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*Review*

# Immunomodulatory Potential and Biocompatibility of Chitosan–Hydroxyapatite Biocomposites for Tissue Engineering

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**Abstract:** Chitosan–hydroxyapatite (CS-HAp) biocomposites, combining the biocompatibility and bioactivity of chitosan with the osteoconductive properties of hydroxyapatite, are emerging as promising candidates for tissue engineering applications. These materials consistently exhibit excellent cytocompatibility, with cell viability rates greater than 95% in MTT and Neutral Red Uptake assays, and minimal cytotoxicity, as demonstrated by low levels of cell death in DAPI and Trypan blue staining. More importantly, CS-HAp biocomposites modulate the immune environment by enhancing the expression of anti-inflammatory cytokines (IL-10 and IL-4) and the pro-inflammatory cytokine TGF-β, while avoiding significant increases in TNF-α, IL-6, or NF-κB expression in fibroblast cells exposed to HAC and HACF scaffolds. In an in vivo dermatitis model, these biocomposites reduced mast cell counts and plasma histamine levels and significantly decreased pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), JAK1/3, VEGF, and AnxA1 levels. Structurally, HACF scaffolds demonstrated larger average pore sizes (95 μm) compared to HAC scaffolds (74 μm), with porosities of 77.37 ± 2.4% and 65.26 ± 3.1%, respectively. These materials exhibited high swelling ability, equilibrium water content, and controlled degradation over a week in culture media. In addition to their immunomodulatory effects, CS-HAp composites promote essential cellular activities, such as attachment, proliferation, and differentiation, thereby supporting tissue integration and healing. Despite these promising findings, significant gaps remain in understanding the underlying mechanisms of immune modulation by CS-HAp biocomposites, and formulation-dependent variability raises concerns about reproducibility and clinical application. Therefore, a comprehensive review is essential to consolidate existing data, identify key knowledge gaps, and standardize the design of CS/HAp composites for broader clinical use, particularly in immunomodulatory and regenerative medicine contexts.

**Keywords:** biocomposites; tissue engineering; bioremediation; immunomodulation; nanocomposites

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## 1. Introduction

Inflammatory skin diseases, such as atopic dermatitis, contact dermatitis, and psoriasis, are prevalent chronic conditions marked by complex and dysregulated immune responses in the skin. These disorders affect approximately 20% of the global population, representing a significant public health concern due to their persistent nature, high recurrence rates, and impact on patients' quality of life. The pathophysiology of these conditions typically involves an imbalance in pro-inflammatory and anti-inflammatory cytokine activity, barrier

dysfunction, and aberrant immune cell activation, leading to sustained inflammation and impaired skin regeneration.

Conventional treatments for these diseases primarily aim to suppress the inflammatory response using corticosteroids, immunosuppressants, or biologic agents targeting specific cytokines. While these therapies can offer symptomatic relief, they are often associated with substantial limitations, including systemic side effects, increased risk of infection, tolerance development, and diminished efficacy over time. Moreover, these treatments rarely address the underlying tissue damage or contribute to the restoration of normal skin architecture and function.

In this context, tissue engineering has emerged as a compelling alternative approach. This field focuses on designing and fabricating biomimetic scaffolds and biomaterials that not only support structural repair but also interact favorably with biological systems to promote tissue regeneration. By integrating principles of material science, cellular biology, and immunology, tissue engineering offers the potential to create multifunctional therapeutic platforms. These scaffolds aim to mimic the native extracellular matrix (ECM), providing an environment conducive to cellular activities such as adhesion, proliferation, differentiation, and migration, all of which are essential for effective skin regeneration. In particular, the development of bioactive, biocompatible materials capable of modulating the local immune environment represents a promising strategy to both repair tissue and resolve chronic inflammation in inflammatory skin diseases [1–4].

Biomaterials serve as the foundational element in tissue engineering, offering a three-dimensional structural framework designed to replicate the architecture and biological functionality of the native extracellular matrix (ECM). This synthetic or natural scaffold is critical for supporting and directing key cellular behaviors necessary for tissue repair and regeneration. Specifically, it provides a conducive microenvironment that promotes cell adhesion, sustains cellular proliferation, and enables lineage-specific differentiation, ultimately guiding the formation of new, functional tissue.

To effectively fulfill these roles, an ideal scaffold must meet several stringent physicochemical and biological criteria. It should exhibit an appropriate level of porosity and pore interconnectivity, which are essential for facilitating the infiltration and migration of cells, as well as for enabling the exchange of nutrients, gases, and metabolic waste. Pore size must be tailored to the specific tissue type being targeted, as different cells and tissues have distinct spatial requirements. Additionally, the scaffold must be structurally stable yet capable of gradual degradation, with a resorption rate that closely aligns with the pace of new tissue formation. This ensures that the scaffold provides initial mechanical support without leaving behind residual foreign material that could provoke chronic inflammation or fibrosis.

Equally important are the material's biocompatibility and immunological profile. The scaffold must be non-toxic and non-immunogenic, avoiding adverse immune reactions that could compromise tissue integration or lead to implant rejection. It should also facilitate molecular signaling processes that naturally occur within the ECM, including interactions with integrins and other cell-surface receptors that regulate cellular behavior.

Among the various materials explored for scaffold development, natural polymers—such as chitosan, alginate, collagen, and hyaluronic acid—have gained particular interest for constructing hydrogel-based scaffolds. These biopolymers are highly regarded for their intrinsic bioactivity, biodegradability, and structural resemblance to the native ECM. Their hydrophilic nature allows them to retain high water content, creating a hydrated and soft matrix that closely mimics the mechanical and biochemical characteristics of natural tissue. Moreover, their chemical versatility enables easy functionalization and cross-linking, which facilitates customization of mechanical strength, degradation rate, and biological

signaling properties. Importantly, these polymers can be processed under mild conditions, preserving the activity of incorporated cells or bioactive molecules and allowing for facile fabrication into diverse scaffold architectures.

Biomaterials, particularly hydrogel-forming natural polymers, are indispensable in tissue engineering due to their ability to replicate the ECM, support vital cellular functions, and integrate seamlessly into biological systems—all of which are prerequisites for successful tissue regeneration [5–9].

