Innovative Strategies in Bone Healing: The Role of Graphene Oxide Nanoparticles in Tissue-Engineered Scaffolds

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INTRODUCTION: Bone grafting is a widely performed orthopedic procedure, making bone the second most commonly transplanted tissue globally. Annually, there are around 2.2 million bone graft procedures performed worldwide, with the United States accounting for over 1 million of these surgeries [2], [3]. The urgent and rising demand for a sustainable and stable source of bone suited for orthopedic and trauma applications is driven by the expected yearly growth rate of 13%. The existing alternatives for bone grafts encompass natural, synthetic, and composite materials. In essence, current bone grafting procedures are complex, demanding, and despite the utilization of modern technologies**,** have potential for post-operative complications. Synthetic grafts, although serving as a viable alternative that mimics characteristics of real bone [3], are constrained by insufficient mechanical strength and vascularization. To tackle these issues, the suggested methodology entails the integration of natural and synthetic polymers as continuous matrices and nanoparticles as fillers to fabricate a nanocomposite interpenetrating polymeric network (IPN) scaffold. This scaffold incorporates Graphene Oxide (GO) nanoparticles to facilitate the synthesis of artificial bone. Graphene Oxide has been found to have a positive impact on the mechanical strength and angiogenic characteristics of the scaffold. Pectin has a role in promoting gelation, osteogenic differentiation, and bioactivity. Polyvinyl alcohol, on the other hand, contributes to enhancing mechanical strength. Additionally, chitosan is known to promote bioactivity and hemostasis, while also exhibiting qualities that inhibit the growth of bacteria and fungi [1]. The study aims to achieve two specific objectives. Firstly, it aims to synthesize a nanocomposite interpenetrating polymer network (IPN) scaffold with an optimized concentration of the nanoparticles and polymers using a freeze-drying technique. Afterward, the physicochemical properties, porosity, and swelling characteristics of the scaffold will be evaluated. Secondly, the study aims to assess the *in vivo* osseointegration properties of the scaffolds by examining their mechanical strength and bioactivity attributes in an *in vitro* setting. This study signifies a critical milestone in the progression of bone tissue engineering with graphene oxide nanoparticles and the innovation of bone grafting techniques that are both more efficient and environmentally sustainable.

METHODS: **(i) Optimization and Scaffold Fabrication:** The scaffolds were carefully adjusted using molecular ratios of 2:1:1 (Pectin: Chitosan: PVA-S1) and 1:2:1 (Pectin: Chitosan: PVA-S2) depending on the concentration of chosen polymers. The nanoparticle concentration varied between 0.5% and 2%. In the end, it was found that the optimum concentration was 2% w/v of the 2:1:1 polymer blend in addition to 2% GO nanosheet colloids (Millipore Sigma, United States). Using a 2% w/v solution combination, two different scaffolds were made: a Pectin-dominant scaffold (S1) with a molecular ratio of 1:2:1, and a Chitosan-dominant scaffold (S2) with a ratio of 2:1:1 using a freeze-drying technique**. (ii) Physicochemical Characterization:** Following scaffold fabrication, molecular-level interactions were examined and the porous structure of the interpenetrating network (IPN) scaffolds was verified by scanning electron microscopy (SEM) analysis, which ensured the presence of physical crosslinking. The liquid displacement technique was utilized to measure porosity, and the PBS absorption assay was employed to evaluate swelling. **(iii) *In Vitro* Cell Culture Assays:** The osseointegration and osteoconduction capabilities were evaluated using MG-63 osteosarcoma cells. Several experiments were carried out, such as the live-dead staining method to distinguish between living and dead cells, the alamarBlue assay to evaluate cell viability, and the 4',6-diamidino-2-phenylindole (DAPI) staining method to evaluate nuclear integrity. To monitor the effectiveness of cell attachment on the scaffolds, SEM analysis was used. Furthermore, Alizarin Red staining was carried out every 7 and 14 days to determine the scaffolds' mineralization properties. The alkaline phosphatase activity was assessed at intervals of 7 and 14 days to ascertain the potential for osteogenic differentiation. **(iv) Mechanical Characterization:** Lastly, a Universal Testing Machine was used to assess the scaffolds' mechanical strength to calculate the scaffolds' compressive strength and Young's moduli. The purpose of these evaluations was to determine whether the IPN scaffold was effective in real-world bone tissue applications.

