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**HIDROGÉIS DE QUITOSANA LIVRE DE CÉLULAS E REFORÇADOS POR
NANOTUBOS DE CARBONO E NANOPARTÍCULAS DE HIDROXIAPATITA
PARA APLICAÇÃO EM TECIDO ÓSSEO**

CELL-FREE CHITOSAN HYDROGELS REINFORCED BY CARBON
NANOTUBES AND HYDROXYAPATITE NANOPARTICLES FOR BONE TISSUE
APPLICATION

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**CELL-FREE CHITOSAN HYDROGELS REINFORCED BY CARBON
NANOTUBES AND HYDROXYAPATITE NANOPARTICLES FOR BONE TISSUE
APPLICATION**

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“To be a scientist is to be naive.

We are so focused on our search for truth,

We fail to consider how few actually want us to find it.

But it is always there, whether we see it or not,

Whether we choose to or not.

The truth does not care about our needs or wants.

It does not care about our governments, our ideologies, our religions.

It will lie in wait for all time.”

- Valery Legasov

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Abbreviation Index

ANOVA – Analysis of Variance

CAD – Computer-Aided Design

CAM – Computer-Aided Manufacturing

CV – Crystal Violet

DI – Deionized Water

DTA – Differential Thermal Analysis

EDS – Energy Dispersive X-ray Scattering

ECM – Extracellular Matrix

FDM – Fused Deposition Modeling

MWCNT – Multi-Walled Carbon Nanotubes

FTIR – Fourier Transform Infrared

MTT - Mitochondrial Dehydrogenase Enzymatic

nHA – hydroxyapatite nanoparticles

PBS – Phosphate Buffer Saline

TGA – Thermogravimetry Analysis

SEM – Scanning Electron Microscopy

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RESUMO

Este estudo apresenta o desenvolvimento e caracterização de um hidrogel de quitosana injetável, livre de células, reforçado com nanotubos de carbono de paredes múltiplas (MWCNT) e nanopartículas de hidroxiapatita (nHA) para aplicação em engenharia de tecido ósseo. O hidrogel visa melhorar as propriedades mecânicas, biocompatibilidade e osteocondutividade, oferecendo uma solução potencial para a regeneração óssea. A funcionalização dos MWCNTs foi realizada por meio de um ataque ácido assistido por banho ultrassônico, aumentando significativamente a carga de superfície e reduzindo o tamanho das partículas. Este processo garantiu melhor interação com a matriz de quitosana, prevenindo a floculação e promovendo a formação uniforme do hidrogel. A Espectroscopia de Infravermelho por Transformada de Fourier (FTIR) confirmou a incorporação bem-sucedida de grupos funcionais da quitosana, MWCNT e nHA, indicando a integração eficaz dos materiais. A estabilidade física e química dos hidrogéis foi avaliada por meio de testes de intumescimento e degradação. Os hidrogéis exibiram propriedades de intumescimento significativas e taxas de degradação controladas em água deionizada e solução salina tamponada com fosfato (PBS), essenciais para manter a integridade estrutural enquanto suportam o crescimento celular. A análise termogravimétrica (TGA/DTA) demonstrou ainda a estabilidade térmica do hidrogel, confirmando sua adequação para aplicações biomédicas. As análises morfológicas e elementares, utilizando Microscopia Eletrônica de Varredura (SEM) e Espectroscopia de Dispersão de Energia (EDS), revelaram uma estrutura porosa bem definida e a presença de cálcio e fósforo, indicando a integração bem-sucedida da nHA na matriz do hidrogel. Essas características são cruciais para mimetizar o ambiente ósseo natural e promover a adesão e proliferação celular. A biocompatibilidade foi avaliada por meio de testes de citotoxicidade e adesão celular utilizando pré-osteoblastos MC3T3-E1. Os resultados mostraram alta viabilidade celular e forte adesão, destacando o potencial do hidrogel para suportar a atividade dos osteoblastos e a regeneração do tecido ósseo. A viabilidade celular foi mantida acima do limiar definido pelas diretrizes ISO 10993-5, confirmando a segurança do material para uso biomédico. A análise da expressão gênica, utilizando a Reação em Cadeia da Polimerase Quantitativa por Transcrição Reversa (RT-qPCR), monitorou a expressão de marcadores osteogênicos chave, incluindo *Alp*, *Tgf- β 2*, *Runx2*, *Opn*, *Bglap* e *Col1a1*. A análise demonstrou a regulação positiva desses marcadores, indicando que o hidrogel promove a diferenciação dos osteoblastos e a remodelação da matriz extracelular, processos essenciais para a regeneração óssea eficaz. A atividade das metaloproteinases de matriz (MMP), crucial para a remodelação da matriz extracelular, foi avaliada por ensaios de zimografia. Os resultados mostraram MMPs ativas, particularmente MMP-2 e MMP-9, no ambiente do hidrogel, apoiando a remodelação dinâmica necessária para a regeneração do tecido ósseo. Em conclusão, o hidrogel de quitosana desenvolvido, reforçado com MWCNT e nHA, apresenta propriedades promissoras para aplicações em engenharia de tecido ósseo. Sua robustez mecânica, biocompatibilidade e osteocondutividade fazem dele um candidato viável para uso clínico na regeneração óssea. Este hidrogel oferece uma

