FABRICATION AND CHARACTERIZATION OF FISH-DERIVED COLLAGEN SCAFFOLD LOADED WITH METRONIDAZOLE NANOPARTICLE FOR PERIODONTAL BONE REGENERATION

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Abstract. Periodontal disease poses a significant challenge to oral health, affecting the tissue and bone supporting the teeth. Tissue engineering emerges as a promising approach for restoring periodontal tissue and preventing bone loss using scaffolds. However, concern arises when using collagen sourced from mammals like porcine and bovine in scaffolds regarding halal status and disease transmission. Additionally, conventional treatment involves systemic antibiotics to control infection, leading to adverse side effects. This study aims to develop a scaffold using fish-derived collagen incorporated with metronidazole nanoparticles (MNP) and analyze scaffold properties while indirectly addressing safety and halal concerns. The scaffold was fabricated by physically cross-linking collagen derived from the tilapia fish (*Tilapia mossambica*) and chitosan, with metronidazole nanoparticles (MNP) incorporated into the blend. The scaffold underwent analysis of its physical characteristics, morphology, and pore size using a scanning electron microscope (SEM), swelling, and biodegradability in phosphate buffer solutions (pH 7.4, 37 °C). The fish-derived collagen-chitosan exhibited a consistent three-dimensional (3D) physical structure and optimal pore sizes (>100 μm). Scaffolds with MNP concentrations ranging from 0 to 40 w/t% displayed excellent swelling ability and biodegradability, exceeding 80%. As the concentration of MNP increased, the scaffold's biodegradation rate slowed, suggesting potential as a controlled drug release vehicle aligned with the rates of new bone formation *in vivo*. In conclusion, the 3D porous scaffold with metronidazole nanoparticles met important criteria for physical structure, pore size, swelling ability, and biodegradability. These halal-compliant scaffolds hold promising potential for applications in tissue engineering and drug delivery and are subject to further *in vivo* and *in vitro* studies.

Keywords: Periodontal disease, metronidazole, nanoparticle, scaffold, controlled release

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Introduction

The 2019 Global Burden Disease Study reports that 14.5% of the world's population faces severe periodontal diseases. This emphasizes periodontitis as a major dental health issue, with symptoms like gum inflammation, discomfort, bad breath, and the formation of periodontal pockets. In severe cases, it may lead to teeth migrating and eventual tooth loss [1]. Guided bone regeneration therapy uses scaffolds to restore lost tissue and prevent disease progression.

Tissue engineering, combining biology, engineering, and materials science, is crucial for tissue repair. It provides porous scaffolds with growth factors, mimicking the extracellular matrix, to enhance cell adhesion, support differentiated function, promote proliferation, and guide new tissue development for regeneration [2]. Porous, biocompatible, and biodegradable 3D scaffolds are essential tools in tissue engineering, offering an ideal environment for optimal cell growth and function. Collagen, renowned for its exceptional biocompatibility and natural biodegradability, plays a vital role in medical device development, particularly in crafting 3D scaffolds for tissue regeneration. To address concerns about halal status and disease transmission from mammalian sources, the focus has shifted to safer alternatives, such as fish-derived collagen [3]. Fish-derived collagen is gaining attention as a promising halal and safer alternative for various applications. However, non-crosslinked collagen scaffolds encounter challenges like rapid biodegradation and limited mechanical strength. Combining chitosan with collagen forms an ionic complex, enhancing the scaffold's mechanical strength [4]. Yet, infection risk in periodontal therapy is a concern, making metronidazole (MET) a promising antibiotic due to its broad-spectrum activity, and proven effectiveness against obligate anaerobes [5]. However, systemic antibiotic administration can cause side effects and bacterial resistance. With the evolution of tissue engineering, scaffolds are now recognized as efficient delivery systems for specific agents [6]. Incorporating antibacterial agents into scaffolds shows promise for bone tissue repair, with notable advancements seen in recent years through the incorporation of nanocarriers. [2].

