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FABRICATION OF SCHIFF BASE CROSSLINKED *N,O*-CARBOXYMETHYL CHITOSAN-BASED HYDROGELS FOR THE TREATMENT OF BONE DEFECTS

APPENDIX – THESIS SUMMARY

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THESIS SUMMARY

Abstract

The large shortage and problems arising from autologous and allogeneic grafts posed the need for an alternative source of grafts to help treat bone defects of varying degrees of complexity. Among the approaches that have been studied, *in situ* crosslinking hydrogels show great potential in controlling the spatial structure of the engineered tissue in accordance with the patient-specific defect. Various materials have been used for hydrogels, such as natural polymers, synthetic polymers, and bioceramics, with different advantages and disadvantages. Among them, natural polymers dominate with the ability to form a hydrated hydrogel network that mimics the structure of the extracellular matrix in the organism and supports the loading of cells or growth factors. Most of the hybrid hydrogel components have natural polymers present to improve the biocompatibility of the three-dimensional structure. For gelation of natural polymers, traditional physical methods such as changing pH and temperature are often problematic because the cell loading conditions are not suitable, therefore new methods such as ionic bonding, which utilizes ultrasonic waves or supramolecular assemblies attract much research. However, hydrogels formed from chemical bonds still show higher stability and allow easier control of binding capacity, thereby achieving the necessary physicochemical properties for the structure. A large amount of research and commercial products have focused on photochemical hydrogels, although a remain of photochemical activators or the formation of free radicals has the potential to damage and even cause cell death. The same problem is encountered with traditional crosslinking agents. The dynamic covalent method, which relies only on the hydrogel component for bond formation, especially Schiff base, thus attracts many studies. This method based on the advantages of natural polysaccharide sources is essential to produce a new method that is safer and more effective in the field of bone tissue engineering. Especially, *in situ* crosslinking hydrogels without the use of regular mass crosslinking agents not only provide a non-toxic option to stem cells but also are an

encouraging resolution for establishing injectable scaffolds. Among different *in situ* crosslinking mechanisms, Schiff base linkage between amine and aldehyde groups is a potential manner for fabricating *in situ* crosslinking hydrogels. Therefore, *in situ* crosslinking hydrogels pave a promising approach in bone tissue engineering. In this study, different hydrogels are fabricated to regenerate bone, which is made from *N,O*-carboxymethyl chitosan crosslinked with oxidized polysaccharides such as aldehyde hyaluronic acid, oxidized alginate, and supplemented with bioceramic-like calcium phosphate. Various hydrogel characterizations such as the ability of hydrogel formation, equilibrium swelling degree, degradability, and compression strength are evaluated. Furthermore, the Schiff base crosslinking between aldehyde groups and amine groups indicating hydrogel formation was confirmed via Fourier transform infrared spectroscopy spectra. The presence of calcium phosphate in hydrogels was also demonstrated by Fourier transform infrared spectroscopy, X-ray diffraction, scanning electron microscopy, and energy-dispersive X-ray spectroscopy images. Furthermore, *in vitro* cytotoxicity and *in vivo* healing in the bone defect animal model are studied.

Aims

General aims:

This study is conducted with the aim of fabricating *in situ* crosslinking hydrogels based on carboxymethyl chitosan to produce Schiff base linkage with alginate or hyaluronic acid, then supplementing with calcium phosphate to form the bone scaffold.

*Specific aims**:***

Specific aim 1. Fabrication of *in situ* crosslinking hydrogels based on oxidized alginate/*N,*O-carboxymethyl chitosan/β-tricalcium phosphate for bone regeneration

Hydrogel components are fabricated from natural polymers. As mentioned above, in fabricating *in situ* crosslinking hydrogel, the chemical structures of chitosan and alginate

are modified to produce Schiff base reaction – a chemical reaction involving the formation of an imine dynamic covalent bond through the interaction between the amine and aldehyde groups. Specifically, the molecular structure of chitosan contains an amine functional group, while the aldehyde functional group is created by oxidizing alginate with sodium periodate. As a result, the molecular structure of chitosan and alginate can establish crosslinks with each other immediately after mixing the hydrogel materials.

The hydrogel materials are mixed in different volume ratios to obtain different hydrogels and then the chemical and physical properties of the resulting hydrogels, which conclude the applicability of these hydrogels to regenerate bone tissue. FT-IR is applied to confirm the presence of Schiff base bonds in the chemical structure of the hydrogel. Gelation time, which is one of the important parameters, affects the structure retention of hydrogels. In addition, the morphological structure and swelling of the hydrogel are also evaluated because these properties have a great influence on the ability to transport nutrients, diffuse oxygen to cell growth, and eliminate waste products from cell metabolism. The pore size in the structure also affects cell adhesion, migration, and proliferation, as well as providing a microenvironment for cell differentiation. The degradation of the hydrogel also needs to be matched to the rate of new tissue formation for bone tissue regeneration to take place effectively. Finally, cytotoxicity testing and *in vivo* regeneration ability of 3D structure on the mouse calvaria defect model of the hydrogel should be carried out to assess its biocompatibility.

