13
Regenerative Medicine: Reconstruction of Tracheal and Pharyngeal Mucosal Defects in Head and Neck Surgery

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13.1
Introduction

13.1.1
History of Implant Materials

The 20th century can be called the era of synthetical polymers. Poly(methyl methacrylate) (PMMA) was firstly recognized as promising implant material through war-wounded pilots in World War II: Soft tissue and eye injuries induced by and containing small fractions of bursting windows of airplane cockpits (PMMA) led to minute foreign body reactions only. Szilagyi *et al.* reported first clinical experiences with *polyethylene terephthalate* as vascular arterial prostheses in 1958 [1]. In the 1960s, J. Charnley, an orthopedic surgeon from United Kingdom developed a functional and cemented total hip endoprosthesis based on steel and ultrahigh molecular weight polyethylene inlays which were cemented into the femoral bone using PMMA as "cement." Beginning at the end of the 1960s, there was a focus on the development of degradable polymeric implant materials.

Since then the availability of so-called polymer systems allows a large-scale variation of material characteristics, for example, of mechanical properties or hydrolytic degradation and thus to adapt these materials to specific local requirements in the organism [2].

13.1.2
Regenerative Medicine

Due to the shift in morbidity spectrum during the last decades and the recent demographic development in the world, the clinical medicine has to deal more and more with diseases gradually leading to a loss of function of important cell and organ systems. In many cases, these diseases cannot be cured by the currently available therapies and the patients have to remain in permanent therapy resulting in high costs.

Handbook of Biodegradable Polymers: Synthesis, Characterization and Applications, First Edition. Edited by Andreas Lendlein, Adam Sisson.

Regenerative medicine is highly interdisciplinary and deals with the restitution, substitution, regeneration of nonfunctional or more or less functionally impaired cells, tissues, organs through biological replacement, for example, through tissues produced *in vitro* or through the stimulation of the body's own regeneration and/or repair processes [3, 4].

Important success in stem cell research [5, 6] and the extracorporeal tissue growth in bioreactors show the potential of regenerative medicine [7–9]. The euphoric visions to grow complete and functional organs *in vitro* right now, however, were recognized to be very premature. This is also due to a lack in basic research and the development of multifunctional implant materials [10].

13.1.3
Functionalized Implant Materials

The experience with polymer implants used in medicine led to a profile of requirements for future polymeric implant materials. The functionality of implant materials has to be broadened. They should be stimuli sensitive and, for example, change their physicochemical behavior due to external stimuli or to biological processes induced at the site of implantation. Bioactive substances like peptides, proteins, or carbohydrates might be immobilized by polymers or released from implants in a well-defined process. The most up-to-date trend in polymer sciences is the development of degradable biomaterials showing multifunctionality. This implies that specific functionalities like hydrolytic degradation, physiological and biomechanical tissue compatibilities, and shape-memory can be adjusted to regiospecific requirements at the site of implantation [11, 12].

AB-copolymer networks are an example for an implant material that can be functionalized.

These networks are produced by photocrosslinking of *n*-butyl acrylate with oligo(ε-caprolacton)dimethacrylate as macrocrosslinker [13, 14]. The incorporation of flexible polybutylacrylate segments allows, for example, the tailoring of material elasticity, which is an important determinant of the biomechanical functionality of this polymer system in the temperature range between room and body temperature. AB-copolymer networks are slowly biodegradable due to their hydrolytically cleavable polyester chain segments. Another group of multifunctional, degradable polymers are multiblock copolymer systems [15–17] containing poly(*p*-dioxanone) hard segments and crystallizable poly(ε-caprolactone) soft segments. Due to their degradability, stimuli sensitivity, biocompatibility, and functionality, these copolymer networks are termed multifunctional. Biomechanical characteristics as well as types and periods of degradation can be adjusted as well.

13.1.4
Sterilization of Polymer-Based Degradable Implant Materials

The sterilization of implant materials is a precondition for their biomedical use. Polymer-based and especially hydrolytically degradable biomaterials in general

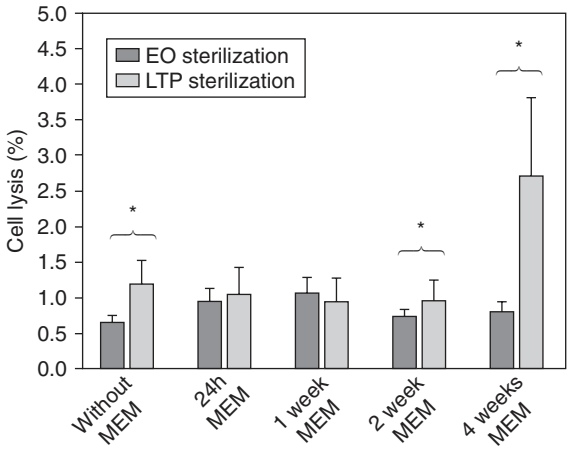


Figure 13.1 Mean rate of cell lysis after different sterilization techniques. Mean rate of cell lysis after EO and LTP sterilization of the polymer samples and different incubation time in physiological solution (MEM). Statistically significant differences of the mean rates of cell lysis were found for the differently sterilized samples without MEM incubation, as well as after 2 and 4 weeks of incubation with MEM. Abbreviations: EO = ethylene-oxide sterilization, LTP = low-temperature plasma sterilization, MEM = minimal essential medium. Reprinted with permission from [20]. Copyright 2003 Wiley Periodicals, Inc.

have a considerably lower thermal and chemical stability as ceramic or metallic materials. They are generally not sterilized with conventional sterilization methods like heat sterilization (temperatures between 160 and 190 °C) or steam sterilization (121 and 134 °C) to avoid a damage of polymers. Sterilization applying ionizing irradiation can change the chemical structure of polymers either by chain degradation or by new crosslinking of chains, so that surface characteristics as well as thermal and mechanical bulk properties can be strongly influenced [18]. A change of the chemical surface structure of implant materials can influence their biocompatibility *in vitro* and *in vivo* [19]. Since the sterilization of polymer-based biomaterials makes high demands on the sterilization method, low-temperature sterilization methods like plasma sterilization (low-temperature plasma sterilization) and sterilization with ethylene oxide are in the focus of intensive contemporary research [20–23] (Figure 13.1).

13.2 Regenerative Medicine for the Reconstruction of the Upper Aerodigestive Tract

Head and neck surgery is concerned with the reconstruction of damaged local tissues like mucosa, cartilage, bone, or skin due to congenital anomalies, progressive diseases, as well as therapeutical interventions. Fistulae of different genesis are associated with most serious complications in the head and neck area [24–26].

