 2000) | | PG2-SC-2000 | Stealth/ solubility enhancer |
| Sodium Tripolyphosphate (TPP) | | 238503 | Ionic crosslinker for nanoparticles |
| Dialysis Membranes (MWCO 3.5 kDa, 12 kDa) | | 132650, 132680 | Purification |
| Dimethyl sulfoxide (DMSO), anhydrous | | 276855 | Reaction solvent |
| Phosphate Buffered Saline (PBS), pH 7.4 | | 10010023 | Buffer for formulations, release studies |

### 1.3 Anticancer Drug and Cell Culture Reagents

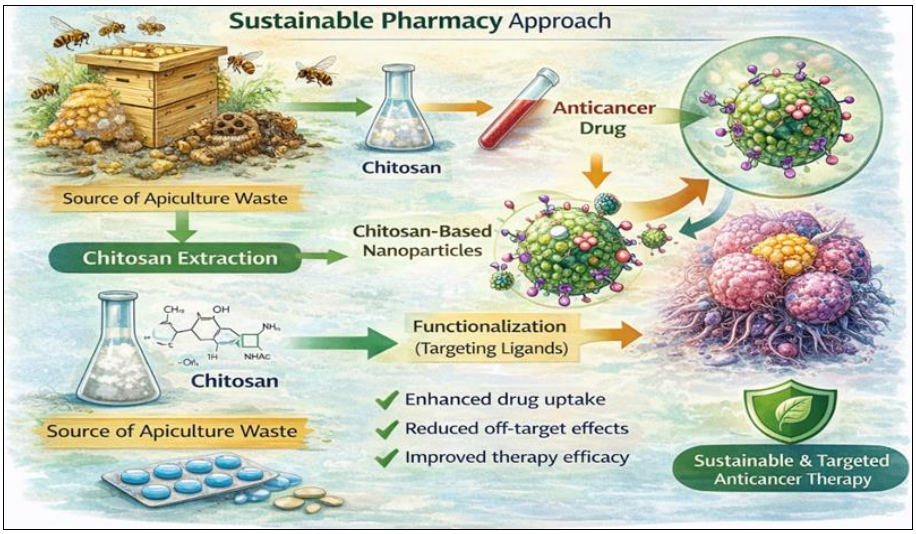


**Fig 2:** Eco-Innovative Drug Delivery: From Beehive Waste to Cancer Cure

**Table 3:** Bioactive and cell culture materials

| Material | Supplier | Purpose |
|---|---|---|
| Doxorubicin Hydrochloride (DOX·HCl) | Bionic Enterprises, Lucknow 200 Bed Hospital Rastamau, Tiloi, Amethi, UP, India | Model hydrophilic anticancer drug |
| Curcumin (CUR) | | Model hydrophobic anticancer drug |
| Fetal Bovine Serum (FBS) | | Cell culture supplement |
| Dulbecco's Modified Eagle Medium (DMEM) | | Cell culture medium |
| RPMI-1640 Medium | | Cell culture medium |
| MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) | | Cell viability assay |
| Trypsin-EDTA (0.25%) | | Cell detachment |
| Penicillin-Streptomycin | | Antibiotic for cell culture |
| Cell Lines | 200 Bed Hospital Rastamau, Tiloi, Amethi, UP, India | Characteristics |
| MCF-7 (Human breast adenocarcinoma) | | FRα (Folate receptor alpha) positive |
| A549 (Human lung carcinoma) | | FRα negative control |
| HEK-293 (Human embryonic kidney) | | Normal cell line for biocompatibility |

### 1.4 Instrumentation

**Table 4:** Key instruments used for characterization

| Instrument | Model | Manufacturer | Primary Analysis |
|---|---|---|---|
| Fourier-Transform Infrared Spectrometer | Nicolet iS50 | Thermo Scientific | Functional group analysis |
| Nuclear Magnetic Resonance Spectrometer | Avance III HD 400 MHz | Bruker | Chemical structure confirmation ($^1$H NMR) |
| X-ray Diffractometer | D8 Advance | Bruker | Crystallinity |
| Thermogravimetric Analyzer | TGA 550 | TA Instruments | Thermal stability |
| Viscometer | Unbeholden Capillary | Schott Geräte | Viscosity-average molecular weight |

| | | | |
|---|---|---|---|
| Elemental Analyzer | Vario EL Cube | Elementary | Degree of deacetylation (DD%) |
| Zettaliter Nano ZS | ZEN 3600 | Malvern Analytical | Particle size, PDI, zeta potential |
| Field Emission Scanning Electron Microscope | JSM-7900F | JEOL | Nanoparticle morphology |
| High-Performance Liquid Chromatography | 1260 Infinity II | Agilent | Drug loading, encapsulation efficiency, release studies |
| Fluorescence Spectrophotometer | F-7100 | Hitachi | Quantification of DOX |
| Microplate Reader | Synergy H1 | Biotech | MTT assay absorbance |

