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Design of injectable bone tissue engineering scaffold consists of ß-tricalcium phosphate beads and alginate

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1. Introduction

Autologous bone is generally considered the gold-standard graft material. The advantage of an autograft is that it contains viable osteoblasts and osteogenic precursor cells that can contribute to the formation of new bone (Arrington et al, 1996). In addition, the autograft possesses the three essential elements of bone regeneration -osteogenesis, osteoinduction, and osteoconduction- that are required for bone regeneration. However, only a minimal amount of bone tissue can be harvested for autografts, the harvesting procedure may lead to donor site discomfort and morbidity, and it may be difficult to form this tissue into the desired shape (Goldberg & Stevenson, 1987; Damien & Parsons, 1991), a problem that is particularly important in the craniofacial region.

Therefore, bone graft substitutes have been used to reconstruct bone defects. A bone graft substitute should be osteoconductive, osteoinductive, biocompatible, biodegradable, structurally similar to bone, easy to use, and cost-effective (Giannoudis et al, 2005). Hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) are both well-known ceramics that possess high tissue compatibility and osteoconductivity. However, neither HA nor β-TCP has osteoinductive or osteogenic abilities, and HA usually shows minimal biodegradation (Spector, 1994; Schmitz et al, 1999).

To overcome these limitations, bone tissue engineering has been promoted as a new way to regenerate bone tissue. This approach combines cells capable of osteogenic activity and osteoinductive signal molecules with an appropriate material (Livingston et al, 2005). For bone tissue engineering to succeed, osteoconductive scaffolding biomaterials must provide a suitable environment for the cells. Furthermore, it is desirable that the scaffolds can control the release of growth factors. Accordingly, functional composite scaffolds for bone tissue engineering have been developed in combination with synthetic ceramics and natural polymers.

Recently, minimally invasive treatments have been developed using an injectable system for bone tissue engineering. Several injectable gels have been used to carry cells in order to engineer bone. Amongst which are collagen (Tsuchida et al, 2003), alginate (Shang et al,

2001; Wang et al 2003), and fibrin (Perka et al 2001) gels, but these substances cannot be molded to the shapes of the bone defects when injected *in situ* or that they cannot follow the shapes of 3D cell culture molds *in vitro*.

In this chapter, I introduce the design of injectable composite scaffold fabricated from β-TCP beads and alginate for bone tissue engineering (Matsuno, 2008). The β-TCP bead/alginate composite is not only injectable, but also instantaneously formed from β-TCP beads and alginate. Therefore, the composite is sufficiently adaptable to the irregularities of bone defects and which possessed adequate mechanical strength. Additionally, the composite act as an appropriate 3D scaffold for osteogenic cells, and function as drug delivery carrier of growth factors.

2. Preparation of injectable composite scaffold

2.1 β-TCP beads

β-tricalcium phosphateis a synthetic calcium phosphate ceramic used as an alternative autologous bone graft. It has been reported that β-TCP has good biodegradability and osteoconductivity as a scaffold material for bone tissue engineering (Le geros, 2002; Matsuno et al, 2006). With regard to shape availability, both block and granular forms are available. However, both shapes pose an injectability problem and are difficult to be molded into a 3D structure.

Thus, we used the β-TCP beads for injectable bone substitute. β-TCP beads were prepared in a manner similar to a previously described method (Ushida et al, 2001; Furukawa et al , 2004; Miyauchi et al , 2004). An aqueous slurry of β-TCP powder (Advance Ltd., Saitama, Japan) and polyvinyl alcohol (Shin-Etsu Chemical Co. Ltd., Tokyo, Japan) aqueous solution as a binder reagent were mixed using an ultrasonic homogenizer. The resultant slurry was added drop-by-drop to liquid nitrogen, whereby slurry drops were frozen, and the frozen beads were freeze-dried. The beads were then sintered at 1100°C for 10 hours. Diameter of the beads was controlled at 100−1,000 μm by regulating the opening and shutting times of an electromagnetic valve.

Figure 1 shows the SEM photographs of β-TCP bead and the surface of the bead. Diameter of the bead was about 500 μm, and micropores were observed on the surface.