These fistulae cause high rates of morbidity and mortality through the development of sepsis, pneumonia, or bleeding from destruction of the carotid wall. The permanent secretion from fistulae and the cervical soft tissue defects (especially of pharyngocutaneous fistulae) is associated with a tremendous reduction of life quality of patients and their stigmatization [24]. Due to postoperative salivary fistulae in oncological patients, their irradiation may not be possible within the planned periods so that therapeutical aims cannot be reached. Contemporary therapeutical options in the treatment of pharyngocutaneous fistulae depend on the size of fistulae and on the indication of a postoperative adjuvant irradiation therapy.

13.2.1 Applications of Different Implant Materials in Tracheal Surgery

In the 1950s, a great number of experiments for the tracheal reconstruction were performed in animals using different materials like acrylresin [27], tantalum [28], stainless steel [29], polyethylene [30], nylon [31],, and teflon [32]. The great number of materials used and the short survival time of the animals demonstrated that the problem of tracheal reconstruction using implant materials could not be solved at this time. The importance of biocompatibility of implant materials and the variable requirements depending on the implantation site became obvious at the end of the 1950s. After the successful application of Dacron™ as arterial prosthesis (1958), it was realized that an appropriate material was not available for the tracheal reconstructive surgery showing the necessary elasticity, rigidity, and biocompatibility. At the end of the 1950s and the beginning of 1960s, there were first trials for the temporary application of polymeric implant materials in the tracheal reconstruction. These materials were covered with mucosa from the urinary or gall bladders to induce growth of connective tissues or bone around tracheal stents. It was called temporary application because the implant material should be removed after the newly grown cartilage or bone in the former tracheal defect zone reached a sufficient stability, so that the reconstructed tracheal tissues would not collapse. Although cartilage and bone tissues could be demonstrated histologically at the site of implantation, a sufficient tracheal stability could not be gained in any one of the animals and all animals died of respiratory insufficiency following tracheal obstruction after the removal of the differently coated implant materials [33, 34]. In the 1960s and 1970s, further materials were tested for tracheal reconstruction, for example, Marlex™ networks (polyethylene/polypropylene networks) [35], silicon rubber [36], and Marlex™ networks covered with cartilage and/or tracheal mucosa [37, 38]. These new materials also did not fulfill the comprehensive requirements for tracheal reconstruction regarding mechanical strength and adequate flexibility to avoid vascular corrosion induced by mechanical irritation. These materials lacked biocompatibility, an air- and liquid tight integration of the implant materials into the adjacent body tissues, an adequate stability against bacterial invasion, and, especially, the epithelialization of the implants with a functional tracheal epithelium [35–38].

Wenig *et al.* showed in 1987 that through application of a fibroblast collagen matrix for the tracheal reconstruction of circumscript defects, the rate of tracheal stenosis could be reduced significantly [39]. In 1989, Schauwecker *et al.* demonstrated the importance of biomechanical properties of implant materials depending on the site of implantation and that the porosity of the material surface was important for the integration of implants in surrounding tissues. These authors applied an isoelastic polyurethane prosthesis with different porosities at the luminal and abluminal surfaces for the reconstruction of 38-mm-long defects of the cervical trachea of 19 dogs. Besides end-to-end anastomosis these authors applied inverted and everted techniques of anastomosis. The mean survival time of animals in case of the inverted technique was 27.7 days, in case of the everted technique 11.3 days, and in case of the end-to-end anastomosis 19.5 days. The worst complications leading to a termination of these trials were local infections and insufficiencies of anastomosis in 12 of the animals and extensive stenoses accompanied by respiratory insufficiency in seven animals. The authors observed that polyurethane prostheses with porous surfaces developed a tight integration into surrounding tissues, but in none of the animals, the luminal prosthetic surface was inhabited by a mucociliary epithelium. The authors attributed the high rate of complications primarily to the animal model chosen because the cervical mobility in dogs was said to be much higher than in humans, pigs, or rats [40].

13.2.2 New Methods and Approaches for Tracheal Reconstruction

Key factors compromising the therapeutical success seem to be the absent regeneration of a functional mucociliary tracheal epithelium enabling the mucociliary clearance, foreign body reactions induced by implant materials, infections, and the necessity of reoperations in preoperated areas. The tissue-engineering technique was described by Langer and Vacanti in 1993 and had three key components: cells for the tissue regeneration, polymer scaffolds as a matrix to support migration, proliferation and differentiation of cells as well as regulating factors which specifically influence the cellular behavior [41]. The following demands on a tracheal prosthesis were made: It should be a flexible construct but able to endure compression which is inhabited by a functional respiratory epithelium [42]. The complete epithelialization of prostheses is thought to be the main condition to allow an adequate mucociliary clearance and to guarantee a reliable barrier against infection and invading connective tissue. There are still very few studies applying the methods of tissue engineering to produce tracheal replacements and to examine these *in vitro* and *in vivo*. Studies introduced by Vacanti *et al.* in 1994 were trend-setting where constructs based on polyglycolic acid and inhabited by bovine chondrocytes and tracheal epithelial cells were applied to close circumferential tracheal defects in rats [43]. In a consecutive study, respiratory epithelial cells were isolated and injected into cartilage cylinders grown *in vitro* [44]. Examinations of these constructs revealed mature cartilage tissues as well as epithelial structures with a submucosal connective tissue. After 3 weeks in culture, different stages of

differentiation of a multilayered highly prismatic epithelium could be documented showing also some ciliary cells. In consecutive experiments, these authors developed a tracheal replacement based on chondrocytes and fibroblasts which was implanted into sheep. The tracheal replacement thus generated could not be shown to develop kinocilia within the respiratory epithelial cells and therefore was not fully functional [45].

Besides the use of different implant materials in experimental and clinical trials during the last 50 years [27–30], there were many other attempts with autologous or allogenic tissues of different origin like fasciae, skin, bone and periost, cartilage and perichondrium, muscle, esophagus, pericardium, intestine, and dura mater [46–50]. Again, high rates of complications were reported, for example, high rates of stenosis and necrosis, of anastomotic insufficiencies, and a lack of mucociliary clearance.

At the end of the 1990s and the beginning of 2000, biodegradable stents were introduced in reconstructive tracheal surgery. Lochbihler *et al.* described in 1997 for the first time the application of a resorbable intratracheal stent made of polyglactine 910 filaments copolymerized with polydioxanone for the temporary stabilization of a tracheal stenosis in rats [51]. Korpela *et al.* applied a spirally shaped and reinforced stent made of poly(L-lactide) to bridge tracheal stenoses in an animal model [52, 53]. Robey *et al.* described in 2000 the application of a biodegradable *poly[(L-lactide)-co-glycolide]* (PLGA) stent for the endotracheal stabilization of reconstructed circumscript defects in the anterior tracheal wall of rabbits using the faszia lata. Stenoses in those animals receiving intratracheal resorbable stents were significantly smaller than those in animals without stents. The high mortality rates of 17% in the implant group and 23% in the control group were mainly caused by the functionally relevant tracheal stenoses. This was the reason why the approach combining the use of autologous materials and biodegradable stents was not accepted. The authors assumed that through controlled release of growth relevant factors from the biodegradable polymeric scaffolds, the potential of this method could be enhanced so that the enhancement especially of cartilage growth would render the reconstructed tracheal segments more stabile [54].