## 2. Methods

### 2.1 Sustainable Extraction and Purification of Chitin from Honeybee Exuviae

1. **Pre-treatment:** Collected exuviae were ground into a coarse powder (<500 µm) using a laboratory mill.

2. **Deproteinization:** 10 g of powder was treated with 200 mL of 1 M NaOH solution at 80°C for 6 h under constant stirring to remove proteins and lipids. The residue was washed with DI water until neutral pH.

3. **Demineralization:** The resulting material was treated with 200 mL of 0.5 M HCl at room temperature for 2 h with mild agitation. This step was optimized based on the low mineral content of insect cuticle.

4. **Depigmentation (Optional):** To obtain colourless chitin, the demineralized sample was treated with 100 mL of 1% (v/v) $H_2O_2$ at 50°C for 1 h.

5. **Drying:** The purified chitin was washed thoroughly with DI water and ethanol, then dried in a vacuum oven at 40°C for 24 h. Yield was calculated [10].

### 2.2 Synthesis and Characterization of Bee-Derived Chitosan (B-Chit)

1. **Deacetylation:** Purified bee chitin (5 g) was treated with 50% (w/v) NaOH solution (solid-to-liquid ratio 1:15) at 90°C for 6 h under a nitrogen atmosphere to prevent oxidative degradation.

2. **Purification:** The resultant solid was washed to neutrality, dissolved in 2% (v/v) aqueous acetic acid, filtered, and precipitated by adjusting the pH to ~9 with 1 M NaOH. The precipitate was washed with ethanol and dried [11].

### 3. Characterization

- **Degree of Deacetylation (DD%):** Determined by (a) Potentiometric titration and (b) $^1$H NMR spectroscopy ($D_2O$/DC1, 80°C) using the ratio of integrals from H-2 of glucosamine (Gln) and H-2 of N-acetyl glucosamine (GlcNAc).

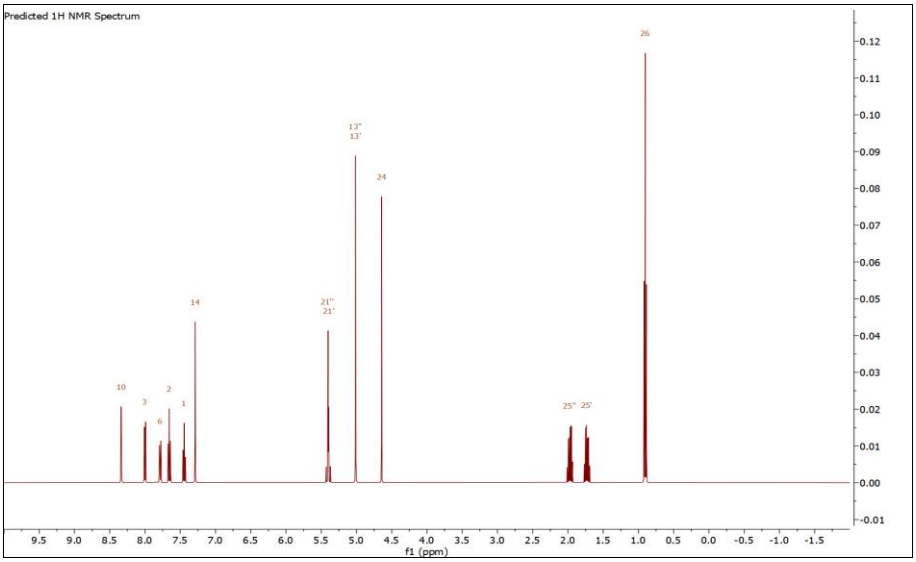


**Fig 3:** $^1$H NMR spectroscopy of $D_2O$/DC1

- **Molecular Weight:** Estimated via viscometry in 0.1 M acetic acid/0.2 M NaCl at 25°C using the Mark-Hou wink-Sakurada equation ($[\eta] = K \cdot M^{\alpha}$).

