BONE FORMATION IN VIVO ON CALCIUM AND PHOSPHATE INDUCED CHITIN MATERIALS

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Abstract
Hydroxyapatite ceramics has been paid attention as bone filler, and bone graft substitutes, due to its biocompatibility and osteo-conduction. Recent studies have found a new surface functionalisation method for creating favorable local conditions that lead to nucleation and growth of calcium phosphate over substrates. Phosphorylation is a surface functionalization method for that purpose. Authors have conducted calcium phosphate coating over chitosan films that were produced by a process based on phosphorylation, Ca(OH)2 treatment and SBF immersion. The obtained samples were commercial chitin, phosphorylated chitin, Ca(OH)2 treated phosphorylated chitin and Ca(OH)2 treated phosphorylated chitin after immersion in SBF solution. These materials were implanted at cranial bone of rats, and the biocompatibility was estimated. 4 weeks after implantation, fibrous tissues were observed between bone and commercial chitin or phosphorylated chitin material. Little fibrous tissue, although was found in the interface between bone and Ca(OH)2 treated phosphorylated chitin after immersion in SBF solution. The Ca(OH)2 treatment and SBF immersion facilitates the formation of a calcium phosphate over the phosphorylated samples in vivo as well as in vitro.

INTRODUCTION
The search for new and better materials for various biomedical applications has been the subject of intensive research as the number of tissue replacement applications increase. Hydroxyapatite ceramics has been paid attention as bone filler, and bone graft substitutes, due to its biocompatibility and osteo-conduction. Research into the stimulation of hydroxyapatite growth on substrate has employed a number of techniques
including the procedure for raising the ionic activity products of hydroxyapatite in the solution containing the substrate to be coated so stimulation precipitation and the creation of apatite nucleation sites [1], and the surface modification by surface graft polymerization of a phosphorous-containing monomer inducing the deposition of Ca and PO₄ ions in the form of a carbonated hydroxyapatite layer firmly bonded with the materials [2]. Recent studies have found a new surface functionalisation method for creating favorable local conditions that lead to nucleation and growth of calcium phosphate over substrates. Authors studied a useful method for creating the favorable local conditions leading to the nucleation and growth of calcium phosphate on fibers [3]. This method of phosphorylation and partial hydrolysis of introduced phosphorous containing functionalities by Ca(OH)₂ treatment resulted in hydrolysis products in intimate contact with the substance. In this present study, phosphorylation as a method for creating favorable local conditions leading to the nucleation and growth of calcium phosphate on cellulose or chitin fiber at body temperature was studied.

In this work, a calcium phosphate coating over chitosan films was produced by a process based on phosphorylation, Ca(OH)₂ treatment and SBF immersion. These materials were implanted at cranial bone of rats, and the biocompatibility was estimated.

**EXPERIMENTAL PROCEDURE**

All chemicals used in this study were supplied by Wako Pure Chemical Industries Ltd. or Katayama Chemicals and used without further purification. Commercial chitin (Beschitin-W) was supplied by Unitika Ltd. Phosphorylation of chitin samples was carried out following the method described in an earlier report [3]. 12 sheets (1-2 g) of chitin were placed into a round bottomed flask equipped with a thermometer, mechanical stirrer, condenser, and N₂ gas inlet tube. 40 g of urea was added to the flask along with 200 mL of DMF. This solution was heated up to a temperature of 110 °C at which point a solution of 32 g of 98 % H₃PO₄ in 50 mL DMF was added. After further heating, the temperature of the reaction was increased to 155°C and was left to reflux for 1 hour. At the end of the refluxing time, the reaction mixture was left to cool under N₂ gas flow. The reaction solution was subsequently filtered off and the fibers washed thoroughly in water to rinse out excess phosphoric acid. Polyvinyl alcohol was added to the phosphorylated chitin fibers, and dried. The dried specimen was uni-axial pressed to make (a) pellets, which were later soaked in saturated Ca(OH)₂ solution (pH=12.4) for about 1 week. The Ca(OH)₂ solution was renewed every 4 days. The Ca(OH)₂-treated phosphorylated chitin samples were placed into plastic screw-top flask to which 10-20 mL of 1.5xSBF solution added. The 1.5xSBF solution was
prepared in the same manner of an earlier publication [1]. The pH of the chitin/1.5xSBF solutions was then measured with the flasks being immersed in a thermostatically controlled 36.5 °C covered water bath for periods of 7 days. For soaking periods of greater than one day, the 1.5xSBF solution was replaced each day and the pH of the old solution measured. These materials were implanted in vivo in subcutaneous back skin or cranial bone of rats at the age of 3 months, sacrificed 2, 4, 8 weeks after implantation, and the biocompatibility and bone formation was estimated.

Scanning electron microscopy and EDX analyses were performed using a Hitachi S-530 scanning electron microscope and a Horiba EMAX-2200 X-ray microanalyzer. All micro-FTIR spectra were recorded of samples encased in a transparent KBr matrix on a Jasco Micro-FTIR Jansen Fourier transform infrared spectrometer and ATR technique also was applied. All 13C and 31P MAS NMR spectra were recorded on a Bruker MSL-200 NMR spectrometer using a magic angle spinning rate of 3 KHz and referencing to 85% H3PO4. Determination of P content of the chitin fiber was analyzed by using a Nippon Jarrell-Ash ICAP-1000S ICP-AES instrument. Each phosphorylated chitin sample was dissolved in a mixture of water, H2O2 and H2SO4 and then diluted to apply to the analysis.