The use of scaffold-loaded nanoparticles offers a notable advantage over traditional drug delivery. Local incorporation of MET enables targeted drug release, potentially avoiding systemic side effects. Nanoparticles allow controlled drug release, various administration routes, and the delivery of both hydrophilic and hydrophobic compounds [7]. Several studies have focused on integrating drugs into scaffolds to enhance physiological performance and support tissue regeneration. In this study, fish-derived collagen and chitosan will be cross-linked with metronidazole nanoparticles to form a porous 3D scaffold. The effects of MNP incorporation (10, 20, 30, and 40 wt%) on scaffold properties will be investigated, analyzing characteristics such as pore size, swelling, and biodegradability.

Materials and Methods

The certified halal fish collagen derived from *Talapia Mossambica* source was purchased from Eva Chemicals, Kuala Lumpur, Malaysia. A solution of collagen and chitosan in a ratio of 30:70 was prepared by dissolving them in a glacial acetic acid solution (1 w/v%). Glycerin, serving as a plasticizer, was introduced to the blend (20%) and stirred for one hour at room temperature. To achieve neutralization, the blends were treated with 5% sodium bicarbonate ($NaHCO_3$). Subsequently, different concentrations of metronidazole nanoparticles (MNP) (10 – 40 w/t%) were added. The mixture was then transferred to 96 wells as a mold and

subjected to slow freezing at −20 °C to −80 °C overnight before undergoing freeze-drying for 24 hours. The lyophilized samples were then crosslinked via dehydrothermal treatment (DHT) at a temperature of 105 °C for 24 hours [4].

Fabrication of Collagen Chitosan Scaffold Loaded with Metronidazole Nanoparticles

The certified halal fish collagen derived from *Talapia Mossambica* source was purchased from Eva Chemicals, Kuala Lumpur, Malaysia. A solution of collagen and chitosan in a ratio of 30:70 was prepared by dissolving them in a glacial acetic acid solution (1 w/v%). Glycerin, serving as a plasticizer, was introduced to the blend (20%) and stirred for one hour at room temperature. To achieve neutralization, the blends were treated with 5% sodium bicarbonate ($NaHCO_3$). Subsequently, different concentrations of metronidazole nanoparticles (MNP) (10 - 40 w/t%) were added. The mixture was then transferred to 96 wells as a mold and subjected to slow freezing at −20 °C to −80 °C overnight before undergoing freeze-drying for 24 hours. The lyophilized samples were then crosslinked via dehydrothermal treatment (DHT) at a temperature of 105 °C for 24 hours [4].

Physical Characterization of Scaffolds

The scaffold's thickness was measured with a micrometer screw gauge was utilized to measure by assessing three randomly selected scaffolds from each group. For the assessment of weight variation, each group was individually weighed using an analytical balance, and the average weight was then calculated (n = 3).

Morphological and Porosity

The scaffold’s morphology was characterized using scanning electron microscopy (SEM), and its pore size was measured randomly (n = 6). Before observation, the surface of the scaffold was coated with a thin layer of gold.

Swelling Test

The swelling ratio was calculated by immersing the scaffold in phosphate buffer saline (PBS) with a pH of 7.4 at 37 °C. After a 24-hour immersion period, the scaffold was gently dried on filter paper and weighed using an analytical balance. The swelling ratio was then calculated using Equation (1) [8].

$$\text{Swelling ratio: } [(Ww - Wd)/Wd] \quad (1)$$

where Wd is the dry weight of Cc-Mn and Ww is the weight of Cc-Mn after swelling

Biodegradation Rate

For assessing the scaffold’s biodegradation characteristics, the scaffold was immersed in 5 mL of PBS with a pH of 7.4 and kept at 37 °C for a period of 28 days. At specific time intervals of 7, 14, 21, and 28 days, the scaffold was removed from the degradation medium and allowed to dry. The degradation rate was expressed as a percentage using Equation (2) [4]:

$$\text{Degradation rate (\%)} = [(Wb - Wa) / Wb] \times 100 \quad (2)$$

where Wb represents the initial weight of the scaffold before degradation, and Wa represents the weight of the scaffold after degradation (N = 3).

