KUSA-A1 골조세포 함유 키토산 지지체를 이용한 생체내 골재생

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In vivo Bone Regeneration by Using Chitosan Scaffolds with KUSA-A1 Oesteoblast Cells

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초록: KUSA-A1 골조세포로부터 골재생을 유도하기 위하여, 스폰지형(CSS) 및 부직포형(CSNW)의 키토산 지지체를 적용하였다. CSNW의 표면적 및 공극 크기는 CSS에 비해 상대적으로 큰 값을 나타낸 반면, 공극 부피는 CSNW의 경우가 CSS에 비해 작은 값을 보였다. 세포고정 시험 결과는 CSNW의 경우가 더 적합한 결과를 나타내었으며, 이는 지지체의 넓은 표면적에 기인한 것으로 판단되었다. *In vivo* 실험을 위하여 세포를 각각의 지지체에 투여 후 일주일간 배양하였으며, BALB/C 무모생쥐의 피하조직에 이식하였다. 이식된 지지체는 각각 수술후 1, 4, 6 및 8주에 채취되어 면역학적 염색을 실시하였다. CSS는 수술후 4주에서 6주 사이에 붕괴되기는 하였으나, 조직의 안정성은 CSNW에 비해 우수한 것으로 관찰되었다. 골조직의 생성은 CSNW와 CSS에 대해 각각 4주 및 8주에서 이루어짐을 확인하였다.

Abstract: For bone regeneration from KUSA-A1 oesteoblast cells (KUSA), chitosan (CS) scaffolds possessing different surface properties, sponge-type (CSS) and nonwoven-type (CSNW), were manufactured. Surface area and pore size of CSNW were larger than those of CSS. On the other hand, the pore volume of CSNW was smaller than that of CSS. Cell attachment evaluation showed CSNW was more adequate then CSS, and this was attributed to the large surface area. For *in vivo* investigation, KUSA were seeded into CS scaffolds in wells followed by a week of cell culture. Obtained CS scaffolds with KUSA were implanted on the subcutaneous tissue of BALB/C nude mice. After surgery, implanted scaffolds were harvested and assayed by immunological staining. Network stability of CSS was better than that of CSNW, even if CSS scaffolds were destroyed between 4 and 6 weeks. Calcification was observed after 4 and 8 weeks for CSNW and CSS, respectively.

Keywords: chitosan, scaffold, bone regeneration, KUSA-A1 oesteoblast cell, surface area.

Introduction

Tissue engineering approach to bone regeneration, having obtained serious interest and research recently, requires an acceptable cell supporting matrix, scaffold, to supply a 3-dimensional substrate for cells to populate on and function appropriately during the newly bone formation.[1-4] In the scaffolds, a parameter that is important factor of the scaffolds is constructive property such as surface area, porosity, and the percentage of void space. Pores are necessary for bone regeneration because that they permit for migration and proliferation of osteoblasts.[5] In recent reports, based on results by Ragetly *et al*.,[6] chitosan (CS) scaffolds were improved in fibrous these compared to sponges for chondrogenic differentiation of mesenchymal stem cells (MSCs) as assessed by glycosaminoglycans (GAGs) content and collagen II mRNA expression after 21 days of culture, and this increased matrix production is not related to a difference in cell number between constructs of MSCs as assessed by DNA content and scanning electron microscopy (SEM) evaluation after seeding. In results by Nehrer *et al*.,[7] cells specific phenotype and biosynthetic activ-

ity were improved in collagen matrices containing smaller pores. These results were attributed to the greater surface area of macroporous matrices, spreading cells over the relatively flat surfaces of pore walls, thereby affecting cell-cell interaction. Small pores (<50 μm) have also been recommended to improve biomechanical strength of engineered constructs.[8] In the other hand, it had been reported that constructs produced on macroporous CS sponges contained more GAG and collagen II than those engineered on CS scaffolds with pores measuring less than 50 μm in diameter.[9] According to few studies show higher porosity is usually associated with greater bone regeneration.[10,11] Polymeric fibers that mimic the structure and function of the natural ECM are of great interest in tissue engineering as scaffolding materials to restore, maintain or improve the function of human tissue.