The treatment of subglottic stenoses, especially in children, still is a high challenge in spite of all the progress in surgery. Cotton and Seid in 1980 introduced the anterior cricoid split [55]. After several modifications of this technique and bearing in mind the contraindications, more than 90% of the children can nowadays be extubated without problems. In spite of the progress, in children undergoing single-step surgical therapy to treat subglottic stenoses, it is necessary to use postoperative intubation over several days as an intratracheal splinting. An external splinting by metallic microplates in the surgical tracheal reconstruction was described first time by Zalzal and Deutch in 1991 [56]. Weisberger and Nguyen applied metallic Vitallium™ miniplates for the external splinting of cartilage transplants in the reconstructive tracheal surgery, and 10 of 13 patients (77%) were successfully extubated immediately after surgery [57]. Willner and Modlin introduced resorbable miniplates in the reconstructive tracheal surgery. These resorbable plates were fixed by sutures in the region of the tracheal defect which

diminished the stability in comparison to fixation by screws [58]. Following the successful application of resorbable plates and screws made of PLGA in the pediatric craniofacial surgery [59, 60], Long *et al.* described the external fixation of rib cartilage transplants by PLGA miniplates and screws in the tracheal reconstruction of subglottic stenoses in dogs in 2001. All of the 10 animals could be extubated without problems directly postoperatively. In all of these animals, there was an adequate widening of the subglottic stenoses over the whole period of observation (up to 90 days postoperatively). Two of the animals developed necroses in the cartilage transplants but in spite of this an endoluminal epithelialization was demonstrated histologically. The eight other animals showed a complete epithelialization of the transplants [61]. Since the degradation of PLGA *in vivo* [60] clearly exceeds an observation period of 90 days like in this study, long-term results are missing concerning the resorption of PLGA in tracheal applications and also the influence of degradation products of PLGA on the mucociliary clearance.

Kojima *et al.* described the production of tissue-engineered tracheal equivalents from cylindrical pieces of cartilage and equipped with an endoluminal epithelium in 2003. Cartilage and epithelial cells were harvested from the septal cartilage of sheep and grown *in vitro*. After proliferation and cultivation *in vitro*, the cartilage cells were seeded on a polyglycolic acid matrix. To shape the construct, the cell polymer scaffold was fixed around a silicon tube and then, for cultivation under *in vivo*, conditions, implanted under the skin in the back of nude mice. Precultivated epithelial cells were suspended in a hydrogel and injected into the cartilage cylinders. After removal of the stabilizing silicon tubes, the tissue-engineered constructs were harvested after 4 weeks of implantation. The morphology of the constructs produced by tissue engineering was described to be similar to the native sheep trachea. Maturated cartilage and the generation of a pseudolayered epithelium were demonstrated histologically. Proteoglycanes and hydroxyproline contents of the constructs were comparable to native cartilage so that the authors assumed that there might be a sufficient stability of such a construct *in vivo* [62]. It is thought that such a tissue-engineered construct in comparison to the earlier applied methods might have the potential to further growth after implantation *in vivo*, which could open new perspectives for the tracheal reconstruction in children. Cartilage was harvested so far from ribs, nasal septum, and ears, and also from tracheal and joint cartilage. While Kojima *et al.* assumed that the elastic cartilage from ears might not have the ideal biomechanical properties needed to produce tracheal constructs [62], other authors were less critical in the application of elastic cartilage from ears for the tissue engineering of cartilage in tracheal reconstruction [63].

Tracheal resection with the following end-to-end anastomosis is currently the therapeutical "gold standard" in the treatment of tracheal stenoses, when less than 50% of the tracheal length in adults and less than 1/3 of the tracheal length in small children have to be removed [64, 65]. The reconstruction of longer stenoses is a therapeutical challenge not solved at the moment. The tracheal reconstruction of such long segments by transplants necessitates an adequate blood supply to avoid the necrosis of the transplants. Jaquet *et al.* examined different

three-component grafts in animals to simulate the anatomical structure of the trachea composed of mucosa, cartilage, and adventitia. Transplants consisting of cartilage from the ear and oral mucosa were revascularized through the laterothoracic fascia in rabbits. The epithelialization of three-component grafts was significantly enhanced through the application of perforated mucosa (40% epithelialization of the constructs after application of perforated mucosa versus 10% epithelialization after application of nonperforated mucosa). In all of the 20 operated animals, there was a sufficient vascularization, and necroses were not detected in the transplants [66]. The authors assumed that the production of vascularized composite grafts is an option for the reconstruction of longer tracheal stenoses. A successful application of these constructs in animals and clinical studies is missing, however.

A completely different approach for the reconstruction of longer tracheal segments was chosen by other groups who applied aortal autografts for the tracheal reconstruction in pigs [67] and in sheep [68, 69]. In both animals, the implants were stabilized postoperatively by silicon stents. Immunosuppression was not applied in either of the animal models. In pig implants, an epithelialization with metaplastic epithelial cells, newly grown cartilage, and nonorganized elastic fibers were demonstrated. In sheep implants, there were initial inflammatory reactions followed by the growth of a mucociliary epithelium and the development of new cartilaginous tracheal rings [69]. In 2006, this group published results from the tracheal reconstruction of a longer segment in a human patient applying an aortal autograft. After the resection of a 7-cm-long cervical tracheal segment due to a tracheal carcinoma situated directly caudal of the cricoid cartilage and localized clearly intratracheally without regional lymph nodes or distant metastases, there was a tracheal reconstruction applying a segment of the autologous, infrarenal aorta of this 68-year-old patient. The excised aortal segment was replaced by a Dacron™ prosthesis. A chronical obstructive pulmonary disease, a peripheral arterial occlusive disease, and a myocardial infarction (17 years before the tracheal reconstruction) were known from this patient. The patient was extubated without problems 12 h postoperatively. There was an endotracheal stabilization applying a silicon stent 3 days postoperatively. An adjuvant irradiation of the whole trachea with 30 Gy was started on the 15th day postoperatively. Four weeks postoperatively, an acute dyspnea appeared in the patient due to granulation in the region of the proximal anastomosis which was treated with a further stent application proximal to the first stent. Both stents could be removed without problems 3 months later. Afterward no further granulomatous tissues could be diagnosed endoscopically at the anastomotic sites. Clinically no more states of dyspnea appeared. The patient died due to septical shock in the course of pneumonia in both lungs 6 months postoperatively. Since family members did not accept autopsy, no further details of the performance of the aorta-based tracheal construct could be revealed [70].