Statistical Analysis

A one-way analysis of variance (ANOVA) was conducted using GraphPad Prism (Version 9.01) to determine the significance value (P value) for differences among the means.

Results and Discussion

Physical Characteristics

Collagen-chitosan scaffolds typically exhibit an off-white or beige color due to the natural color of the collagen and chitosan materials. A prior study also reported that collagen-chitosan scaffolds loaded with L-glutamic acid exhibited an off-white scaffold color [9]. Furthermore, collagen-chitosan-hydroxyapatite scaffolds for bone repair in ovariectomized rats were noted to exhibit similar white three-dimensional structures [10]. Similar to this study, both the drug-free scaffold and scaffold containing different concentrations of MNP (10 - 40 w/t%) appeared beige in color with no noticeable physical appearance among the different amounts of the MNP in the scaffolds, as shown in Figure 1. In this study, the scaffold without the MNP (0 w/t%) served as a blank scaffold, while the collagen-chitosan scaffold with the drug (ranging from 10 - 40 w/t%) was also examined. The thickness and weight variation of the scaffold were directly associated with mass uniformity and dosing accuracy [11]. The average thickness of all prepared scaffolds ranged from 5.003 ± 0.004 mm to 5.017 ± 0.012 mm in length, 6.037 ± 0.010 mm to 6.072 ± 0.007 mm in width, showing a consistent three-dimensional structure of similar size. The weight variation values increased as the concentration of MNP increased, ranging from 5.96 ± 0.07 mg to 6.31 ± 0.08 mg. However, each group exhibited uniform weight, indicating that the drug was uniformly distributed. The consistent thickness and weight values obtained in this study depicted the scaffold's uniformity in their physical appearance.

Morphological Properties

The SEM micrographs of both the prepared free-drug scaffold and the scaffold containing different concentrations of MNP in the range of 10 - 40 w/t% showed an open porous structure with a uniformly interconnected network, as shown in Figure 1. In tissue engineering, this structural characteristic plays a critical role in supporting cell nutrition and proliferation migration, aiding in tissue vascularization and the formation of tissue. Instead of the pore network structure that guides and promotes the development of new tissue, the porous surface also facilitates mechanical interlocking, thereby enhancing the implant's stability [12]. In this study, interconnected open pores were observed, a crucial feature facilitating the exchange of gas, waste, and nutrients for cells inside the scaffolds [21]. Furthermore, scaffold morphology can be noticed in elongated or round shapes [13]. In this study, an empty scaffold and drug (Figure 1(a)) loaded with drug (Figure 1(b) to (e)) exhibited similar structure and geometry of the surface of the scaffold with similar rounded pores. Thus, the drug-loaded scaffold did not influence the structure and geometry of the surface of the scaffold.