CS is biocompatible, biodegradable, non-toxic, and used for anti-microbial and hydrating agents.[12] Moreover, CS is easily processed into gels,[13] membranes,[14] beads,[15] nanofibers,[16] micro-,[17] nanoparticles,[18] and sponge[19] for tissue engineering. KUSA-A1 oesteoblast cells (KUSA), immortalized stromal cell line, established from murine bone marrow cultures. They are unique, mature osteoblast cell line and will serve as a very suitable model for *in vivo* osteogenesis.[20]

In this study, for effectively bone regeneration from KUSA, CS scaffolds possessing different surface properties were manufactured in as sponge-type (CSS) and as nonwoven-type (CSNW). The properties of scaffolds were evaluated by analytical tools including SEM and Bunauer, Emmet and Teller (BET).

Experimental

Material. CS (chitosan, M_n ~5000, 82% deacetylated) and CS nonwoven web were supplied from Texan Meditech, Korea. Acetic acid and disodium hydrogen phosphate were purchased from Sigma (UAS). 95% ethanol (Duksan, Korea) was used as received. Distilled water was of Milli-Q quality (Millipore, USA). All the other chemicals used in this research were HPLC grade and used as received.

Preparation of Scaffolds. CSNW was manufactured by using 5 sheets of 0.8×0.8 cm^2 CS nonwoven web bonded by needle punching. To prepare CSS, 1 g of CS was dissolved in 100 mL of 0.1 M acetic acid aqueous solution at room temperature. This solution was stirred for a day. This homogeneous solution was filtered, debubbled, and cast into polyethylene petri dish. Then this specimen was frozen at -80 °C for a day

followed by freeze drying. This lyophilized specimens were immersed into ethanol at room temperature for a day and washed with distilled water for 5 times. Neutralization was performed with 0.1 M disodium hydrogen phosphate aqueous solution at room temperature for 24 hrs. Prepared scaffolds were washed with distilled water for 5 times and lyophilized in a freeze dryer.

Cell Attachment. KUSA were obtained from Korea Bank of Cells and cultured at 37 °C by using α-minimal essential medium (α-MEM) with 10% fetal bovine serum (FBS) and penicillin/streptomycin mixture (pen/strep) (Hyclon, USA) under 5% CO_2 environment. CS scaffolds were sterilized with ethylene oxide gas and placed in 6-well cell culture plate. Approximately 2×10^5 cells in 50 μL α-MEM were injected into each scaffold by 1 mL syringe. After cell injection, CS scaffolds were incubated at 37 °C for 4 hrs under 5% CO_2 environment. Proliferation of KUSA on CS scaffolds was performed for a week after adding α-MEM, FBS, pen/strep, and osteogenic reagents (10^{-2} M β-glycerophosphate, 50 μg/mL *L*-ascorbic acid, and 10^{-8} M dexamethason) (Sigma, USA).

Surgical Procedure. The surgical procedures for this study were performed according to protocols approved by the Kyungpook National University Hospital Animal Center. BALB/C nude female 5-week old mice were used for *in vivo* tests of this study. Each mouse was premeditated according to their weight with intramuscular injection of solution (2 cc/kg) composed with ketamin (1.5 cc), rumpun (0.164 cc), and phosphate buffer solution (PBS) (2 cc). Anesthetized mice were made an incision on back. CSNW and CSS scaffolds with KUSA were implanted in subcutaneous flank of mice.

Immunological Assays. Mice were sacrificed at 1, 4, 6, and 8 weeks. Implanted specimens on back were harvested and fixed by 10% formalin (Duksan, Korea) at 4 °C for 24 hrs, followed by washing, dehydration, and embedding in paraffin. Paraffin blocks were cut into slices of 10 μm in thickness by microtome (Leica, USA) and attached on glass slides. The slides were deparaffinized with xylene (Merck, USA) with 3 times. Remaining xylene was removed by soaking with alcohols and washing with flourished water. For hematoxylin and eosin (H&E) staining, the slides transferred into Harris's hematoxylin (Muto, Japan) for 2 min. Over-stained dyes were removed by dipping into 0.5% HCl (Merck, USA) aqueous solution. After washing in distilled water, eosin staining was performed for 30 s. For von kossa staining, the slides were stained with 5% $AgNO_3$ aqueous solution under sunlight for 30 min and washed with distilled water. After 20 min of devel-

opment, specimens were washed with distilled water again. The slides were fixed with 5% sodium thiosulfate (Sigma, USA) solution for 2 min. Stained specimens were washed with xylene twice and mounted with malinol mounting solution (Muto, Japan).

