Study of Mechanical and Thermal Properties in Nano-Hydroxyapatite/Chitosan/Carboxymethyl Cellulose Nanocomposite-Based Scaffold for Bone Tissue Engineering: The Roles of Carboxymethyl Cellulose

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Abstract: Synthetic scaffolding for bone tissue engineering (BTE) has been widely utilized. The scaffold for BTE requires sufficient porosity as a template for bone cell development and growth so that it can be used in the treatment of bone defects and fractures. Nevertheless, the porosity significantly influences the compressive strength of the scaffold. Hence, controlling the porosity is a pivotal role to obtain a proper scaffold for practical BTE application. Herein, we fabricated the nanocomposite-based scaffold utilizing nano-hydroxyapatite (n-HA). The scaffold was prepared in combination with chitosan (Ch) and carboxymethyl cellulose (CMC). The ratios of n-HA, Ch, and CMC used were 40:60:0, 40:55:5, 40:50:10, 40:45:15, and 40:40:20, respectively. By controlling the Ch and CMC composition, we can tune the porosity of the nanocomposite. We found that the interpolation of the CMC prevails, as a crosslinker reinforces the nanocomposite. In addition, the binding to Ch enhanced the compressive strength of the scaffold. Thermal characteristics revealed the coefficient of thermal expansion decreases with increasing CMC content. The nanocomposite does not expand at 25–75 °C, which is suitable for human body temperature. Therefore, this nanocomposite-based scaffold is feasible for BTE application.

Keywords: BTE; nanocomposite; scaffold; porosity; compressive strength; body temperature

1. Introduction

Various biomedical techniques have been developed to overcome the bone defect. This includes the so-called bone tissue engineering (BTE) method [1]. BTE is a principle of biological discipline and technique to create a temporary matrix. This matrix will be used as a place for bone cells to grow using a scaffold. The scaffold will act as a template for bone cell regeneration, and support the formation of new tissue [2]. Therefore, the scaffold should be biodegradable, biocompatible, and osteoconductive [3]. The scaffold should have a porosity of about 30–90% [4], with minimal pore sizes of 100 µm to serve
as a template for bone cell regeneration and growth [5]. Apart from scaffold, the microenvironment, such as extracellular matrix or the employed cells, also plays an important role in the regenerative processes. Synergism of scaffold and stem cells offers advances and provides biological alternatives for restoring bone defects with improved tissue functions. Currently, researchers are trying to create scaffolds which can enhance the osteogenic differentiation [6]. Stem cells are necessary in the field of translational regenerative medicine. This is owing to the capability of the cells in differentiating into the osteogenic lineage. The differentiation will, in turn, influence the bone treatment. The safety related to in vitro and in vivo models in regenerative medicine is associated with the usage of animal serum. Instead of fetal bovine serum (FBS), human platelet lysate (PL) is now considered. Stem cells cultured in the presence of PL exhibiting a higher healing rate after injury revealed the appropriate option for its clinical-grade use [7].

Polymeric scaffolds are the most popular scaffolds as a model in vitro, and in vivo for bone tissue engineering applications. The selection is made due to ease in altering the physiochemical characteristics. The tunability will support the demands for a comprehensive range of applications in orthopedics. The synthetic scaffold with the predefined properties can be fabricated using nano-hydroxyapatite (n-HA) and chitosan (Ch). This is due to the fact that n-HA is identical to a component of bone [8,9]. The n-HA are bioactive, biodegradable, and osteoconductive, hence widely utilized as an implant and in bone regeneration applications [10,11]. The HA has been widely combined with Ch as a composite to form a scaffold. Ch could be formed as porous for bone cell growth, and we can control pore size in the scaffold by merely controlling the compositions of n-HA and Ch [12]. Ch is a natural polymer with excellent biocompatibility and biodegradability. Ch also offers bioresorbable, antibacterial, non-antigenic, biofunctional, and osteoconductive properties. These make Ch suitable for the primary material of composite-based scaffold [12–14].