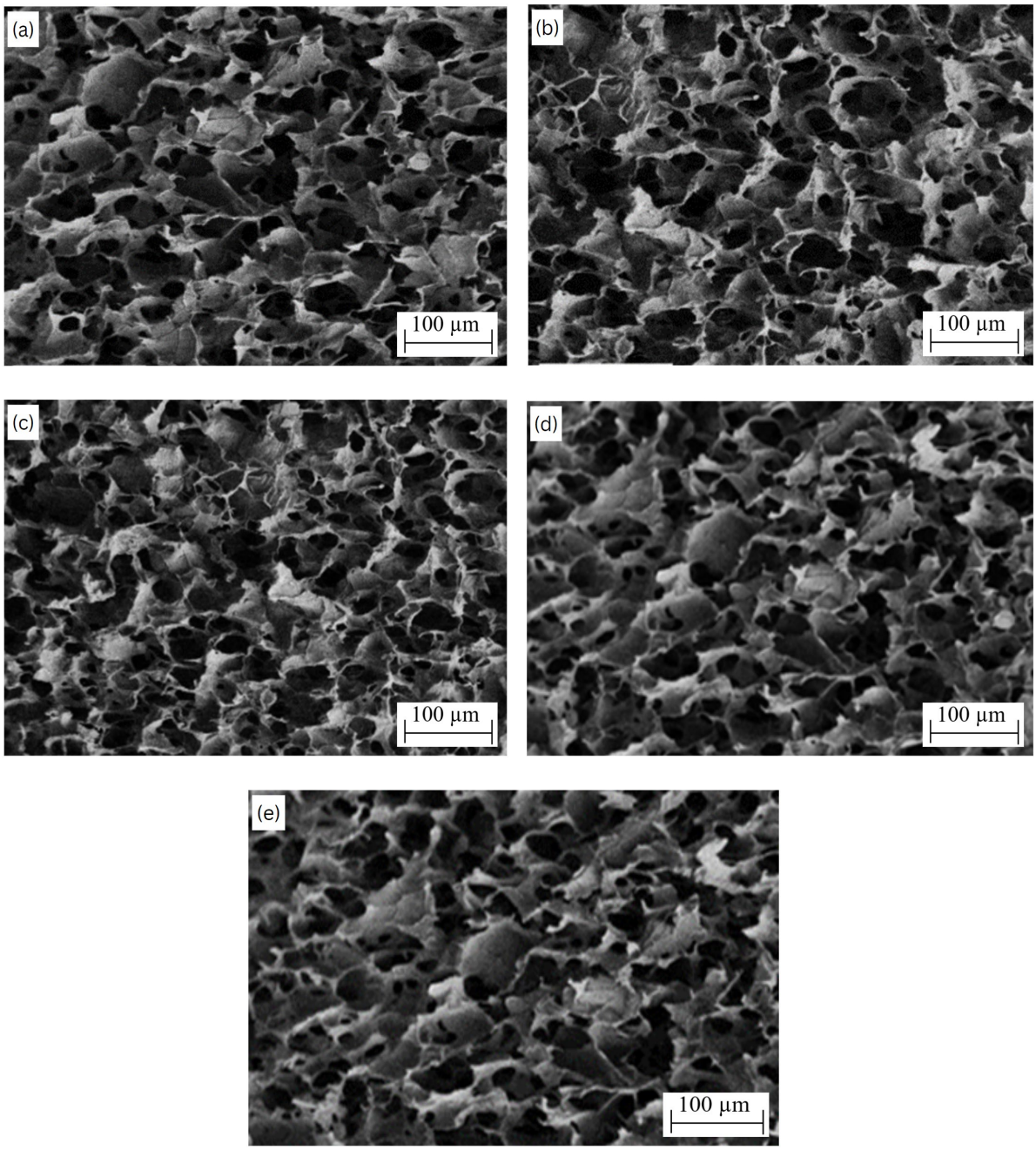

Figure 1: The photograph and SEM micrographs of a scaffold containing different amounts of MNP (a) 0 w/t%, (b) 10 w/t%, (c) 20 w/t%, (d) 30 w/t% and (e) 40 w/t%.

Pore Size

In this study, the mean values of pore size displayed a gradual decline as the MNP concentration incorporated into the scaffold increased, as shown in Figure 2. The control group exhibited the highest mean value pore size of 110.9 ± 5.8, followed by the MNP-loaded scaffolds (105.2 µm ± 3.2, 104.8 µm ± 4.8, 101.9 µm ± 5.2, and 101.5 µm ± 4.8 for 10 mg, 20 mg, 30 mg, and 40 mg, respectively). Thus, the pore size of both the empty scaffold and the drug-loaded scaffold met the minimum requirement of pore size for 3D bone regeneration. This is because the scaffold's macropore size is important for cell seeding, distribution, migration,