Specific aim 2. Fabrication of novel bone substitute alginate – *N,O*-carboxymethyl chitosan – Aldehyde hyaluronic acid – Biphasic calcium phosphate for bone regeneration

Oxidizing alginate accelerates the degradation process of hydrogel due to the breakdown of polymer chains as well as the appearance of aldehyde functional groups, causing alginate to lose its initial inertness. This makes the hydrogel system unsuitable for bone scaffold applications that require longer regeneration times. Therefore, to improve the rapid degradation time of the hydrogel system mentioned above, I used alginate instead of

oxidized alginate. Furthermore, BCP which is considered to overcome the rapid solubility of β-TC was used as a bioceramic. Additionally, apart from the Schiff base linkage between NOCC and AHA, the ionic bond between alginate and calcium phosphate also contributes to improving both the degradation time, mechanical properties, and cell interaction.

Specific aim 3. Polycaprolactone hybrid scaffold loaded with *N,O*-carboxymethyl chitosan/Aldehyde hyaluronic acid/Hydroxyapatite hydrogel for bone regeneration

The fabricated hydrogels demonstrate good bone regeneration capability; however, they are suitable for non-load-bearing bone defects, in line with the compressive strength of these hydrogels. To enhance the bone regeneration application of *in situ* crosslinking hydrogel, a porous PCL scaffold is used to load the hydrogel. Thanks to the combination of synthetic polymer and composite hydrogel, the compressive strength of this hybrid scaffold is significantly increased, allowing it to regenerate tibial defects in a rabbit model.

Hypotheses

To achieve the abovementioned aim, this study makes three following hypotheses:

Hypothesis 1: Hydrogels are fabricated through Schiff base reaction between *N,O*-carboxymethyl chitosan and oxidized alginate, and the addition of bioceramic further enhances the hydrogel's mechanical properties.

1. The first hydrogel research is "Fabrication of *in situ* crosslinking hydrogels based on oxidized alginate/*N,O*-carboxymethyl chitosan/β-tricalcium phosphate for bone regeneration", which was published in December 2022 in Journal of Science: Advanced Materials and Devices (Web of Science, Q1, Impact Factor: 7.382)

This initial study aimed to fabricate and evaluate an injectable hydrogel composed of oxidized alginate (OAlg), *N,O*-carboxymethyl chitosan (NOCC), and β-tricalcium phosphate (β-TCP) for the treatment of non-load-bearing bone defects. The successful

formation of hydrogels through Schiff base crosslinking between the amine groups of NOCC and the aldehyde groups of OAlg was confirmed by the presence of characteristic C=N linkage peaks around 1641 cm^{-1} in the Fourier transform infrared spectroscopy (FT-IR) spectra. The study investigated the influence of OAlg concentration and the OAlg:NOCC ratio on various hydrogel properties, including gelation time, morphology, swelling behavior, degradation rate, and compressive strength. It was observed that a lower OAlg concentration (3%) led to faster gelation and the formation of more stable structures. The inclusion of β-TCP significantly enhanced the compressibility of the hydrogels, a crucial factor for their potential as bone substitutes. *In vitro* cytocompatibility assays demonstrated that the hydrogels were non-cytotoxic, and *in vivo* studies using a mouse calvarial defect model showed promising bone regeneration, with the OA3NO13 hydrogel formulation exhibiting the most superior outcomes in promoting the formation of new bone tissue. This initial study laid the groundwork by establishing the feasibility of using this ternary hydrogel system for bone regeneration in a non-load-bearing scenario.

Overall, in this work, the *in situ* crosslinking hydrogels based on OAlg, NOCC, and β-TCP were prepared with two hydrogel systems of OA6NO and OA3NO and at four ratios of OAlg:NOCC in each system, ranging from 1:1 to 1:4. The formation of Schiff base linkages was confirmed by FT-IR analysis and the integration of β-TCP into hydrogel was also proven. Concentrations of OAlg and different OAlg:NOCC ratios did affect hydrogel formation, characteristics, *in vitro* cytocompatibility, and *in vivo* bone repair. Specifically, the gelation ability of OA3NO hydrogels was predominant compared to OA6NO, leading to the exclusion of OA6NO hydrogels in further experiments. Generally, most hydrogels were porous, biodegradable, biocompatible, and non-cytotoxic. In most results, the OA3NO13 hydrogel yielded the most superior outcomes, including quick gelation time, interconnected porous structure with the highest porosity, acceptable swelling capacity and degradability, high compressive strength, high *in vitro* cytocompatibility and promising *in vivo* bone regeneration, demonstrating its potential in support, repair, and regeneration of non-displaced or non-load-bearing bone injuries.