Although the aorta-based allogenic tracheal constructs did not perform too well in the pig, this approach in two animal models and in humans was remarkable both from clinical and from scientific perspectives. From a clinical perspective, the use of aortal segments offers a tubular structure, comparable in diameter to

the trachea, which is air and fluid tight, flexible and with high mechanical strength, and is available in the afforded amount. There are problems, however, with the lack of biomechanical stability not avoiding the collapse of airways and with the missing epithelialization. From a scientific perspective, this approach allows the use of decellularized tissues, even of allogenic ones, as preformed, long-distance scaffolds in tracheal reconstruction, which enable the ingrowth and differentiation of the patient's own precursor/stem cells assumed to be needed for the regeneration of functional tissues. The application of tracheal-based allogenic constructs exploiting a decellularized donated human trachea was successfully applied by Macchiarini *et al.* in the reconstruction of a main bronchus of a 13-year-old female patient with a severe bronchio malacia. All cellular and MAC antigens are removed from the trachea which was then feeded with epithelial cells and chondrocytes developed *in vitro* from mesenchymal stem cells of the recipient. The scaffold allowed the unobstructed function of the patient's airways directly after surgery. Now almost 1 year later, the bronchoscopical findings are still regular with appropriate mechanical characteristics and a sufficient bronchociliary clearance. An immunosuppressive therapy was not necessary. The combination of autologous cells with appropriate implant scaffolds is thought to be a well applicable therapeutical option for the reconstruction of the airways [71]. A lot of efforts in basic science and clinical research have still to be spent until the growth of biomechanically loadable segmental cartilage can be engineered on demand and tissue-engineered tracheal constructs will be inhabited by fully functional epithelial cells [72].

13.2.2.1 Epithelialization of Tracheal Scaffolds

The first application in humans of an artificial trachea produced according to principles of regenerative medicine was published by Omori in 2005. A papillary carcinoma in the thyroid of a 78-year-old woman necessitated a hemithyroidectomy together with the resection of the anterior tracheal wall. The tracheal wall defect was reconstructed by a patch based on a Marlex™ net covered with collagen. Two months postoperatively, endoscopic analysis revealed the epithelialization of the scaffold. And there was also a sufficient mechanical stability in the scaffold. Two years after surgery, there were still no respiratory complications or insufficiencies. In spite of missing long-term results, the authors were convinced that new therapeutical options will be offered for the reconstructive tracheal surgery by regenerative medicine [73].

The relatively long period of 2 months needed to epithelialize the patch, which was applied in the tracheal reconstruction, points to a problem that could not get adequately solved. After application of novel polypropylene collagen scaffolds for the reconstruction of circumscript tracheal defects in dogs, the complete epithelialization of the scaffold could be demonstrated 8 months postoperatively only [74]. A fully functional tracheal epithelium is essential as a physical barrier against the extratracheal milieu, as regulator for the comprehensive metabolic functions of the airways including transport of fluids and ions and for the mucociliary clearance and the patency of the airways [75]. The early development of a complete and

functionally adequate epithelialization of tracheal scaffolds is of critical importance for the biofunctionality of implants and constructs produced following the principles of tissue engineering. The research on mechanisms of regeneration and differentiation of respiratory epithelial cells in contact with tissue-engineered constructs started only recently. Before that, the research concerning the differentiation mechanisms of respiratory epithelial cells was focused on their differentiation in the embryonic phase [76] and on the development and differentiation of epithelial cells from precursor/stem cells [77]. It was shown that basal cells of the human trachea probably are precursors of respiratory epithelial cells [77, 78]. The tracheal epithelium is mainly composed of ciliary cells, goblet cells, and basal cells [79–81]. Basal cells are essential for the generation of precursor cells which are fundamental for the regeneration of epithelial damage [77, 78, 82–84].

Nomoto *et al.* seeded the scaffold material used by Omori with tracheal epithelial cells of rats *in vitro*. These epithelial cells expressed *in vitro* the cytokeratines 14 and 18 as typical intermediate filaments of epithelial cells as well as occludin, a constituent of tight junctions in epithelial cells which is a main component of the barrier against diffusion of soluble substances into the intercellular space. The cell-seeded scaffolds were applied for the reconstruction of cervical tracheal defects of 3 mm length in rats. Over the whole period of observation (30 days) *in vivo*, the artificial trachea was covered with epithelium. Partially, a single- or double-layered epithelium was found not carrying cilia, whereas other parts displayed prismatic epithelial cells with functional cilia [85]. In a further development of this technique, a thin collagen matrix (Vitrigel™) was applied for 3D growth of cells in the scaffold. This 3D matrix enhanced the growth of epithelial cells as well as the invasion of mesenchymal cells. There was a clearly accelerated regeneration of functional epithelial cells carrying cilia after tracheal reconstruction in rats using Vitrigel-coated scaffolds compared to noncoated scaffolds [86].

The importance of epithelial–mesenchymal interactions for morphogenesis, homeostasis, and regeneration of the epithelium are well known from literature since several years [87–89]. During epithelial regeneration, epithelial precursors arrived from the borders of epithelial damage to proliferate and differentiate there. Mesenchymal cells situated below the epithelium regulate epithelial growth and differentiation through generation of an appropriate biomatrix and through synthesis and release of growth relevant factors [90, 91]. Fibroblasts are also important participants in the interactions between epithelial and mesenchymal cells and strongly influence epithelial regeneration in wound healing. They are able to secrete a variety of growth factors like keratinocyte growth factor, epidermal growth factor, and hepatocyte growth factor [92, 93]. The importance of fibroblasts was shown already for epidermal wound healing [93], oral [94] and corneal epithelial regeneration [95], and also for tracheal epithelial regeneration [96]. The cocultivation of epithelial cells and tracheal fibroblasts *in vitro* induced the generation of a layered epithelium containing epithelial cells with cilia, goblet cells, and basal cells. Moreover, a basal membrane was constituted *in vitro* between epithelial cells and fibroblasts where the presence of integrin-β4 was demonstrated, which is a specific marker of basal membranes and of epithelial mucin secretion [96].

In further studies, the authors demonstrated the potential of heterotopic fibroblasts (from dermis, nasal, and oral mucosa) for tracheal epithelial regeneration. Regeneration of epithelial cells in contact with different heterotopic fibroblasts showed different characteristics in structure, development of cilia, secretion of mucins, and expression of ion and water channels, for example, aquaphorines and Na^+/K^+ ATPase. In contact with nasal fibroblasts, however, no mature and fully functional tracheal epithelium was generated *in vitro*. Dermal fibroblasts induced the generation of an epidermal like epithelium. Especially the cocultivation with fibroblasts from the oral mucosa induced the regeneration of a morphologically and functionally regular tracheal epithelium. This was comparable to the regeneration of epithelium *in vitro* after cocultivation with tracheal fibroblasts. Fibroblasts from the tracheal and the oral mucosa expressed keratinocyte growth factor, epidermal growth factor, and hepatocyte growth factor. Fibroblasts from the oral mucosa enhanced proliferation and migration of epithelial cells *in vitro* similarly to the tracheal fibroblasts. Since the explantation of oral mucosa is clearly less invasive than that of tracheal mucosa, there seems to be a very promising method available now to develop scaffolds with a functionally adequate epithelium for the tracheal reconstruction [97].