and further neovascularization in vivo [15]. Further analysis of variance (ANOVA) was conducted to compare the means of the pore sizes between the scaffold without the drug (control) and the scaffold with the drug. However, the highest concentrations of the drug did not show any significant difference in the pore size compared to the empty scaffold, and the pore size falls within the range mentioned in the previous study of 50 to 300 µm achieved through freeze-drying techniques [14]. This indicates the effectiveness of the physical crosslinking technique employed in the fabrication of the scaffold. The scaffold's pores can be broken down into nanosize (100 nm), micropore size (100 nm – 100 µm), and macro-roughness (100 µm–millimeters) [13]. This suggests that the 100 µm pore size of the scaffold will not hinder the release of the drug, making it suitable for the efficient release of metronidazole in nanometer dimensions. This can be supported by previous studies where melatonin in the range of 110 - 200 nm was able to sustain the release of the drug from the scaffold for 21 days [16]. Thus, the pore size of the scaffold is not only suitable to facilitate tissue growth and regeneration but also the release of nanoparticle drugs, providing a sustained and localized drug delivery system.

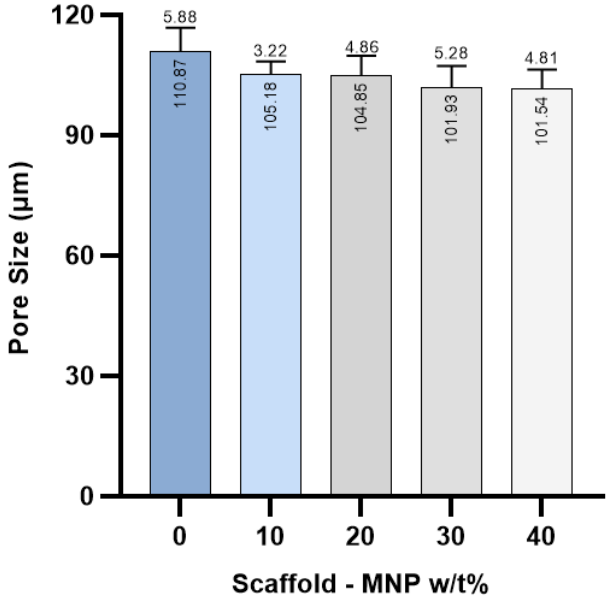

Figure 2: The average pore size of scaffolds at different concentrations of MNP (0 - 40 w/t%). Error bars indicate the mean ± S.D. (n = 6).

Swelling Test

The swelling capability of the scaffold is crucial as it enables the absorption and retention of a significant amount of water or biological fluids and facilitates biological interaction [17]. Previous research has shown that silk fibroin, collagen, and chitosan can be crosslinked and lyophilized to make a 3D scaffold that swells up to 3000% after 1 hour of immersion. This characteristic makes them well-suited for tissue engineering applications [8]. The highest swelling ratio was observed in the scaffold without the incorporation of MNP at 0% w/t. At 10 w/t%, the swelling ratio reduced to 77.51% ± 0.63. This trend continued as the nanoparticle concentration increased at 20 w/t% and the swelling decreased to 51.86% ± 0.60, while at 30 – 40 w/t%, it further reduced to 43.42% ± 0.45 and 42.26% ± 1.06 as shown in Figure 3.

