Investigating the Mechanical Behaviors of Organic/Inorganic Composite Bone Scaffolds

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Investigating the mechanical behaviors of organic/inorganic composite bone scaffolds

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Abstract

The regeneration of bone over a large area cannot occur without a structure for the bone cells to bind and divide. The use of an organic/inorganic composite bone scaffold appears to be a promising alternative to the current clinical standard of bone grafting. Bone grafting is very limited, in that the size and shape of the area are hard to replicate and the use of donor tissue can trigger an immunologic response resulting in rejection of the bone tissue. This study experimented with composite bone scaffolds which can be made to fit the shape of the area in which bone must be regenerated with high mechanical strength and properties that enhance bone growth and mineralization. Graphene oxide was functionalized with bisphosphonate (Bis-GO) to be used in the scaffolds to promote bone mineralization and increase the mechanical strength of the scaffold. Sodium-titanate nanofibers were fabricated to maintain the mechanical strength of the scaffold during biodegradation. Bis-GO and sodium-titanate nanofibers (1.3 mg/mL or 2.6 mg/mL) were integrated into chitosan at different levels, and the mechanical strength of the scaffolds was tested using Instron (Single Column 5944, USA). Compression tests of the scaffold samples showed that composite chitosan/Bis-GO/1.3 mg titanate and chitosan/Bis-GO scaffolds had a significantly higher compressive modulus (p<0.05) than the 4 scaffolds of other combinations.
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1. Introduction

The regeneration of bone occurs in response to injury, such as a small bone fracture, but the process is unsuccessful in larger bone defects occurring from certain injury and disease, such as craniofacial abnormalities. The growth of bone cells over a large damaged area is hindered by the lack of a construct to allow for the cells to grow. Bone is able to grow to a certain critical size, but growth beyond the critical size requires surgical intervention. A current standard clinical practice is bone grafting, which has many limitations, including limited donors, biocompatibility and immunologic rejection, and proper size and shape of the graft to fit the damaged area. Due to these limitations, an alternative method of promoting bone regeneration over a large area is needed which can be used to treat bone injury and disease more accurately and efficiently.

The overall goal of this research is to develop a 3-D artificial bone scaffold on which bone cells will be able to grow to form new tissue. Bone tissue consists of cells, an organic matrix made up of collagen and non-collagenous proteins, and an inorganic matrix made up mostly of hydroxyapatite \((\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2)\). The organic collagen fibrils give bone flexibility, and the inorganic minerals give bone its mechanical strength. The process of bone mineralization is thought to require \(\text{Ca}^{2+}\) and \(\text{PO}_4^{3-}\) ions to interact with phospholipids to form hydroxyapatite nanocrystals. Therefore, important aspects of these bioscaffolds are bioactivity to promote bone mineralization and mechanical strength suitable for bone tissue regeneration.

Bisphosphonate functionalized graphene oxide and sodium-titanate nanofibers were used in this project, and their effects on mechanical behavior of the bone scaffolds were observed. Bisphosphonate functionalized graphene oxide was used in the scaffold due to the
necessity of phosphorous to living organisms in DNA, RNA, cell membranes, bone and teeth, and the fact that bisphosphonates have already been used successfully in tissue engineering\(^4,5\). Sodium-titanate fibers were used for both their strong interwoven structure and the use of sodium ions in the formation of hydroxyapatite. The hypothesis for this portion of the project is that a composite bone scaffold made of the organic and inorganic materials above can be made to increase mechanical integrity of the scaffold and ultimately result in bone regeneration.
2. Materials and Methods

2.1. Graphene oxide synthesis and functionalization

Graphene oxide was synthesized using a modified Hummer’s method. Graphene oxide was then treated to convert surface hydroxyl groups into carboxyl groups. Bisphosphonate (alendronate) was then covalently bonded with the carboxylic acid groups on the graphene oxide.

2.2 Fabrication of sodium-titanate nanofibers

Sodium-titanate nanofibers were synthesized using a modified Stober method consisting of the combination of silicon dioxide, sodium hydroxide and deionized water.