Characterization. Morphologies of CSNW and CSS scaffolds, with and without attached KUSA, were evaluated by using SEM S-4300 (Hitachi, Japan). For SEM observation of CS scaffolds with KUSA, specimens were washed with PBS and sunk in glutaraldehyde solution (Sigma, USA) for cell fixation. Fixed scaffolds were washed twice with 0.1 M sodium cacodylate buffer (Fluka, USA) and dehydrated in ethanol (Merck, USA) with stepwise increase in ethanol concentration from 25 to 100%. Then, tetramethylsilane (Sigma, USA) was added to remove ethanol and dry CS scaffolds. CS scaffolds were put on a sample holder and coated with a thin layer of platinum for SEM observation. Surface area, pore volume, and pore size of CS scaffolds were measured by using BET (Quanpachrome, USA). To evaluate cell viability by 5-(3-caroboxymethy-oxyphenyl)-2n-tetrazolium inner salt (MTS) (Promega, USA) assay, 1×10^5 KUSA cells were seeded on 96-well with 100 μL of α-MEM. After 24 hrs of cell adhesion on 96-well, CS scaffolds were placed on attached cells. Cell culture was sustained for scheduled times. Cultured KUSA was stained by MTS solution after removing CS scaffolds. Absorbance of stained cells was measured spectrophotometrically at 490 nm by using microplate reader and compared with that of the control, same number of cells without scaffold.

Results and Discussion

Table 1 shows the summarized BET results of CS scaffolds. From the BET results summarized in Table 1, CSNW is more surface and larger pore size than CSS. Surface area, pore volume, and pore size of CSS are 6.846 m²/g, 1.274 cc/g, and 2.172 Å, respectively. CSNW's are 7.965 m²/g, 1.031 cc/g, and 2.661 Å, respectively. According these results, CSNW has 16% and 22% more surface area and pore size than CSS,

Table 1. Surface Characteristics of CS Scaffolds

Characteristics	CSS	CSNW
Surface area(m²/g)	6.846	7.965
Pore volume(cc/g)	1.274	1.031
Pore size(desorption, Å)	2.172	2.661

respectively. However, CSS is 20% larger pore volume than CSNW. It analogizes that CSNW could have cell adhesiveness and expected good cell proliferation, spreading after cell seeding, moreover, CSNW possessing larger surface area and pore size will be expected great cell-cell interconnection and scaffold in bone tissue engineering.

