Osteoinductive Calcium Phosphate Clay Nanoparticle Bone Cements (CPCs) with Enhanced Mechanical Properties

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Abstract—**Calcium Phosphate Cements (CPCs) with osteoconductive properties are limited in their applications because of their poor mechanical properties. This study investigated the additive effect of Dexamethasone-doped Halloysite Nanotubes (HNTs on the mechanical properties of CPCs. HNTs are nanosized tubes with alumino-silicate composition. Physico-chemical properties, cytocompatability and cellular functionality of the nanocomposites were assayed. Results suggest that these nanoenhanced composites have a huge potential to broaden the applications of CPCs.**

1. INTRODUCTION

Bone cements are used in setting the bone implants in place and in a variety of dental and orthopedic surgeries. Two commonly used bone cements are acrylic cements and CPCs. Among acrylic cements, the most widely used bone cements are the Poly Methylmethacrylate (PMMA) cements. PMMA cements have been used in orthopedic surgeries since the 1950s because of their mechanical properties and in-situ setting properties [1]. Since its introduction it has been widely used in fixing metallic implants, joint replacement and bone repair surgeries. PMMA bone cements, however, suffer two major limitations. These cements set via polymerization reaction that is exothermic. These temperatures can reach up to 100^{o}C [2] causing surrounding tissue necrosis, implant failure and other post operative complications. The other limitation is monomer toxicity that leaks into surrounding tissues and then into systemic circulation [3].

CPCs have good compatibility because of their composition and biodegradability. These cements are classified as brushite or apatite cements based on the final setting product. Brushites have dicalcium phosphate and apatites have hydroxyapatites as final product, respectively. These two compounds are essentially the inorganic components of bone tissue. These cements are resorbed by the body after the surgery without causing any toxic reactions. CPCs are generally comprised of two calcium phosphate salts and a setting liquid. These salts dissolve in the setting liquid, form nuclei of crystallization and crystallize into hydroxyapatite. These are the hypothesized steps in the process of setting and are not exothermic in nature [4]. The only disadvantage of using CPCs is their mechanical properties. These cements are brittle and do not have good compressional or flexural strengths.

Addition of nanoparticles have been examined as a means to enhance these properties [5] [6]. In this study, HNTs were used as these nanoparticles have an open inner lumen that can be loaded with a diverse set of chemicals.. These chemicals can be growth factors, antibiotics, chemotherapeutic agents, etc. Chemically, HNTs are sheets of alumino-silicate rolled in the form of hollow tubes with the outer surface negatively charged (due to presence of

silica) and lumen having positive charge (due to alumina). These nanotubes have dimension of 0.5- 5µ length and luminal diameter of 15- 100nm [7]. Cytocompatiblity of HNTs has been shown to be cytocompatible with cell viability of 70% at 75µg/mL exposure [8].

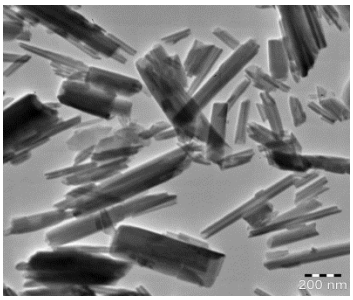

Figure1: Scanning electron micrograph of HNTs.

HNTs loaded with different drugs show an extended release profile [9]. Dexamethasone (Dex) is steroidal anti-inflammatory drug in treatment of autoimmune and other inflammatory diseases. But, at low concentrations of 10^{-7} to 10^{-9} M, Dex was shown to induce osteoblast differentiation. Introduction of growth factors (e.g., Bone Morphogenetic proteins) and osteoblast differentiating agents from bone cements can be an important treatment modality, as it enhances the rate of bone deposition and repair.

HNTs loaded with Dex were added to CPCs and the material properties, chemical composition, and cytocompatibility of this formulation was tested. Compression testing was performed on the specimens chosen. Surface topography was observed with scanning electron microscopy. Using FT-IR, the chemical composition of the CPCs was analyzed. Cytocompatability and cell compatibility of CPCs(with and without doped HNTs) was assessed for changes in cell viablility, proliferation and osteoconductive properties. Osteoinductive CPCs were fabricated with HNTs loaded with Dex and assessed in a similar manner.

