골조직 공학에서의 BMP가 코팅된 스캐폴드의 XPS 및 FT-IR 연구

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XPS and FT-IR Study on BMP Coated Scaffolds for Bone Tissue Engineering

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ABSTRACT

수산화 아파타이트 스캐폴드를 스펀지 방법을 이용하여 제조 하였다. 제조한 수산화 아파타이트 스캐폴드를 침적코팅을 이용하여 표면에 젤라틴으로 코팅 하였다. 젤라틴을 코팅한 수산화 아파타이트 스캐폴드에 두 가지 다른 코팅 방법인 침적코팅과 항원-항체반응을 이용하여 골형성단백질을 코팅하였다. 제조한 스캐폴드의 상분석과 생물학적 특성을 코팅하지 않은 순수 수산화 아파타이트 스캐폴드와 비교, 분석 하였다. Fourier transform infrared spectroscopy와 X-ray photoelectron spectroscopy를 이용하여 상분석을 하였고, 세포의 증식 및 분화를 측정 하기 위해 cell counting kit와 alkaline phosphatase시험을 진행 하였다. 상분석 결과 수산화 아파타이트 표면에 젤라틴 및 골형성 단백질이 코팅이 되었다는 것을 알 수 있었으며, X-ray photoelectron spectroscopy결과에서 탄소와 칼슘의 양을 확인하여 침적코팅에 비해 항원-항체반응을 이용한 코팅 방법에서 골형성 단백질이 더 잘 코팅 되었다는 것을 확인 할 수 있었다. 또한, cell counting kit와 alkaline phosphatase시험에서 골형성 단백질을 코팅한 수산화 아파타이트 스캐폴드가 세포의 증식 및 분화에서 더 좋은 결과를 나타내는 것을 알 수 있었으며, 특히 3일 이후부터, 세포 분화에서는 항원-항체 반응을 이용한 코팅 방법에서의 스캐폴드에서 더 높은 세포 분화를 확인 할 수 있었다. 이 연구를 통해 수산화 아파타이트 스캐폴드에 골형성 단백질을 코팅 할 경우, 침적코팅 방법 보다 항원-항체 반응을 이용하여 코팅 했을 때, 세포의 분화가 더 향상 된다는 것을 알 수 있었다.

KEY WORDS: Bone morphogenic proteins, Fourier-transform infrared spectroscopy, Hydroxyapatite, Scaffold, X-ray photoelectron spectroscopy

Introduction

The biomaterial for bone tissue engineering demands three properties; biocompatibility, osteoconductivity and osteoinductivity. The autografts have the advantages of being histocompatible and non-immunogenic, where they have minimal risk of transferring infectious diseases, while being osteoinductive and osteoconductive(Malak et al., 2008). However, such autografts have disadvantages of being limited in quantity, and relatively expensive. Hence the synthetic biomaterials have been researched to replace the autografts.

Synthetic bioceramics were widely used as the biomaterial for bone tissue engineering. The hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, and β-tricalcium phosphate (β-TCP), $Ca_3(PO_4)_2$, are well-known bioceramics which are biocompatible and bioactive. The β-TCP has a stoichiometry similar to amorphous bone precursors, whereas HA has a stoichiometry similar to bone mineral(Giannoudis et al., 2005).

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These materials exhibit high biocompatibility with surrounding living tissue and high osteoconductivity characteristics(Descamps et al., 2008). However, they still lack one of the important requirements as the synthetic bone graft substitutes, the osteoinductivity. One method of enhancing the bone formation and reconstruction is to perform protein therapy, which involves the local application of osteoinductive growth factors, such as bone morphogenic proteins (BMP)(Crouzier et al., 2011). The BMP has been used for biomaterials by means of coating and releasing methods(Nie et al., 2007; Kang et al., 2011; Takahashi et al., 2005). Some of research showed that the control of BMP releasing affects the osteogenesis and bone growth(Zhao et al., 2010). The slowly releasing of BMP was shown to enhance the bone growth and osteogenesis compare with fast releasing BMP.