Figures 1(a) and 1(b) showed SEM photographs of CSS and
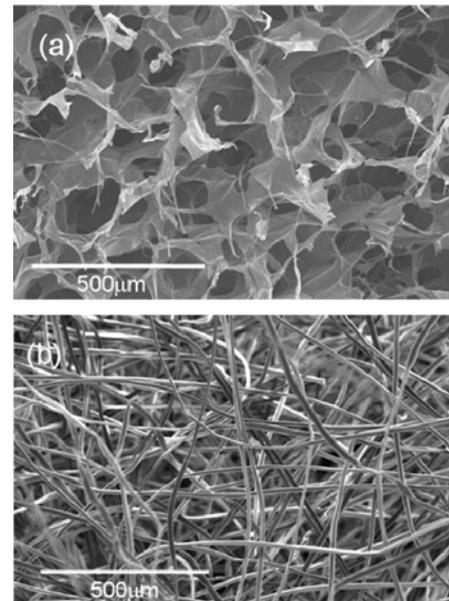
Figure 1. SEM micrographs of CS scaffold surfaces: (a) CSS; (b) CSNW.
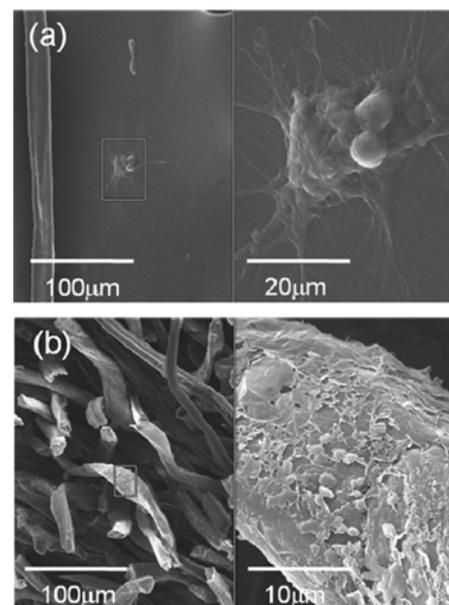
Figure 2. SEM micrographs showing cell adhesion on CS scaffold surfaces: (a) CSS; (b) CSNW.
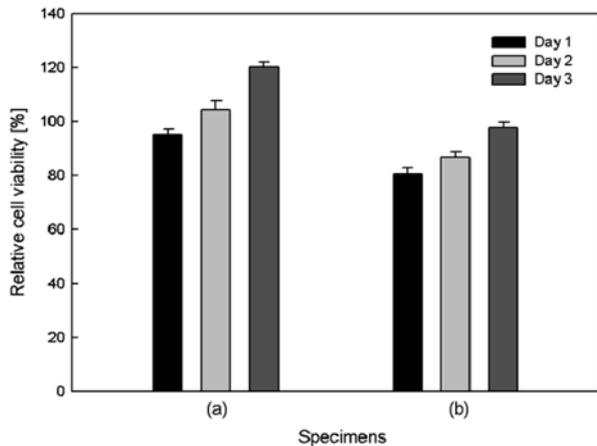
Figure 3. Cell viability by MTS assay: (a) CSS; (b) CSNW (n=5, p<0.05).

Figure 4. Photograph of nude mouse with two type scaffolds seeded on the subcutaneous tissue.

CSNW scaffolds, respectively. Surface morphologies of CS scaffolds are similar in microstructure. As shown Figure 1, even if many pores have existed in CSS, so CSS consists of one-piece because of interconnection among nonwoven. In other hands, CSNW has composed of fiber bundles, and then many pores were constructed of space between fibers. KUSA on CSS and CSNW were shown on Figure 2. Cell attachment of KUSA on CSNW is greater than CSS in Figure 2. Although CSS scaffold possessed a little cell adhesiveness, attached cells were spreading extra cellular matrix (ECM) proteins on wide area. However, CSNW had great cell-cell interactions on each fiber. Microstructures should be considered when designed scaffolds for tissue engineering because respective cells recognize structures with dimensions comparable to 13-85 μm.[7,9,21] More specifically, fiber diameter and surface topology were found to affect cell adhesion, proliferation and spreading. Similar results were gained compared other reports.[4,5,10]

To effect of released substrates from scaffolds to cells, we observed cell viabilities compared to control group at a scheduled time. As Figure 3, cell viabilities of CSS are 95.16%, 104.52%, and 120.16%, and these of CSNW are 79.4%, 86.52%, and 95.1%, respectively, after 1, 2, and 3 days of placed scaffolds on cells. Cell viabilities of CSS and CSNW are growing up as time goes. CSNW was manufactured by CS and many additives such as adhesive, and plasticizer agents. Additives have a little toxicity. However, these were not impacted on cell viability as long time. Recent studies showed that the biocompatibility of chitin and CS nanofibers were found to promote cell attachment and spreading of normal human keratinocytes and fibroblasts.[22] This may be a con-

sequence of the high surface are available for cell attachment and growing.[12] Therefore two types of CS scaffolds can be considered as biocompatible material biologically. In addition, CS with fibrous forms, CSNW, is more expected to effective scaffolds than CSS for cell adhesion and proliferation.
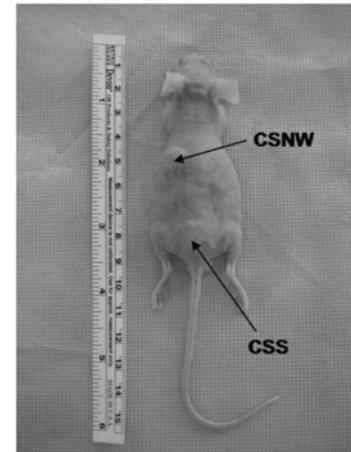
Two types of CS scaffolds with KUSA cells were implanted on subcutaneous tissue of back in nude mice. Upper scaffold is CSNW and under this is CSS as shown in Figure 4. CS scaffolds were harvested on 1, 4, 6, and 8 weeks after surgery, and then the specimens embedded in paraffin were assayed by immunological staining to confirm calcification.