To date, various fabrication techniques have been conducted to fabricate the composite-based scaffold. For instance, the freeze-drying method is considered the method of choice. This method is chosen to synthesize the composite-based scaffold, due to simplicity, low cost, and ease of preparation. This method offers an easy way to control the constituent compositions of the composite, including the formation of pores. In this method, the replication of ice crystal dendrites is trapped [15]. Following that, the ice crystal is allowed to evaporate, in order to form pores. In this present research, n-HA/Ch composite-based scaffold is fabricated by the freeze-drying method. The findings reveal lower compressive strength with good cell biocompatibility, compared to a single Ch-based scaffold [16]. The scaffold is required to have good compressive strength at about 2–12 MPa, and, hence, it can be used as a synthetic bone [17]. Herein, we propose embedding a natural polymer carboxymethyl cellulose (CMC) as a composite filler to enhance the mechanical strength of the composite-based scaffold. The CMC has an opposite charge to Ch, therefore, they will create stronger crosslinks via ionic bonds [18,19]. The nanocomposite-based scaffold is fabricated by using a variation of Ch and CMC, while the n-HA compositions are kept constant at 40 wt%, as the basic component of the scaffold. Various physical property characterizations are conducted to understand the effect of CMC in the n-HA/Ch/CMC nanocomposite. In short, the preparation of a composite-based scaffold with excellent compressive strength, sufficient porosity, and biodegradation rate can be achieved. Thermal analysis is also carried out, to understand its thermal response to the human body temperature. This study indicates that the nanocomposite scaffold fabricated, using the freeze-drying method, with modifications in their strength, could serve as a novel and promising bone substitute for tissue engineering.

2. Materials and Methods

The scaffolds were fabricated with variations of n-HA, Ch, and CMC of about 40:60:0, 40:55:5, 40:50:10, 40:45:15, and 40:40:20, respectively, as shown in Table 1. Scaffolds were prepared using a freeze-drying method by dissolving n-HA, Ch, and CMC in polyvinyl alcohol aqueous solution as a solvent. These were then followed by the addition of an acetic acid of 2 weight%. After pouring this mixture into a mold, n-HA/Ch/CMC solvents were frozen at −80 °C for 12 h and dried for 48 h.
Samples were then immersed in NaOH (10%) for 2 h, to neutralize the acetic acid residue. These samples were then washed with water up to pH 7, and dried in the oven at 60 °C.

**Table 1.** Composition details of nano-hydroxyapatite (n-HA), chitosan (Ch), and carboxymethyl cellulose (CMC).

| Samples | n-HA:Ch:CMC (wt%) | n-HA (g) | Ch (g) | CMC (g) |
|---------|------------------|---------|-------|--------|
| A       | 40:60:0          | 4       | 6     | 0      |
| B       | 40:55:5          | 4       | 5.5   | 0.5    |
| C       | 40:50:10         | 4       | 5     | 1      |
| D       | 40:45:15         | 4       | 4.5   | 1.5    |
| E       | 40:40:20         | 4       | 4     | 2      |

The samples were then characterized by Fourier-transform infrared (FTIR), to identify the functional group and to determine the composition of the samples. The porosity of the samples was identified using the liquid displacement method [20]. For this, samples were immersed in a solution consisting of 8 mL of alcohol (96%) for 48 h. Henceforth, the porosity can be calculated using Equation (1).  

\[
\text{Porosity} = \left( \frac{m_1 - m_0}{pV_0} \right) \times 100\%
\]  

wherein \( m_0 \) is initial mass and \( m_1 \) is the final mass after immersion in alcohol, \( V_0 \) is the volume of alcohol, and \( p \) is the density. Following that, the porosity was visualized by scanning electron microscopy (SEM) characterization, to shed a light on the effect of Ch and CMC compositions on the porosity. The compressive strengths of the samples were tested using a compressive strength machine via Equation (2), below.  

\[
\sigma = \frac{F}{A}
\]  

Meanwhile, the biodegradation of the samples was identified via immersing the samples in 5 mL of simulated body fluid (SBF) [21] solution for 4 weeks, and the data were taken at the 1st, 3rd, and 4th week. The biodegradation was estimated by Equation (3).  

\[
W_L = \left( \frac{W_0 - W_1}{W_0} \right) \times 100\%
\]  

where \( W_0 \) is initial weight, \( W_1 \) is final weight, and \( W_L \) is the weight loss. To understand the thermal properties, we used a differential scanning calorimeter (DSC) to determine steam temperature (Tb), glass transition temperature (Tg), decomposition temperature (Td), and crystallization temperature (Tc) at temperature ranges of 25–900 °C. Thermogravimetry analysis (TGA) was also used to determine the mass changes with a heat velocity of 10 °C/minute at 25–900 °C. Meanwhile, the thermomechanical analysis (TMA) was utilized to determine the thermal expansion during heating at 25–800 °C, with a heat velocity of 10 °C/minute.

