International Journal of Trend in Scientific Research and Development (IJTSRD)
Volume 9 Issue 6, Nov-Dec 2025 Available Online: www.ijtsrd.com e-ISSN: 2456 – 6470

Comparative Toxicity and Long-Term Biocompatibility of Crosslinkers in Herbal Nanosponges

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ABSTRACT

Herbal nanosponges are nanoscale porous carriers that enhance the solubility and therapeutic efficiency of plant-derived bioactives. Among several formulation variables, the choice of crosslinker plays a decisive role in determining biodegradation, immune response, and overall safety. Although most studies focus on drug loading and release kinetics, relatively few have addressed their long-term or immunological safety.

This review summarizes and compares the toxicity profiles of widely used crosslinkers-diphenyl carbonate (DPC), carbonyldiimidazole (CDI), citric acid, and glutaraldehyde-drawing attention to data gaps and safety concerns. Finally, a structured toxicity-testing framework is proposed to promote safer, biocompatible, and sustainable crosslinker choices for herbal nanosponge systems.

KEYWORDS: *Nanosponges, Crosslinker toxicity, Biocompatibility, Herbal drug delivery, Immunogenicity, Chronic exposure.*

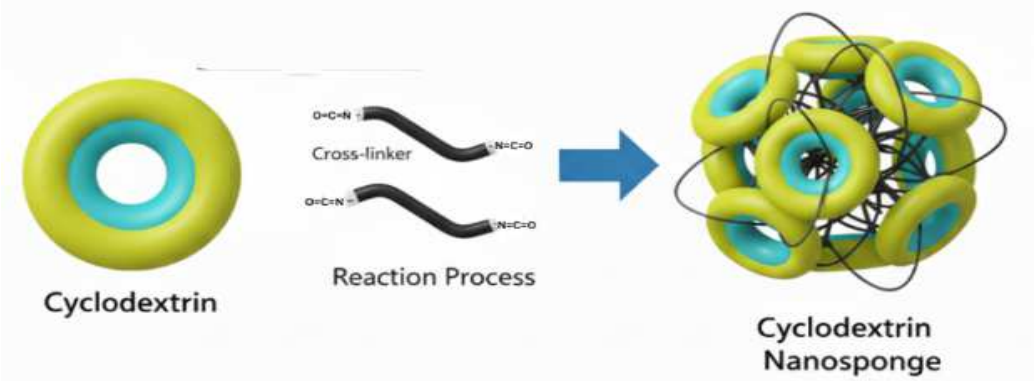
How to cite this paper: Jeevan Kunde | Vaishnavi Gite | Vaishnavi Lokhande | Dipti Sonawane | Sakshi Sontakke "Comparative Toxicity and Long-Term Biocompatibility of Crosslinkers in Herbal Nanosponges" Published in International Journal of Trend in Scientific Research and Development (ijtsrd), ISSN: 2456-6470, Volume-9 | Issue-6, December 2025, pp.668-670, URL: www.ijtsrd.com/papers/ijtsrd99949.pdf

IJTSRD99949

INTRODUCTION

Herbal bioactives often suffer from poor water solubility and limited absorption, which restrict their therapeutic potential. Nanosponges, formed through crosslinking of cyclodextrins or other biodegradable polymers, have emerged as an efficient solution. Their three-dimensional porous structure allows encapsulation of both hydrophilic and lipophilic drugs, offering controlled release and better protection of sensitive compounds.

However, despite impressive advances in formulation technology, toxicological evaluation of these systems has not received equal attention. The safety of nanosponges is inherently linked to the chemical nature of their crosslinkers, as unreacted residues or degradative fragments can cause cellular or immunological stress. Therefore, a comparative assessment of crosslinker toxicity is necessary to design herbal nanosponge formulations that are not only effective but also biologically safe.



Crosslinker Overview

Crosslinking can be achieved either chemically or physically. Among chemical options, DPC, CDI, citric acid, and glutaraldehyde are the most common.

DPC is widely used in β-cyclodextrin nanosponges, creating stable carbonate linkages that are largely inert post-reaction, though minor residues may persist. CDI forms imidazole-based linkages and offers high reactivity, yet traces of its residues can influence cell compatibility. Citric acid and other polyacids are gaining attention as greener, biodegradable alternatives. Glutaraldehyde, while efficient, is known for its strong reactivity and potential cytotoxicity, making it less suitable for biocompatible systems.

Physical or polymeric crosslinking—using agents like ethyl cellulose—bypasses the need for reactive chemicals and can enhance safety, though it sometimes results in lower mechanical stability.

Physicochemical Influence of Crosslinkers

The chemistry of the crosslinker governs nanosponge size, porosity, and surface properties. A higher crosslink density generally leads to smaller pores and slower drug release, while a lower density favors higher drug loading but may cause burst release.

The surface charge and hydrophilicity also depend on the linker type. For instance, carbonate bonds contribute to neutral or hydrophobic surfaces, whereas amide or imidazole linkers increase hydrophilicity and protein interaction potential.