Also, the rate of osteogenesis is known to be affected by the quantification of BMP, and the amount of BMP coated on the scaffold is different according to the coating methods. The coating of the BMP on scaffold can be achieved either physically or chemically. One of the physical methods of coating is the dip coating method, which is the simple process for depositing a thin film of solution onto matrix. The liquid film formation is achieved by two main mechanisms; gravity draining of liquid solution and evaporation of solvent(Yimsiria et al., 2006). The chemical coating of BMP on the scaffold is achieved by creating chemical bond between matrix and BMP. Among the chemical coating method, the antibody-antigen coating method has been used for the protein coating, which involves a gamma globulin protein known as the antibody that is used in the immune system to identify foreign antigen. Such binding of antibody with antigen is a highly specific interaction, which allows antibody to identify and bind only to their unique antigen(Zhao et al., 2010).

Several synthetic scaffolds, such as biodegradable polymers and bioactive ceramics, which have been developed to sustain the wound site and subsequently replace and regenerate new tissues, have proven to be applicable clinically(Kim et al., 2005). Some of the essential features of the scaffolds are; the interconnected highly porous network for cell growth and nutrients flux(Seyed-Iman et al., 2010), the appropriate surface chemistry for cell adhesion and proliferation, and the mechanical properties for the support of the tissues as they grow(Jack et al., 2009). In addition to these, the osteoinductivity is also important features for tissue engineering scaffold.

In this study, the HA scaffold having biocompatibility and osteoconductivity was used as the matrix for BMP coating. After the gelatin coating of HA scaffold for high strength and chemical bonding with BMP, the BMP and antibody were coated on HA scaffold by chemical covalent bond. Hence the purpose of this study was to create HA scaffold with enhanced biological response. In addition, the osteogenic properties were maintained for long period of time as the BMP coating methods were used.

Material and method

1. Fabrication of hydroxyapatite scaffold

The HA scaffold was fabricated by template casting method. The HA slurry was prepared by mixing of HA nano powder (Ossgen, Korea) and organic additives (5% polyvinyl alcohol, 1% methyl cellulose, 5% ammonium polyacrylate dispersant, and 5% N, N-dimethylformamide drying agent) into distilled water. Before the HA slurry coating, the polyurethane sponge was treated by NaOH solution to generate the OH group on the sponge. The sponge was then coated with slurry and dried at room temperature for 12 h and HA scaffold was sintered at 1250℃ for 3 h.

2. Physical coating of BMP-2 and gelatin on hydroxyapatite scaffold

The 3 wt% gelatin from the bovine skin was melted in distilled water at 45℃ and the 0.2% glu-

taraldehyde was added to the gelatin solution for the crosslinking of gelatin. After the coating of HA scaffold with the gelatin using the dip coating method, gelatin coated scaffold was dried at 40℃ vacuum drying oven for removal of the glutaraldehyde.

The scaffold coated with gelatin was then coated with BMP-2 using the dip coating method. The scaffold was soaked into BMP-2 solution at vacuum environment and following 1 h, the scaffold coated by BMP-2 was dried at room temperature for 24 h. The BMP-2 was coated physically by the mechanical forces of gravity through draining of liquid solution and evaporation of solvent.

3. Chemical coating of BMP-2 and gelatin on hydroxyapatite scaffold

For the chemical coating of BMP-2 and gelatin on HA scaffold, first the gelatin was coated by same method as the physical coating method. The coating of the gelatin on HA scaffold resulted in formation of amine group on its surface which allow bonding with the Traut's reagent (2-Iminothiolane·HCl) (Sigma-Aldrich, USA) that was applied to the gelatin coated scaffold. The sulfo-succinimidyl derivative (Sulfo-SMCC, Sigma-Aldrich, USA) was treated with BMP-2 antibody (Zhao et al., 2010) which also formed strong bond due to the amine group present on the Sulfo-SMCC. The mixture of the Sulfo-SMCC and BMP-2 antibody was then applied to the gelatin coated HA scaffold that was treated with the Traut's reagent, which allowed formation of reaction bond between Traut's reagent and the Sulfo-SMCC. Such coated scaffold was then soaked into BMP-2 solution for the conjugation between BMP-2 and antibody on the scaffold's surface. After 3 hours of reaction between BMP-2 and antibody, the scaffold was washed with phosphate buffer saline (PBS).