RESULTS: The fabrication of the IPN scaffolds using the freeze-drying method was successfully achieved, resulting in highly interconnected porous structures, as demonstrated by the SEM images. Through meticulous ImageJ analysis, the pore diameter of the scaffold was calculated, falling within the range of 120 µm, deemed suitable for effective bone implants (pending SEM measurements) (**Figure 1-a&b**). The porosity and swelling characteristics of the Chitosan-dominant scaffold (S1) were determined to be 62% and 2112%, respectively, whereas the Pectin-dominant scaffold (S2) exhibited 58% porosity and 1478% swelling. These percentages align with previously reported literature and are deemed suitable for applications in bone tissue engineering. In the *in vitro* cytocompatibility assessments, the Pectin-dominant scaffold (S1) displayed a 1.7-fold increase, and the Chitosan-dominant scaffold (S2) exhibited a 1.4-fold increase in percent cell viability compared to the control group (Tissue culture plate)(**Figure 1-c**). These findings signify the non-toxic nature of the scaffold and its ability to effectively support cell proliferation. The results from DAPI and Live/Dead staining were consistent with the alamarBlue assay, as DAPI staining revealed the preservation of nuclear integrity in cells with the scaffolds compared to the control group(**Figure 1-d-i**). Moreover, the alizarin red staining indicated enhanced calcium deposition in the presence of the IPN scaffolds at both the 7-day and 14-day intervals, highlighting their potential for promoting mineralization. Assessment of osteogenic differentiation via ALP activity demonstrated a notable increase of around 2.1-fold in the Pectin-dominant scaffold (S1) and a 2.9-fold increase in the Chitosan-dominant scaffold (S2) over the 7-day and 14-day periods compared to the control group(**Figure 1-j**). The evaluation of the mechanical properties of the scaffold revealed Young's modulus of 80 and compressive strength of 2.5 MPa, falling within the range of cancellous bone (**Figure 1-k&l**). Statistical analyses were conducted using GraphPad Prism Software, employing ordinary two-way ANOVA followed by a Tukey post hoc test. A significance level of $p < 0.05$ was applied for all analyses.

DISCUSSION: Graphene Oxide (GO) has attracted a lot of interest in modern biomedical applications, especially when it comes to solving the urgent needs and constraints in orthopedics. The need for bone grafts is increasing, and there is a pressing need to find more sustainable and effective alternatives to traditional grafting methods. Interpenetrating polymeric network (IPN) scaffolds made of nanocomposite materials have emerged as a creative and promising approach that may be able to address the shortcomings of conventional bone grafting techniques. This work represents a significant advancement in the use of graphene oxide (GO) in tissue engineering applications, intending to significantly improve the scaffold's angiogenic and mechanical strengths. The thorough assessment of the scaffold's physicochemical and biological attributes, such as pore diameter, porosity, and swelling characteristics, highlights its exceptional suitability for use with bone implants and supports its potential use in orthopedics. The scaffold's capacity to withstand physiological stresses is reinforced by the careful mechanical characterization that emphasizes its Young's modulus and compressive strength, which fall within the range of cancellous bone. Therefore, demonstrating its suitability for a range of bone tissue applications.

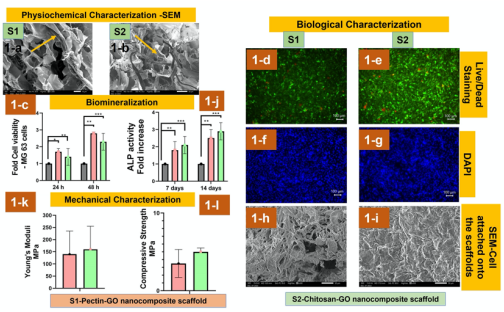

Figure 1: The major observations of the study: (a&b) **SEM image** revealed the porous nature of the GO-incorporated scaffolds. Presence of GO nanoribbons were captured (c) **Cytocompatibility assays** prove the biocompatibility of GO incorporation and supports proliferation of cells in the scaffolds (d&e) **Live/Dead staining** corroborates the AlamarBlue assay (f&g) Scaffolds supports the cells to maintains its nuclear integrity (h&i) **SEM imaging** confirms the osseointegration property of the scaffolds (j) **ALP assay** confirms the biomineralization of the scaffolds (k&l) **Mechanical Characterization** Young's modulus and compressive strength falling within the range of cancellous bone, hence showing its suitability for a range of bone tissue applications

SIGNIFICANCE: In summary, the results obtained from this research work demonstrate a notable progression in the domain of bone tissue engineering. These findings provide a full comprehension of the physicochemical, cytocompatibility, mineralization, and mechanical characteristics of the nanocomposite scaffold that was developed. This study exhibits potential for the advancement of innovative and environmentally friendly bone grafting methodologies, which has the potential to enhance orthopedic treatment outcomes while addressing the growing global need for efficacious bone graft remedies. Additional research, encompassing *in vivo* tests, is essential to substantiate the effectiveness and compatibility of the scaffold within a more intricate biological milieu.**ACKNOWLEDGEMENTS**: Dr. Sriram, College of Dentistry, Chicago, Bridge Grant Funding- UIC, and Blazer Foundation.

REFERENCES: [1] H Kanniyappan *et al., 2021,* [2] Van der Stok *et al*.,2011, [3] Bone Grafting - Basic Science – Orthobullets, Aug 2023.