In 2008, the same group used this technique of cocultivation of epithelial cells and tracheal fibroblasts to produce a tracheal scaffold seeded with cells *in vitro* and applied the tissue-engineered scaffold for the tracheal reconstruction in rats [98]. The authors could demonstrate a fully functional epithelium *in vivo*. Besides the cocultivation of tracheal epithelial cells and fibroblasts, also the cocultivation of tracheal epithelial cells and mesenchymal stem cells for the "*in vitro*" reconstruction of a fully functional tracheal epithelium is described in the literature. The epithelium thus produced showed morphological, histological, and functional characteristics of the tracheal mucosa. The authors assumed that the cocultivation with mesenchymal stem cells could play a main role in tissue engineering in future [99].

13.2.2.2 **Vascular Supply of Tracheal Constructs**

A problem not adequately solved so far is the vascular supply of scaffolds and of tissue constructs developed from these scaffolds *in vivo*. In contrast to other parenchymal organs, the trachea is supplied by a network of small blood vessels which is evidently not easy to generate. Microanastomoses were not successful in animal models [100, 101] and therefore not further persecuted. It is known from the literature that after tracheal reconstruction, the capillary network present at the anastomosis proceeded in the direction of the implant only 2 cm at maximum and that this process of revascularization took several months [102]. In tracheal implants, which were longer than 3 cm, there was a lysis of the epithelium with a consecutive destruction of the basal membrane followed by the development of granulomatous tissues producing a tracheal stenosis. While bioreactors allow the growth of autologous cells [103] and functional tissues and are routinely used for the generation of osteochondral constructs, and tissue-engineered heart valves, there are very few studies showing the application of bioreactors for the generation

of tracheal scaffolds. Decisive problems hindering the application of tracheal scaffolds in humans are the missing epithelialization and revascularization of the constructs. Tan *et al.* published in 2006 the concept of a so-called *in vivo* bioreactor for the generation of tracheal constructs. They proposed layered scaffolds with a porous catheter within the inner layer of the scaffold for a continuous supply of cells and nutrition media and an outer layer of the construct granting the necessary stability. In contrast to traditional bioreactors in which nutrition media mainly flow around the constructs, now a perfusion system was planned within the scaffolds similar to the blood vessel distribution *in vivo* [104]. This group seeded in a next step a phase-segregated multiblock copolymer (DegraPol™) [105] with human tracheal epithelial cells and offered a continuous supply of cells and nutrition media via a porous catheter within the scaffolds. The continuous perfusion of the tubular biodegradable scaffolds coincided with an adequate epithelialization of the constructs and an accelerated vascularization in the chorioallantois membrane assay. The authors assumed that the concept of the *in vivo* bioreactor allows a more physiological process in the reconstruction of tissues and that better initial conditions are granted for the problem so far not solved, the vascularization of tracheal scaffolds [106].

13.2.3
Regenerative Medicine for Reconstruction of Pharyngeal Defects

The reconstruction of the pharynx by degradable, multifunctional polymeric materials would be a novel therapeutical option in head and neck surgery. The use of implant materials for the reconstruction of pharyngeal defects is currently at the early beginning. Until now, there are only data concerning the use of implant materials in the area of the oral mucosa and the palate available. Hallén *et al.* injected crosslinked hyaluronic acid in rats in the dorsal pharynx wall to treat velopharyngeal insufficiency. In all animals, an early inflammatory reaction due to the hyaluronic acid was found. Six months after injection, the hyaluronic acid was still detectable at the original localization of injection and surrounded by connective tissues. Despite lacking of long-term results, the authors assumed that the injection of crosslinked hyaluronic acid is appropriate for the augmentation of a slight velopharyngeal insufficiency in humans [107]. Ophof *et al.* implanted skin substrates after cell seeding with oral keratinocytes *in vitro* into palatinal wounds in dogs as a model for closure of cleft palate by tissue-engineered constructs. In all six animals, the loss of the epithelium and a distinctive degradation of the skin substrates were detectable. The authors concluded that an adequate integration of these tissue-engineered constructs required an early and sufficient revascularization of the scaffolds *in vivo* [108]. A main focus in tissue engineering of oral mucosa is currently the use of novel dermal scaffolds and epithelial cell culture methods including 3D models. An updated review is given by Moharamzadeh *et al.* [109].

Despite numerous biomedical applications of tissue-engineered constructs in almost all medical fields, up to now there are no literature data available regarding the pharyngeal reconstruction with implant materials after tumor resection neither

in animal models nor in humans. The availability of multifunctional polymeric implant materials, which can be adapted according to the anatomical, physiological, biomechanical, and surgical requirements [12, 16], facilitates the development of novel therapeutical options also in head and neck surgery. A main scientific topic of the own group is the biocompatibility testing of an elastic degradable AB-copolymer networks [13, 14] *in vitro* and *in vivo*, which seems to be appropriate for the reconstruction of pharyngeal defects due to its physicochemical characteristics.

13.3
Methods and Novel Therapeutical Options in Head and Neck Surgery

13.3.1
Primary Cell Cultures of the Upper Aerodigestive Tract

The use of cell cultures is an essential tool in nearly all biological and medical research laboratories. The biocompatibility testing should be conducted with cultures of site-specific cells depending on the biomedical application to assess the specific interaction between the biomaterial and site-specific different cells [110]. Thus, the biocompatibility testing of a polymeric material which seems to be appropriate for the reconstruction of pharyngeal defects should be conducted with primary cell cultures of the pharynx. The knowledge about the interactions between the implant materials and cells/tissues is a basic requirement for an ideal adaptation of a polymeric material according to the specific needs of the upper aerodigestive tract (ADT). In our studies, primary cell cultures of the oral cavity, the pharynx, and the esophagus were established and biochemically characterized. Immunocytological investigations showed different relative amounts of epithelial, fibroblastic, and smooth muscle cells depending on the anatomical site of explantation [111]. Relatively little is known about the mechanisms of regular and delayed wound healing of the pharyngeal epithelium. Therefore, a comprehensive characterization of primary cell cultures of the pharynx was a first step for the development and establishment of novel therapeutical options [111, 112].