The two types of sample (HAP and HAC) were prepared by above processes as well as control sample (uncoated HA scaffold, HA) and designated with code for the purpose of this paper (Table 1.).

4. Surface characterization

The surface functional group of gelatin coated HA scaffold was analyzed by Fourier-transform infrared (FT-IR) spectroscopy where the presence of gelatin was measured by identifying amide groups. After the BMP-2 coating on HA scaffold, the surface composition of scaffold was measured by X-ray photoelectron spectroscopy (XPS). The presence of the BMP-2 on the coating layer was analyzed by the peaks of C, N, O et al. and the intensity of C and Ca peaks were used to quantify the BMP-2.

5. Biological evaluation

The MC3T3-E1 cells (mouse osteoblast cells, ATCC, USA) were cultured in α-MEM medium (WelGene, Korea) with 10% fetal bovine serum (Gibco, USA), 1% penicillin (Gibco, USA) in 37℃, 5% CO_2 incubator and used for *in vitro* tests. The cells (1×10^5 cells/100 μl) were seeded on samples for 1, 3, 7, 14 days. The cell proliferation was measured by cell counting kit (CCK-8, Dojindo Laboratories, Japan). The absorbance was read at 450 nm with an ELISA reader (Benchmark Plus, USA). The cell differentiation was measured by measuring the level of Alkaline Phosphatase (ALP) activity using Sensolyte®

Table 1. The designated code and description of samples

Designated Code	Description of the sample	BMP-2 coating methods
HA	HA scaffold	Control
HAP	HA scaffold/Gelatin and BMP-2 coating by physical bonding	Dipping coating
HAC	HA scaffold/Gelatin and BMP-2 coating by chemical bonding	Antigen-Antibody coating

pNPP ALP assay kit (Anaspec Inc., USA). The cells were lysed using Triton X-100 from the kit and reacted with working solution. The absorbance was measured at 405 nm.

Results and Discussion

Fig. 1 shows the FT-IR results of all of test samples. For HA, the spectra showed OH^- absorption band at 3575 and 637 cm^{-1} with the v_1, v_3 and v_4 bands of phosphate groups at 959, 1049-1094 and 570-600 cm^{-1}, respectively. Addition to bands present for HA, the amide I band and the amide A band was evident at 1638 cm^{-1} and 3440 cm^{-1} , respectively for the both HAP and HAC which indicated that the gelatin was well-coated at HA scaffold surface on these samples, concurring with other papers(Muyonga et al., 2004; Pereda et al., 2011). However, presence of BMP-2 coating on HAP and HAC was difficult to be analyzed using the FT-IR.

Hence the XPS examination was carried out for above reason, focusing the bonding energy (BE) between 0-1100 eV as shown in Fig. 2. The Fig. 2 showed that the surface components of BMP-2 and BMP-2 coated HA scaffolds (HAP and HAC) by XPS analysis. The C 1s, N 1s, O 1s, Na 1s and Cl 2p peaks were present at BMP-2 surface (Fig. 2 (a)) whereas the similar peaks were identified for the HAP and HAC surfaces (Fig. 2 (b)), along with additional peak of the Ca 2s, Ca 2p, P 2s and P 2p peaks that corresponds to the surface composition of hydroxyapatite(Yana et al., 2003). Hence the XPS results showed that the BMP-2 was well-coated on both HAP and HAC. Fig.3 showed the C 1s and Ca 2p XPS survey spectra of HAP and HAC. At C 1s spectra (Fig. 3 (a)), the intensity of HAC peak was higher than HAP. In contrast with C 1s, the intensity of Ca 2p peak was higher at HAP (Fig. 3 (b)). Hence these results show that the HAC was the better method at BMP-2 coating.

Fig. 4 shows the cell proliferation result following 1, 3, 7, and 14 days of cell culturing that was measured using CCK-8. The optical density (OD) value was similar between HAP and HAC, which were both higher than the OD value of HA. This result showed that the BMP-2 coated scaffold resulted in better cell viability than uncoated scaffold, though method of the BMP-2 coating did not seem to affect the cell viability.