### 3. Results

The identification of the functional group by FTIR is shown in Figure 1. The redshift of the absorption peak of the FTIR spectrum is observed. The spectra from 1031.92 cm\(^{-1}\) to 1035.77 cm\(^{-1}\) corresponds to the P–O stretching vibrations. This indicates a chemical interaction of n-HA (PO\(_4^{3-}\) functional group) with Ch and CMC. The blueshift is detected from the absorption peak of the FTIR spectrum from 1656.85 cm\(^{-1}\) to 1591.27 cm\(^{-1}\). This peak corresponds to N–H stretching vibrations. This peak has resulted from the chemical interaction of Ch (NH\(_3^+\) functional group) with n-HA and CMC. The CH\(_2\) functional group of CMC in the samples is detected at 1467.83 cm\(^{-1}\) for sample B,
and at 1460.11 cm$^{-1}$ for samples C, D, and E. The COO$^-$ functional group of CMC has disappeared in samples C, D, and E, at 1591.27, 1589.34, and 1591.27 cm$^{-1}$, respectively. The COO$^-$ functional group of CMC is undetectable in sample B, probably due to its small composition (CMC 5 wt%). It is important to note that the COO$^-$ in the CMC acts as a crosslink with NH$_3^+$ in the Ch. According to FTIR results, the COO$^-$ have similar spectrum energy with NH$_3^+$ around 1610–1580 cm$^{-1}$. This indicates strong bonding to enhance the compressive strength of the samples.

![FTIR spectrum of nanocomposite n-HA/Ch/CMC with variation in CMC composition.](image)

**Figure 1.** FTIR spectrum of nanocomposite n-HA/Ch/CMC with variation in CMC composition.

To understand the porosity of the samples, we used a liquid displacement method to quantify the porosity. Figure 2 (black line) shows the relations of porosity in the samples with CMC contents. The samples (B, C, D, and E) have porosity around 37.51–61.72%. This is predicted as a standard value of porosity required for the scaffold to use as a template for bone cell regeneration and growth [5]. High porosity is found in the sample without CMC (sample A) at about 85.92%, due to its high Ch content. We found that the porosity decreases with increasing CMC contents. The mechanical strength of the samples increases with increasing CMC content. The highest compressive strength coefficient is found at 5.47 MPa for sample E, as depicted in Figure 2 (blue line). A minimum mechanical strength of the scaffold for cancellous bone application is about 2 MPa. This condition is fulfilled in samples C, D, and E. Moreover, according to Figure 2, we found that the porosity and mechanical strength are strongly correlated. The Ch, which acts as pore-forming [12], has hydrophilic ability to generate a strong bonding with CMC. Consequently, the porosity decreases [18], but it can enhance the compressive strength.
Figure 2. The porosity (black line) and compressive strength (blue line) of nanocomposite-based scaffold n-HA/Ch/CMC.

We visualized the porosity of the samples by SEM measurements, as depicted in Figure 3. The SEM images are taken from the surface (Figure 3a–e) and cross-section (Figure 3f–j) areas of the samples. The SEM results of both surface and cross-sectional areas of the sample reveal the average pore sizes of ~100 µm. This is known as the minimum size required for bone cell regeneration and growth templates [2]. These results explicitly show that the porosity decreases with increasing CMC content, in agreement with the quantification of porosity using liquid displacement method results, as shown in Figure 2.

Figure 3. The surface (a–e) and cross-sectional (f–j) SEM results of the nanocomposite-based scaffold n-HA/Ch/CMC, having average pore sizes of ~100 µm.