13.3.2
Assessment and Regulation of Matrix Metalloproteases and Wound Healing

The amount and organization of the extracellular matrix in normal wounds is determined by a dynamic balance between overall matrix synthesis, deposition, and degradation [113]. A strictly controlled degradation of the extracellular matrix is an important process for the regular wound healing. An imbalance between degradation and synthesis of the matrix during wound healing would cause a delayed wound healing with fistulae and ulcerations in case of outbalanced degradation of the extracellular matrix or hypertrophic scars and keloids in case of outbalanced synthesis of the extracellular matrix [114].

Matrix metalloproteases (MMPs) are a class of structurally related, zinc-dependent endopeptidases that are collectively responsible for the degradation of extracellular matrix proteins. MMPs have an important function in wound healing [115, 116]. Under regular conditions *in vivo*, the expression and activation of MMPs is strictly controlled. The activity of MMPs is regulated at the level of transcription and zymogen activation and can be inhibited by specific inhibitors: the tissue inhibitors of metalloproteases TIMPs. Recently, four different TIMPs (TIMP 1–4) were identified and cloned [117]. In the literature, different MMP- and TIMP levels were reported in regular and delayed wound healing [118, 119]. The delicate balance between the activity of MMPs and TIMPs plays a key role in building a functional extracellular matrix. Up to now, little is known about the mechanisms of wound healing and MMP expression of cells of the upper ADT *in vitro* and *in vivo* [120, 121].

A comprehensive characterization of the MMP- and TIMP expressions of cells of the upper ADT is a basic requirement to develop and establish novel therapeutical options in head and neck surgery in case of delayed wound healing after surgical treatment. A main focus of the own biocompatibility testing was the analysis of the MMP- and TIMP expressions of primary cell cultures of the upper ADT after cell seeding on different modifications of the polymeric implant material to gain the knowledge for an optimal adaptation of these materials to the specific requirements of the upper ADT.

Among the primary cell cultures investigated, cells of the pharynx were seeded on the surface of a multifunctional copolymer as well as on the surface of commercially available polystyrene cell culture dishes as control. On both surfaces, cells became adherent, proliferated, and reached confluence. No statistically significant differences of the mean cell numbers were found on Day 1, 3, 6, 9, and 12 of cell growth after cell seeding [112]. The highest MMP-1-, MMP-2-, and TIMP levels were found on Day 1 of cells' growth on both surfaces. There were decreasing levels during the following time of the investigation (Figure 13.2). No statistically significant differences of the MMP- and TIMP expressions were detectable between the polymer and the control surfaces. The kinetics of MMP-2 expression were analyzed on the protein level and by RT-PCR on the mRNA level (Figure 13.2) [112]. Based on the current results, the adhesion, proliferation, and differentiation of the primary cell cultures of the pharynx were not influenced by the multifunctional copolymer.

13.3.3 Influence of Implant Topography

The integration of a material in the surrounding tissues is a basic requirement for a successful clinical application of an implant material *in vivo*. The surface characteristics of materials including their surface topography and chemical composition are of very high importance for the interaction between the material and cells and tissues [122, 123]. Until now, some cellular processes are known, which could be useful to assess the cellular behavior on implant materials. Most of this knowl-

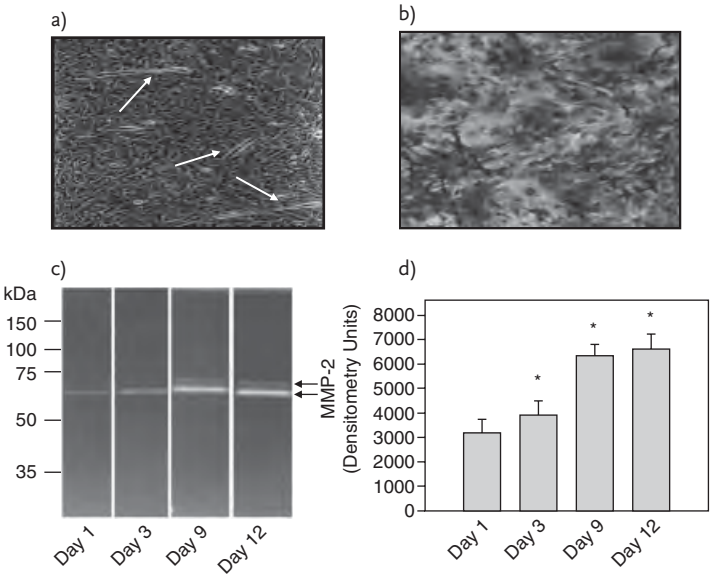


Figure 13.2 Histological findings of pharyngeal cells and results of zymography of MMP-2 of pharyngeal cells grown on a polymer surface. (a) Phase-contrast microscopy of pharyngeal cells grown on polystyrene surface of commercially available cell culture dishes is shown. Pharyngeal cells showed a confluent monolayer on the surface of 35-mm cell cultures dishes after 3 days with the typical cuboid morphology of epithelial cells. Smooth muscle cells of the pharyngeal epithelium are labeled by white arrows (magnification ×20). (b) In order to better visualize the pharyngeal cells after cell seeding on the polymer surface, Coomassie Blue staining was used. Pharyngeal cells began to form colonies after cell seeding and started to become confluent on Day 3 of cell growth (magnification ×20). (c) SDS-substrate gel electrophoresis (zymography) of primary cell cultures of the pharynx grown on the polymer surface is shown. The kinetics of appearance and activity levels of 72 kDa (MMP-2) band of pharyngeal cells are shown on Day 1, 3, 9, and 12 of cell growth. Bands are marked by arrows. The gelatinolytic activities of media conditioned by pharyngeal cells grown on the polymer surface were normalized to equal cell numbers. (d) Scanning densitometry units of the gelantinolytic bands are shown. Statistical analysis was performed to determine differences of MMP-2 levels between Day 1 and the subsequent days of cell growth. Statistically significant differences ($P \leq 0.05$) are marked by a star. Data taken from three independent experiments (values are mean ± SD). Parts c and d reprinted from [111], Copyright 2007, with permission from IOS Press.

edge is based on cell culture investigations and it is unknown if these mechanisms are also found *in vivo* [124, 125]. A fundamental requirement for a successful application of degradable implant materials for the pharyngeal reconstruction *in vivo* is a saliva-tight integration of the material in surrounding tissues to avoid salivary fistulae with destruction of neighboring soft tissue. The development of long-term degradable polymeric scaffolds for pharyngeal reconstruction has to guarantee an adequate biocompatibility and biofunctionality as well as growth of a functional tissue formation considering the specific physiological and

mechanical requirements of the upper ADT. Important progress in biomaterial research of the last years was made in the improvement of cell adhesion and proliferation by the optimization of scaffold design with respect to specific requirements of the different implantation sites *in vivo* [126]. Main aspects of the research work were focused on the influence of different macroscopical and microscopical design parameters on the local differentiation of variable cells. Other aspects dealt with the controlled release of growth factors [127, 128]. Until now, relatively little is known about the influence of different surface topographies of polymeric implant materials on the gene expression and synthesis of enzymes that are directly involved in extracellular matrix remodeling [129, 130].