Figure 5 shows the H&E staining results for CS scaffold; Figures 5(a)-(d) and 5(e)-(h) are for CSS and CSNW, respectively. As time goes by, CS scaffolds were swelled, migrated to other cells, and appeared some inflammation on out of scaffolds in Figure 5. Especially, CSNW was swelled until 4 weeks, however, was expressed breakaway of each fiber, as shown Figure 5(g) and 5(f). On the other hand, although breakaway was partially shown, CSS was swelled until 8 weeks, steadily. According to other results, biodegradation of CS was occurred from 4 weeks after surgery *in vivo*. It seems that the breakdown of structure of CS scaffolds was started among 4 and 6 weeks because of biodegradation. CSS was occurred to be loosed structure because of partial CS biodegradation from one-piece. Nevertheless, the scaffold structure was destroyed because that non-degradation fiber also was breakaway as degradation of CS fiber structure. It seemed that differentiated cells were disappeared together non-degradation fiber. So network stability of CSNW was more decreased than CSS.

Results of von kossa staining for CS scaffolds were shown
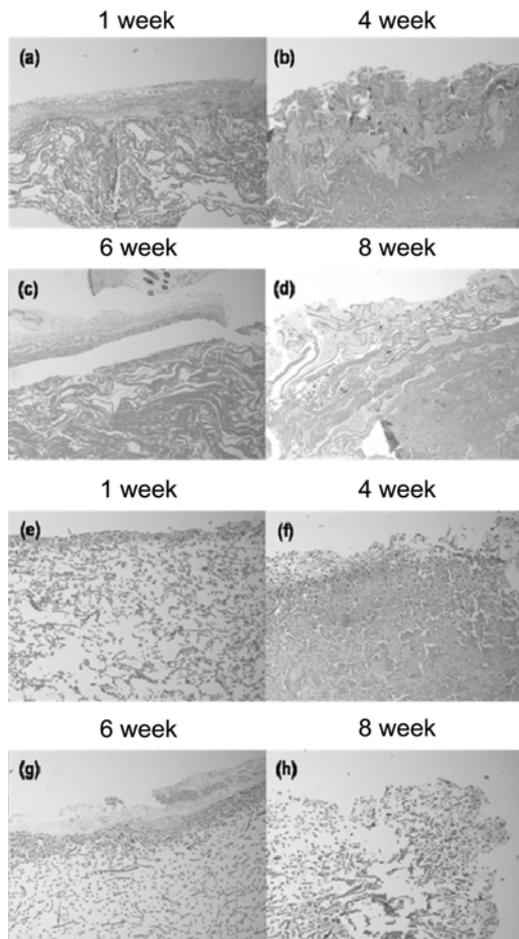
Figure 5. Microscopic appearance of H&E staining results for CS scaffolds *in vivo*. (a)-(d); (e)-(h) are for CSS and CSNW, respectively (×200).
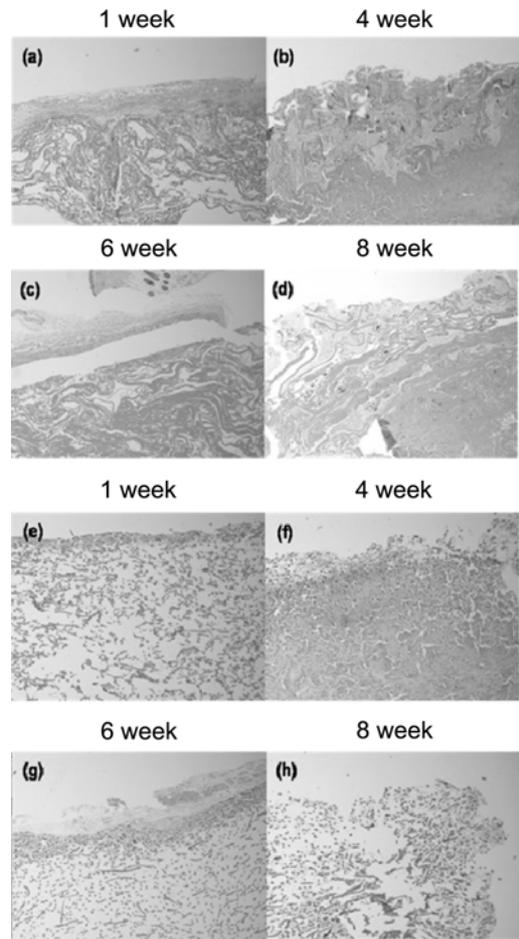
Figure 6. Von kossa staining results for CS scaffolds by microscope *in vivo.* (a)-(d); (e)-(h) are for CSS and CSNW, respectively (×200).