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2. MATERIALS AND METHODS

A. CPC Fabrication

CPCs specimens were made using different calcium salts to determine the best composition with respect to mechanical properties. The most suitable formulation was achieved when tetracalcium phosphate (TTCP provided by CaP Biomaterials) and Dicalcium phosphate (DCPA provided by Sigma-Aldrich) were used in solid phase and Chitosan lactate (CL) was used as the setting liquid. Equimolar ratio of TTCP and DCPA were mixed and to this 10% w/v solution of CL was added. This mixture is thoroughly mixed using mortar and pestle to form a soft mass. This mass is allowed to set in different molds depending on assay being performed in humidified chamber maintained at 37^0C.

B. CPC Material Testing

Compression test was performed on cylindrical shape specimens (12 mm length and 6 mm diameter). Compression test was performed on dry specimens and also on specimens placed in simulated biological fluid (SBF) for 24 hrs before testing. Testing was done using ADMET tensile tester with a load speed of 1mm/min.

FTIR spectroscopy was done to analyze the composition of CPCs after setting. Potassium bromide (KBr) pellet method was used for FTIR spectroscopy. Thin discs of KBr were prepared by placing small amount of KBr into the die set and were subjected to a pressure of 8 tons. This pressure caused the KBr to re-crystallize to form thin transparent discs. These discs were used to obtain the background. Small amount of test sample powder was added to KBr and thin discs were prepared by a similar procedure.

C. Cell Culture

CPC scaffolds were fabricated to evaluate osteoconductive nature of CPCs. This set contained different concentration of HNTs (0%, 5%, 10% and 15% of HNTs w/w of TTCP and DCPA powder) that were not loaded with any drug/growth factor. Scaffolds were made as thin discs of 2mm thickness and 10mm diameter. Before seeding cells onto scaffolds they were immersed in 95% ethanol for 5 min and air dried under hood for sterilization. Mouse pre-osteoblast cell lines MC3T3–E1 (ATCC® CRL-2593™) were used for seeding on scaffolds without Dex to evaluate osteoconductive property of CPC fabricated.

HNTs were loaded with Dex using vacuum loading technique [7]. These loaded HNTs were added to CPCs in varied proportions. For these scaffolds, 10% HNTs w/w were used where 5% and 50% of the HNTs used were loaded with Dex. These osteoinductive scaffolds were seeded with mouse bone marrow stromal cells (BSCs) (ATCC® CRL-12424™).

Cells were seeded onto scaffolds and were analyzed for collagen, acidic mucopolysaccharides and alkaline phosphatase (ALPase) activity. Collagen content was estimated using Picrosirus red (PSR) staining while the presence of acidic mucopolysaccrides were determined using Alcian blue. These stains were destained using glacial acetic acid. ALPase assay was done to access the phenotype of the cells as it is characteristic of osteoblasts. BCIP/NBT substrate was procured from Sigma-Aldrich and manufacturer's protocol was followed to assay ALPase activity. These assays were done on 7th and 14th day and for osteoinductive scaffolds, assays were done on 0th, 3rd, 7th and 14th day after seeding them.

3. RESULTS AND DISCUSSION

A. CPC Material Testing

Addition of HNTs resulted in 12% increase of compression strength of CPC/HNT when 10% w/w of HNTs was added. Compressive strengths of different compositions of CPCs is shown in Table 1 below. Particle size of TTCP was observed to play important role in determining the compression strength. Figure 2 shows the plot of compression strength with different TTCP particle size. These specimens did not have HNTs added to their composition.

Table 1: Compressive strenghts of CPCs with different concentration of HNTs.

Composition	Peakload (kg)	Pressure (MPa)
0 % HNTs	63.41±2	17.59±1
5% HNTs	60.10±7	17.18±2
10% HNTs	70.12±5	19.83
15% HNTs	52.09±5	14.72

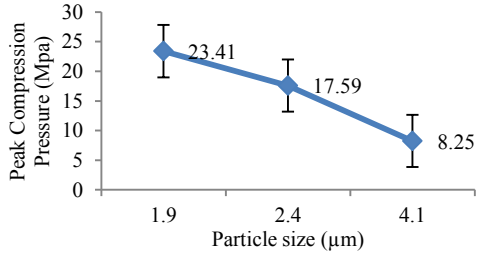

Figure 2 Plot of compression strength with respect to particle size

CPCs specimens were tested for compressive strength after they were immersed in SBF for 24 hr duration. Results suggest that CPCs were resilient. Compression test was stopped when 10% reduction is size was observed. With increase in HNTs concentration, slight increase in compressive strength as shown in Table 2.