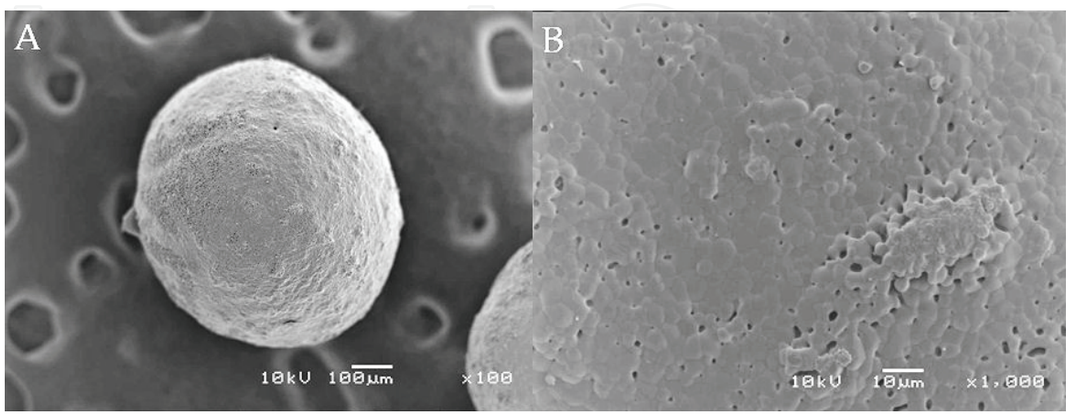

Fig. 1. SEM photographs of a β-TCP bead (A) and the surface of the bead (B).

Figure 2 shows the XRD patterns of the synthesized β-TCP beads and commercially available β-TCP (OSferion®; Olympus, Tokyo, Japan). There were no significant differences in the intensity of β-TCP peaks between β-TCP beads and commercially available β-TCP.

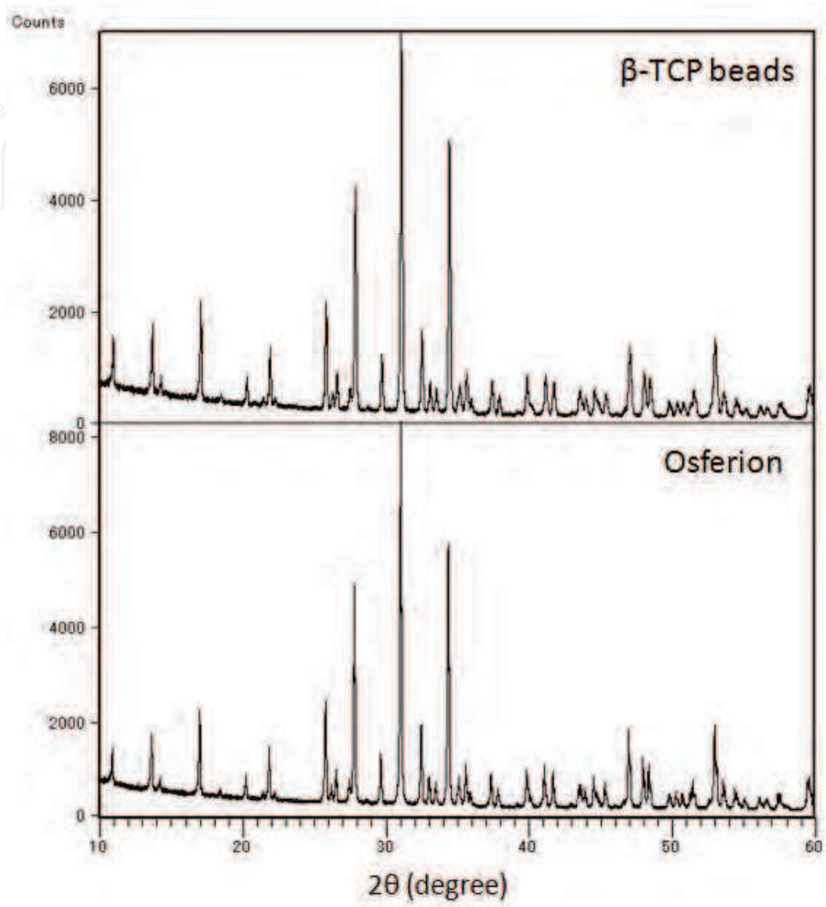

Fig. 2. X-ray diffraction patterns of β-TCP beads and commercially available β-TCP