Biodegradation is another critical factor. Hydrolysable linkers yield harmless products such as carbon dioxide or carboxylic acids, while amide and urethane linkages ensure structural stability under physiological conditions. Selecting the appropriate linker thus involves balancing mechanical strength, biodegradability, and compatibility.

In Vitro Toxicity and Biocompatibility

Toxicity screening typically begins with in vitro assays such as MTT, Alamar Blue, and Trypan Blue tests that measure cell viability. Most reports show that β-cyclodextrin nanosponges crosslinked with DPC or CDI are biocompatible at moderate concentrations. Still, excessive residues can lower cell survival. Hemolysis tests usually reveal less than 5 % red-cell lysis, confirming good blood compatibility.

Nonetheless, many studies omit key analyses such as oxidative stress, mitochondrial impairment, or complement activation. Chronic-exposure data are limited, and direct cross-comparisons under identical conditions are rare. A more comprehensive in vitro framework should therefore include dose-response analysis, oxidative stress biomarkers, cytokine evaluation, and genotoxicity tests to predict long-term safety more reliably.

In Vivo Safety and Immunotoxicity

In vivo research on nanosponge safety remains fragmented. Short-term studies generally indicate acceptable tolerance for DPC- and CDI-based systems, but data on chronic exposure, biodistribution, and immune response are insufficient. Degradation fragments such as phenolic or imidazole residues could provoke inflammation or oxidative imbalance when exposure is prolonged.

Well-designed comparative studies employing standardized animal models, dose levels, and exposure durations are essential. Including hematological, histopathological, and cytokine analyses would provide a clearer view of systemic and immunological effects.

Interactions with Herbal Actives

The interplay between the nanosponge matrix and encapsulated herbal compounds may influence both therapeutic performance and toxicity. Herbal molecules can undergo oxidation or partial degradation inside the nanosponge, and any reactive crosslinker residues could intensify this effect.

To separate these variables, experiments should compare three groups: unloaded nanosponges, drug-loaded nanosponges, and pure herbal extracts. This distinction helps identify whether observed toxicity arises from the nanosponge system itself or from the herbal active ingredient.

Analytical Approaches for Safety Evaluation

Reliable safety assessment combines analytical chemistry with biological testing. High-performance liquid chromatography (HPLC) or gas chromatography (GC) can quantify unreacted crosslinkers, while LC-MS/MS assists in identifying degradation products.

Fluorescent or radiolabeled tracking techniques reveal organ distribution and clearance patterns. Additional toxicological endpoints—hematology, serum biochemistry, and histopathology—help evaluate systemic effects. Immunotoxicity testing, including complement and cytokine assays, and genotoxicity assessments such as DNA-damage or oxidative-stress markers, should be integrated for a complete toxicity profile.

Applying these standardized protocols under Good Laboratory Practice (GLP) conditions ensures reproducible and comparable safety data.

Standardized Toxicity-Testing Framework

A tier-based framework is proposed for nanosponge crosslinker evaluation:

Tier I: Basic in vitro cytotoxicity and hemolysis screening.

Tier II: Degradation-kinetic and metabolite profiling.

Tier III: Acute in vivo toxicity studies.

Tier IV: Repeated-dose (28-90 day) sub-chronic assessments.

Tier V: Long-term exposure studies evaluating reversibility and delayed effects.

This structured roadmap aids in determining the no-observed-adverse-effect level (NOAEL) and supports cross-comparison among different nanosponge systems.

Safer Crosslinker Alternatives

Available evidence indicates the following order of safety:

Citric acid > polymeric/physical crosslinking > DPC > CDI > glutaraldehyde.

Citric acid and other polyacids are biodegradable and generate minimal toxic residues. Polymeric physical crosslinking avoids chemical residues entirely, though it may reduce rigidity. DPC and CDI provide efficient bonding but need careful purification to remove residual agents. Glutaraldehyde, owing to its strong reactivity and potential cytotoxicity, is best avoided for herbal formulations.

Careful optimization of the crosslinker-to-polymer ratio remains essential to minimize toxicity without compromising nanosponge structure.

Limitations and Future Perspectives

Research on the toxicology of herbal nanosponges is still limited by non-uniform testing methods, variable particle sizes, and inter-species differences. The absence of specific regulatory standards for herbal nanocarriers further complicates evaluation.

Future work should emphasize harmonized testing protocols, chronic-exposure models, and use of computational toxicology and in silico prediction tools to anticipate long-term outcomes.

Conclusion

A comprehensive understanding of crosslinker toxicity is fundamental for developing safe and effective herbal nanosponge formulations. Comparative and long-term studies are urgently needed to close current knowledge gaps. Emphasis on biodegradable and biocompatible linkers, combined with standardized testing and regulatory alignment, will help establish nanosponges as reliable carriers for herbal therapeutics in modern medicine.

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