Chitosan and hydroxyapatite are two naturally derived biomaterials that have garnered significant attention in biomedical research, particularly in the field of tissue engineering, due to their complementary structural and biological properties. Both materials originate from natural sources—hydroxyapatite (HA) from bone and mineral deposits, and chitosan from the exoskeletons of crustaceans such as shrimp and crabs—and offer distinct yet synergistic advantages when applied in regenerative medicine.

Hydroxyapatite (HAp) is a calcium phosphate mineral that closely resembles the inorganic component of natural bone. Its chemical similarity to human hard tissues grants it excellent biocompatibility and osteoconductivity, meaning it not only integrates well with the body but also supports the growth and attachment of bone-forming cells (osteoblasts). One of HAp's most important features is its ability to form direct chemical bonds with living bone tissue, which enhances implant stability and facilitates long-term integration. Additionally, HAp is known for its bioactivity, as it can induce the formation of apatite layers on its surface in physiological environments—an important factor for initiating bone regeneration. These properties make it particularly well-suited for applications in orthopedic implants, bone graft substitutes, dental restorations, and load-bearing scaffolds.

Chitosan, on the other hand, is a linear polysaccharide obtained through the deacetylation of chitin, the second most abundant natural polymer after cellulose. It possesses a unique combination of physicochemical and biological characteristics that are highly desirable for soft tissue applications. As a cationic polymer, chitosan interacts electrostatically with negatively charged cell membranes and biomolecules, promoting cell adhesion, migration, and proliferation—all vital processes for tissue repair and regeneration. Furthermore, chitosan is biodegradable, breaking down into non-toxic, bioresorbable by-products that are easily eliminated from the body. Its biocompatibility, non-antigenicity, and low toxicity ensure it does not provoke harmful immune responses upon implantation.

Beyond these basic attributes, chitosan exhibits a diverse range of biofunctional properties that extend its utility in tissue engineering [10–13]:

- it supports hemostasis, aiding in the rapid cessation of bleeding by promoting platelet aggregation and clot formation.
- it enhances angiogenesis, the formation of new blood vessels, which is critical for supplying nutrients and oxygen to regenerating tissue.
- it can activate macrophages and influence their polarization toward a regenerative (M2) phenotype, thereby contributing to immune modulation and wound healing.
- its intrinsic antimicrobial activity provides a barrier against bacterial colonization, reducing the risk of infection during tissue repair.

Given the individual advantageous properties of chitosan and hydroxyapatite, combining them into composite biomaterials has attracted significant attention [14]. The aim is to leverage the strengths of each component, potentially leading to synergistic effects that enhance bioactivity, mechanical properties, and ultimately, the regenerative capacity of the material [15]. Furthermore, such biocomposites designed for tissue engineering, especially for inflammatory conditions, must demonstrate favorable interactions with the host immune system, specifically exhibiting immunomodulatory or anti-inflammatory properties [16].

This paper focuses on the immunomodulatory properties of chitosan–hydroxyapatite biocomposites based on findings presented in the provided sources. It examines in vitro studies evaluating the scaffolds' influence on inflammatory marker expression and in vivo studies assessing their impact on inflammation and tissue repair in a dermatitis model [17].

*Novelty*

The novelty of this study lies in its integrative approach, combining tissue engineering, immunology, and biomaterials science to address inflammatory skin diseases like dermatitis. It introduces chitosan–hydroxyapatite (CS-HAp) biocomposites as bio-instructive scaffolds that go beyond structural support to actively modulate the immune response. Unlike conventional anti-inflammatory treatments, this strategy promotes regenerative healing through immune modulation rather than suppression. Key innovations include dual-function biomaterials (chitosan for immune modulation, HAp for structural support), emphasis on immunomodulation as a core design principle, use of natural, ECM-mimicking hydrogels for enhanced biocompatibility, demonstrated efficacy in in vivo dermatitis models, including synergy with stem cell-derived factors, and potential for clinical translation in treating chronic, non-healing inflammatory skin conditions.

## 2. Materials and Composition of Biocomposites

The studies discussed in the sources utilize biocomposites incorporating chitosan and hydroxyapatite, although with variations in additional components and fabrication methods.

One study describes the synthesis of two marine biopolymer-based hydrogel scaffolds: HAC (hydroxyapatite, alginate, and chitosan) and HACF (hydroxyapatite, alginate, chitosan, and fucoidan) [18]. The materials used included sodium alginate from brown algae (medium viscosity), chitosan (low molecular weight, degree of deacetylation 75–85%), hydroxyapatite, and fucoidan [19]. Please note that, since alginate and hydroxyapatite are chain-like polymers, their physicochemical properties may vary slightly depending on the study examined, among those cited in this paper. The ratios used for HACF construction were 5:5:3:0.1 weight ratios of HAp, alginate, chitosan, and fucoidan, respectively [20].

Alginate is a biocompatible, hydrophilic, and biodegradable anionic polymer derived from brown seaweed and bacteria, known for its ability to form gels with divalent cations [21]. Fucoidan is a sulphate-rich polysaccharide from brown seaweeds, possessing anti-inflammatory, anti-angiogenic, and anti-adhesive actions, and can reinforce cellular activities for tissue regeneration [22].

Another study evaluated a biomaterial composed of hydroxyapatite and chitosan associated with conditioned medium from dental pulp stem cells for treating dermatitis. This biomaterial was a chitosan–xanthan–hydroxyapatite composite membrane. Xanthan gum is also a polysaccharide, used here along with chitosan and hydroxyapatite [23].

These compositions highlight the use of natural polysaccharides (chitosan, alginate, xanthan, fucoidan) in conjunction with hydroxyapatite to create composite biomaterials aimed at tissue engineering applications, including those involving inflammation [24].

## 3. Fabrication and Characterization

The fabrication of the HAC and HACF hydrogel scaffolds was achieved through a co-precipitation method involving mechanical stirring and thermal processing. Alginate and hydroxyapatite were dissolved together, and then chitosan dissolved in acetic acid was added. This mixture was stirred, heated, treated with sodium phosphate, and cross-linked with calcium chloride. The resulting slurry was cast, dried, further cross-linked, washed, and freeze-dried to obtain the scaffolds. The incorporation of fucoidan in HACF followed a similar procedure, adding fucoidan to the alginate solution before the addition of hydrox-

yapatite and chitosan. This process is designed to create a structured composite through molecular interactions, including ester bond formation between alginate and chitosan, chelation of alginate's guluronic acid residues with $Ca^{2+}$ ions, electrostatic interactions between cationic chitosan and anionic alginate/fucoidan, and hydrogen bonding between HAp and the polymers [25–28].