2.2 Alginate hydrogel

Alginate has many uses in bioengineering, such as in polymer films, cell encapsulation, wound dressings, and surgical sponges (De Vos et al, 1997; Matthew et al, 1997). Alginic acid is soluble in water and can be ionically crosslinked with a non-toxic divalent cation solution, such as calcium chloride (De Vos et al, 1997). The calcium ions bind the guluronic acid sites of alginate strands together to form a stable alginate gel (Becker et al, 2001). However, alginate lacks the initial mechanical strength needed for bone tissue engineering.
Alginate hydrogel was made by dissolving alginic acid sodium (Protanal LF 10/60, FMC BioPolymer, PA, USA) in phosphate-buffer saline (PBS) to give two final concentrations of 1.0 and 2.0 wt%.

2.3 Injectable 3D-formed composite scaffold

The β-TCP beads, with a diameter of 710−850 μm, were dipped in 1.0% $CaCl_2$ solution (Sigma-Aldrich Co.) and then dried. β-TCP beads in the syringe were sterilized with ethylenoxide gas and alginate hydrogel with Stericup® (0.22 μm; Millipore Co., MA, USA). As alginate hydrogel was pushed out from the tip of the syringe, it passed through the $CaCl_2$- treated β-TCP beads. As soon as a 3D-formed composite of β-TCP beads and alginate was obtained due to the instantaneous crosslinking that occurred in the syringe.

Figure 3 shows a photograph of injectable 3Dformed composite of β-TCP beads and alginate and a light microscope photograph of the composite. Each bead was wrapped in the anastomosing network of the alginate gel, and the beads were bonded tightly to form a 3D structure. Minute crystal projections were observed on the cross-sectional surface of the β-TCP beads treated with $CaCl_2$ (Fig. 4).

Fig. 3. Injectable 3D-formed composite composite.

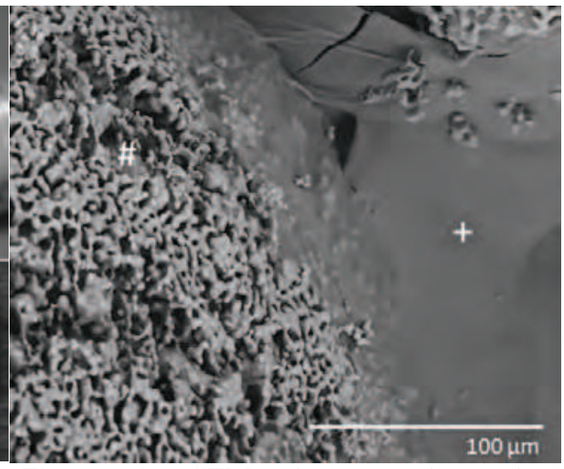

Figure. 4 SEM of the surface of the β-TCP beads (#), alginate (+)

3. Evaluation of β-TCP bead/alginate composite

3.1 Mechanical strength of the composite

The compression tests were performed in the two types of composites (1.0 and 2.0 wt% alginate hydrogels; 5.0 mm in diameter and 10.0 mm in length). The compressive strength increased with increased alginate concentration. The composite of 2.0 wt % alginate concentration exhibited a compressive strength of 69.0±4.6 kPa, which was significantly greater ($P<0.05$) than the strength seen with 1.0 wt% (6.11±2.28 kPa).

3.2 *In vitro* evaluation of 3D mesenchymal stem cell culture

Human mesenchymal stem cells (MSCs) were dispersed into the alginate hydrogel. Following which, MSC loaded alginate hydrogel was added into the syringe which was packed with $CaCl_2$-treated beads. Consequently, MSC-loaded alginate was crosslinked and MSCs were loaded into the 3D scaffold at about 2×10^5 cells/scaffold. They were cultured in an osteogenic differentiation medium (10mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbic acid) for 1, 7, and 14 days.

Figure 5 shows the SEM photographs of MSC-loaded composite after 1 day and 14 days in the osteogenic differentiation culture. MSCs were observed in the composite at day 1 (Fig. 5A). After 14 days, many calcified nodules appeared on the surface of β-TCP beads and alginate (Fig. 5B).