nova abordagem para o tratamento de lesões e condições relacionadas ao osso, potencialmente melhorando os resultados dos pacientes por meio da reparação e regeneração óssea aprimoradas. A avaliação abrangente de suas propriedades sugere que este hidrogel pode efetivamente suportar a atividade dos osteoblastos e facilitar o complexo processo de remodelação do tecido ósseo, marcando um avanço significativo no campo da medicina regenerativa e engenharia de tecidos.

Palavras-chave: biomateriais, medicina regenerativa, biotinta, bioimpressão 3D, engenharia de tecidos, quitosana, hidroxiapatita, nanotubos de carbono.

ABSTRACT

This study presents the development and characterization of a cell-free chitosan hydrogel reinforced with multi-walled carbon nanotubes (MWCNT) and hydroxyapatite nanoparticles (nHA) for application in bone tissue engineering. The hydrogel aims to enhance mechanical properties, biocompatibility, and osteoconductivity, providing a potential solution for bone regeneration. The functionalization of MWCNT was achieved through an acid attack assisted by an ultrasonic bath, significantly increasing the surface charge and reducing particle size. This process ensured better interaction with the chitosan matrix, preventing flocculation and promoting uniform hydrogel formation. Fourier Transformed Infrared Spectroscopy (FTIR) confirmed the successful incorporation of functional groups from chitosan, MWCNT, and nHA, indicating effective material integration. Physical and chemical stability of the hydrogels was assessed through swelling and degradation tests. The hydrogels exhibited significant swelling properties and controlled degradation rates in both deionized water and phosphate-buffered saline (PBS), essential for maintaining structural integrity while supporting cell growth. Thermogravimetric analysis (TGA/DTA) further demonstrated the hydrogel's thermal stability, confirming its suitability for biomedical applications. Morphological and elemental analyses using Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) revealed a well-defined porous structure and the presence of calcium and phosphorus, indicative of successful nHA integration into the hydrogel matrix. These characteristics are crucial for mimicking the natural bone environment and promoting cell attachment and proliferation. Biocompatibility was evaluated through cytotoxicity and cell adhesion tests using MC3T3-E1 pre-osteoblasts. The results showed high cell viability and strong adhesion, highlighting the hydrogel's potential to support osteoblast activity and bone tissue regeneration. The cell viability was maintained above the threshold defined by ISO 10993-5 guidelines, confirming the material's safety for biomedical use. Gene expression analysis using Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) monitored the expression of key osteogenic markers, including *Alp*, *Tgf- β 2*, *Runx2*, *Opn*, *Bglap*, and *Colla1*. The analysis demonstrated the upregulation of these markers, indicating that the hydrogel promotes osteoblast differentiation and extracellular matrix (ECM) remodeling, essential processes for effective bone regeneration. Matrix metalloproteinase (MMP) activity, crucial for extracellular matrix remodeling, was assessed using zymography assays. The results showed active MMPs within the hydrogel environment, particularly MMP-2 and MMP-9, supporting the dynamic remodeling required for bone tissue regeneration. In conclusion, the developed chitosan hydrogel reinforced with MWCNT and nHA exhibits promising properties for bone tissue engineering applications. Its mechanical robustness, biocompatibility, and osteoconductivity make it a viable candidate for clinical use in bone regeneration. This hydrogel provides a new avenue for treating bone-related injuries and conditions, potentially improving patient outcomes through enhanced bone repair and regeneration. The comprehensive evaluation of its properties suggests that this hydrogel can effectively support osteoblast activity and facilitate the complex process of bone

tissue remodeling, marking a significant advancement in the field of regenerative medicine and tissue engineering.