The scaffold for BTE application should be able to degrade gradually, since it is implanted in the human body. This gradual degradation is important, because the cells of bone need some time to grow in the scaffold. To observe the biodegradable ability of the samples, we immersed the samples in the SBF solution, and the data was taken at the 1st, 3rd, and 4th weeks. As shown in Figure 4, the CMC strongly affects the biodegradable ability of the samples, wherein, the increase of CMC content can increase the percentage of weight loss. We speculate that this is due to the fact that both Ch and CMC are hydrophilic, and generated a degradation from the hydrolysis process when the samples were...
immersed in SBF solution. The weak C–O bonding (reactive) in the CMC is easy to break when they interact with molecules from the SBF solution.

![Figure 4. Percentage of weight loss of nanocomposite-based scaffold n-HA/Ch/CMC taken in the 1st, 3rd, and 4th weeks.](image)

It is worthwhile to mention that the nanocomposite-based scaffold n-HA/Ch/CMC should be bioactive, biodegradable, containing 30–90% porosity, and require a minimum compressive strength of 2 MPa. According to the compilation data made in Table 2 and the literature study, we conclude that sample C, with 10 wt% of CMC, is considered as the best sample. This sample contains 49.77% porosity, the compressive strength of 3.89 MPa, percent of weight loss at the 4th week of about 29.92%, nearly constant degradation rates, and average porosity sizes of about 92.10–136.00 µm. In this case, the CMC gives a significant impact on the quality of the scaffold, wherein it has a pivotal role to control the porosity and mechanical strength of the composite-based scaffold.

**Table 2.** Compilations of porosity, mechanical strength, and biodegradability data from experiments.

| Parameters                          | CMC Variations   |
|-------------------------------------|------------------|
|                                     | A (0 wt%) | B (5 wt%) | C (10 wt%) | D (15 wt%) | E (20 wt%) |
| Porous diameters (µm)               | 24.80–136.00 | 46.10–155.00 | 92.10–136.00 | 25.46–150.10 | 26.82–177.30 |
| Porosity (%)                        | 85.92 | 61.72 | 49.77 | 44.65 | 37.51 |
| Compression strength (MPa)          | 1.49 | 1.57 | 3.89 | 4.14 | 5.47 |
| Percent weight loss (%)             | First week | 4.66 | 18.49 | 22.90 | 35.40 | 31.60 |
|                                     | Third weeks | 10.91 | 20.94 | 27.37 | 36.82 | 34.61 |
|                                     | Fourth weeks | 11.60 | 21.70 | 29.92 | 37.31 | 35.61 |

After realizing the scaffold with desirable porosity and mechanical strength for BTE application, we also analyzed the thermal properties of this nanocomposite-based scaffold. Here, we used DSC measurement to determine the steam temperature (Tb), glass transition temperature (Tg), decomposition temperature (Td), and crystallization temperature (Tc) at temperature ranges of 25–900 °C, as shown in Figure S1 of the supporting material. We compiled the observed data we obtained in DSC measurements, as shown in Figure 5. We found that the Tb, Td, and Tc tend to increase with increasing CMC, conversely, the Tg tends to decrease with increasing CMC. As previously explained, Ch has opposite charge with CMC, which forms an ionic bonding and induces a cross-link between them [18]. Henceforth, the CMC enhances the chemical bond of n-HA and Ch, generating the reduction of porosity and enhancing the
mechanical strength. In this sense, it may be required a higher thermal energy to perturb the molecules in the samples with higher CMC composition. Therefore, this leads to the variation of steaming ability (Tb), chemical decompositions (Td), and crystallization (Tc) of the samples with a variation of CMC composition. Nevertheless, Td of Ch and CMC components in the nanocomposite is constant at around 290–300 °C, close to the Tm of Ch and CMC at 270 °C and 284 °C, respectively [22,23]. Finally, the Tg particularly describes the flexibility of the polymer chain in the samples. Specifically, the Ch has a rigid structure [24], and its polymer chain is difficult to stretch. Therefore, the Ch content tends to increase the Tg; with sample A (60 wt% Ch) rendering higher Tg compared to the samples with lower Ch composition, and thus, the trend of Tg is the inverse of the variation of CMC composition.