Characterization techniques confirmed the successful incorporation of components and revealed critical physical properties relevant to tissue engineering and biological interaction [29]. Fourier Transform-Infrared (FTIR) spectroscopy verified the presence of characteristic functional groups from chitosan, alginate, hydroxyapatite, and fucoidan in the composite scaffolds, indicating chemical interactions and associations between the components [30].

Scanning electron microscopy (SEM) analysis of the HAC and HACF hydrogel scaffolds displayed characteristic porous morphologies. Pore size and distribution are significant for cell penetration, nutrient/metabolite transport, and ultimately, tissue formation and neovascularization. The HACF hydrogel, containing fucoidan, exhibited larger average pore sizes (95 µm) compared to HAC (74 µm), signifying differences possibly related to composition and cross-linking interactions. These pore sizes were within ranges considered suitable for various cellular activities and tissue engineering applications like chondrogenesis (70–150 µm) and fibrovascular tissue formation (around 500 µm), supporting cell infiltration and nutrient traffic (Figure 1) [31–33].

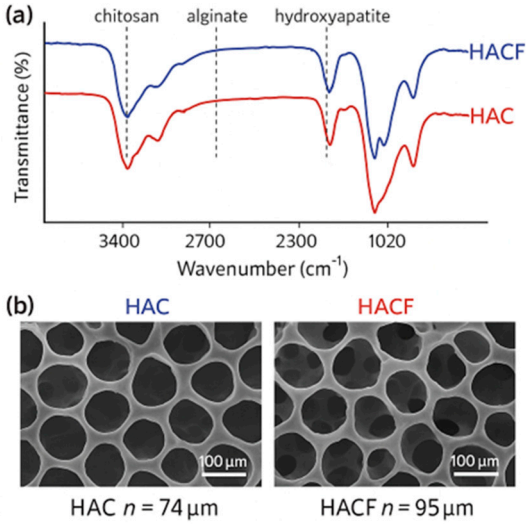



**Figure 1.** (**a**,**b**) HAC (hydroxyapatite, alginate, and chitosan) and HACF (hydroxyapatite, alginate, chitosan, and fucoidan) properties.

Porosity measurements, determined by the solvent replacement method, indicated that HAC and HACF hydrogels possessed significant porosity (65.26 ± 3.1% and 77.37 ± 2.4% respectively), further confirming their suitability for tissue engineering by facilitating cell attachment and mass accommodation [34]. Apparent density measurements showed similar values for both scaffolds, suggesting uniform pore size and distribution, which can influence mechanical properties [35].

Swelling ability and equilibrium water content (EWC) are critical properties, as the highly hydrated state of hydrogels mimics the native ECM and influences the diffusion of solutes [36]. The swelling ratio and EWC are proportional to the hydrogel's affinity for water and are influenced by ionic groups and microstructure [37]. HACF hydrogels demonstrated higher swelling and EWC compared to HAC, suggesting greater hydrophilicity. High water

content, soft consistency, and channeling porosity contribute to hydrogels being ideal ECM substitutes, enhancing cell growth and infiltration by facilitating nutrient transport [38].

Biodegradation studies in cell culture medium (DMEM with FBS) and PBS evaluated the scaffolds' stability. Both HAC and HACF hydrogels remained stable for over a week in DMEM. In PBS, both showed progressive weight loss, with HACF degrading at a more controlled rate than HAC, a potentially advantageous characteristic for tissue engineering where scaffold degradation should match the rate of new tissue formation. A slight drop to acidic pH was observed, possibly due to ester bond cleavage, which could be buffered in vivo [39–42].

These physiochemical characteristics—porosity, pore size, swelling ability, water content, and controlled biodegradation—are crucial for the biological performance of the scaffolds and their interaction with cells and the host environment, including immune responses (Figure 2) [43].

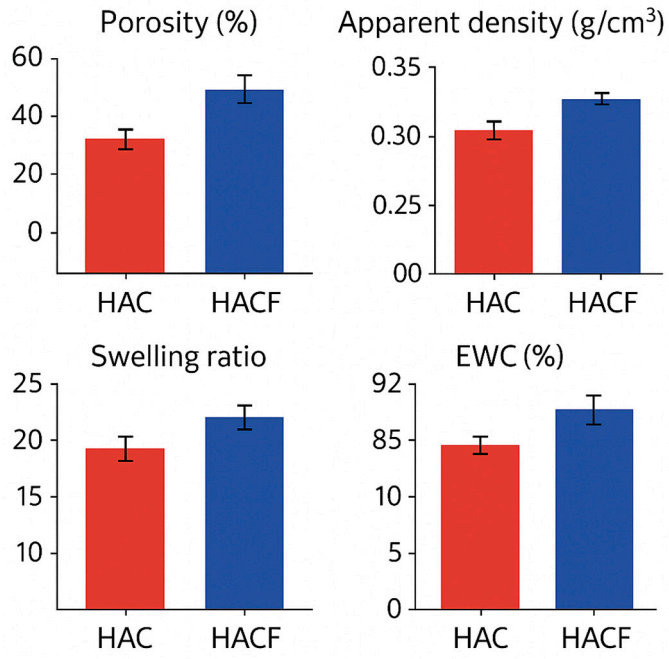



**Figure 2.** HAC (hydroxyapatite, alginate, and chitosan) and HACF (hydroxyapatite, alginate, chitosan, and fucoidan) porosity and apparent density data.

## 4. Immunomodulatory Properties: In Vitro and In Vivo Evidence

The immunomodulatory properties of chitosan–hydroxyapatite biocomposites can be inferred from their interaction with biological systems, assessed through biocompatibility tests and specific analyses of inflammatory markers.

### *4.1. In Vitro Immunomodulatory Assessment (HAC and HACF Scaffolds)*

The study on marine biopolymer hydrogels directly investigated the immunomodulatory and anti-inflammatory properties of HAC and HACF scaffolds using in vitro methods. This was done by assessing the mRNA expression of major inflammatory markers: Tumor Necrosis Factor-alpha (TNF-α), Interleukin-6 (IL-6), and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB). These markers were measured in L929 mouse fibroblast cells under cytotoxic conditions [44–46].