Table 2: Compressive strenghts of wet samples.

Composition	Peak load (kg)	Pressure (MPa)
0 % HNTs	9.89	3.12
5% HNTs	10.53	3.13
10% HNTs	8.39	3.23
15% HNTs	11.72	3.33

FTIR

Band of peaks seen at Wavenumber at 900-1200 cm^{-1} are due to PO^{+}_{4} groups present in all the test samples.Peaks at 1450 cm^{-1} are characteristic of CO_3^{2-} group found in HA. Peaks at 3100- 3700 cm^{-1} are due to –OH groups HA [10]. Comparing peaks for –OH group we can observe that CPCs with HNTs have stronger peak than just CPCs. This could be that, presence of nanoparticles acted as seeding agents facilitating precipitation of HA crystals. Figure 3 is the FTIR spectra of CPCs.

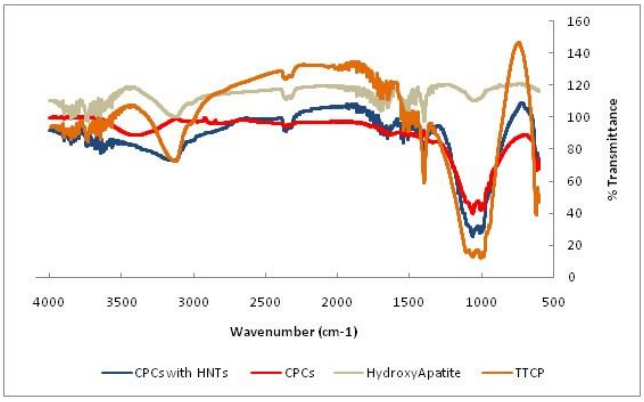

Figure 3 FTIR spectra of CPCs, HA and TTCP

Electron micrographs of the CPCs were taken to access the surface topography. The roughness of the surface promotes cell adhesion and proliferation. In the figure 4, it can be observed that the TTCP and DCPA particles are covered with chitosan lactate (sheet like structures).

Figure 4: SEM image of CPC with 10% HNT.

B. *Cell Culture*

Osteoconductive scaffolds

Scaffolds stained deeply with PSR for 7th and 14th day suggesting collagen was being produced and secreted. Day 7 scaffolds had almost same content of collagen secreted on all concentrations of HNTs. Day 14 collagen content was higher than that of day 7 and maximum collagen staining was observed for CPC scaffold with 15% HNTs w/w.

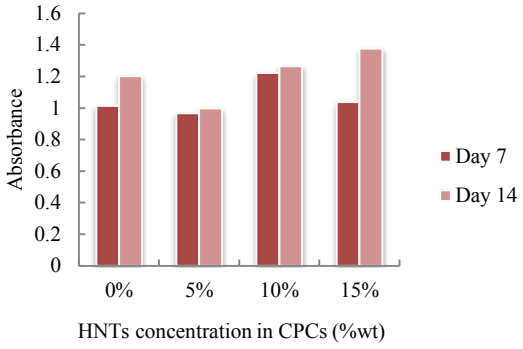

Figure 4: PSR staining for analyzing collagen content.

Similar results were observed for Alcian blue for polysaccharides as shown in Figure 5. Results suggest that preosteoblasts were not negatively affected by the presence of HNTs in the scaffolds. The CPCs showed good biocompability.

ALPase assay supports that cells were osteoblastic and their activity was also as consistent as other staining results. ALPase enzyme activity was quantified and results are compared in the Figure 6

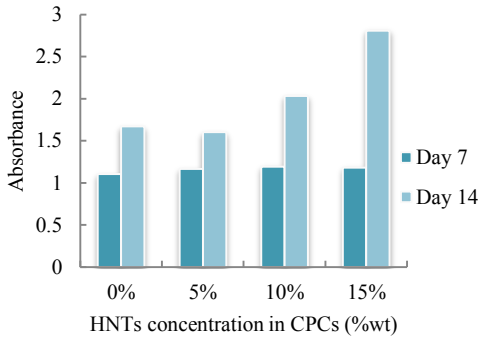

Figure 5: Alcian blue staining for evaluating mucopolysaccharides content.