In summary, through the first research, hydrogels are fabricated through Schiff base reaction between NOCC and OAlg, proven by FT-IR spectra. Specifically, the expected C=N linkage peaks were observable at 1641 cm^{-1} in all hydrogel samples. Furthermore, the results reported a significant improvement in compressibility when bioceramic (β-TCP) was added and mixed with the polymers. Although the fabricated hydrogels possess lower compressive strength than native bone, they can be applied to reconstruct non-load-bearing bone defects and non-displaced fractures. Therefore, the first research fully addressed the Hypothesis 1.

Hypothesis 2: The use of alginate instead of oxidized alginate extends the degradation time of *in situ* crosslinking hydrogel, making it more suitable for bone regeneration applications.

2. The second hydrogel research is "Fabrication of novel bone substitute alginate – *N,O*-carboxymethyl chitosan – Aldehyde hyaluronic acid – Biphasic calcium phosphate for bone regeneration", which was published in October 2023 in Reactive and Functional Polymers (Web of Science, Q1, Impact Factor: 5.1)

Building upon the first study, the second research endeavor focused on developing a novel hydrogel composed of alginate, *N,O*-carboxymethyl chitosan (NOCC), aldehyde hyaluronic acid (AHA), and biphasic calcium phosphate (BCP) for bone tissue engineering. A key aim of this study was to extend the degradation time of the hydrogel and improve its mechanical properties. The hydrogels formed rapidly within 60-70 seconds, making them suitable for injectable applications. Scanning electron microscopy (SEM) revealed a highly interconnected porous structure with pore sizes ranging from 100 to 200 μm, a morphology known to be beneficial for cell activity. The successful formation of Schiff base linkages between NOCC and AHA, as well as the presence of BCP, was confirmed using FT-IR and X-ray diffraction (XRD) analyses. Notably, the incorporation of BCP significantly improved the hydrogels' resistance to degradation, with certain formulations retaining over 40% of their mass after 14 days. The compressive strength of the hydrogels was also

significantly enhanced by the addition of BCP, with the Alg-NOCC-AHA-BCP40 formulation demonstrating the highest strength. *In vitro* cytotoxicity tests showed high cell viability, and *in vivo* studies using a murine calvarial defect model demonstrated promising bone regeneration, with the Alg-NOCC-AHA-BCP40 hydrogel again showing the most favorable results. This study identified an optimal BCP loading of 40% that provided a balanced combination of degradation rate, mechanical properties, and cell interaction.

Throughout all experiments, we could conclude that 40% BCP was the most optimal proportion of the hydrogel-CaP composite. This percentage offered a sufficient amount of BCP to react with Alg and integrate into the hydrogel matrix with minimal aggregation, optimized the crosslinking density, and stabilized the porous structure, thereby preserving the highest hydrogel mass in the physiological medium, enhancing the compressive strength, promoting cell viability and proliferation, and facilitating the best regeneration at the bone defect site.

In summary, through the second research, *in situ* crosslinking hydrogels are fabricated through Schiff base reaction between NOCC and AHA, the use of alginate instead of oxidized alginate extends the degradation time of the fabricated hydrogels, as evidenced by the fact that they could retain their masses over 40% after 14 days of immersion in PBS at 37°C. Furthermore, the results showed that the addition of BCP to Alg-NOCC-AHA significantly increased the compressive strength of the hydrogel. Therefore, the second research fully addressed the Hypothesis 2.

Hypothesis 3: The use of a support scaffold made from biocompatible synthetic polymer significantly improves the mechanical properties of *in situ* crosslinking hydrogel.

3. The third hydrogel research is "Polycaprolactone hybrid scaffold loaded with *N,O*-carboxymethyl chitosan/Aldehyde hyaluronic acid/Hydroxyapatite hydrogel for bone regeneration", which was published in September 2024 in Journal of Biomedical Materials Research Part B: Applied Biomaterials (Web of Science, Q2, Impact Factor: 3.2)

Hydrogel has emerged as a potential material for bone grafting thanks to its biocompatibility, biodegradation, and flexibility in filling irregular bone defects. In the previous study, I incorporated bioceramic (β-TCP) in the NOCC/OAlg to enhance the hydrogel's osteoconductivity and osteoinductivity. In this study, I used another type of bioceramic, HAp, since it is more stable than β-TCP and possesses a composition closest to bone minerals. Therefore, I fabricated a novel NAH hydrogel system, composed of NOCC, AHA, and HAp. To improve the mechanical strength of the fabricated hydrogel, a porous PCL matrix was synthesized and used as a 3D support template for NAH hydrogel loading, forming a novel PCL/NAH hybrid scaffold. A mixture of monosodium glutamate (M) and sucrose (S) at varied weight ratios (5M:5S, 7M:3S, and 9M:1S) was used for the fabrication of 3D PCL matrix.