Our own results demonstrated the importance of the surface structure of polymeric implant materials on the cellular behavior depending on surface roughness (smooth versus rough surfaces). The cell adhesion, proliferation, as well as the kinetics of secretion and activity of MMP-1, MMP-2-, and TIMPs differed significantly depending on the type of cells and on the surface structure of the copolymer. Significantly greater average total cell numbers of oral and pharyngeal primary cells were found after cell seeding on the rough surface compared to the smooth polymer surface. Esophageal cells showed the highest cell numbers on the control (polystyrene). Oral and pharyngeal cells revealed similar kinetics of appearance and activity of MMP-1, MMP-2, and TIMPs with the highest values on Day 1, followed by a decrease of the activity levels on the rough polymer and the control surface. Oral and pharyngeal cells seeded on the smooth polymer surface displayed an opposite pattern with the lowest activity of MMP-1, MMP-2, and TIMPs on Day 1 and the highest values on Day 12. Esophageal primary cell cultures showed a comparable kinetic pattern of appearance and activities on all three different surfaces (smooth and rough polymer surface, control surface) with the lowest MMP-1-, MMP-2,- and TIMP expression on Day 1 and the highest values on Day 12 [131].

The presence or absence of the extracellular matrix or components of it govern the proliferation, differentiation, and biochemical activities of different primary cell cultures of the upper ADT. These results were confirmed by data from the literature, which also showed the influence of the surface topography on the gene expression and synthesis of the enzymes directly involved in extracellular matrix remodeling [132].

The results of these experiments suggest a specific influence of surface topography on the behavior of cells in contact with implant materials. The knowledge of the exact mechanisms of the cell–biomaterial interactions is a basic requirement for the development of an "ideal" implant material to establish cell- and tissue-optimized novel therapeutical options in head and neck surgery based on polymeric implant materials.

13.3.4 Application of New Implant Materials in Animal Models

The use of degradable implant materials in the area of the upper ADT makes high demands on the chemical, enzymatic, bacterial, and mechanical stability of a material. A premature degradation of the implant material would probably cause exten-

sive salivary fistulae with high mortality potentially culminating in carotid artery rupture. Because of the chemical conditions in the upper ADT with changing pH values, enzymatical, bacterial, and particular mechanical load during deglutition and digestion, the reconstruction of the upper ADT by a degradable implant material requires adequate chemical, enzymatical, bacterial, and mechanical stabilities of the scaffold material. We established a standardized radical critical defect in the gastric wall of rats which was closed by an elastic long-term degradable polymeric implant. The stomach was used as a "worst-case" application site to test the stability of the implant material under extreme chemical, enzymatical, bacterial, and mechanical load. In this model, the mortality of the gastric breakdown of sutures with fistulae and local or generalized peritonitis in the follow-up is comparable to the mortality of insufficiencies and salivary fistulae of the pharynx. The implantation group included 42 animals. A primary wound closure of the gastric wall defect without biomaterial implantation was conducted in the control group ($n = 21$). Furthermore, a so-called baseline group which included animals kept under the same housing conditions without any surgical procedure was investigated ($n = 21$). The implantation periods or times of observation were 1 week, 4 weeks, and 6 months [133, 134].

Fundamental parameters investigated in this animal model were a tight closure between the polymer and surrounding tissues, the chemical and mechanical stability of the implant material, and the integration of the polymer in the surrounding tissue as well as the question of tissue regeneration after reconstruction of the defect with the polymeric implant material. Gastrointestinal complications like fistulae, perforation, or peritonitis did not occur in any of the animals. A liquid- and gas-tight anastomosis between the polymer and the adjacent stomach wall existed in all animals of the implantation group [133]. To test the impermeability between the implant material and adjacent gastric wall, the intragastric pressure was measured after maximal dilatation of the stomach by air insufflation (Figure 13.3) [133]. Neither in the implantation group nor in the control group a delayed wound healing was observed macroscopically or microscopically after 1 week, 4 weeks, and 6 months of implantation time after primary wound closure. After 1 week, a beginning regeneration of the gastric wall was detected starting from the border area of the gastric wall defect. After 4 weeks and 6 months, a regular multilayered stomach tissue as known from histology was found in the former defect zone of the gastric wall (Figure 13.4). In the control group, the defect was replaced by scar tissue [134]. Furthermore, the systemical influence of the AB-copolymer network was investigated. It is well known from literature that the peritoneum is a very sensitive compartment for inflammatory reactions in the organism depending on the biocompatibility of implant materials [135]. Incompatibilities of implant materials, a too early degradation, or the accumulation of degradation products are expected to cause local inflammatory reactions originating acute-phase reactions concomitant with the induction of gene expression of acute-phase proteins. The concentrations of the acute-phase proteins α_1-acid glycoprotein and haptoglobin, however, did not show statistically significant differences between the AB-copolymer network and the control group [136]. The analyses of the mechanisms of the integration of the implant material in the adjacent tissues as well as the mechanisms of material

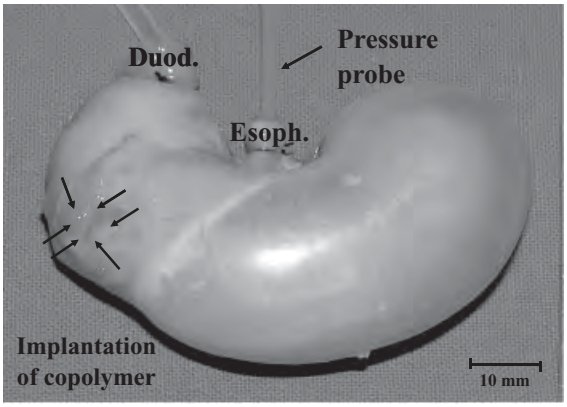
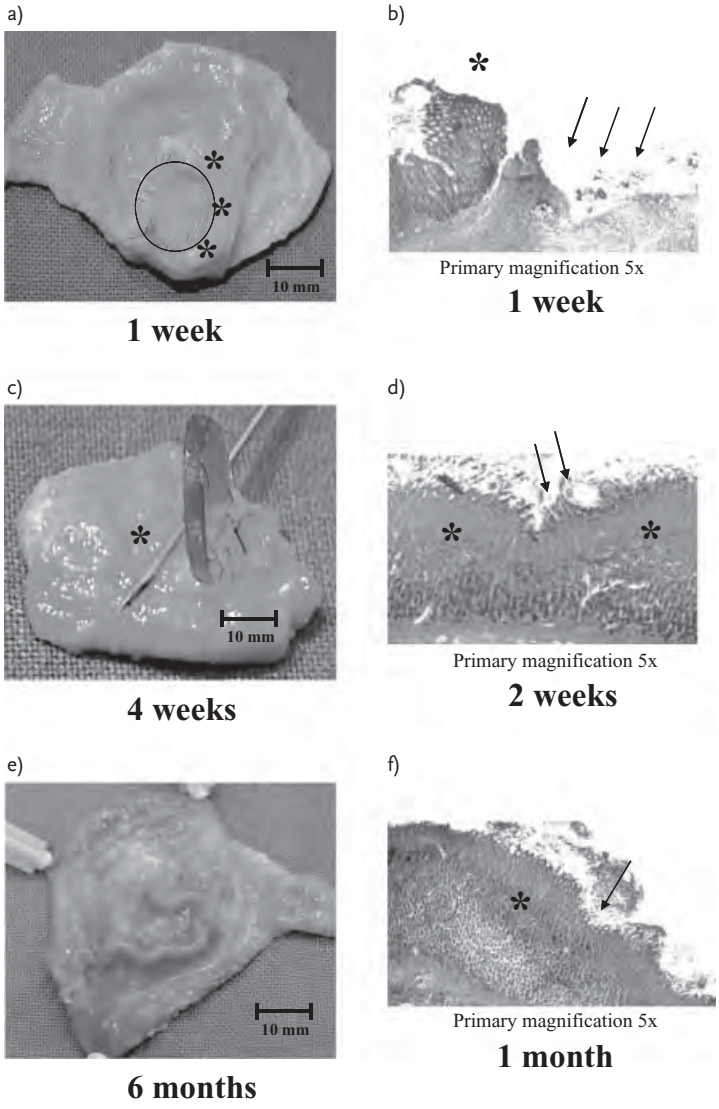