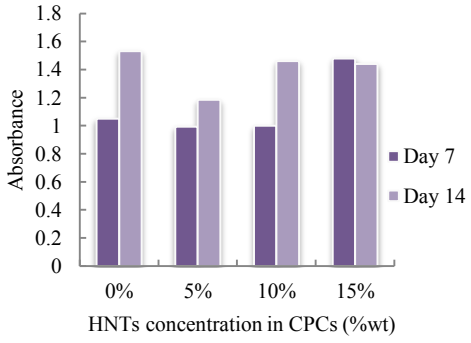

Figure 6: ALPase assay results for day 7 and 14.

- Osteoinductive scaffolds

Four sets of scaffolds were fabricated to evaluate osteoinductive property of CPCs. Setting liquid remains

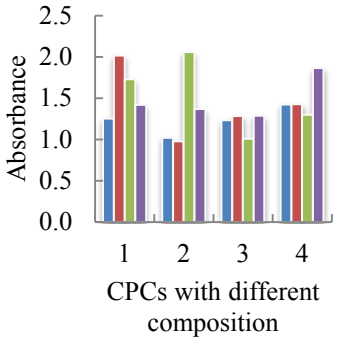

Figure 7: PSR staining for osteoinductive CPCs.

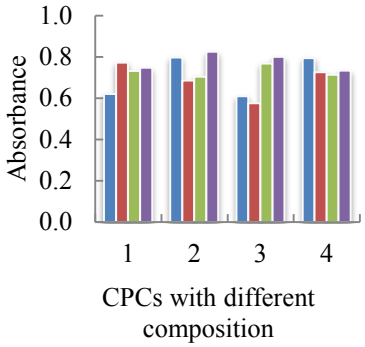

Figure 8: Alcian blue staining

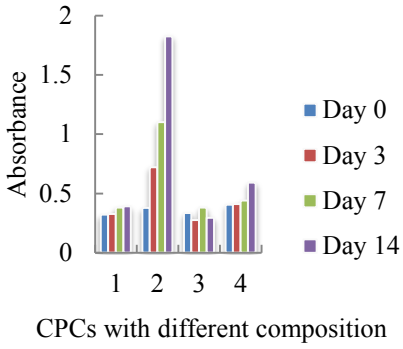

Figure 9: ALPase assay

same for all the formulations except for changes in solid components.

Set 1: CPC scaffolds containing TTCP and DCPA
Set 2: CPC scaffolds with TTCP, DCPA and 10% w/w empty HNTs
Set 3: CPC scaffolds with TTCP, DCPA and 10% w/w HNTs of which 5% are loaded with Dex
Set 4: CPC scaffolds with TTCP, DCPA and 10% w/w HNTs of which 50% are loaded with Dex

These scaffolds were seeded with bone marrow stromal cells. These cells are pluripotent undifferentiatied cells that upon right stimulation can differentiate into osteoblasts or adipocytes or chondrocytes.

Collagen content secreted by BSCs was analyzed with PSR staining. Scaffolds with Dex loaded HNTs showed less collagen secretion compared to other two scaffolds where no differentiating agent in them. Staining of seeded scaffolds was done on day 0, 3, 7 and 14 to evaluate collagen secretion. It can be observed that all the scaffolds secreted good amounts of collagen except for scaffolds with 5% Dex as shown in Figure 7.

Results for alcian blue staining were similar to that of PSR staining, suggesting that same trend was followed on scaffolds for both collagen and acidic mucopolysaccharides secretion. Plot of ECM produced by cells on different scaffolds is shown in Figure 8. Even thought the amount of extracellular matrix (ECM) secretions is different for different CPCs, it is important to consider that all the scaffolds are supporting cell attachment, proliferation and cell secretions are normal and no toxicity is seen on cells.

Interesting results were observed in measurement of ALPase activity. It was predicted that scaffolds with Dex loaded HNTs would promote BSCs differentiation, but the results were something else. Differentiation was more pronounced on scaffolds with blank HNTs and not significant on scaffolds with Dex loaded HNTs. HNTs promoting BSCs to differentiate into osteoblastic phenotype was also observed in scaffolds with HA-chitosan/polygalacturonic acid [11]. This supports that HNTs inherently have differentiating potential in particular for osteoblasts.

5. Conclusions and Future Work

We conclude that CPCs fabricated using TTCP and DCPA as solid phase and 10% chitosan lactate solution as setting liquid have good osteoconductive nature with improved mechanical properties. Scaffolds with Dex loaded HNTs were osteoinductive and have good cytocompatibility. HNTs loaded can be loaded with different agents like chemotherapeutic agents, growth

6. References

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