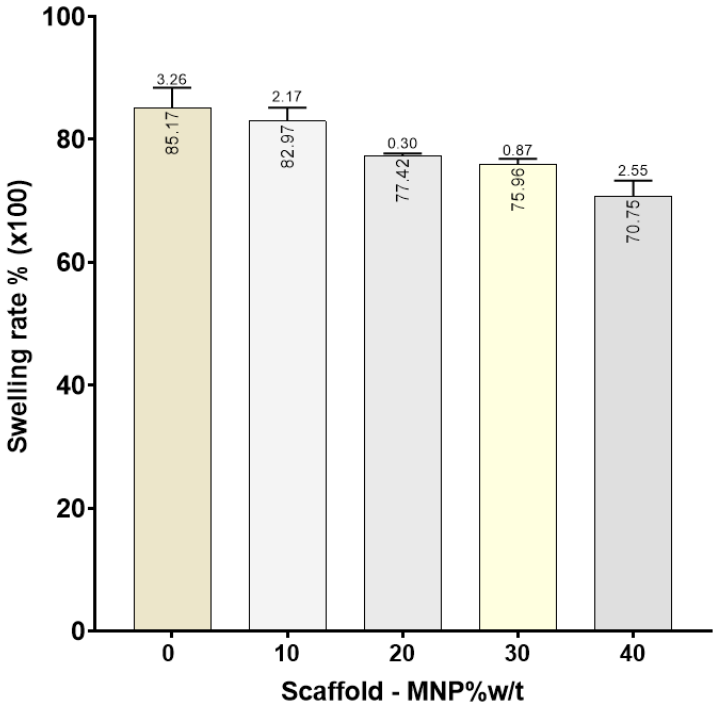

Figure 3: The swelling rate of the scaffold at different concentrations of MNP (0 - 40 w/t%). Error bars depict the mean ± S.D. (n = 6).

The reduction in swelling ratio with increasing nanoparticle concentrations can be correlated with the nanoparticles modifying the water flow through the scaffold. As shown in Figure 4, water molecules diffuse into the scaffold, causing swelling until it reaches equilibrium. However, the presence of nanoparticles might decrease the available holes within the scaffold, leading to less water diffusion into the drug-loaded scaffold and a subsequent decrease in swelling compared to the empty scaffold [18]. This observation aligns with prior research involving chitosan-collagen scaffolds with zinc oxide nanoparticles, where reduced swelling was noted due to nanoparticle-restricted water penetration [4].

Another phenomenon can be noticed in a previous study where ZnO nanoparticles were added to swollen oxidized starch hydrogel and there was a decrease in the swelling ratio due to ZnO nanoparticles as crosslinking [19]. Furthermore, metronidazole nanoparticles exhibit poor solubility in water at pH 7.4 [20], thereby reducing the water retention capability in the scaffold. This is similar to a previous study where the incorporation of ibuprofen in scaffolds may reduce water retention capability because ibuprofen is non-polar and insoluble in water [21]. Despite these considerations, water retention is well-suited for sustained drug delivery over an extended period, allowing for the control of the swelling behavior of scaffolds and aiding in filling specific tissue defects for stable implantation [21]. This efficiency in delivering bioactive molecules, such as drugs or growth factors, also enables targeted drug delivery at the site of action, indirectly minimizing systemic side effects and maximizing therapeutic efficiency [17]. However, both the scaffolds with different MNP concentrations (10 - 40 w/t%) and the MNP-free scaffold (0 w/t%) displayed significant swelling capability, exceeding 3000%. As mentioned in a prior study, a swelling score (> 3000%) is considered an excellent indicator of the scaffold's swellability. Therefore, all scaffolds exhibited good swelling abilities when immersed in a PBS solution for 24 hours. Thus, this drug-loaded scaffold possesses a remarkable ability to absorb and retain water while maintaining the 3D structure. This

highlights its suitability for tissue engineering applications, aligning with the swelling behavior indicated in prior studies [17].