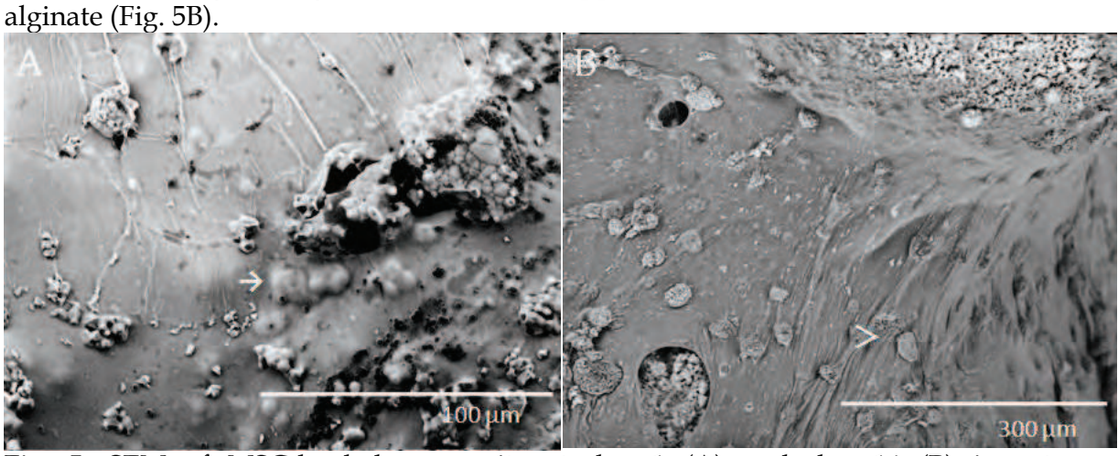

Fig. 5. SEM of MSC-loaded composite at day 1 (A) and day 14 (B) in osteogenic differentiation culture. MSCs (arrow), calcified nodules (>)

Figure 6 shows that the ALP activity of MSCs grown within the composite of osteogenic differentiation culture increased from day 1 to day 7, but decreased from day 7 to day 14. There was a significant increase between day 1 and day 7(*P<0.05), and similarly a significant decrease between day 7 and day 14 (**P<0.05).

No OCN mRNA expression was detected at day 1. However, OCN mRNA expression increased from day 1 to day 7, and increase between day 7 and day 14 was statistically significant (*P<0.05) (Fig. 7).

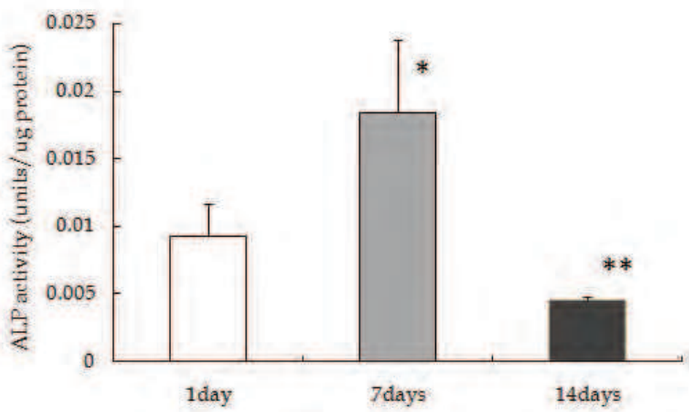

Fig. 6. ALP activity of MSCs grown within the composite in osteogenic differentiation culture.

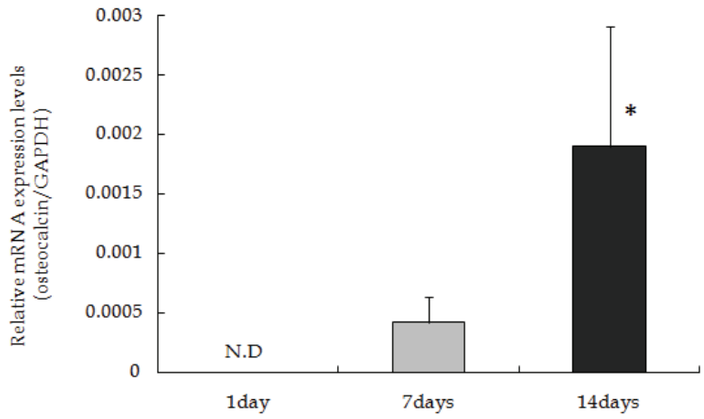

Fig. 7. OCN mRNA expression levels of MSCs grown within the composite in osteogenetic differentiation culture.