RESULTS AND DISCUSSION

EDX spectra of the phosphorylated chitin fibers clearly showed that P was present in the fibers. The ICP measured phosphorous content of the phosphorylated chitin was found to be at 2.95 wt%. The appearance of the phosphorylated chitin under the electron microscope also appeared unchanged from that of unphosphorylated chitin fibers. The Micro-FTIR spectra of unphosphorylated chitin and phosphorylated chitin indicated that the typical chitin band at 1000 cm⁻¹ band due to C=O stretching on the unphosphorylated chitin was overlapped by P-O stretching band at 1050 cm⁻¹ from PO4 groups chemically bonded to chitin. The presence of a small peak at 800 cm⁻¹ may also be attributed to the P-O bending band after phosphorylation of the chitin fiber. 13C MAS NMR spectra of the phosphorylated chitin fibers indicated that C-6 chemical shift position are obvious indicating that phosphorylation takes place in this region. The 31P MAS NMR spectrum of the phosphorylated chitin fibers showed an intense and narrow peak at 0.91 ppm was observed indicating the PO4 functionalities attached near the C-6. In addition, presence of a small peak at -9.9 ppm indicates that another compound is formed which is believed to be ammonium hydrogen phosphate.

Phosphorylated chitin soaked in Ca(OH)₂ solution appeared to be covered with a thin yet observable coating. When EDX analysis over large area was carried out, a Ca:P
ratio of 1.78 was obtained indicating that a large amount of Ca in the sample which can be either attributed to Ca(OH)$_2$ or CaCO$_3$. High magnification EDX analyses of the coated fibers gave Ca:P ratio of 1.29. It has been speculated that at pH>6.3, octacalcium phosphate (OCP) is the preferred precursor for supersaturation in solutions containing Ca$^{2+}$ and HPO$_4^{2-}$ ions. It is assumed that Ca(OH)$_2$ has partially hydrolyzed the chitin-PO$_4$ groups to initially produce perhaps OCP as an initial phase that has then rapidly transformed to calcium deficient apatite. In contrast, phosphorylated fibers not subjected to the Ca(OH)$_2$ treatment did not exhibit calcium phosphate growth upon immersion in 1.5xSBF solution.

Soaking of Ca(OH)$_2$-treated phosphorylated chitin fibers in 1.5xSBF solution was found to lead to the deposition of a calcium phosphate layer. It is believed that the thin coatings of calcium phosphate material on the fibers produced by partial hydrolysis of the chitin PO$_4$ functionalities by soaking in saturated Ca(OH)$_4$ solution act as a nucleation layer upon which the calcium phosphate can grow from 1.5xSBF solution. In general, the growth of calcium phosphate from 1.5xSBF solution begins immediately after 1 day of soaking. This suggests that clusters have partially dissolved upon introduction of the chitin into the 1.5xSBF solution. The growth of calcium phosphate layer after soaking for 1-6 days appears to proceed by nucleation on the existing coating in the form of circular flakes which then grow in number and size on the surface. After soaking for 9-17 days, a thicker coating was observed. Fig.1 depicts the bending strength of chitin, phosphorylated chitin, Ca(OH)$_2$ treated phosphorylated chitin and Ca(OH)$_2$ treated phosphorylated chitin after soaking in SBF for 7 days. The Ca(OH)$_2$ treated phosphorylated chitin soaked in SBF solution for 7 days shows the improvement in bending strength, which may be due to the formation of calcium phosphate and chitin composite material. The micro-FTIR spectrum of the coating material grown on the chitin fiber after 9 days soaking in 1.5xSBF solution. Characteristic phosphate associated bands at 1031, 601 and 563 cm$^{-1}$ are observed. The
EDX measured Ca:P ratio over a large area of the calcium phosphate coating suggests Ca-enrichment due to the presence of calcium carbonate. The Ca:P ratios measured for individual coatings deposited on Ca(OH)2-treated phosphorylated chitin fibers were within 1.29-1.55, suggesting Ca-deficient apatite.

Fig.2 shows the micrographs of the histologic samples after 4 weeks (in vivo) in subcutaneous back skin of rats at the age of 3 months where original magnification was 10 and samples was alizarin red strained. No inflammatory cells and giant cells were found, and these samples shows good biocompatibility. The fibrous layer was not found in Ca(OH)2-treated phosphorylated chitin sample but found in phosphorylated chitin sample. So the latter was recognized as alien substances, but calcium phosphate and chitin composite can directly adhere to tissue. Fig.3 shows the micrographs the histologic samples after 4 weeks (in vivo) in the cranial bone. Much amount of bone was formed around Ca(OH)2-treated phosphorylated chitin sample than phosphorylated chitin sample. Calcium phosphate of the sample may induce calcification in vivo.

Fig.2 Micrographs of the histologic samples after 2 weeks in vivo in subcutaneous back skin of rats at the age of 3 months. (a) Phosphorylated chitin and (b) Ca(OH)2-treated phosphorylated chitin. Magnification x10, alizarin red stain.

Fig.3 Micrographs of the histologic samples after 4 weeks in vivo in cranial subcutaneous back skin of rats at the age of 3 months. (a) Phosphorylated chitin and (b) Ca(OH)2-treated phosphorylated chitin. Magnification x40, alizarin red stain.
CONCLUSION

The present study has demonstrated that the technique of phosphorylation and partial hydrolysis of introduced containing functionalities by Ca(OH)₂ treatment to create hydrolysis products in intimate contact with the substrate has been found to be a useful method for creating the favorable local conditions leading to the nucleation and growth of calcium phosphate. The obtained calcium phosphate and chitin composite materials showed good biocompatibility and bone formation ability in vivo.

REFERENCES