in Figure 6; Figures 6(a)-(d) and 6(e)-(h) are for CSS and CSNW, respectively. As the arrows, Figures 6(d) and 6(f) were expressed calcium deposits on CSS and CSNW, respectively. Based on the report by Karp *et al*.,[23] beginning approximately 2 weeks after surgery progressive calcification of the matrix-bound collagen bundles is observed. The collagen fibrils become organized along the implanted region and calcium salts are deposited in the lamellar phase after 8 weeks.[24] However, Komuro *et al*.,[25] reported new bone generation after irregular osteogenesis, based on a process of remodeling and resulting in cortical bone 10 weeks after surgery.[26] CSNW to possess more abilities of cell attachment, larger surface area, and dense structure was faster appeared alkaline phosphate deposit on 4 weeks than CSS one's on 8 weeks. However, alkaline phosphate deposit of CSNW was disappeared on 6 and 8 weeks, and it analogized that produced deposit was

breakaway together cells and non-degradation fiber because of network stability destroys of CSNW.

High surface area, large pore size, good cell viability, and network stability are important factors on the scaffold for bone regeneration. CSNW shows good properties, higher surface area and pore size, more cell-cell interaction, and faster formation ratio of calcification than CSS. However, relative low cell viability and dimensional stability are serious trouble on CSNW. On the other hand, CSS is shown larger pore volume and more effective cell viability than CSNW, although appearance of calcium mineral on CSNW for 4 weeks was faster than CSS's for 8 weeks *in vivo*. Dimensional stability of the scaffold is one of the serious factors for large tissue formation. Fiber formation of CSNW is promoted breakaway and degradation of a scaffold with adherent cells and formed minerals as the results of *in vivo*. Thus, CSS is effective construct for

stimulating and growing KUSA to osteocyte for long time regeneration.

Conclusions

The CS scaffolds, CSS and CSNW, possessing different surface area, pore size, and pore volume were prepared. CSNW is larger surface area and pore size, however, smaller pore volume than CSS by using BET. Follow by cell attachment of CSNW was higher by using SEM. On the other hand, cell viability of CSNW a little lower than CSS due to additives, nevertheless, these data were belonged on biosafety region for using on tissue engineering. Moreover, alkaline phosphate deposits of CSNW were earlier formed on 4 weeks then CSS on 8 week, nevertheless, structure stability of CSS was higher than CSS using by immunological staining *in vivo*. From this result, it can be concluded that network stability is an important factor for large bone regeneration as well as surface area and pore size for cell-cell interconnection. Therefore, CSS can be a more effective scaffold for long time bone regeneration because of good dimensional stability. For future work, it will be necessary to study CSNW having high network stability. Furthermore, CS scaffolds combined growth factor delivery system could be adopted for another regeneration such as myogenic or chondrogenic differentiation using MSCs.