TNF-α, IL-6, and IL-1β are pro-inflammatory cytokines that activate inflammatory cells during acute responses. NF-κB is a key transcription factor involved in the signaling pathways leading to the production of these cytokines. Therefore, evaluating the expression

levels of these markers in contact with the biomaterial can indicate whether the material induces a pro-inflammatory response [47,48].

The results showed no significant changes in the mRNA expressions of TNF-α, IL-6, and NF-κB in both HAC and HACF hydrogels when compared to control cells. This is a strong indication of the non-toxic and biocompatible nature of HAC and HACF. The authors conclude that this favorable response may be attributed to the immunomodulatory and anti-inflammatory activities exerted by the constituent natural polysaccharides present in the synthesized hydrogels, including chitosan and alginate, and fucoidan in the case of HACF [49]. This finding suggests that these hydrogels can provide good biocompatibility without inducing inflammation. The study noted a slightly better effect with HACF compared to HAC, possibly due to the negatively charged sulphated fucoidan enhancing its interaction with biological systems [50,51].

These in vitro findings demonstrate that HAC and HACF biocomposites do not appear to trigger a significant inflammatory response mediated by the tested cytokines in fibroblast cells, which is a crucial aspect for materials intended for implantation or application in tissue engineering (Figure 3).

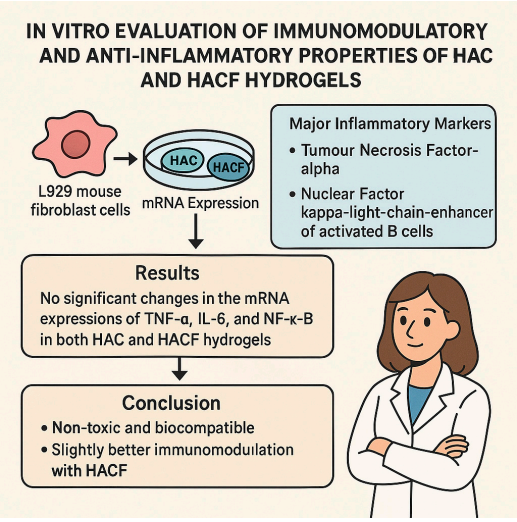



**Figure 3.** HAC (hydroxyapatite, alginate, and chitosan) and HACF (hydroxyapatite, alginate, chitosan, and fucoidan) in vitro evaluations.

Standard practices in such in vitro experiments typically involve the following:

- Negative Control (Untreated Cells): Cells cultured without any treatment to establish baseline gene expression levels.
- Positive Control (Pro-inflammatory Stimulus): Cells treated with known inducers of inflammation, such as lipopolysaccharide (LPS), to confirm the assay's sensitivity and the cells' responsiveness to inflammatory stimuli. For instance, LPS has been used to activate macrophages and fibroblasts, leading to increased production of cytokines like TNF-α and IL-6.

These controls ensure that any observed effects on cytokine expression are attributable to the biomaterial's properties rather than experimental artifacts [44–51].

### *4.2. In Vivo Assessment in a Dermatitis Model (Chitosan–Xanthan–Hydroxyapatite Composite)*

The study investigating the biomaterial composed of hydroxyapatite and chitosan (along with xanthan gum) for skin tissue repair in a rat dermatitis model provides in vivo evidence related to its impact on inflammation. Dermatitis is characterized by uncontrolled

inflammatory responses. The model used involved inducing skin injury and inflammation on the backs of rats using acetone and friction [52–54].

The study compared different treatment groups: induced without treatment (G1), induced and treated with standard hydrocortisone ointment (G2), induced and treated with the biomaterial without conditioned medium (G3), and induced and treated with the biomaterial with conditioned medium (G4). Treatments were applied once daily or as a single dressing (biomaterial) for 5 days, and evaluations were performed on day 8 post-induction [54].

Analysis of skin fragments revealed significant differences in inflammatory markers and tissue structure between the groups. Groups G1 (untreated) and G2 (hydrocortisone) showed rupture and hyperplasia of the epidermis and a significant inflammatory influx. G1 also had the highest number of mast cells, many degranulated. Mast cells are key players in allergic reactions and dermatitis, releasing mediators like histamine that contribute to inflammation, itching, and angiogenesis. Plasma histamine levels were also reduced in the G4 group (biomaterial + CM (conditioned medium)), suggesting reduced mast cell activity. Crucially, Groups G3 (biomaterial without CM) and G4 (biomaterial with CM) showed less thickened skin, a better tissue regeneration process, and reduced mast cells compared to G1 and G2 [54–56].

Furthermore, the study measured the expression of several inflammatory mediators. Annexin A1 (AnxA1) is an endogenous anti-inflammatory mediator known for inhibiting mast cell degranulation and histamine release. Janus kinases (JAKs) are a family of intracellular enzymes that transmit signals from cytokine receptors on the cell surface to the nucleus. Janus kinase (JAK)-1 and JAK-3 are involved in the JAK/STAT signaling pathway triggered by inflammatory cytokines like IL-31, which is strongly linked to itching in dermatitis. Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1β, and IL-6 are pro-inflammatory cytokines central to the inflammatory cascade. Vascular Endothelial Growth Factor (VEGF) is a pro-angiogenic factor, with mast cells being a main source stimulating angiogenesis in dermatitis lesions [57,58].

The results showed that the expression of AnxA1 and positive cells for JAK-1 and JAK-3 were increased in G1 and G2 but reduced in the groups treated with biomaterials (G3 and G4), mainly G4. Likewise, the treatments in G3 and G4 lowered levels of TNF-α, IL-1β, IL-6, and VEGF compared to G1 and G2. These in vivo findings provide compelling evidence that the chitosan–xanthan–hydroxyapatite composite biomaterial itself (G3), and even more so when combined with conditioned medium (G4), can significantly reduce the inflammatory process and promote tissue regeneration in a dermatitis model [58]. The reduction in mast cells, plasma histamine, pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), and factors involved in itching/inflammation pathways (JAK1/3) and angiogenesis (VEGF) directly demonstrate an anti-inflammatory and immunomodulatory effect. The improved tissue regeneration observed in G3 and G4 correlates with this reduction in inflammation. This aligns with the general understanding that chitosan-based biomaterials can accelerate wound healing, partly by enhancing the functions of cells involved in inflammation like macrophages and fibroblasts [59].