Keywords: biomaterials, regenerative medicine, bio-ink, 3D bioprinting, tissue engineering, chitosan, hydroxyapatite, carbon nanotubes.

1. INTRODUCTION

1.1 Bone Structure and Physiology

The human skeletal system is a dynamic organ comprising about 200 bones that provide structural support and protection to vital organs, facilitate movement, store minerals, and house bone marrow for hematopoiesis (HART et al., 2020; OLIVEIRA; LEEUWENBURGH; MANO, 2021). Bone injuries, categorized as critical or non-critical, pose a significant clinical challenge due to their varying requirements for repair and regeneration. Non-critical injuries, such as simple fractures, typically heal through the body's natural reparative processes, while critical injuries, like large bone defects, may not heal adequately without intervention (GEMINI-PIPERNI et al., 2014). A deeper understanding of bone structure and physiology, including the roles of differentiation and ECM remodeling gene markers, is essential for developing effective treatments.

Two main categories divide the bone tissue, which are the macroscopic and microscopic. Subdividing the macroscopic organization, we have the cortical and trabecular bones. The first comprehend the dense outer layer forms approximately 80% of the skeletal mass and provides mechanical strength, particularly in weight-bearing bones. Cortical bone is organized into osteons, or Haversian systems, cylindrical structures aligned with the bone's longitudinal axis, enabling resistance to compressive forces (HART et al., 2020). The second is localized predominantly at the ends of long bones, and within the vertebrae, trabecular bone has a porous architecture that reduces weight while maintaining structural integrity. Its mesh-like network of trabeculae is vital for the absorption and distribution of mechanical loads and serves as a critical site for hematopoiesis due to its housing of red bone marrow (TOMASZEWSKA et al., 2021). Besides, the cell organization in bone tissues is very dynamic, containing highly specialized matrices that work together to maintain bone integrity and function.

Histologically, three specific cell types constitute bone, as follows: **1. Osteoblasts**, mesenchymal-derived cells that actively participate in the synthesis of the bone matrix by producing organic components and subsequently mineralizing them (BOSKEY; ROBEY, 2019) – osteoblasts an essential role during bone tissue regeneration and because that proper relevance has been highlighted this cell during bone engineering; **2. Osteoclasts**

are hematological-derived cells that become multinucleated during osteoclastogenesis in response to RANKL and MCSF. Osteoclasts perform the opposite function to osteoblasts, as they break down the bone matrix for mineral and protein reabsorption (BOSKEY; ROBEY, 2019); and 3. Osteocytes, osteoblast-derived cells embedded in the mineralized bone matrix and responsible for orchestrating bone remodeling, also serve as relevant reserves of RANKL (BOSKEY; ROBEY, 2019), as illustrated below in **Figure 1**:

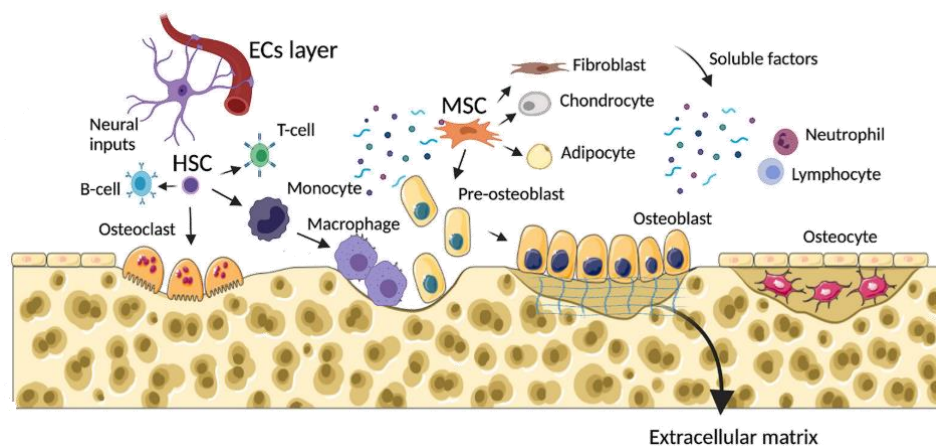


Figure 1: Example of the microenvironment of bone tissue regarding different hierarchical cell sources and their respective differentiation and orchestration. (Taken and adapted from OLIVEIRA; LEEUWENBURGH; MANO, 2021)

Thus, it is essential to consider biological events such as cell adhesion, proliferation, and differentiation in the complex organization of bone tissue to design a bio-ink that adequately mimics the desired environmental features of native tissue.