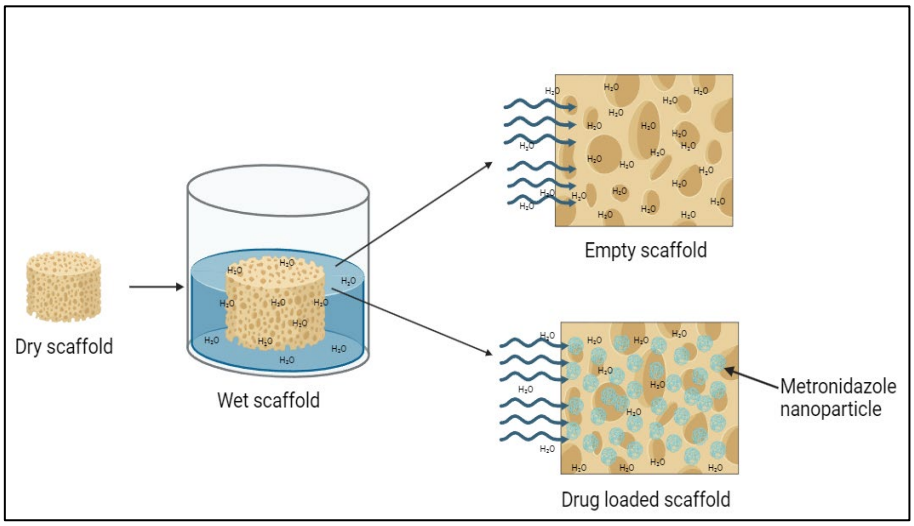

Figure 4: The illustration of the swelling behavior of the scaffold was created with Bio Render.

Biodegradation Rate

The scaffold with varying concentrations of 0 – 40 w/t% exhibited degradation rates of 69%, 68%, 60%, and 57% after 1 week of immersion in PBS solution, as shown in Figure 5. Notably, scaffolds with 0 w/t% and 10 w/t% MNP degraded more rapidly, reaching 92% and 90% degradation within 2 weeks of immersion. In contrast, the scaffolds loaded with 20 - 40 w/t% showed a slower and more gradual degradation trend over 4 weeks. By week 2, the degradation rates for scaffolds with MNP at 20 w/t%, 30 w/t%, and 40 w/t% MNP were 78%, 75%, and 73%, respectively. By the end of week 4, these rates had increased to 88%, 88%, and 87%. Although the physiological fluid supports the breakdown of the scaffold, a slower degradation rate was observed when more MNP was incorporated into the scaffolds. This deceleration is attributed to the limited penetration of water due to the poor solubility of nanoparticles in water at pH 7.4 [4]. Nevertheless, all the scaffolds demonstrated excellent biodegradability properties, with biodegradation percentages exceeding 80% at the end of the experiment.

Based on the previous study, when the drug was incorporated into the scaffold, the drug release was influenced by the diffusion and degradation of the scaffold [22]. The observed gradual degradation trend in this study provides an advantage in drug delivery systems, enabling extended and sustained drug release from the scaffold. Furthermore, natural materials such as collagen, fibrin, and hyaluronic acid are characterized by high biological activity and good biocompatibility. Unfortunately, their biodegradation rate often leads to faster structure degradation than required for regeneration. In the previous study, the DHT-treated collagen-chitosan scaffold demonstrated a prolonged degradation and a good biodegradability rate, which also aligned with the rate of new bone formation *in vivo* occurring over 4 weeks [23]. This was attributed to crosslinking within the polymer [24]. This aligns with the present study, where varied concentrations of drug-loaded collagen chitosan scaffold showed extended degradation, preserving the 3D porous structure for about 4 weeks. This indicates that the drug-

loaded scaffold exhibits good biodegradability, and biostability and could be effectively used for tissue engineering and regeneration.