In order to facilitate bone regeneration, the fabricated scaffolds are required to possess interconnected porous structure with various pore sizes, at least 100 μm to establish a biological bond between the bone and the implant. Furthermore, pore sizes are required to reach 200 μm for both osteon development within the bone implant and bone formation. Therefore, in this study, different porogen ratios between sucrose and monosodium glutamate were used to fabricate PCL scaffolds with suitable pore sizes. Given its excellent mechanical qualities and extremely slow degradation, PCL is one of the synthetic polymers utilized in bone tissue engineering the most frequently. However, with its nature as an aliphatic polyester, PCL produces hydrophobic environment which is not ideal for cell adhesion and proliferation. In order to promote native cells recruiting to the defect, hydrogel loading to PCL is a promising strategy. Therefore, this study was conducted with

the goal of fabricating hybrid scaffolds that not only possess porous structures but also improve cell attachment.

The third research study aimed to address the mechanical limitations of the previously developed hydrogels by fabricating a hybrid scaffold composed of polycaprolactone (PCL) loaded with a hydrogel made from *N,O*-carboxymethyl chitosan (NOCC), aldehyde hyaluronic acid (AHA), and hydroxyapatite (HAp). The goal was to create a scaffold suitable for load-bearing bone defects. The hydrogel component in this study utilized HAp instead of β-TCP or BCP, chosen for its closer compositional similarity to bone minerals and higher stability. The PCL scaffold structure was controlled by using different ratios of monosodium glutamate and sucrose as porogens, which influenced the interconnectivity and hydrogel loading efficiency. The hybrid scaffolds exhibited significantly increased compressive strength compared to the hydrogel alone, with the PCL/NAH_5:5 formulation showing strength comparable to human cancellous bone. *In vitro* cytotoxicity assays indicated that all hybrid scaffolds were biocompatible with L929 mouse fibroblasts. *In vivo* bone healing was assessed using a rabbit tibia defect model, a more clinically relevant model for load-bearing applications. The results showed promising bone regeneration, with the PCL/NAH_7:3 scaffold demonstrating the best overall performance. This study successfully demonstrated a strategy to overcome the mechanical limitations of hydrogels for bone regeneration in load-bearing scenarios.

With characteristics including the largest area of woven bone, which was significantly larger than other samples, the quantity and distribution of fibroblast in the fibrous tissue, and few chronic inflammatory cells, the PCL/NAH_7:3 exhibited the best regeneration.

In summary, through the third research, the use of a support scaffold made from PCL significantly improves the mechanical properties of *in situ* crosslinking hydrogel, making it more suitable for bone regeneration applications, demonstrated by effectively regenerating tibia defects in rabbit model. Therefore, the third research fully addressed the Hypothesis 3.

Conclusion

In conclusion, this comprehensive study demonstrated the potential of different hydrogel systems and hybrid scaffolds in bone repair and regeneration. The *in situ* crosslinking hydrogels based on OAlg, NOCC, and β-TCP, particularly the OA3NO13 hydrogel, exhibited quick gelation times of approximately 1 minute, interconnected porous structures with a porosity of 70%, acceptable swelling capacity, and degradability, high compressive strength of 2.7 kPa, and high *in vitro* cytocompatibility with cell viability above 90%. Additionally, *in vivo* studies showed promising bone regeneration with a significant increase in new bone formation after 4 weeks.

To improve the degradation time and compressive strength of the hydrogel, alginate was used as a substitute for oxidized alginate. The *in situ* crosslinking hydrogel continues to be developed through Schiff base linkage between NOCC and AHA. The Alg-NOCC-AHA-BCP hydrogels demonstrated that an optimal BCP loading of 40% provided a suitable degradation rate, and compressive strength of 80 kPa, and supported cell proliferation with a significant increase in cell number over 7 days. These properties facilitated the bone regeneration process effectively.

Furthermore, the novel hybrid PCL/NAH_7:3 scaffold showed superior interconnectivity and good compressive strength of 623.8 ± 6.8 kPa, which is comparable to cancellous bone. The scaffold also exhibited evenly distributed pores with an average size of 193.0 ± 23.7 μm. The *in vivo* results indicated promising bone regeneration capability, with the largest area of woven bone (782 ± 12 μm) after 8 weeks of grafting. The hybrid scaffold was non-cytotoxic and supported the in-growth of cells and neo-tissues effectively.

These findings suggest that the developed hydrogels and hybrid scaffolds have significant potential for supporting, repairing, and regenerating bone injuries, contributing to advancements in BTE.