The scaffold composed of hydroxyapatite, chitosan, and xanthan gum demonstrated functional integration with host tissue, particularly in terms of tissue regeneration and modulation of inflammatory and angiogenic responses, as assessed in a rat model of dermatitis. Multiple functional endpoints were evaluated to determine its integration with host tissue and therapeutic potential. Tissue regeneration was confirmed through histological analysis showing improved skin structure, while inflammatory regulation was demonstrated by reduced levels of TNF-α, IL-1β, and IL-6 via immunohistochemistry and

ELISA, indicating good biocompatibility. Angiogenesis was indirectly assessed through decreased VEGF expression, suggesting controlled vascular response [52–59].

Conditioned medium is typically obtained by culturing cells, such as mesenchymal stem cells (MSCs), in serum-free media for 24–48 h. The supernatant is then collected, filtered to remove cell debris, and concentrated if necessary. In some studies, the conditioned medium is freeze-dried to enhance stability before incorporation into scaffolds, typically through methods such as soaking, rehydration, or encapsulation. The dosing of CM is often determined based on the concentration of bioactive factors and the desired therapeutic effect. For instance, in the development of a secretome-enriched alginate/extracellular matrix hydrogel patch, the CM was rehydrated into the scaffold to achieve a high concentration of bioactive factors.

The retention and release of bioactive factors from scaffolds are commonly evaluated using techniques like enzyme-linked immunosorbent assay (ELISA) or quantitative polymerase chain reaction (qPCR) to measure the levels of specific proteins or mRNA over time. For instance, in the development of a secretome-enriched alginate/extracellular matrix hydrogel patch, the release of bioactive factors such as VEGF, HGF, IGFBPs (insulin-like growth factor binding proteins), IL-6, and IL-8 was assessed to evaluate the sustained release profile and therapeutic efficacy.

## 5. Mechanisms Contributing to Immunomodulation

Drawing from the properties of the constituent materials and the observed biological responses, several mechanisms may contribute to the immunomodulatory properties of chitosan–hydroxyapatite biocomposites.

- Intrinsic Properties of Chitosan: Chitosan is described as non-antigenic and biocompatible. It is also stated to have significant biochemical importance in processes like macrophage activation and recruitment of neutrophils to sites of infection. While macrophages are inflammatory cells, their activation phenotype can shift from pro-inflammatory (M1 macrophages) to pro-resolving/regenerative (M2 macrophages). Its role in enhancing the function of inflammatory cells suggests a potential to guide the inflammatory response towards a regenerative phase rather than simply suppressing it [60–62].
- Intrinsic Properties of Hydroxyapatite: Hydroxyapatite is described as non-inflammatory and non-immunogenic. Its ability to form direct bonds with living tissue facilitates integration and minimizes foreign body reactions that can trigger chronic inflammation [63,64].
- Synergistic Effects in Composites: The combination of components like chitosan, alginate, hydroxyapatite, and fucoidan may lead to synergistic actions that enhance the overall immunomodulatory effect. The negatively charged sulphated fucoidan in HACF, for instance, was suggested to make it more effective than HAC. While not detailed in the sources, complex molecular interactions (electrostatic, hydrogen bonding) within the composite structure could influence the release of components or the material's surface properties, affecting cell interactions [65,66].
- Modulation of Inflammatory Cytokine Expression: The in vitro study showed no increase in TNF-α, IL-6, and NF-κB expression by fibroblasts in contact with the scaffolds. The in vivo study showed a reduction in TNF-α, IL-1β, and IL-6 levels in the skin tissue. This suggests the biomaterials can directly or indirectly downregulate the production or activity of key pro-inflammatory mediators [67,68].
- Influence on Mast Cells and Associated Pathways: The reduction in mast cells and plasma histamine levels observed in vivo is a direct anti-inflammatory effect. The reduced expression of JAK1 and JAK3, components of the IL-31 pathway involved

in itching and inflammation, further points to the material's ability to interfere with key inflammatory signaling cascades in dermatitis [69,70]. The anti-allergic actions of AnxA1, which were shown to be modulated by the biomaterial treatment (reduced expression compared to controls, though the meaning of reduced expression vs. increased expression in controls is complex based solely on the provided text structure) [64], also indicate an interaction with allergy/inflammatory mediators.

- Protein Adsorption Profile: The adsorption of plasma proteins onto the scaffold surface immediately upon contact is a critical initial event influencing cell interaction and subsequent biological responses, including inflammation [71,72].
- Controlled Degradation and Non-Toxic Products: A suitable degradation rate ensures the scaffold is replaced by new tissue without causing chronic inflammation due to persistent foreign material. The biodegradation products should also be non-toxic and biocompatible. The studies indicate the scaffolds are non-toxic in vitro and their degradation products did not induce significant inflammatory marker expression [73,74].
- Support for Tissue Regeneration: By providing a favorable microenvironment (porosity, water content) for cell adhesion, proliferation, and migration, and by potentially attracting key cells involved in healing (fibroblasts, keratinocytes, potentially macrophages), the biomaterials facilitate tissue repair [75].

## 6. Other Biocompatibility Aspects

Beyond direct immunomodulation tests, the studies assessed general biocompatibility, which is intrinsically linked to the material's interaction with the immune system (Figure 4).

