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Composition of chitosan-hydroxyapatite-collagen composite scaffold evaluation after simulated body fluid immersion as reconstruction material

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Abstract. Hydroxyapatite (HA) formation is one of the most important aspects of bone regeneration. Because domestically made chitosan-hydroxyapatite-collagen composite scaffolding from crab shell and bovine bone and tendon has potential as a maxillofacial reconstruction material, the material's HA-forming ability requires evaluation. The aim of this research is to investigate chitosan-hydroxyapatite-collagen composite scaffold's potential as a maxillofacial reconstruction material by observing the scaffold's compositional changes. Scaffold specimens were immersed in 37°C simulated body fluid (SBF) for periods of 2, 4, 6, and 8 days. Scaffold composition was then evaluated by using energy dispersive spectroscopy (EDS). The calcium (Ca) and phosphorus (P) percentages of the scaffold were found to increase following SBF immersion. The high Ca/P ratio (3.82) on the scaffold indicated HA formation. Ion exchange played a significant role in the increased percentages of Ca and P, which led to new HA layer formation. The scaffold's HA acted as a nucleation site of Ca and P from the SBF, with collagen and chitosan as the scaffold's matrix. Chitosan-hydroxyapatite-collagen composite scaffold shows potential as a maxillofacial reconstruction material, since its composition favors HA formation.

1. Introduction

Tissue defects in the maxillofacial region may be caused by trauma, neoplasm, infection, or congenital abnormality. Autogenous bone grafting is the gold standard for reconstructing bone defects [1]. Despite the advantages of the technique, harvesting autogenous bone graft is an invasive method that often results in patient morbidity. Limited bone volume is another disadvantage of grafting, especially for large-scale ablative surgery [2]. Alternative methods are thus necessary to overcome this problem. Bone tissue engineering is one promising option in maxillofacial reconstruction. The technique aims to create artificial construction to regenerate new tissue. Isolated cells are cultured in three-dimensional scaffolds and placed in the patient's body to restore the defect [3].

Three essential components of tissue engineering include cell, scaffold, and growth factors. Scaffolding acts as a matrix where the cells can proliferate; the scaffold serves as water, nutrition, and growth-factor reservoir until the scaffold is replaced by mature bone tissue [4]. Scaffolding material must comply with certain requirements, such as good biocompatibility, adequate mechanical strength, sufficient biodegradability, and the ability to bond with bone tissue, especially in the case of bone

tissue engineering. This last ability is also known as bioactivity [5]. Certain materials may be bioactive due to their hydroxyapatite composition, which comprises 60 percent of human bone. Nevertheless, hydroxyapatite exhibits slow biodegradation [6]. On the other hand, collagen shows rapid biodegradation and poor mechanical strength [7]. Natural polymers, such as collagen and chitosan, also show favorable bioactivity, and chitosan exhibits acceptable biodegradation [8]. Chitosan is a material from invertebrata like crab, shrimp skin, and lobster, which has been tested as bone graft material and it has the characteristic of nondigestibility, high viscosity, and water binding properties, biocompatible, biodegradable and low cytotoxicity [9].

The combination of chitosan, hydroxyapatite, and collagen as a scaffold has the potential to optimize each material's superior traits. Bone-like composite was initially created based on this notion. Several earlier studies have reported that composite scaffold increases osteoblast proliferation due to the combination of properties from hydroxyapatite, collagen, and chitosan [10].

National Nuclear Energy Agency of Indonesia produces chitosan-hydroxyapatite-collagen scaffold from abundant natural resources. Hydroxyapatite is extracted from bovine bone, whereas bovine tendon provides collagen; chitosan is obtained from crab shells. The scaffolds may be produced in various shapes and sizes—with uses ranging from reconstructing small dental extraction wounds to large tumor resection defects—in a cost-efficient manner.

This composite scaffold should undergo in vitro bioactivity testing to observe the scaffold's ability to form a hydroxyapatite layer before proceeding to the clinical application. This study thus aimed to investigate chitosan-hydroxyapatite-collagen composite scaffolding's potential as a maxillofacial reconstruction material by observing the scaffold's compositional changes. The study includes the measurement of calcium (Ca) and phosphorus (P) percentages before and after SBF immersion, as well as Ca/P ratio changes.

2. Materials and Methods

The scaffolding was produced by National Nuclear Energy Agency of Indonesia. Crab shells were diluted using acetic acid for 24 hours to extract chitosan. After obtaining chitosan in gel form, collagen gel and hydroxyapatite powder from bovine tendon and bone (respectively) were incorporated and mixed for 10 minutes. The chitosan: hydroxyapatite: collagen ratio was 2:3:3. This compound was then molded into the desired shape and size. The freeze-dry method was applied for 2 x 24 hours. The final result was a white and sponge-like scaffold.