![Graph showing variations in steam temperature (Tb), glass transition temperature (Tg), decomposition temperature (Td) (n-HA, Ch and CMC), and crystallization temperature (Tc) of the samples taken from observation of DSC measurements.](image)

**Figure 5.** The variations in steam temperature (Tb), glass transition temperature (Tg), decomposition temperature (Td) (n-HA, Ch and CMC), and crystallization temperature (Tc) of the samples taken from TGA measurements.

Furthermore, TGA measurements were conducted, to understand the mass changes resulting from applied heating to the samples at 25–900 °C, as depicted in Figure S2 of the supporting materials. Thus, we show the compilation of the observed data we obtained in TGA measurements in Table 3. We found that mass degradation is divided into four regions of temperature ranges. The first range is at 25–178 °C, with the mass degradation of 0.36–3.85% occurring in relation to the water steam point, which agrees with DSC measurement. This determines the Tb of the scaffold at 117–178 °C. The second range, at 178–400 °C, indicates the decomposition of Ch and CMC of 0.70–14.94%. The third range, 400–660 °C, indicates decomposition of HA of 0.71–11.05%. The fourth range, at 660–800 °C, is presumably due to the decomposition of CO$_3^{2-}$ and HPO$_4^{2-}$ in the HA [25]. In general, the percentage of mass degradations decreases with the increase of CMC compositions. We speculate that the CMC reinforces chemical bonding of the scaffold compounds. The functional group of NH$_3^+$ in Ch is strongly connected with the functional group of COO$^-$ in CMC [26]. Thus, it affects the compound degradation.

**Table 3.** The changes of scaffold mass, from TGA measurement.

| Samples | Initial Mass (mg) | Mass Degradations (%) | Total Mass Degradation (%) |
|---------|------------------|------------------------|---------------------------|
|         | 25–178 °C        | 178–400 °C             | 400–660 °C                | 660–800 °C                |
| A       | 45.67            | 3.85                   | 14.94                     | 5.39                      | 0.93                      | 25.12                     |
| B       | 48.50            | 0.59                   | 1.76                      | 11.05                     | 0.65                      | 14.05                     |
| C       | 61.70            | 0.40                   | 1.00                      | 1.58                      | 0.37                      | 3.33                      |
| D       | 80.74            | 0.36                   | 0.70                      | 1.11                      | -                         | 2.17                      |
| E       | 51.00            | 0.52                   | 0.82                      | 0.71                      | -                         | 2.05                      |
We continued the thermal analysis of the samples by using TMA measurements, as depicted in Figure S3 of the supporting materials. Figure 6 depicts a compilation of the observed data we obtained in TMA measurements. It is clear that sample A has the highest shrinkage, due to no CMC content. The scaffold with no CMC is weak when they are mechanically pressed during TMA measurement. These TMA measurements show that CMC has a significant effect on the sample dimension reduction and the mechanical strength of the samples.

![Figure 6. The shrinkage percentage of the scaffold.](image)

Table 4. The expansion coefficient of the scaffold from TMA measurement.

| Samples | Expansion Coefficient (m/m °C) |
|---------|--------------------------------|
|         | 25 °C     | 110 °C    | 282 °C    | 713 °C    |
| A       | $0.11 \times 10^{-4}$ | $-2.01 \times 10^{-4}$ | $-9.37 \times 10^{-4}$ | $-34.11 \times 10^{-4}$ |
| B       | $0.07 \times 10^{-4}$ | $-3.99 \times 10^{-4}$ | $-0.62 \times 10^{-4}$ | $-17.37 \times 10^{-4}$ |
| C       | $0.12 \times 10^{-4}$ | $-3.47 \times 10^{-4}$ | $-5.02 \times 10^{-4}$ | $-11.64 \times 10^{-4}$ |
| D       | $0.12 \times 10^{-4}$ | $-4.25 \times 10^{-4}$ | $-2.24 \times 10^{-4}$ | $-11.38 \times 10^{-4}$ |
| E       | $0.06 \times 10^{-4}$ | $-3.17 \times 10^{-4}$ | $-2.22 \times 10^{-4}$ | $-13.57 \times 10^{-4}$ |

The information from DSC, DTA, and TMA measurements is important to understand the thermal properties of the samples. It is due to the viability of the scaffold, which should be compatible to be implanted in the human body. From the DSC result, the scaffold has no thermal properties by exotherm and/or endoderm. Hence, the scaffold can be used in those temperature ranges similar to that of human body temperature. Moreover, the TGA results show a small degradation at high temperatures, indicating that the samples tend to prevent their compound compositions. The n-HA/Ch/CMC 