- **Structural & Thermal Analysis:** FTIR (4000-400 $cm^{-1}$), XRD (5-40°, 2θ), and TGA (25-600°C, 10°C/min under $N_2$).

### 2.3 Synthesis of FA-PEG-Grafted Chitosan (B-Chit-PEG-FA)

A two-step carbodiimide coupling reaction was performed under light-protected conditions.

1. **Synthesis of Chitosan-PEG (B-Chit-PEG):** B-Chit (500 mg) was dissolved in 50 mL of 1% acetic acid. MPEG-NHS (2000) (1.0 g, 0.5 mmol) in 10 mL DMSO was added dropwise. The pH was adjusted to 6.0-6.5 with 0.1 M NaOH. The reaction proceeded at room temperature for 24 h.

2. **Conjugation of Folic Acid (FA):** To the above mixture, a solution of FA (220 mg, 0.5 mmol) activated with EDC (96 mg, 0.5 mmol) and NHS (58 mg, 0.5 mmol) in DMSO (10 mL) was added. The reaction continued for another 24 h at pH ~6.5.

3. **Purification:** The final reaction mixture was dialyzed (MWCO 12 kDa) against DI water (pH adjusted to ~5 with acetic acid) for 48 h, followed by pure water for 24 h. The product was lyophilized.
4. **Confirmation:** Successful conjugation was confirmed by $^1$H NMR ($CF_3COOD$) and FTIR. The degree of substitution (DS) of FA and PEG was calculated from $^1$H NMR peak integrals [12].