2.3 Chitosan preparation

2% (w/v) chitosan was prepared with 1% (v/v) acetic acid and stirred overnight. The resulting chitosan solution was then partially set aside, and partially combined with Bis-GO. The chitosan that was combined with Bis-GO was stirred for 15 minutes followed by sonication for 5 minutes.

2.4 Scaffold sample preparation

1 mL of chitosan or chitosan/Bis-GO was poured into 5 mL syringes. Sodium-titanate fibers were then added at different concentrations (0, 1.3 and 2.6 mg/mL) to the syringes, creating 6 samples groups. Once the sodium-titanate fibers were well distributed throughout the solution, the syringes were placed in the refrigerator prior to...
freezing at -80°C. The samples were then placed into the lyophilizer (Labconco, FreeZone 4.5) for 5 days. After retrieval of the scaffold samples from the lyophilizer, each sample (n=5) was cut into cylinders (7.5 mm diameter and 3 mm thickness).

2.5 Imaging and analysis

Fourier transform infrared spectroscopy (FTIR) from 4000−650 cm⁻¹ was used to characterize the synthesized graphene oxide and Bis-GO. Transmission electron microscopy (TEM) was used to observe the graphene oxide. Energy dispersive X-ray analysis (EDX) was used to analyze the elemental composition of the graphene oxide products. Scanning electron microscopy (SEM) was used to observe the properties of the developed scaffolds containing only chitosan, sodium-titanate fibers or Bis-GO.

2.6 Mechanical testing

Once the scaffold samples were cut into cylinders, stress, strain, and elastic modulus was then evaluated to measure mechanical strengths of the scaffolds. Instron (Single Column 5944, USA) was used along with BlueHill 3 software to create a mechanical compression test with the crosshead speed of 0.5 mm/min and 5kN load cell. Each sample was compressed to 25% (0.75 mm) of the original thickness (3 mm).
3. Results and Discussion

After the synthesis and functionalization of graphene oxide, EDX was used to analyze the elemental composition of the graphene oxide products. Bisphosphonate functionalization of graphene oxide was successful, as seen by the presence of phosphate and sodium compounds from alendronate.

![Figure 1](image_url)

**Figure 1.** (a) Photographic image of GO, GO-COOH, and Bis-GO in distilled water. (b) TEM image of graphene oxide. (c) FTIR spectra of GO, GO-COOH, and Bis-GO. (d) EDX results of GO-COOH (d) and Bis-GO.

The proper functionalization of graphene oxide with bisphosphonate should allow for enhanced mineralization of bone within the scaffold due to the bone mineralization processes requiring the interaction of Ca\(^{2+}\) and PO\(_4^{3-}\) ions to further interact with phospholipids to form hydroxyapatite nanocrystals\(^3\).
Sodium-titanate nanofiber synthesis resulted in a network of fibers creating a porous, net-like structure with high mechanical integrity.

**Figure 2.** (a) Photographic image of self-assembled sodium-titanate nanofibers. (b) SEM image of entangled, self-assembled structure of sodium-titanate nanofibers at low magnification (10µm). (c) EDX results of sodium-titanate nanofibers.

The EDX spectrum of the sodium-titanate nanofibers displayed the presence of titanium, oxygen and sodium, as expected. The network of fibers, seen in Figure 2(b), should be beneficial in increasing the mechanical strength of the bone scaffold as well as aiding in the mineralization process.

The scaffolds, created by lyophilization of chitosan mixed with different amounts of sodium-titanate fibers and Bis-GO, had different observable characteristics. Upon integration of chitosan and Bis-GO, the solution assumed a darker brown color when compared to the typical opaque yellow chitosan solution. The characteristics of the scaffolds composed of just chitosan, chitosan/sodium-titanate nanofibers and chitosan/Bis-GO can be seen in Figure 3 below.
The SEM images of the scaffold composed of only chitosan (Figure 3a, d, e) can be seen with a limited, poorly integrated structure; there is not a porous network to promote bone growth. The SEM images of the chitosan/sodium-titanate fiber scaffold (Figure 3b, f, g) display a scaffold with a highly integrated porous structure; this is likely much more suitable to allow bone to grow and spread throughout the scaffold. The SEM images of the chitosan/Bis-GO scaffold (Figure 3c, h, i) show a scaffold that exhibits a more densely layered network.