Fig. 5 shows the ALP activity of each sample. At day 1, all of the samples showed similar ALP activity level. However, on day 3, the HAP and HAC have shown higher level of ALP activity compared to HA, where the HAC have shown to result in enhanced ALP activity compare to HAP. Finally on day 14, the HAC showed the highest ALP activity than others. This result indicated that the BMP-2 coating enhanced the cell differentiation and use of antigen-antibody coating method for BMP-2 resulted in better ALP activity level compare to those samples prepared by dip coating method. The reason of enhanced cell proliferation and differentiation rate on the chemically coated scaffold was due to the maintenance of BMP-2 release for sufficient period of time due to the chemical bond that was present between BMP-2 and the antibody (Zhao et al., 2010).

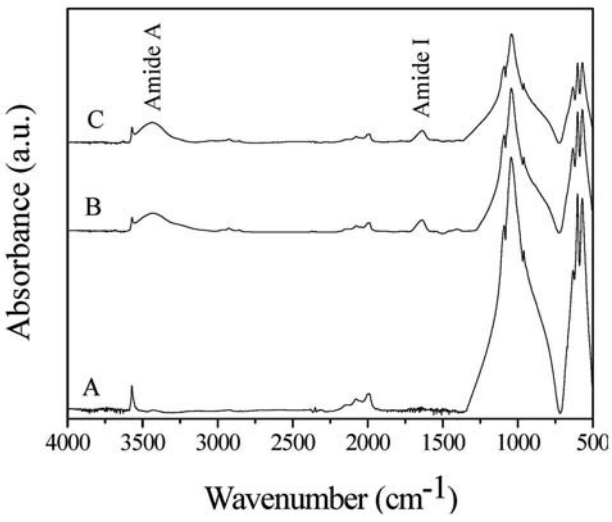

Fig 1. The FT-IR results of (A) HA, (B) HAP and (c) HAC.

Conclusion

The BMP-2 coated scaffold was successfully fabricated using two different methods of dip coating and antigen-antibody coating methods. It was shown that both methods were adequate in terms of coating BMP-2 on the scaffolds as shown by FT-IR and XPS, though antigen-antibody coating method re-

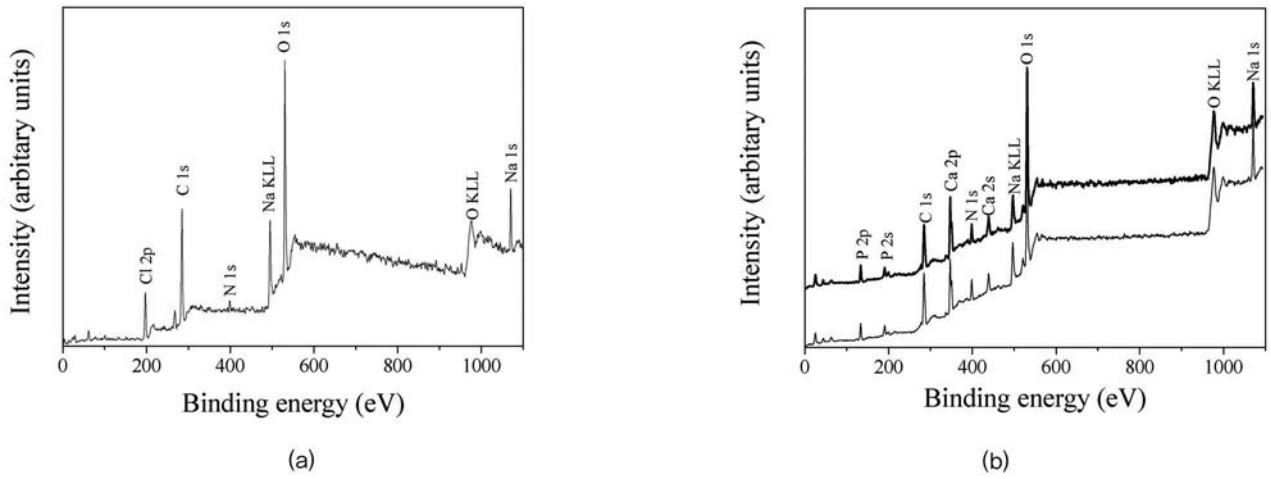

Fig 2. The XPS survey spectra of the HA scaffold before and after BMP-2 coating (a) BMP-2 (b) HAP (upper line) and HAC (under line).