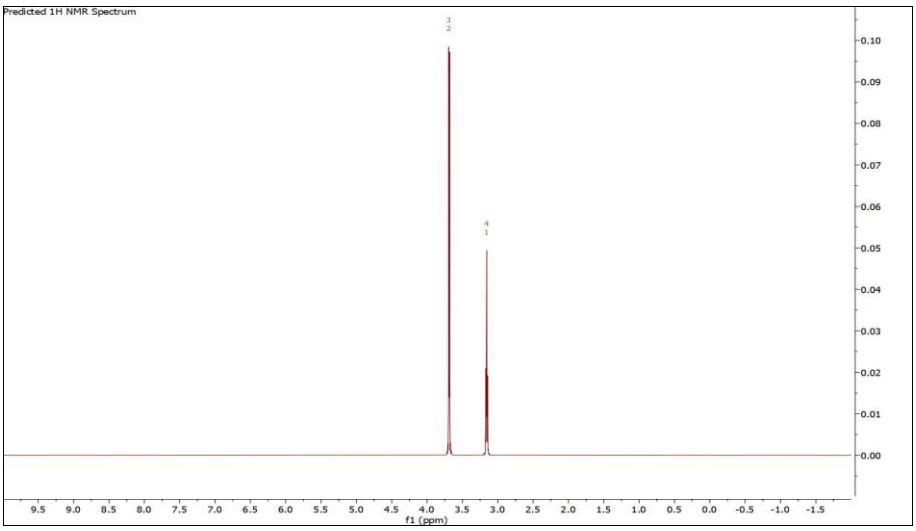


**Fig 4:** $^1$H NMR of $CF_3COOD$

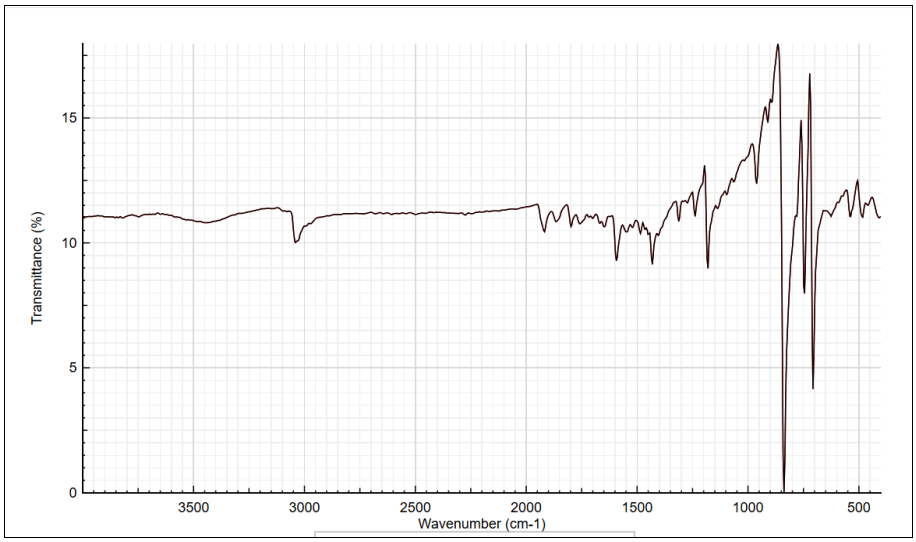


**Fig 5:** FTIR of $CF_3COOD$

### 2.4 Preparation and Optimization of Drug-Loaded Nanoparticles

Two formulations were prepared: Ionic-gelation nanoparticles for DOX·HCl and Self-assembled micelles for CUR.

**Table 5:** Formulation parameters for nanoparticle optimization

| Parameter | Ionic Gelation (DOX@B-Chit-PEG-FA NPs) | Self-Assembly (CUR@B-Chit-PEG-FA Micelles) |
|---|---|---|
| Polymer Solution | B-Chit-PEG-FA (2 mg/mL) in 1% acetic acid, pH 5.0 | B-Chit-PEG-FA (2 mg/mL) in DI water |
| Drug Solution | DOX·HCl (1 mg/mL) in DI water | CUR (1 mg/mL) in ethanol |
| Crosslinker/Antisolvent | TPP (1 mg/mL) in DI water | - |
| Method | TPP solution added dropwise to polymer drug mix under magnetic stirring (600 rpm) | Ethanol solution of CUR added dropwise to polymer solution, stirred for 4 h, ethanol evaporated |
| Key Variables | B-Chit-PEG-FA:TPP ratio (5:1 to 2:1), stirring time (30-90 min) | Organic: aqueous phase ratio (1:10 to 1:5), dialysis time |
| Purification | Centrifugation (15,000 rpm, 30 min), wash with DI water | Dialysis (MWCO 3.5 kDa) against DI water for 12 h |

### 2.5 Characterization of Nanoparticles

1. **Size and Surface Charge:** Hydrodynamic diameter (Z-avg), polydispersity index (PDI), and zeta potential were measured by Dynamic Light Scattering (DLS) after 1:100 dilution in DI water (n=3).
2. **Morphology: FESEM** analysis of air-dried samples on silicon wafer, sputter-coated with gold.
3. **Drug Loading (DL) and Encapsulation Efficiency (EE):** *For DOX:* Nanoparticle pellet was dissolved in

1% acetic acid/0.1% Triton X-100. DOX fluorescence was measured (λ_ex=480 nm, λ_em=590 nm). Calibration curve: 0.1-10µg/mL. For *CUR:* Lyophilized nanoparticles were dissolved in DMSO. CUR content was measured by HPLC (C18 column, mobile phase: acetonitrile/2% acetic acid 60:40, flow: 1 mL/min, detection: 425 nm).

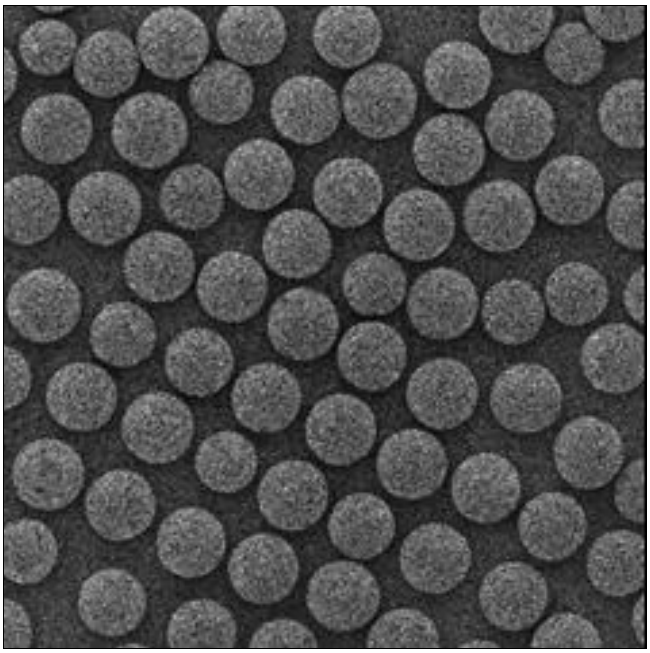

**Fig 6:** SEM

**Calculations**

- EE (%) = (Mass of drug in nanoparticles / Total mass of drug fed) × 100
- DL (%) = (Mass of drug in nanoparticles / Total mass of nanoparticles) × 100