3.3 *In vivo* evaluation of MSCs loaded composite scaffold

Non-osteogenic-differentiated MSCs loaded into the 3D composite scaffold (5×10^6 cells/scaffold) and MSC-free composite as control were subcutaneously implanted into the backs of 6-week-old KSN nude mice. The mice were killed at 8 weeks after implantation.

Figure 8 shows the histological sections of the 3D composite scaffold loaded with non-osteogenicdifferentiated MSCs at 8 weeks after implantation. Newly formed, bone-like calcified tissue was directly deposited on the surface of the β-TCP beads. Interconnected alginate was completely degraded, and bone-like calcified tissue was observed in the connective tissue. The surface of the β-TCP beads was slightly degraded and vacuolization was observed in the beads. On the other hand, cell-free composite contained only fibrovascular tissue around nondegraded β-TCP beads (data was not shown).

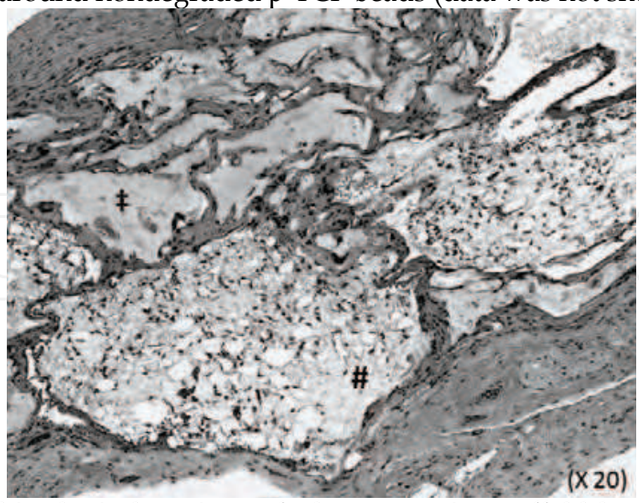

Fig. 8. HE-stained histological sections of the composite scaffold loaded with MSCs at 8 weeks after implantation.
Newly formed, bone-like calcified tissue (‡), β-TCP beads (#)

3.4 *In vitro* release of growth factor from composite

A solution of basic fibroblast growth factor (bFGF) was dispersed into the alginate hydrogel. Following which, bFGF incorporated alginate hydrogel was added into the syringe which was packed with $CaCl_2$-treated beads. Consequently, bFGF-incorporated composite scaffold was obtained. In vitro release tests were performed as follows. The composite scaffolds completely sank in the Hank's balanced solution, and incubated at 37°C . bFGF concentration was determined using ELISA.

Figure 9 shows that the release of bFGF from the composite. There was a significant increase between day 1 and day 4 (*P<0.05).

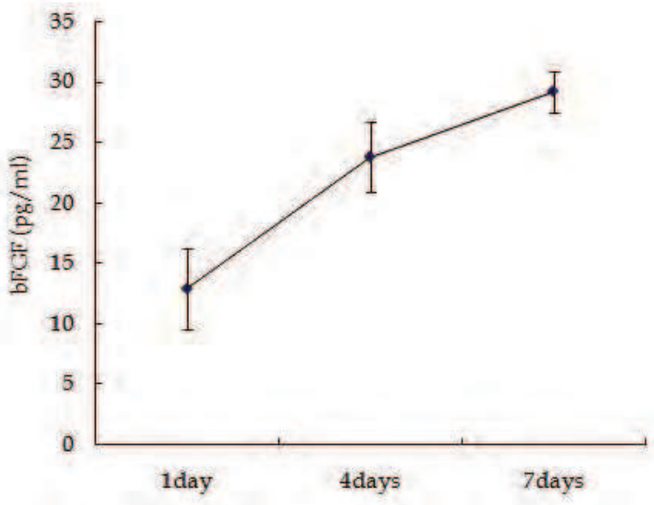

Fig. 9. The release of bFGF from the composite.

4. Conclusion

Both ceramics and polymers have their own merits and drawbacks, and a better solution may be to synergize the advantageous properties of both materials for composite scaffolds. Therefore, many different composite forms such as sponges, gels, films, and blocks have been developed using different methods (Wahl & Czernuszka, 2006). However, multi functional composite such as our developed composite are little or no.