![Sample A (40:56:0), Sample B (40:56:5), Sample C (40:50:10), Sample D (40:48:15), Sample E (40:40:20)](image)
4. Discussion

The n-HA had been well-known as potential material for bone implant applications, because it has similar component with bone [25]. However, several limitations of n-HA have necessitated the development of HA-based composite with various reinforcements [3]. Here, we combined n-HA with Ch and CMC to form a composite. Ch, in this case, could be formed as a porous structure for bone cell growth, while CMC serves to increase the mechanical strength of the composite.

The samples were fabricated by various Ch/CMC compositions, to understand the effects of this compound on the physical and biocompatibility properties of the composites, whereas the freeze-drying method was chosen to synthesize this composite-based scaffold due to simplicity, low cost, and ease of preparation. According to the chemical and structural examinations, we found that the porosity and mechanical strength are strongly correlated. The Ch, which is pore-forming [12], has the hydrophilic ability to generate a strong bonding with CMC. Consequently, the porosity decreases [18], but it can enhance the compressive strength. This conjecture is also supported by mechanical property examinations, where the compressive strength of 3.89 MPa was obtained, higher than the required minimum mechanical strength for scaffold for BTE. Moreover, the best sample, containing 10 wt% of CMC, has 49.77% porosity, with average sizes of about 92.10–136.00 µm. This value is adequate for the template for bone cell regeneration. The thermal analysis also shows that the n-HA/Ch/CMC nanocomposite is suitable as a scaffold, since this nanocomposite has a much lower expansion coefficient, compared to human bone, at 25 to 75 °C.

The information from DSC measurements is very important for the feasibility of the composite as a scaffold to be implanted in the human body. The feasibility and quality of the scaffold could be determined from the values of the Tb, Tg, Tc, and Td of the nanocomposite. In particular, the Td is important to determine, because it is related to the stability of the chemical composition of the composite, which further influences the functionality of the scaffold during implantation in the human body. Moreover, the TGA results show that the composite did not show any significant changes. The expansion coefficient of the composite, derived from TMA measurements, is also relatively small, compared to the coefficient expansion of bone, therefore the nanocomposite is implantable.

The abovementioned results imply that the CMC gives significant impact to the feasibility of the composite. In fact, various material combinations with HA are possible to increase the feasibility of HA-based composites and to increase the functionality, especially for BTE application. In this present research, the CMC serves as a composite filler to enhance the mechanical strength of the composite-based scaffold, while keeping the functionality intact. Hence, the n-HA/Ch/CMC nanocomposite is suitable for BTE application. In the present study, we analyzed the morphology, mechanical, and thermal behavior of the prepared n-HA/Ch/CMC nanocomposite for bone application, focusing on the effect of varying the percentage of CMC. The results of this research indicate that attention should be given to the preparation procedure of preparing nanocomposite, with required compressive strength, sufficient porosity, and biodegradation rate being observed. The n-HA/Ch/CMC composite-based scaffolds are the most promising for bone tissue engineering application, because of their transformation in strengthening and degradation capabilities [28].

5. Conclusions

This work demonstrates that it is possible to fabricate the n-HA/Ch/CMC composite scaffolds by a simple, low-cost, and eco-friendly freeze-drying method. The composite scaffolds were evaluated for their morphology, mechanical, degradation, and thermal behavior. CMC played a vital role in improving the compressive strength and bioactivity of the prepared scaffolds. The success of a bone scaffold for bone tissue engineering can be determined by its ability to stimulate and aid in both the commencement and completion of bone defect repair. The biodegradability test designates that the
scaffold composites are degradable naturally over time. In conclusion, the above results indicate that the n-HA/Ch/CMC composite scaffold is a unique biodegradable porous scaffold with essential physicochemical and biological properties for bone tissue engineering applications.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2076-3417/10/19/6970/s1, Figure S1: DSC characterization result of the samples with variation CMC composition; Figure S2: TGA characterization result of the samples with variation CMC composition; Figure S3: TMA characterization result of the samples with variation CMC composition.

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