Next, scaffold specimens were immersed in 37°C SBF (made by following the Kokubo method) for periods of 2, 4, 6, and 8 days [5]. After the immersion, the specimens were air-dried for 24 hours at room temperature. The scaffold's composition was evaluated using EDS (Carl Zeiss-Bruker type EVO MA10). The Ca and P percentages were monitored and then converted into a Ca/P ratio.

3. Results and Discussion

3.1 Results

The pre-immersion scaffold specimens showed carbon, oxygen, sodium, phosphorus, and calcium, while scaffolds that had been immersed in SBF exhibited additional elements, such as nitrogen, magnesium, and chlorine (Table 1).Calcium concentrations increased in accordance with the immersion period length, although a decrease was observed on day 4. Furthermore, the phosphorus percentages fluctuated, reaching a peak at day 6 (10.44%), while the day 8 specimen showed a slight decrease (8.08%) compared to the previous period. Although the ratio had lowered, it was still higher than the pre-immersion concentration. As Table 2 shows, the post-immersion Ca/P ratio experienced a gradual decrease on days 2 (2.70), 4 (2.62), and 6 (2.57) compared to the pre-immersion scaffold (2.84) and then steeply increased on day 8 (3.82). These scaffolds' Ca/P ratios corresponded to non-stoichiometric biological apatite, with a Ca/P ratio >1.67.

Table 1. Composition of chitosan-hydroxyapatite-collagen composite scaffold pre- and post SBF immersion

	Day 0	Day 2	Day 4	Day 6	Day 8
Carbon	29.26	24.37	27.10	13.69	-
Nitrogen	-	7.54	6.43	4.90	-
Oxygen	40.85	39.01	43.28	38.04	51.68
Sodium	21.06	1.00	1.45	1.96	2.40
Magnesium	-	0.17	0.17	0.29	0.50
Phosphorus	2.30	7.10	5.22	10.44	8.08
Chlorine	-	1.64	2.55	3.87	6.44
Calcium	6.53	19.15	13.70	26.81	30.84

Table 2. Ca/P ratio of chitosan-hydroxyapatite-collagen composite scaffold pre- and post SBF immersion

Duration	Ca/P Ratio
Day 0	2.84
Day 2	2.70
Day 4	2.62
Day 6	2.57
Day 8	3.82

3.2 Discussion

The chitosan-hydroxyapatite-collagen composite scaffold examined in this study showed an increase in calcium and phosphorus percentages; the Ca/P ratio also reached 3.82. These results indicate a newly formed hydroxyapatite layer on the surface of the scaffold. Chitosan-hydroxyapatite-collagen composite scaffold includes OH^- and PO_4^{3-} on its surface. The negative surface attracts Ca^{2+} in SBF and forms calcium-rich calcium phosphate; calcium aggregation attracts PO_4^{3-}, and the process continues [11]. Calcium phosphate layers increase the Ca/P ratio and attract minor ions from the SBF, such as Na^+, Mg^{2+}, and Cl^-[12]. This increase occurred in our study: neither Mg^{2+} nor Cl^- was initially detected, but the percentage rose after SBF immersion.

The phosphorus and calcium percentages dropped on day 4, however. This condition may have been caused by ion release from the composite material due to SBF ion attraction [13]. When a scaffold was immersed in SBF, ion exchange between the scaffold and the SBF occurred. Calcium release also occurred along with phosphate release, apatite layer re-precipitation, and surface topography alteration [13]. On day 4, an imbalance is assumed to have occurred between the ion release and the apatite layer re-precipitation on the scaffold specimen's surface. Nonetheless, the calcium and phosphorus percentages increased on day 6. Ion release affects scaffold degradation, which is expected to be in line with mature bone formation [14].

The increase of calcium and phosphorus in this study is in accordance with other investigations that have reported calcium and phosphorus deposition on polymer and hydroxyapatite composite following12 hours of SBF immersion [15]. Bioactive glass and polymer composition are essential in the increase of Ca and P percentages [16]. Hydroxyapatite in the scaffold plays an important role in attracting Ca and P, while the polymers act as the matrix for new hydroxyapatite accumulation [17]. In

addition, the non-stoichiometric Ca/P ratio of the scaffold indicates the presence of carbonated hydroxyapatite, which resembles human bone apatite composition rather than pure hydroxyapatite. These results thus support the bioactivity potential of chitosan-hydroxyapatite-collagen composite scaffold.

4. Conclusion

In conclusion, chitosan-hydroxyapatite-collagen composite scaffold showed bioactivity potential in this study because of the increased calcium and phosphorus percentages we found. The Ca/P ratio also indicated carbonated hydroxyapatite layer formation. This study is limited to compositional analysis of chitosan-hydroxyapatite-collagen composite scaffold, however; further study will be required to confirm the scaffold's bioactivity and other important requirements as a maxillofacial reconstruction material.

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