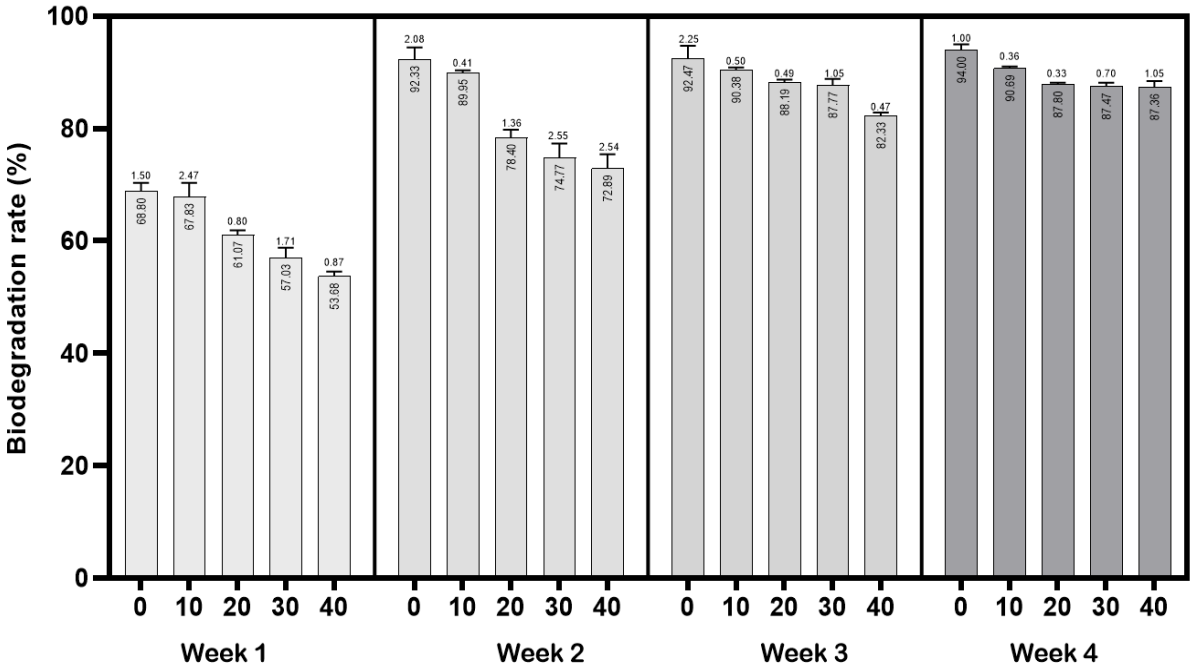

Figure 5: The scaffold's biodegradation rate (%) at different concentrations of MNP (0 - 40 w/t%). Error bars indicate the mean ± S.D. (n = 3).

In recent years, the development of tissue engineering and nanotechnology has captivated a great deal of attention, leading to numerous approaches toward designing scaffolds. In a previous study, the term "scaffold" was referred to as the 3D biomaterial before cells were incorporated, either in vitro or in vivo [25]. These scaffolds are designed to act as host biomaterials for incubating cells, guiding their attachment, growth, differentiation, proliferation, phenotype, and migration to facilitate the development of new tissue. However, achieving a well-defined drug-loaded scaffold with specific properties, sizes, and shapes remains an ongoing challenge. This is due to the variety and complexity of biological tissues, where suitable materials are required to produce cell-friendly biomaterials and 3D scaffolds that can mimic the composition of native tissue. To address current limitations and the demand for safe scaffolds in periodontal therapy, tissue engineering, and nanotechnology, they are emerging as future applications in biomedical fields. For optimal functionality, scaffolds must meet various requirements, including appropriate pore size, swelling ability, biodegradability, and morphological and structural characteristics that promote cellular adhesion, integration, and interaction with the biological environment prior to in vitro and in vivo application [18].

In this study, the scaffold exhibits structural consistency, a suitable pore size, and the ability to absorb and retain water while maintaining its 3D structure. This creates a favorable environment for essential biological interactions, such as the exchange of nutrients and oxygen crucial for cell proliferation, adhesion, differentiation, and the formation of new tissue [17]. Furthermore, numerous factors, such as physical loading, scaffold environment, structure, surface, and chemical modification, influenced the degradation rate of the scaffold, and as observed in this study, the degradation rate of the scaffold decelerated in the initial stages of this study due to the presence of MNP. This crucial property offers the potential to act as a

vehicle for drug release, allowing precise control of drug release for an extended duration. Consequently, drug-loaded scaffolds have emerged as a valuable candidate in the development of advanced therapeutic and regenerative strategies.

Conclusions

In conclusion, this study has successfully fabricated three-dimensional porous scaffolds loaded with metronidazole nanoparticles. The scaffold showed notable primary and crucial features and criteria, including excellent physical structure, pore size and morphology, swelling ability, and gradual and good biodegradability properties. Additionally, it complies with halal materials, making it potentially suitable for tissue engineering applications and drug delivery systems, thereby addressing concerns with non-halal scaffolds. However, further in vitro and in vivo studies on the collagen-chitosan scaffold with metronidazole nanoparticles will be conducted.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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