Recently, the increasing popularity of minimally invasive techniques has led to the development of an injectable system that can be molded to the shape of the bone defect and which polymerizes when injected into the site (Niemeyer et al, 2004). Such devices should be easy to implant, thereby shortening the surgical time and minimizing the damage to healthy tissue. β-TCP has been used for bone tissue engineering. However, it is difficult to fit into the surgical sites around implants or to be shaped to the desired forms using β-TCP blocks or granules. The composites consisting of β-TCP beads and alginate can be injects into bone defect site. On the other hand, the composite can simply be cut with scissors or a sharp knife, and can therefore be easily molded for use for various tissue disorders.

Bone tissue engineering approach is to combine osteogenetic cells with an appropriate composite scaffold. In keeping with the tissue engineering concept, cells are cultivated on an appropriate 3D scaffolds to replace 3D tissue defects (Liu et al, 2006). However, elaborate

seeding and culture conditions are needed to achieve uniform cellular distribution, sustain cell viability, and provide nutrients for tissue formation in scaffolds (Weinand et al, 2006).
In this study, alginate hydrogel was used to facilitate the delivery and distribution of MSCs. It has been previously demonstrated that alginate hydrogels induce bone formation *in vitro* (Weinand et al, 2006; Alsberg et al, 2001). However, the lack of adequate initial strength of hydrogel/cell constructs requires additional mechanical support for implantation (Niemeyer et al, 2004). Therefore, a uniform size of $CaCl_2$-coated β-TCP beads was selected to reinforce the initial strength. Calcium ions can be crosslinked with the guluronic acid sites of alginate strands to form a stable alginate gel (Becker et al, 2001). Leveraging on all these properties in this study, an instantaneously formed, injectable 3D scaffold was obtained after alginate hydrogel reacted with $CaCl_2$-coated β-TCP beads and then loaded with MSCs.
In vitro evaluation, ALP activity at 7 days was significantly increased as compared to day 1, but decreased from day 7 to day 14. As a marker of early osteogenic differentiation, the ALP activity of the construct was measured on days 1, 7, and 14 of the culture period. The typical rise and fall of ALP activity was characteristic of osteogenic differentiation and common to all culture systems (Shin et al, 2004). Furthermore, OCN mRNA expression, another marker of osteogenic differentiation, was significantly increased at day 14 as compared to day 7. Therefore, it is conceivable that calcification progressed in the composite.
In vivo experiment demonstrated that the β-TCP bead/alginate composite had an osteoinductive effect in soft tissue — without an osteogenic differentiation medium for MSCs. Similarly, Livingston *et al*. (2005) demonstrated that hydroxyapatite/β-TCP ceramic scaffold promoted bone formation in soft tissue, which was induced by non-osteogenic-differentiated MSCs. Furthermore, this study demonstrated that β-TCP bead/alginate composite induced the formation of bone-like calcified tissue. Taken together, these results suggested that a 3D structure consisting of β-TCP beads and alginate allowed an appropriate spatial arrangement of osteogenic cells as well as vascular invasion. These findings also indicated that injectable β-TCP bead/alginate composites supported bone regeneration with a minimally invasive technique.
In addition, the β-TCP bead/alginate composite can locally release growth factors from the composite, used as drug delivery carriers. It will be enhance bone formation to treat bone defects.
In light of the encouraging results obtained for osteogenesis, the novel composite developed in this study may be useful as an injectable scaffold for bone tissue engineering.

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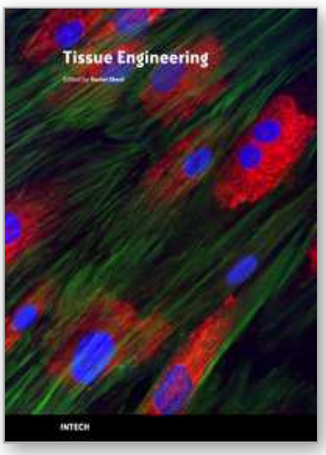

Tissue Engineering

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The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues that closely match the patient's needs can be reconstructed from readily available biopsies and subsequently be implanted with minimal or no immunogenicity. This eventually conquers several limitations encountered in tissue transplantation approaches. This book serves as a good starting point for anyone interested in the application of Tissue Engineering. It offers a colorful mix of topics, which explain the obstacles and possible solutions for TE applications.

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