Cell adhesion is currently a well-studied event in normal physiological processes, pathological conditions, and/or other cell-cell and cell-matrix interactions (OKEGAWA et al., 2004). The components governing cell adhesion contribute decisively to regulating cell differentiation, proliferation, and death. Therefore, proper cell adhesion and subsequent integration with the host tissue are crucial for homeostasis maintenance. Over the last decades, our research group has investigated cell-material interaction to map biomarkers to better comprehend this biological and essential step during bone regeneration, also because it can guide researchers to have previsibility during novel material's propositions. For instance, we showed that within the first 2 hours of adhesion, physiological osteoblast adaptation culminates in different signaling pathways responsible for cytoskeleton rearrangement, with signals triggered from the activation of

proteins such as MAPKp38 and FAK (ZAMBUZZI et al., 2009) and it is finely modulated when those cells are adapted on ceramic or metallic materials (FERNANDES et al., 2023; DE ALMEIDA et al., 2024; FERNANDES et al., 2023; GOMES et al., 2023). In this scenario, we have gained experience also demonstrating cell signaling during osteoblast adhesion, showing that reactive oxygen species (ROS) modulates the action of protein tyrosine phosphatases (PTPs) during cellular mechanisms involved in osteoblast adhesion, and it needs to be considered during predictable biomaterial development (FERNANDES et al., 2014). Our research group's investigation of the adhesion transition to the proliferative metabolism of osteoblasts showed significant findings that brought light to understanding the entrance to differentiation metabolism.

As mentioned earlier, once injured, bone tissue undergoes a healing process that can occur through two distinct mechanisms: repair and regeneration. Regeneration occurs when the damaged tissue is repopulated based on the proliferation and differentiation of the original tissue's cells. Consequently, the regenerated tissue should have the same functional and morphological properties as the native tissue. On the other hand, in the repair process, the damaged tissue is sometimes replaced by fibrous connective tissue, resulting in a collagen scar, and it leads to a loss of the original characteristics and functions and bone regeneration is considered a priority over repair in trauma cases, thus restoring the bone's original shape and functions (ZAMBUZZI et al., 2011a, 2011b). The process of bone regeneration comprises distinct stages that may assemble, including inflammation, hard callus formation, and other remodeling processes (KALFAS, 2001). Non-critical injuries, such as minor fractures or small bone defects, typically heal naturally through the bone's intrinsic repair mechanisms. Monitoring markers like alkaline phosphatase (ALP) and osteocalcin (BGLAP) are essential in assessing the healing progression in non-critical injuries (KARSENTY, 2017; NAKAMURA et al., 2020; VIMALRAJ, 2020). Meanwhile, critical bone injuries, such as segmental bone defects or comminuted fractures, pose a significant challenge due to the extensive bone loss or severe damage that the body's natural processes cannot adequately repair. These injuries, such as grafts or biomaterials, require external intervention to facilitate healing. Then, it often necessitates monitoring gene markers such as BMP-2, RUNX2, and other essential genes regarding the remodeling of bone inorganic crystal deposition involved in bone cell proliferation, osteogenic differentiation, and extracellular matrix (ECM)

remodeling activities (BERGERON et al., 2007; CARREIRA et al., 2014; HALLORAN; DURBANO; NOHE, 2020; RAIS et al., 2023).