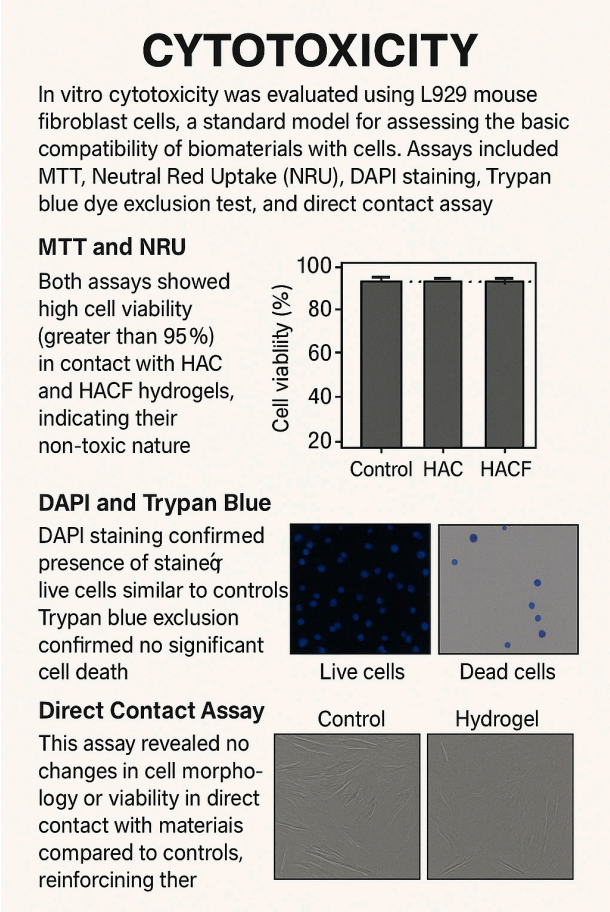



**Figure 4.** Biocomposites cytotoxicity evaluations.

- Hemocompatibility: For biomaterials potentially contacting blood, hemocompatibility is crucial. Tests included hemolysis, RBC (Red Blood Cells) aggregation, and plasma protein adsorption [76,77].
- Hemolysis: Both HAC and HACF hydrogels exhibited very low hemolysis rates, well within the acceptable range for biomaterials. This indicates minimal damage to red blood cells upon contact [78].
- RBC Aggregation: Microscopic analysis showed no aggregation of red blood cells in contact with the hydrogel extracts, demonstrating no adverse effect on blood fluidity [79].
- Plasma Protein Adsorption: As discussed above, significant adsorption was observed, particularly of albumin, which is favorable for biocompatibility. These results confirm that the HAC and HACF hydrogels are hemocompatible and do not evoke adverse effects in blood flow or compatibility [80,81].
- Cytotoxicity: In vitro cytotoxicity was evaluated using L929 mouse fibroblast cells, a standard model for assessing the basic compatibility of biomaterials with cells. Assays included MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), Neutral Red Uptake (NRU), DAPI (4′,6-Diamidino-2-Phenylindole) staining, Trypan blue dye exclusion test, and direct contact assay [82,83].
- MTT and NRU: Both assays showed high cell viability (greater than 95%) in contact with HAC and HACF hydrogels, indicating their non-toxic nature and that of their degradation products [84].
- DAPI and Trypan Blue: DAPI staining confirmed the presence of stained live cells similar to controls. Trypan blue exclusion confirmed no significant cell death, as dead cells, permeable to the dye, were not observed in significant numbers [85,86].
- Direct Contact Assay: This assay revealed no changes in cell morphology or viability in direct contact with the materials compared to controls, reinforcing their biocompatibility. These comprehensive in vitro cytotoxicity evaluations demonstrate that the HAC and HACF hydrogels are cytocompatible and non-toxic [87,88].

Taken together, the hemocompatibility and cytotoxicity studies provide strong support for the biocompatibility of these chitosan–hydroxyapatite-based composites, a prerequisite for any material intended to interact favorably with the immune system and host tissue.

## 7. Potential Applications

Based on the demonstrated immunomodulatory properties, biocompatibility, and ability to promote tissue regeneration, chitosan–hydroxyapatite biocomposites hold significant potential for various applications in tissue engineering and therapeutic treatment of inflammatory conditions.

- Tissue Engineering Scaffolds: Their porous architecture, suitable pore size, swelling capacity, biocompatibility, and controlled degradation make them promising candidates as scaffolds for regenerating various tissues. The absence of significant inflammatory response observed in vitro is critical for the success of tissue regeneration, as chronic inflammation can impede healing and lead to scar tissue formation [89].
- Treatment of Inflammatory Skin Diseases: The in vivo findings in the dermatitis model are particularly relevant [90]. The ability of the chitosan–xanthan–hydroxyapatite composite to reduce inflammation, decrease mast cell numbers, lower pro-inflammatory cytokine levels, and improve tissue regeneration suggests its potential as a therapeutic dressing or material for managing inflammatory skin conditions like dermatitis [90,91]. This approach offers an alternative to conventional therapies that primarily suppress inflammation. The enhanced effect when combined with conditioned medium also

points to strategies combining these biomaterials with bioactive molecules for improved outcomes [92,93].

- Regenerative Dentistry and Bone Regeneration: Although not the primary focus on immunomodulation in this paper, the sources also mention the traditional association of hydroxyapatite–chitosan composites with bone and periodontal tissue engineering [94]. The HAC and HACF compositions were investigated partly based on previous favorable results for adhesion and growth of mesenchymal cells from dental pulp and bone tissue engineering applications [95]. The immunomodulatory and biocompatibility profiles are essential for these applications to ensure successful integration and remodeling of bone tissue without adverse immune reactions.

The studies collectively suggest that these biocomposites, particularly those incorporating additional bioactive components like fucoidan or used in conjunction with paracrine factors from stem cells, represent a promising strategy for modulating the local environment to enhance tissue repair and resolve inflammation [96].

Biocompatibility aspects are summarized in Table 1.

**Table 1.** Biocompatibility aspects.

| Assessment | Test/Method | Result |
|---|---|---|
| Hemolysis | Hemolysis assay | Very low hemolysis rates; within acceptable biomaterial range |
| RBC Aggregation | Microscopic analysis | No red blood cell aggregation; normal blood fluidity |
| Plasma Protein Adsorption | Protein adsorption assay | High albumin adsorption; favorable for biocompatibility |
| General Cytotoxicity | L929 fibroblast cell model | Comprehensive in vitro evaluation using multiple assays |
| MTT and NRU | MTT and Neutral Red Uptake assays | Cell viability >95%; non-toxic |
| DAPI and Trypan Blue | DAPI staining and Trypan Blue dye exclusion | Viable cells observed; minimal cell death |
| Direct Contact Assay | Direct material–cell contact | No changes in morphology or viability; confirms cytocompatibility |

## 8. Limitations

Chitosan–hydroxyapatite hydrogels encounter various limitations that render them less favorable when compared to newer materials like graphene and bioactive glasses. Regarding mechanical properties, these hydrogels typically exhibit inadequate mechanical strength for applications that require load-bearing capabilities. This lack of strength may result in early failure under demanding physical conditions. Conversely, graphene, with its remarkable mechanical properties, has the potential to significantly improve the strength and flexibility of hydrogels. Studies have shown that graphene oxide can enhance the mechanical properties of chitosan-based hydrogels, and this improvement in performance could enable their application in load-bearing scenarios. Furthermore, the degradation rates of chitosan–hydroxyapatite hydrogels may not correspond effectively with the natural bone remodeling process. Such a discrepancy can diminish treatment effectiveness, as the hydrogel needs to degrade within a suitable timeframe to offer adequate mechanical

support. In contrast, bioactive glasses can be tailored to achieve appropriate degradation rates, thus promoting the healing process [97–99].