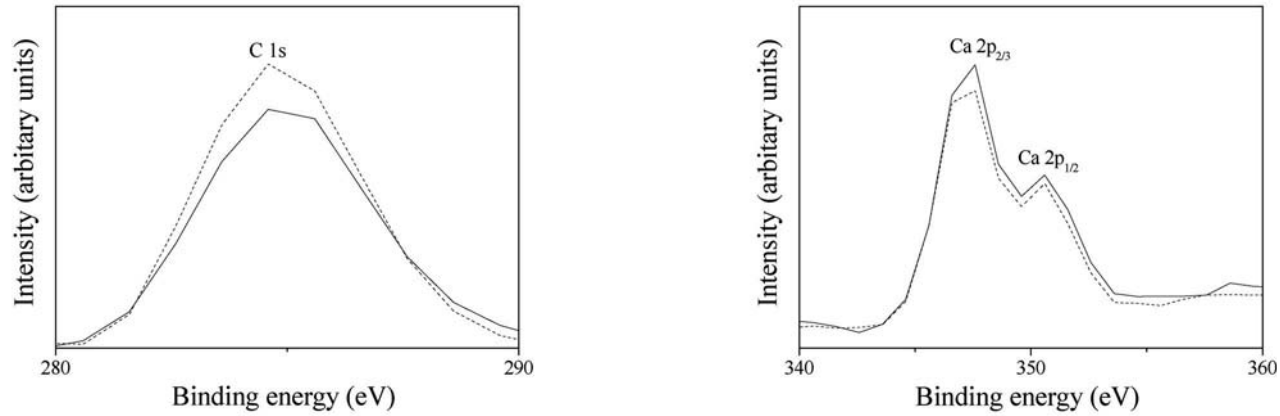

Fig 3. The XPS survey spectra of the HA scaffold after BMP-2 coating (a) C 1s and (b) Ca 2p2/3 and Ca 2p1/2 (solid line: HAP, dotted line: HAC).

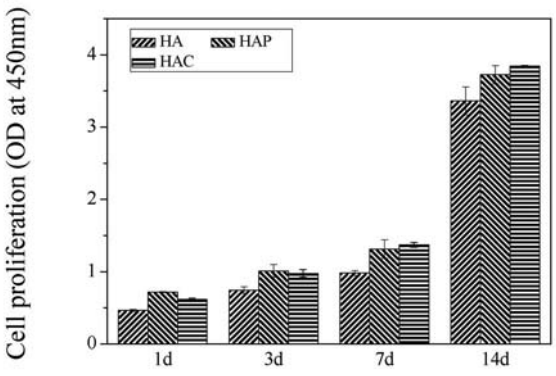

Fig 4. The proliferation of MCT3-E1 cells on the HA scaffolds after 1, 3, 7 and 14 days.

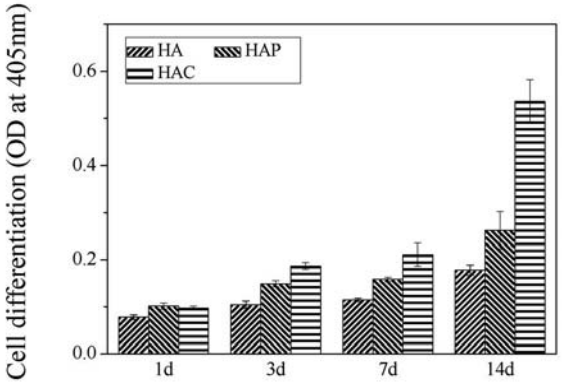

Fig 5. The differentiation of MCT3-E1 cells on the HA scaffolds after 1, 3, 7 and 14 days.

sulted in better coating of BMP-2 than those coated by dip coating method. Also the scaffold coated by BMP-2 using both of methods resulted in improved cell proliferation and differentiation compare to uncoated control sample, whereas the samples prepared by antigen-antibody method resulted in enhanced cell differentiation compare to samples prepared by dip coating method. This study provides significant data regarding the biological properties of HA scaffold according to the presence of BMP-2 in coating layer and the method of BMP-2 coating.

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