CHARACTERIZATION OF BIOMIMETIC CALCIUM PHOSPHATE DEPOSITED ON CHITOSAN FOR ORTHOPEDIC IMPLANT APPLICATIONS

+Chesnutt, BC; +Yuan YL; *Yang Y; *Ong JL; +Haggard WO; +Bumgardner JD
Joint Program in Biomedical Engineering,+University of Memphis and *University of Tennessee-Memphis, Memphis, TN;
bcmann@memphis.edu

Introduction
Chitosan is a deacetylated form of chitin, a natural polysaccharide found mainly in the exoskeletons of crustaceans. Chitosan is an attractive biomaterial because it is very biocompatible, and has been shown to enhance wound healing, promote cell adhesion and migration, and to have antimicrobial properties [1]. Calcium phosphate is widely used as an orthopedic biomaterial because of its osteoconductive properties [2,3]. Recently, biomimetic deposition of calcium phosphate over natural polymers such as chitosan has shown promise as an approach to produce composite scaffolds for bone tissue engineering or orthopedic implant coatings [4]. However, many factors may affect the structure and function of these materials, including the degree of deacetylation of the chitosan, the composition of the solution used to create the biomimetic calcium-phosphate coatings, and the length of time biomimetic deposition is allowed to occur. In this study, we investigated the effect of these parameters on the composition and crystallinity of calcium phosphate biomimetically deposited on chitosan, as well as the effect on osteoblast cell growth and mineralization.

Materials and Methods
Films were made from 92.3% and 80.6% de-acetylated chitosan by dissolving 1 g chitosan powder in 100 mL 1% acetic acid. The resulting solutions were poured into round Teflon dishes and allowed to air dry for five days. The films were then neutralized with NaOH and washed three times with de-ionized water. To form the biomimetic calcium-phosphate coating, the films were first phosphorylated and then rinsed with de-ionized water [4]. The films were then soaked in saturated Ca(OH)2 for eight days. The lume-soaked films were suspended vertically on cotton threads in a glass beaker filled with 1.5X or 1.0X simulated body fluid [4]. SBF was replaced every other day, and films were removed at 7, 14, and 21 days.

The mineralized films were viewed with a scanning electron microscope equipped with an energy dispersive spectrophotometer. An EDS spectrum of each film was taken to determine the Ca/P ratio, and elemental mapping was used to examine the location of calcium and phosphorus atoms on the surface. X-ray diffraction (XRD) was used to characterize the crystal structure of the coating. The dissolution of the coatings was investigated by incubating 6 of each type of film with 0.5 mL DMEM. Media was removed on Days 1, 3, 5, 7, 9, 11, and 13, and the amount of calcium in the solution was quantified (Calcium reagent kit, Pointe Scientific). Plain chitosan films were used as controls. Films were also removed for examination by SEM after 7 and 13 days.

To measure osteoblast attachment and growth on the mineralized films, 3 samples of each film were placed in a 24-well plate and sterilized by exposure to ethylene oxide gas. UMR106 cells were seeded onto the films at a density of 1x10^4 cells/cm^2 and cultured for one week in DMEM with 10% FBS and 1% antibiotic/antimycotic. After seven days, the cells were lysed and the total dsDNA was measured as an estimate of cell growth. Alkaline phosphatase activity was also measured as a marker of bone cell phenotype. To examine osteoblast extracellular matrix production, human osteoblast cells (NHOSa, Clonetics) were seeded onto sterilized films at a density of 1x10^5 cells/cm^2. Cells were cultured for 14 days in OBM cell media supplemented with 10%FBS, 1% antibiotic/antimycotic, ascorbic acid, hydrocortisone, and β-glycerophosphate. After 2 weeks, the matrix was dissolved by incubating the films in 0.5 M acetic acid containing 1mg/mL pepsin for 24 hours. Collagen released into the solution was then measured with a sandwich ELISA. Total protein produced was also measured using the Bradford reagent.

Results
XRD analysis of the mineralized films revealed that the crystallinity of the calcium phosphate coatings was influenced by the degree of deacetylation (DDA) of the chitosan substrate as well as the concentration of SBF. Coatings on chitosan soaked for 21 days in 1.5X SBF had a crystal structure similar to hydroxyapatite, while the calcium phosphate deposited on chitosan soaked in 1.0X SBF was primarily amorphous. In addition, coatings on 92.3% DDA chitosan were also more crystalline than coatings on 80.6% DDA chitosan. As shown in Figure 1, SEM images of the mineralized films supported this, with the coatings on films soaked in 1.5X SBF appearing more crystalline than those soaked in 1.0X SBF. The average Ca:P ratio was 1.8 which is slightly higher than the Ca:P ratio of hydroxyapatite, and there were no significant differences in Ca:P ratio observed between the different films. Elemental mapping revealed that calcium and phosphorus were uniformly present and co-localized on the surface of the films. Dissolution studies revealed that the smallest amount of calcium was released from 92.3% DDA films soaked in 1.5X SBF for 21 days, although some reprecipitation of calcium phosphate may have occurred because the cell culture media used in this study also contained calcium and phosphates. This corresponds to the coatings with the highest crystallinity. In addition, several films soaked in 1.0X SBF had areas where the coating appeared to be completely removed after 2 weeks in cell media, and no similar areas were found on any film soaked in 1.5X SBF.

There were significant differences in cell growth between the films. Although cells were able to grow on all films at least as well as on uncoated chitosan, there were significantly more cells on 92.3% DDA films soaked in 1.0X SBF for 7 or 21 days (Figure 2) than on any other films (p<0.05). Collagen produced by NHOSa cells was similar on all films, although, in contrast to cell growth, total protein production was highest on 80.6% DDA films soaked in 1.5X SBF for 21 days.

Conclusions
The degree of deacetylation of chitosan and concentration of SBF has a significant effect on the crystallinity of biomimetically deposited calcium phosphate coatings. The most crystalline coating developed on 92.3% DDA chitosan soaked in 1.5X SBF, and this coating also dissolved the least in cell culture media. Cells grew best on coatings developed in 1.0X SBF, but protein production was higher on films soaked in 1.5X SBF. This unique response may allow orthopedic implant surfaces or composite tissue engineering scaffolds to be customized to selectively induce osteoblast growth or matrix production.

References

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