## 9. Protocols

### *9.1. HAC Formulation: Chitosan–Hydroxyapatite Composite Preparation Protocol*

- Chitosan Solution: Dissolve 1.5% ($w/v$) medium molecular weight chitosan (degree of deacetylation: 75–85%) in 0.1 M acetic acid at 40 °C.
- Hydroxyapatite Precursors: Prepare separate aqueous solutions of calcium nitrate tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$] and diammonium hydrogen phosphate [$(NH_4)_2HPO_4$].
- Mixing: Add the calcium solution to the chitosan solution and stir at 250 rpm for 2 h at 40 °C. Then, introduce the phosphate solution into the mixture.
- pH Adjustment: Adjust the pH to an alkaline range using ammonium hydroxide ($NH_4OH$), monitoring with a pH meter.
- Aging and Sonication: Maintain the mixture at 40 °C with stirring at 85 rpm for 24 h, followed by sonication for 3 h to ensure homogeneity.
- Scaffold Formation: Pour the resulting gel into Teflon molds (5 × 10 mm), freeze at −80 °C, and lyophilize for 48 h to obtain 3D scaffolds.

Four different compositions can be produced by varying the hydroxyapatite content to 0%, 50%, 60%, and 70% $v/v$ relative to the composite [100].

Characterization

- Morphology: Scanning Electron Microscopy (SEM) was used to assess the porous structure and distribution of hydroxyapatite within the chitosan matrix.
- Structural Analysis: Fourier Transform-Infrared Spectroscopy (FTIR) and X-ray Diffraction (XRD) analyses confirmed the formation of hydroxyapatite and its interaction with chitosan.
- Mechanical Properties: Compression testing evaluated the mechanical strength suitable for bone tissue engineering applications [100].

### *9.2. HACF Formulation: Cross-Linked Chitosan–Hydroxyapatite Composite*

- Chitosan Solution: Dissolve 2.0 g of chitosan in 50 mL of 2% ($v/v$) acetic acid, stirring for 6 h.
- Hydroxyapatite Addition: Add a specific amount of hydroxyapatite powder to the chitosan solution and stir for 1 h at room temperature to achieve a homogeneous mixture.
- Molding and Freezing: Transfer the mixture into molds and freeze at −18 °C for 24 h.
- Freeze-Drying: Lyophilize the frozen samples at −50 °C for 72 h to create porous scaffolds.
- Neutralization: Immerse the scaffolds in a 10 wt% sodium hydroxide (NaOH) solution for 12 h to neutralize residual acetic acid, followed by washing with deionized water.
- Final Freeze-Drying: Freeze-dry the neutralized scaffolds to obtain the final product.

Scaffolds can be prepared with varying hydroxyapatite content, marked as 1%, 2%, 3%, and 4% HAp/CS, based on the ratio of hydroxyapatite mass to the volume of the chitosan solution [101].

Characterization

- Microstructure: Field Emission Scanning Electron Microscopy (FESEM) and Transmission Electron Microscopy (TEM) analyzed the scaffold's morphology and hydroxyapatite distribution.

- Phase Composition: X-ray Diffraction (XRD) assessed the crystalline phases present in the composite.
- Chemical Interactions: Fourier Transform-Infrared Spectroscopy (FT-IR) identified functional groups and interactions between chitosan and hydroxyapatite [101].

## 10. Batch-to-Batch Variability and Potential Immunogenicity

The physicochemical properties of chitosan can vary significantly based on its molecular weight, degree of deacetylation, and source material. These variations can influence the performance of CS–HAp composites in biomedical applications. For instance, a study on chitosan-coated PLGA nanoparticles highlighted that different chitosan derivatives exhibited substantial differences in adsorption efficiency, zeta potential, and immune cell activation markers (CD40 and CD86) in vitro. Specifically, chitosan HCl demonstrated the highest adsorption efficiency and immune activation, while other derivatives like carboxymethylchitosan and oligomers showed lower immunogenicity. Additionally, the process of integrating chitosan with hydroxyapatite can lead to challenges in achieving uniform distribution within the polymeric matrix. This lack of uniformity can affect the mechanical properties and biological responses of the composite, potentially leading to inconsistent clinical outcomes [102].

Chitosan's cationic nature allows it to interact with immune cells and enhance antigen delivery, which is beneficial in vaccine development. However, these same properties can also elicit immune responses. For example, chitosan-coated nanoparticles have been shown to activate dendritic cells and increase the expression of co-stimulatory molecules, which are indicative of immune activation [103]. The immunogenicity of chitosan can be influenced by its molecular characteristics and the presence of other components. Modifications such as quaternization or thiolation can enhance its mucoadhesiveness and immunostimulatory properties, which may be advantageous in vaccine formulations. However, these modifications could also lead to varying immune responses across genetically diverse populations, as individual genetic makeup can influence the recognition and processing of such materials [104]. In the context of CS–HAp composites, the immunogenicity is not solely dependent on chitosan but also on the hydroxyapatite content. It has been have shown that increasing the hydroxyapatite concentration in CS–HAp scaffolds can promote osteogenic differentiation and modulate inflammatory cytokine production, suggesting a complex interplay between the components that could influence immune responses [105].