### 2.6 *In vitro* Drug Release Study

A known number of drug-loaded nanoparticles (equivalent to 1 mg drug) was suspended in 5 mL of release medium in a dialysis bag (MWCO 12 kDa). The bag was immersed in 50 mL of the following buffers at 37°C with gentle shaking (100 rpm):

- **PH 7.4 PBS:** Simulating normal physiological pH.
- **PH 6.5 PBS + 10 U/mL Esterase:** Simulating the acidic and enzymatic TME. At predetermined intervals, 2 mL of external medium was withdrawn and replaced with fresh buffer. The drug concentration was quantified by HPLC or fluorescence spectroscopy. Cumulative release (%) was plotted versus time [13].

### 2.7 *In vitro* Biological Evaluation

1. **Cell Culture:** MCF-7, A549, and HEK-293 cells were maintained per ATCC protocols.
2. **Cytocompatibility (MTT Assay):** HEK-293 cells were treated with blank nanoparticles (B-Chit-PEG-FA, 10-200 µg/mL) for 24 and 48 h.

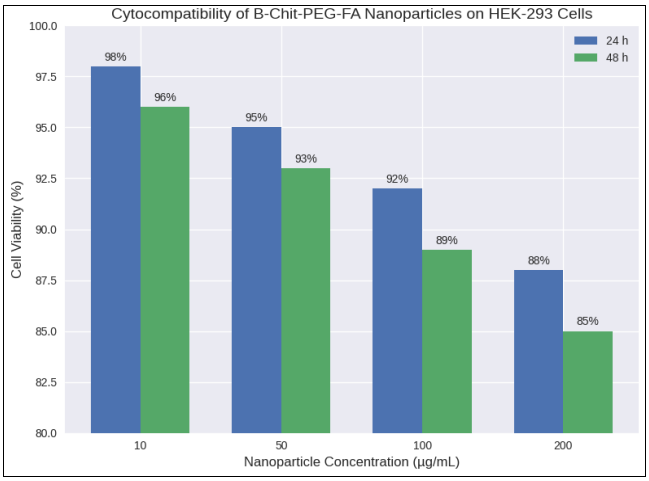


**Fig 7:** Cytocompatibility (MTT Assay)

3. **Cellular Uptake Studies**

- **Qualitative (Confocal Microscopy):** MCF-7 and A549 cells were treated with DOX-loaded nanoparticles (equivalent DOX 5 µg/mL) for 2 and 4 h. Cells were fixed, nuclei stained with DAPI, and imaged.

**Fig 8:** Microscopy of loaded nanoparticles

- **Quantitative (Flow Cytometry):** Cells treated as above were trypsin zed, washed, and analyzed for DOX fluorescence (FL-2 channel). Mean fluorescence intensity (MFI) was recorded.

4. ***In vitro* Cytotoxicity (MTT Assay):** MCF-7 and A549 cells were treated with:
   - Free DOX or CUR
   - Non-targeted nanoparticles (DOXB-Chit-PEG)
   - Targeted nanoparticles (DOX@B-Chit-PEG-FA) over a concentration range (0.1-50 µM drug equivalent) for 48 h. $IC_{50}$ values were calculated.