Before mechanical testing, the scaffolds were cut into cylinders 7.5 mm in diameter and 3 mm thick. The scaffolds were made in 6 groups: chitosan only, chitosan/1.3 mg sodium-titanate fibers, chitosan/2.6 mg sodium-titanate fibers, chitosan/Bis-GO, chitosan/Bis-GO/1.3 mg sodium-titanate fibers, and chitosan/Bis-GO/2.6 mg sodium-titanate fibers. The latter 3 scaffolds had a very notable structural difference when view by the naked eye (Figure 4).
The chitosan/Bis-GO scaffold seen in Figure 4a has a very dense and firm-feeling structure with very little porosity. The chitosan/Bis-GO/1.3 mg sodium-titanate fibers scaffold seen in Figure 4b is more porous than the chitosan/Bis-GO scaffold, but is still firm. The chitosan/Bis-GO/2.6 mg sodium-titanate fibers scaffold seen in Figure 4c lost most of its density and was very easily compressed. This, along with the mechanical strength results seen below in Figures 5f and 6 suggest that there is a threshold at which the sodium-titanate nanofibers or the Bis-GO will have too much electrostatic interaction and compromise the mechanical strength of the scaffold. Further tests are required and will be done to assess this threshold.

The stress-strain curves from the compression tests of the samples are seen in Figure 5. The mean elastic modulus was calculated and a comparison of each sample’s compressive modulus is seen in Figure 6. Statistical analysis was performed by means of a one-way ANOVA test, and the results are outlined below Figure 6.
Figure 5. Compression test results of each series of scaffold samples (n=5). (a) chitosan only, (b) chitosan/1.3 mg sodium-titanate fibers, (c) chitosan/2.6 mg sodium-titanate fibers, (d) chitosan/Bis-GO, (e) chitosan/Bis-GO/1.3 mg sodium-titanate fibers, and (f) chitosan/Bis-GO/2.6 mg sodium-titanate fibers.
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Figure 6. Mean elastic modulus with standard deviation for each of the 6 tested groups.

When compared to the compressive moduli of Chitosan, Chitosan/1.3mg sodium-titanate nanofibers and Chitosan/Bis-GO/2.6mg sodium-titanate nanofibers, **Chitosan/Bis-GO** had p-values less than 0.01 (p<0.01), indicating that the compressive modulus of **Chitosan/Bis-GO** was significantly higher than the 3 other samples mentioned.

When compared to the compressive modulus of Chitosan, **Chitosan/Bis-GO/1.3mg sodium-titanate nanofibers** had p-values less than 0.01 (p<0.01), indicating that the compressive modulus of **Chitosan/Bis-GO/1.3mg Titanate** was significantly higher than the sample with Chitosan alone. When compared to Chitosan/1.3mg Titanate and Chitosan/Bis-
GO/2.6mg sodium-titanate nanofibers, Chitosan/Bis-GO/1.3mg sodium-titanate nanofibers had p-values less than 0.05 (p<0.05), indicating that the compressive modulus of Chitosan/Bis-GO/1.3mg sodium-titanate nanofibers was also significantly higher than the samples with Chitosan/1.3mg sodium-titanate nanofibers and Chitosan/Bis-GO/2.6mg sodium-titanate nanofibers.

4. Conclusion

The use of bisphosphonate functionalized graphene oxide and sodium-titanate nanofibers looks to be a promising combination for creating a composite bone scaffold with high mechanical strength that is also able to cover a large area and promote bone mineralization throughout the scaffold. The threshold for Bis-GO and sodium-titanate nanofibers in the chitosan scaffold solution needs to be determined to find a reproducible method of creating a scaffold with the proper structure and mechanical strength required to support the entire bone regeneration process. Once a proper ratio of chitosan to Bis-GO to sodium-titanate nanofibers is determined, bone cell seeding tests need to be performed in the scaffold, and the growth and mineralization of bone throughout the scaffold will need to be determined. With further study, bisphosphonate functionalized graphene oxide and sodium-titanate nanofibers might be used to create a composite bone scaffold that could improve or even save the lives of many people with different bone injuries and diseases.
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