## 11. Future Approaches

While the current studies demonstrate broad anti-inflammatory and regenerative effects in a dermatitis model, future research should focus on more detailed immunological analyses using flow cytometry, immunofluorescence, or single-cell RNA sequencing to profile the behavior of macrophages (M1/M2 phenotypes), T lymphocytes (e.g., regulatory T cells, Th17 cells), dendritic cells, and neutrophils in the presence of the biomaterial. These investigations could determine whether the biocomposite actively polarizes macrophages toward a pro-regenerative (M2) phenotype, suppresses pro-inflammatory T cell responses, or modulates antigen-presenting cell functions. Additionally, time-course studies should be incorporated to assess how immune cell recruitment, activation, and resolution phases evolve in response to the scaffold. Co-culture models in vitro using specific immune cells or human peripheral blood mononuclear cells (PBMCs) could further clarify direct cell–material interactions before in vivo validation. These approaches would provide mechanistic insights into how the scaffold achieves its immunomodulatory effects and support its rational design for tailored immune responses in tissue engineering applications. Chitosan–hydroxyapatite (CS–HAp) biocomposites are emerging as multifunctional materials in

tissue engineering, particularly for bone repair, due to their synergistic combination of chitosan's biocompatibility and biodegradability with hydroxyapatite's osteoconductivity and bioactivity. These scaffolds exhibit strong performance strategies by leveraging the immunomodulatory potential of chitosan, which helps reduce pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6, while enhancing anti-inflammatory mediators such as IL-4 and IL-10. This capacity to shape the local immune response is crucial in promoting a regenerative microenvironment, especially in early wound healing phases. In addition to their immunomodulatory action, CS–HAp scaffolds demonstrate superior biocompatibility and osteoinductivity, supporting robust cell adhesion, proliferation, and differentiation. Studies have shown their ability to promote osteoblast and mesenchymal stem cell activity, facilitating bone matrix deposition and mineralization [105–107].

When compared to commonly used biomaterials, CS–HAp composites outperform many synthetic options like poly(lactic-co-glycolic acid) (PLGA), which, while mechanically robust, often lack intrinsic bioactivity and may cause acidic degradation by-products that hinder healing. In contrast, CS–HAp scaffolds present an inherently bioactive surface that better supports bone regeneration. Natural polymers such as collagen and gelatin are also widely explored, but they frequently suffer from inadequate mechanical integrity for load-bearing applications. CS–HAp's mineral phase enhances mechanical stability, making it more suitable for orthopedic applications requiring strength and resilience [108,109].

From a strategic development perspective, recent innovations have focused on incorporating controlled-release systems within CS–HAp scaffolds for the delivery of bioactive factors such as BMP-2, VEGF, or stem cell-derived conditioned media, thereby enhancing their regenerative performance. These multifunctional systems not only accelerate healing but also tailor the immune and cellular responses over time. Moreover, efforts are underway to optimize scaffold porosity, degradation kinetics, and mechanical behavior to better mimic native bone properties and support vascularization—an essential feature for large defect repair [110,111].

## 12. Conclusions

Chitosan–hydroxyapatite (CS/HAp) biocomposites, often incorporated into hydrogel scaffolds or membranes and combined with other natural polymers or bioactive molecules, demonstrate significant potential for applications in tissue engineering and the treatment of inflammatory conditions. The growing body of research indicates that these materials possess favorable immunomodulatory properties, offering a novel approach to both tissue regeneration and inflammation control. In vitro studies on HAC and HACF hydrogels show excellent biocompatibility, with no significant induction of pro-inflammatory markers (TNF-α, IL-6, NF-κB) in fibroblast cells, suggesting they do not provoke inflammatory responses and can provide a conducive environment for cellular processes. These properties are likely due to the presence of natural polysaccharides such as chitosan, which plays a pivotal role in modulating immune responses.

In vivo studies using a chitosan–xanthan–hydroxyapatite composite in a rat dermatitis model demonstrate its ability to reduce inflammation and promote tissue regeneration. Key outcomes include reduced skin thickening, fewer mast cells, lower plasma histamine levels, decreased expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), and modulation of critical inflammatory and angiogenesis pathways (JAK1/3, AnxA1, VEGF). These findings, supported by thorough biocompatibility assessments (hemocompatibility, cytotoxicity), underscore the capacity of CS/HAp biocomposites to interact favorably with the host immune system.

However, despite the promising therapeutic potential, significant gaps remain in understanding the precise mechanisms underlying these immunomodulatory effects, as well

as the impact of different composite formulations on immune responses. A comprehensive review is imperative to consolidate the growing body of evidence, identify key research gaps, and provide a clearer pathway for optimizing CS/HAp biocomposites for clinical use in inflammatory diseases and regenerative medicine. Such a review will not only inform future material design but also advance our understanding of their therapeutic potential, especially in tailoring immune modulation for a pro-regenerative environment.

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## Abbreviations

The following abbreviations are used in this manuscript:

| | |
|---|---|
| AnxA1 | Annexin A1 |
| BMP-2 | Bone morphogenetic protein-2 |
| CD40 and CD86 | Cluster of Differentiation 40 and Cluster of Differentiation 86 |
| CS | Chitosan |
| CS-HAp | Chitosan–hydroxyapatite |
| CM | Conditioned medium |
| DAPI | 4′,6-Diamidino-2-Phenylindole |
| ECM | Native extracellular matrix |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EWC | Equilibrium water content |
| FESEM | Field Emission Scanning Electron Microscopy |
| FTIR | Fourier Transform Infrared Spectroscopy |
| G1 | Induced without treatment |
| G2 | Induced and treated with standard hydrocortisone ointment |
| G3 | Induced and treated with the biomaterial without conditioned medium |
| G4 | Induced and treated with the biomaterial with conditioned medium |
| HAC | Hydroxyapatite, alginate, and chitosan |
| HACF | Hydroxyapatite, alginate, chitosan, and fucoidan |
| HAp | Hydroxyapatite |
| IL-10 and IL-4 | Anti-inflammatory cytokines |
| IL-1β and IL-6 | Interleukin-1 beta and Interleukin-6 |
| IGFBPs | Insulin-like Growth Factor Binding Proteins |
| JAKs | Janus kinase |
| JAK1/3 | Itching/inflammation pathways |
| LPS | Lipopolysaccharide |
| mRNA | Messenger RNA |
| M1 | Pro-inflammatory M1 macrophages |
| M2 | Pro-resolving/regenerative M2 macrophages |
| MSCs | Mesenchymal stem cells |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide |

| | |
|---|---|
| NF-κB | Nuclear Factor kappa-light-chain-enhancer of activated B cells |
| NRU | Neutral Red Uptake |
| PBMCs | Peripheral blood mononuclear cells |
| qPCR | Quantitative polymerase chain reaction |
| RBC | Red blood cells |
| SEM | Scanning electron microscopy |
| STAT | Signal Transducer and Activator of Transcription |
| TEM | Transmission Electron Microscopy |
| TGF-β | Pro-inflammatory cytokine |
| TNF-α | Tumour Necrosis Factor-alpha |
| VEGF | Vascular endothelial growth factor |
| XRD | X-ray Diffraction |

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