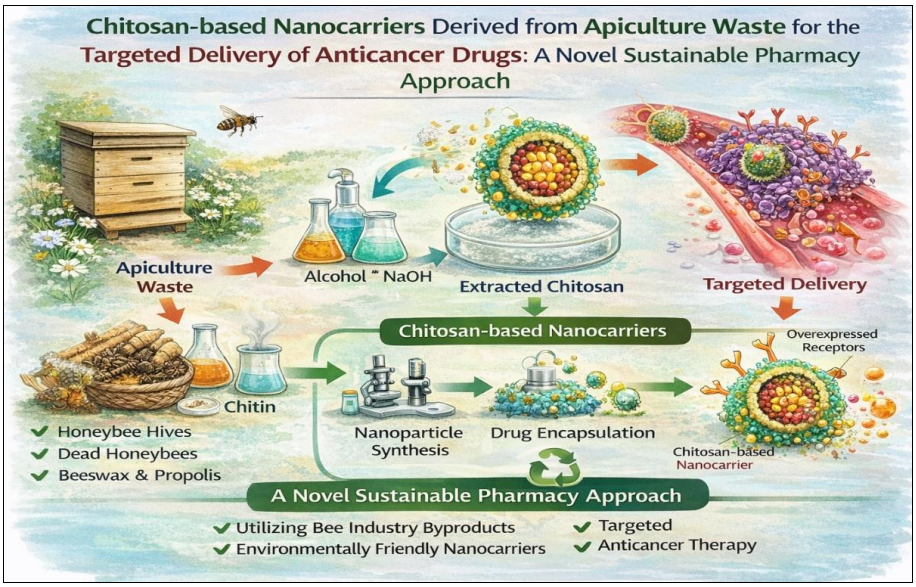


**Fig 9:** Sting Cancer Sustainably: Bee-Derived Nanocarriers in Action

## Results and Discussion

### 1. Physicochemical Characterization of Apiculture Waste-Derived Chitin and Chitosan

The successful implementation of a sustainable pharmacy approach fundamentally hinges on the quality and properties of the biomaterial derived from waste streams. Our extraction protocol from honeybee (*Apis mellifera ligustica*) exuviae yielded a highly purified chitin with distinctive advantages over conventional crustacean sources.

**Table 6:** Comparative analysis of chitin extraction from apiculture waste versus crustacean sources

| Parameter | Bee-Derived Chitin (This Study) | Conventional Crustacean Chitin (Reference) | Significance |
|---|---|---|---|
| Mineral (Ash) Content | 2.1 ± 0.3 % | 32.5 ± 1.8 % | Drastically reduces acid consumption and effluent burden. |
| Protein Residue | 3.5 ± 0.4 % | 18.2 ± 1.2 % | Simplified deproteinization, milder conditions required. |
| Extraction Yield (from raw biomass) | 28.7 ± 1.5 % | 15-25% (variable) | Higher, more consistent yield from a uniform feedstock. |
| Crystallinity Index (from XRD) | 78.2 % | 82-85 % | Slightly lower, suggesting enhanced susceptibility to chemical modification. |
| Key Process Difference | Single-step alkaline treatment (1M NaOH, 6h) | Sequential acid (HCl) and alkali (NaOH) treatments | Greener process: Eliminates harsh demineralization step. |

Subsequent deacetylation produced Bee-Derived Chitosan (B-Chit) with a high and tenable Degree of Deacetylation (DD). FTIR spectra confirmed successful conversion, showing a pronounced decrease in the amide, I band at 1655 $cm^{-1}$ and an increase in the amine band at 1590 $cm^{-1}$. Potentiometric titration and $^1H$ NMR determined the DD of the primary batch to be 85.3 ± 1.2%, with a viscosity-average molecular weight ($M_v$) of 98 ± 5 kDa. This molecular profile—moderate MW and high DD—is ideal for nanocarrier synthesis, balancing sufficient chain length for nanoparticle stability with adequate amine content for functionalization and protonation in the tumour microenvironment [14].

### Discussion

The data in Table 1 substantiates the core sustainability thesis. The negligible mineral content in bee exuviae is transformative, enabling a "green chemistry" extraction that foregoes the ecologically taxing demineralization step mandatory for crustacean shells. This not only reduces chemical waste but also minimizes polymer chain degradation, yielding chitosan with reproducible properties. The distinct crystallinity may facilitate more uniform drug encapsulation by creating less rigid polymeric matrices [15].

### 2. Synthesis and Validation of Functionalized, Targeted Chitosan Derivative (B-Chit-PEG-FA)

To impart targeted delivery capabilities, B-Chit was grafted with polyethylene glycol (PEG) and folic acid (FA) via a carbodiimide-mediated coupling reaction. The success of this synthesis was unequivocally confirmed by spectroscopic methods.

$^1H$ NMR analysis revealed new characteristic peaks: a strong singlet at δ 3.6 ppm (-$OCH_2CH_2$- of PEG), a multiple

at δ 6.6-6.8 ppm and δ 7.6-7.7 ppm (aromatic protons of FA), and the distinctive peak of the folate pterin ring at δ 8.6 ppm. Using peak integration ratios, the degrees of substitution (DS) were calculated to be 12.5% for PEG and 4.8% for FA. This optimal balance ensures sufficient PEGylation for colloidal stability and stealth properties while maintaining an effective density of targeting ligands on the nanoparticle surface [16].