Proliferation of bone cells, particularly osteoprogenitor cells, is the foundational step in bone repair and regeneration. This early stage involves the rapid expansion of progenitor cells to ensure a sufficient supply of osteoblasts for subsequent differentiation and matrix production (LI et al., 2019). It highlights the importance of controlled proliferation by regulating several key gene markers involved in cell cycle progression and cellular adhesion. The cyclin-dependent kinases (CDKs) are central regulators of the cell cycle, driving the transition from the G1 phase to the S phase. Together with their respective cyclins, CDK2, CDK4, and CDK6 phosphorylate the retinoblastoma protein (Rb), releasing E2F transcription factors that promote DNA replication and cell proliferation. Proper regulation of these CDKs is essential for ensuring controlled proliferation, which could lead to aberrant tissue formation (AMANI et al., 2021; ASGHAR et al., 2022; MACHADO et al., 2019; WESTON; BARR, 2023). Also, we have the p15 gene marker, a cyclin-dependent kinase inhibitor that binds to and inhibits CDK4 and CDK6, preventing excessive proliferation by blocking cell cycle progression at the G1 phase. Such inhibition is crucial for maintaining a balance between cell proliferation and differentiation during bone healing (AMANI et al., 2021; PAN et al., 2010). Another important marker for bone cell proliferation is focal adhesion kinase (FAK), a non-receptor tyrosine-kinase that mediates cell adhesion, migration, and proliferation. It plays a pivotal role in the initial phase of bone healing by promoting the adhesion and proliferation of osteoprogenitor cells at the injury site. FAK signaling pathways are activated in response to integrin engagement and mechanical stress, facilitating cellular proliferation and tissue repair (FÅHRAEUS; P.LANE, 1999; MISHRA; MANAVATHI, 2021).

Following proliferation, the differentiation of osteoprogenitor cells into mature osteoblasts is critical for bone formation. The expression of osteogenic proteins and matrix components regulates several genes such as RUNX2, BGLAP, osteopontin, Tissue Growth Factor Beta 2 (TGF- β 2), COL1A1, and alkaline phosphatase (ALP), which are pivotal in differentiating osteoblasts from precursor cells. ALP is an early osteoblast differentiation marker crucial for the mineralization process. ALP hydrolyzes phosphate esters, increasing local concentrations of inorganic phosphate, which combines with

calcium to form hydroxyapatite crystals in the bone matrix. Elevated ALP activity indicates active bone formation, commonly used as a marker for osteogenic differentiation (BERGERON et al., 2007; LI et al., 2019; MYGIND et al., 2007). TGF- β 2 is a multifunctional growth factor that regulates both proliferation and differentiation of osteoprogenitor cells. TGF- β 2 promotes the synthesis of collagen and other matrix proteins and modulates the expression of key osteogenic markers like RUNX2, thereby enhancing osteoblast differentiation and bone formation (ELDRED et al., 2012; LI et al., 2022; TOWNSEND et al., 2011). RUNX2 controls the expression of many osteogenic genes, including the ALP, COL1A1, and BGLAP. RUNX2 activity is essential for the maturation of osteoprogenitor cells into functional osteoblasts and is a critical determinant of bone formation capacity (GOMATHI et al., 2020; KOMORI, 2010; LIU et al., 2021; WAGLEY et al., 2020). COL1A1 is the primary component of the organic bone matrix, and type I collagen provides a scaffold for mineral deposition and structural support. COL1A1 expression is upregulated during osteoblast differentiation, facilitating the production of a collagenous matrix critical for bone strength and integrity (KOMORI, 2019). Meanwhile, BGLAP works as a late marker of osteoblast differentiation that regulates bone mineralization, which binds to hydroxyapatite and calcium ions in the bone matrix, contributing to the stabilization and maturation of newly formed bone (KARSENTY, 2017; KARSENTY; FERRON, 2012). Another important gene to monitor is osteopontin, a non-collagenous glycoprotein that promotes cell adhesion, migration, and mineralization (NAKAMURA et al., 2020). Osteopontin is involved in both the initial and later stages of bone formation, facilitating the attachment of osteoblasts to the matrix and contributing to ECM organization.

Regarding the activity of ECM during the bone healing process involving the breakdown of damaged matrix components and the deposition of new ones, other important genes, such as Matrix metalloproteinases 2 and 9 (MMP-2 and -9), Bone Sialoprotein (BSP), Tissue Inhibitors of Metalloproteinases 1 and 2 (TIMP-1 and -2), and Bone Morphogenetic Protein 2 (BMP-2) are critical in its remodeling. MMP-2 and -9 enzymes degrade collagen and other ECM components, allowing for matrix turnover and remodeling. MMP-2 and MMP-9 are particularly important during the early phases of bone healing, where they help clear damaged tissue and create space for new bone formation (CHEN et al., 2021; ELDRED et al., 2012; LA ROCCA et al., 2004; PEETERS-JORIS; HAMMANI; SINGER, 1998; PUCCI-MINAFRA et al., 2001). On