References

1. J. H. Jang, O. Castano, and H. W. Kim, *Adv. Drug Deliv. Rev.*, **61**, 1065 (2009).
2. R. M. Nerem and A. Sambanis, *Tissue Eng.*, **1**, 3 (1995).
3. K. J. L. Burg, S. Porter, and J. F. Kellam, *Biomaterials*, **21**, 2347 (2000).
4. D. W. Hutmacher, *Biomaterials*, **21**, 2529 (2000).
5. P. Kasten, I. Beyen, P. Newmeyer, R. Luginbuhl, M. Bohner, and W. Richter, *Acta Biomater.*, **4**, 1904 (2008).
6. G. R. Ragetly, D. J. Griffon, H. B. Lee, L. P. Fredericks, W. G. Evans, and Y. S. Chung, *Acta Biomater.*, **6**, 1430 (2010).
7. S. Nehrer, H. A. Breinan, A. Ramappa, G. Young, S. Shortkroff, L. K. Louie, C. B. Sledge, I. V. Yannas, and M. Spector, *Biomaterials*, **18**, 769 (1997).
8. M. Kon and A. C. de Visser, *Plast. Reconstr. Surg.*, **67**, 288 (1981).
9. D. J. Griffon, M. R. Sedighi, D. V. Schaeffer, H. A. Eurell, and A. L. Johnson, *Acta Biomater.*, **2**, 313 (2006).
10. K. U. Lewandrowski, J. D. Gresser, S. Bondre, A. E. Silva, D. L. Wise, and D. J. Trantolo, *J. Biomater. Sci. Polym. Ed.*, **11**, 879 (2000).
11. T. D. Roy, J. L. Simon, J. L. Ricci, E. D. Rekow, V. P. Thompson, and J. R. Parsons, *J. Biomater. Master. Res. A*, **66**, 283 (2003).
12. R. Jayakumar, M. Prabaharan, S. V. Nair, and H. Tamura, *Bio. Technol. Adv.*, **28**, 142 (2010).
13. H. Nagahama, T. Kashiki, H. New, R. Jayakumar, T. Furuike, and H. Tamura, *Carbohydr. Polym.*, **73**, 456 (2008).
14. H. Nagahama, H. New, R. Jayakumar, S. Koiwa, T. Furuike, and H. Tamura, *Carbohydr. Polym.*, **73**, 295 (2008).
15. R. Jayakumar, R. L. Reis, and J. F. Mano, *J. Bioact. Compat. Polym.*, **21**, 327 (2006).
16. K. T. Shalumon, N. S. Binulal, N. Selvamurugan, S. V. Nair, D. Menon, and T. Furuike, *Carbohydr. Polym.*, **77**, 863 (2009).
17. M. Prabaharan and J. F. Mano, *Drug Deliv.*, **12**, 41 (2005).
18. A. Anitha, V. V. Divya Rani, R. Krishna, V. Sreeja, N. Selvamurugan, S. V. Nair, H. Tamura, and R. Jayakumar, *Carbohydr. Polym.*, **78**, 672 (2009).
19. K. Muramatsu, S. Masuda, Y. Yoshihara, and A. Fujisawa, *Polym. Degrad. Stab.*, **81**, 327 (2003).
20. S. Matsumoto, I. Shibuya, S. kusakari, K. Segawa, T. Uyama, A. Shimada, and A. Umezawa, *Biochemica. Biophysica. Acta*, **1725**, 57 (2005).
21. J. Bhardwah and R. M. Pilliar, *J. Biomed. Mater. Res.*, **57**, 190 (2001).
22. H. K. Noh, S. W. Lee, J. M. Kim, J. E. Oh, K. H. Kim, C. P. Chung, S. C. Choi, W. H. Park, and B. M. Min, *Biomaterials*, **27**, 3934 (2006).
23. N. S. Karps, J. G. Macarty, and J. S. Scheriver, *Ann. Plast. Surg.*, **89**, 1 (1992).
24. L. Califano, A. Cortese, and A. Zupi, *J. Oral Maxillofac. Surg.*, **52**, 1179 (1994).
25. Y. Komuro, T. Takato, and K. Harii, *Plast. Reconstr. Surg.*, **94**, 152 (1994).
26. B. C. Cho, H. Y. Chung, D. G. Lee, J. D. Yang, J. W. Park, K. H. Roh, G. U. Kim, D. S. Lee, I. C. Kwon, E. H. Bae, K. H. Jang, R. W. Park, and I. S. Kim, *J. Oral Maxillofac. Surg.*, **63**, 1753 (2005).