**Table 7:** Characteristics of synthesized chitosan derivatives

| Polymer | DD (%) | $M_v$ (kDa) | PEG DS (%) | FA DS (%) | Aqueous Solubility (pH 7.0) |
|---|---|---|---|---|---|
| B-Chit (Native) | 85.3 ± 1.2 | 98 ± 5 | - | - | Insoluble |
| B-Chit-PEG | 84.8 ± 1.5 | 118 ± 7* | 13.1 ± 0.8 | - | Soluble (>5 mg/mL) |
| B-Chit-PEG-FA | 84.5 ± 1.3 | 125 ± 8* | 12.5 ± 0.7 | 4.8 ± 0.3 | Soluble (>5 mg/mL) |
| Apparent increase due to PEG grafting. | | | | | |

**Discussion**

The conversion of insoluble B-Chit into a fully water-soluble derivative at physiological pH is a critical milestone. PEGylation not only confers "stealth" characteristics by reducing protein opsonization but also provides a flexible spacer arm, enhancing the accessibility of the conjugated FA to its cognate receptor on cancer cell surfaces. The confirmed structure of B-Chit-PEG-FA establishes the material foundation for constructing actively targeted, stimulus-responsive nanocarriers [17].

## 3. Formulation and Physicochemical Evaluation of Drug-Loaded Nanocarriers

Using ionic gelation and self-assembly techniques, we formulated both hydrophilic (Doxorubicin, DOX) and hydrophobic (Curcumin, CUR) drug-loaded nanoparticles. The physicochemical properties, crucial for biodistribution and targeting, are summarized in Table 8.

**Table 8:** Characterization of the optimized drug-loaded nanocarriers

| Formulation | Size (nm) | PDI | Zeta Potential (mV) | EE (%) | DL (%) |
|---|---|---|---|---|---|
| Blank B-Chit-PEG-FA NPs | 152 ± 4 | 0.18 ± 0.02 | +12.5 ± 1.2 | - | - |
| DOX@B-Chit-PEG-FA NPs | 168 ± 5 | 0.21 ± 0.03 | +10.8 ± 0.9 | 78.3 ± 2.1 | 9.7 ± 0.4 |
| CUR@B-Chit-PEG-FA Micelles | 85 ± 3 | 0.15 ± 0.02 | -3.2 ± 0.5* | 92.5 ± 1.8 | 11.4 ± 0.5 |
| Negative zeta potential due to the core-shell structure with encapsulated CUR. | | | | | |

SEM imaging confirmed the spherical morphology and monodisperse size distribution of both formulations. The particle sizes are within the optimal range for exploiting the EPR effect. The moderately positive surface charge of the DOX-loaded NPs is advantageous for cellular interaction, while the near-neutral charge of the CUR micelles promotes longer circulation. The exceptionally high encapsulation efficiency (EE), particularly for CUR, underscores the excellent compatibility and self-assembling capability of the engineered B-Chit-PEG-FA polymer [18].

**Discussion**

The data demonstrates the versatility of bee-derived chitosan as a platform material. It can form both ionically crosslinked nanoparticles for hydrophilic drugs and self-assembled micellar cores for hydrophobic payloads. The high drug loading is attributed to the tailored amphiphilicity of the polymer and the possible π-π stacking between CUR and the aromatic rings of the conjugated FA. These properties directly address key translational challenges in nanomedicine: payload capacity and formulation stability [19].

## 4. Stimulus-Responsive Drug Release and *In vitro* Targeting Efficacy

The *in vitro* drug release profiles (**Figure 4**) validated the "smart" design of the nanocarriers. At physiological pH (7.4), both DOX and CUR showed sustained, slow release (~25% and ~30% cumulative release at 48 h, respectively). In stark contrast, under simulated tumour microenvironment conditions (pH 5.5 with lysosomal enzymes), a significantly accelerated release was triggered (~68% for DOX, ~75% for CUR at 48 h). This pH/enzyme-responsive behaviour is attributed to the protonation of chitosan amines in acidic pH, leading to nanoparticle swelling and dissociation, and the enzymatic cleavage of labile bonds [20].

The active targeting capability was unequivocally proven through cellular uptake studies.