the other hand, TIMP-1 and -2 balance their activity to prevent excessive matrix degradation (JANSEN et al., 2006; LI; TAY; YIU, 2020; SHIBUTANI et al., 1999; XI et al., 2020), while BSP nucleates hydroxyapatite crystals and the mineralization of the bone matrix, which is highly expressed in areas of active bone remodeling and is essential for proper matrix organization and mineralization (BARTHELEMI et al., 2012; CHAVEZ et al., 2023; HUNTER; GOLDBERG, 1993). Last, BMP-2 is a potent osteogenic factor promoting osteoblast differentiation and ECM synthesis. BMP-2 stimulates the production of collagen and other matrix proteins and enhances mineral deposition, making it a key player in bone regeneration and repair (CARREIRA et al., 2014; HALLORAN; DURBANO; NOHE, 2020; PARK et al., 2020; ZHANG et al., 2018). All of them facilitate the reorganization of the ECM, a necessary step for new bone formation, ensuring a balance between matrix degradation. Monitoring these markers provides insights into the remodeling phase of bone repair and is vital for developing therapeutic interventions.

Often, to improve the healing of bone tissues and recover their natural physiology and functions, surgeons use bone autologous grafts collected from the patient's bones to avoid rejection problems. Although such a strategy is fundamental in daily medical procedures and considered the gold standard, doctors cannot collect significant amounts for grafting critical bony lesions. Then, using natural or synthetic scaffolds in therapeutic interventions plays a crucial role in offering medically feasible routes and improving patient recovery. Natural scaffolds are derived from biocompatible and biodegradable materials like collagen, chitosan, and hyaluronic acid, which provide a favorable environment for cellular functions and facilitate tissue integration. Natural scaffolds can be combined with growth factors and other bioactive molecules to enhance bone regeneration further, while synthetic scaffolds provide structural support and promote cell attachment, proliferation, and differentiation. They combine synthetic materials such as hydroxyapatite, tricalcium phosphate, bioactive glass, and carbon-based materials with many polymer options (i.e., hydrogel-based composites) to create osteoconductive/osteoinductive environments that encourage new bone formation. Therefore, developing new biomaterial synthesis routes is crucial as they can orchestrate active biomolecules derived from different cell types, integrating a biological response that favors tissue regenerative mechanisms and looking for recapitulating the desired bone microenvironment to host osteoprogenitor cells.

5. CONCLUSION

This study successfully developed a cell-free chitosan hydrogel reinforced with multi-walled carbon nanotubes (MWCNT) and hydroxyapatite nanoparticles (nHA) for application in bone tissue engineering. The negative charges from MWCNTs obtained through an acid attack and ultrasonication process and the synthesized hydroxyapatite nanoparticles were successfully incorporated within the chitosan matrix, as confirmed by FTIR spectroscopy, which identified the dynamic interaction among the compounds. The hydrogels demonstrated good short-term swelling properties in deionized water and phosphate-buffered saline (PBS), although improving the hydrogel long-term degradation rates with cell culture medium is necessary. The thermogravimetric analysis further indicates that MWCNT and hydroxyapatite nanoparticles prefer interacting with each other compared to chitosan when coexisting during the formation of the composite hydrogel. Morphological analyses using Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) quantification of Ca/P ratios of hydrogel containing hydroxyapatite showed the distribution of stable apatite crystals within chitosan hydrogel, indicating successful integration of nHA either in the presence or not of MWCNT. At the same time, biocompatibility tests using ISO 10933-5 guidelines

revealed high cell viability and firm adhesion of MC3T3-E1 pre-osteoblasts, underscoring the hydrogel's potential to support osteoblast activity and bone tissue regeneration. Gene expression analysis demonstrated upregulation of key osteogenic gene markers, suggesting that the hydrogel promotes osteoblast differentiation and extracellular matrix remodeling, supported by the confirmation of matrix metalloproteinases (MMPs) activity, which is crucial for ECM remodeling. We labeled such complementary approaches as Quarantine Protocol for Cell-free Bio-ink (QP-BioScreen), also extendable to other bio-ink formulations, where the chitosan-MWCNT-nHA hydrogel was demonstrated to be environmentally safe for 21 days. After monitoring, such protocol can address further assistance with 3D-cell-laden scaffold applications, where using a conditioned cell culture medium by the same cell-free hydrogel can potentially speed up cell adaptation, proliferation, and differentiation.

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