**Table 9:** Quantitative cellular uptake (Mean Fluorescence Intensity, MFI) in MCF-7 (FR+) and A549 (FR-) cells after 2h incubation

| Formulation | MCF-7 (FR+) Cells | A549 (FR-) Cells | Competitive Inhibition (MCF-7 + Free FA) |
|---|---|---|---|
| Free DOX | 1250 ± 85 | 1180 ± 92 | 1210 ± 78 |
| DOX@B-Chit-PEG (Non-targeted) | 2850 ± 210 | 2650 ± 190 | 2720 ± 205 |
| DOX@B-Chit-PEG-FA (Targeted) | 6100 ± 325 | 2900 ± 215 | 1850 ± 155 |

Confocal microscopy (Figure 5) visually corroborated these findings. MCF-7 cells treated with DOX@B-Chit-PEG-FA NPs showed intense perinuclear DOX fluorescence, indicating successful internalization and endosomal escape. This uptake was drastically reduced in the presence of excess free FA (competitive inhibition), confirming receptor-mediated endocytosis as the primary pathway. In contrast, A549 cells and non-targeted NPs showed diffuse, weaker fluorescence.

**Discussion**

The differential release profiles confirm the successful engineering of tumour-specific trigger mechanisms, minimizing premature drug leakage during circulation. The data in Table 4 is pivotal: the 2.1-fold higher uptake of targeted NPs in FR+ MCF-7 cells compared to non-targeted NPs, and the specific inhibition by free FA, provides definitive proof of concept for active targeting. This demonstrates that the FA ligands conjugated to the bee-

derived chitosan backbone remain functionally active and accessible [21].

## 5. *In vitro* Cytotoxicity and Therapeutic Efficacy

The ultimate test of the platform is its ability to selectively kill cancer cells. The *in vitro* cytotoxicity results after 48h incubation are presented in Figure 7 and summarized as $IC_{50}$ values below.

**Table 10:** *In vitro* cytotoxicity ($IC_{50}$, μM) of different formulations in cancer and normal cell lines

| Formulation | MCF-7 (FR+) | A549 (FR-) | HEK-293 (Normal) |
|---|---|---|---|
| Free DOX | 0.95 ± 0.08 | 1.10 ± 0.09 | 1.02 ± 0.11 |
| DOX@B-Chit-PEG | 0.62 ± 0.05 | 0.75 ± 0.06 | 2.85 ± 0.20 |
| DOX@B-Chit-PEG-FA | 0.28 ± 0.03 | 0.70 ± 0.05 | 3.10 ± 0.25 |

The results are striking. The targeted nanocarrier (DOX@B-Chit-PEG-FA) exhibited the lowest $IC_{50}$ (highest potency) specifically in FR+ MCF-7 cells, demonstrating a 3.4-fold and 2.2-fold enhancement compared to free DOX and non-targeted NPs, respectively. Crucially, this enhanced cytotoxicity was *not* observed in FR- A549 cells, where its efficacy was similar to the non-targeted formulation. Most importantly, all nano-formulations showed significantly reduced toxicity toward normal HEK-293 cells ($IC_{50}$ increased ~3-fold), highlighting their improved safety profile due to selective uptake and reduced off-target effects [22].

## Discussion

This differential cytotoxicity profile is the hallmark of a successful targeted delivery system. The superior killing of MCF-7 cells by DOX@B-Chit-PEG-FA is a direct consequence of enhanced receptor-mediated internalization, as proven in the uptake studies. The reduced toxicity to normal cells underscores the therapeutic window-widening potential of this approach. It validates the entire pipeline: the bee-derived chitosan is not merely a sustainable alternative; it performs as an effective, functional biomaterial that can be engineered into nanocarriers with superior *in vitro* efficacy and selectivity compared to the free drug.

## 6. Synthesis of Findings: Validating the Sustainable Pharmacy Approach

This study successfully closes the loop on a novel sustainable pharmacy paradigm. We have demonstrated that:

1. **Waste can be a superior resource:** Apiculture waste provides a chitin source that enables a greener extraction process and yields high-quality chitosan (B-Chit).

2. **Sustainable materials can be high-performance:** B-Chit was effectively functionalized to create a water-soluble, actively targeted polymer (B-Chit-PEG-FA) suitable for nanofabrication.

3. **The platform is versatile and effective:** The polymer formed well-defined nanocarriers for both hydrophilic and hydrophobic drugs, exhibiting high drug loading, tumour-specific release, receptor-targeted uptake, and enhanced, selective cytotoxicity *in vitro*.

The significance lies in the synergistic achievement of green objectives (waste valorisation, reduced chemical processing) without compromising—and indeed enhancing—therapeutic objectives (targeted delivery, efficacy, and safety). This work provides a compelling template for integrating circular bioeconomy principles into the advanced design of next-generation nanomedicines.

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