Figure 13.3 Aspect of the explanted stomach after 1 week of copolymer implantation. The polymer implantation site is marked by arrows. A flexible tube for air insufflation was inserted in the duodenum. The pressure was measured by a probe in the resected esophagus. The pressure probe is marked by an arrow. A special anatomical feature of the rat stomach becomes overt: the stringent separation between the glandular part of the stomach where the copolymer was implanted (marked by arrows) and the nonglandular part. The influence of this special anatomical feature on the biofunctionality of the polymeric material is unknown so far and needs to be investigated in another animal model. Abbreviations: Duod. = Duodenum; Esoph. = Esophagus. Reprinted by permission from [133], available at "http://www.reference-global.com/." Copyright 2006, Walter de Gruyter GmbH & Co. KG.

Figure 13.4 Macroscopical and histological findings after polymer implantation. (a) The explanted stomach is shown after 1 week of implantation. The polymer is marked by a black line. The mucosa started to overgrow the polymer from the border area (marked by stars). (b) Histological findings are shown after 1 week of implantation. The marginal area next to the defect zone showed a regular stomach epithelium marked by stars. According to the macroscopical findings, the beginning of tissue regeneration was detectable from the marginal area next to the defect zone. The polymeric material used for defect closure was removed due to the xylene and ethanol treatment and cutting of paraffin sections and was not detectable on most of the histological sections (defect closures by polymer are marked by arrows). (c) After 4 weeks of implantation time, the polymer was almost detached from the stomach and was just fixed by single sutures. The former defect was closed by regenerated tissue (marked by a star). (d) The histological findings after 2 weeks of implantation time are shown. The marginal areas next to the former defect zone are marked by stars. The former defect zone (marked by arrows) was regenerated by histological regular formated stomach epithelium. (e) After 6 months of implantation time, the polymer was completely detached from the stomach wall in all animals. (f) Histological findings after 1 month of implantation time are shown. Histologically regular formated stomach epithelium was found in the former defect zone (marked by an arrow) in all animals of the implantation group. No differences were detectable between the epithelium of the marginal area next to the former defect zone (marked by a star) and the regenerated epithelium of the former defect zone (marked by an arrow). Reprinted from [134] with permission. Copyright 2007, Georg Thieme Verlag KG, Stuttgart, New York.



degradation and tissue regeneration are topics of currently ongoing examinations. It was recently found that by introducing glycolide–glycolide diads as weak links [105, 137, 138] in the macrodimethaycrylate precursors, a faster and adjustable degradation rate of the rather slowly degrading AB-copolymer networks can be achieved. For semicrystalline partially degradable AB-copolymer networks from oligo([ε-caprolactone]-*co*-glycolide) dimethacrylates and *n*-butylacrylate of different molar glycolide contents *in vitro* higher degradation rates of AB networks with higher χG were measured by mass loss, decrease of G, and increase of Q due to the glycolide containing ester bonds and especially glycolide–glycolide diades in

the oCG, which can be considered as weak links [105, 137]. Upon cleavage of glycolide containing ester bonds, the remaining oligo(ε-caprolactone) segments regain mobility, can rearrange, and crystallize as shown by slightly increasing T_m during degradation, for example, AB-CG(21)-10. The degradation *in vivo* was only slightly accelerated compared to *in vitro* conditions in the studied time frame for glycolide-free AB-CG(0)-10 networks. This suggests that enzymes, which are known to be major contributors to the degradation of poly(ε-caprolactone) [139] could not very well access the semicrystalline poly(ε-caprolactone) segments in the bulk of the AB networks [140].

In the experiments performed with the AB-copolymer networks so far, the chemical, hydrolytical, and enzymatic stability as well as the biomechanical functionality of the polymeric implant material were shown under the extreme conditions of the stomach. The postoperative increase in weight of the animals [133], the impermeability between the implant material and adjacent tissues of the gastric wall [133], the concentrations of the acute-phase proteins α_1-acid glycoprotein and haptoglobin [136], as well as the lack of gastrointestinal complications suggest that the wound healing was not negatively influenced by the degradable AB-copolymer network during the time of investigation. On the contrary, a support of tissue regeneration by the implant material was detected. The results available so far regarding the tissue compatibility allow to regard the AB-copolymer network as a very promising implant material for the development of novel therapeutical options in head and neck surgery based on degradable biomaterials.

13.4 Vascularization of Tissue-Engineered Constructs

The vitality and functionality of tissue-engineered constructs depends on an adequate blood supply with oxygen and nutrients as well as on the removal of metabolites. Most of the tissues/organs successfully tissue engineered until now are relatively thin and/or avascular like cartilage, skin, or urinary bladder. Therefore, wound healing-driven angiogenesis in recipients is thought to be sufficient to supply the tissue-engineered constructs with oxygen and nutrients in many cases. It was suggested that the supply of blood and nutrients of the scaffolds applied for pharyngeal reconstruction could be sufficient because the used implant materials are relatively thin (<100 μm). In any case, the applied scaffolds should support angiogenesis. The investigation of the influence of polymeric implant materials on the angiogenesis is therefore an important aspect of biocompatibility testing.

In our investigations *in vitro*, we showed that bovine capillary endothelial cells (ECs) of the adrenal cortex [141] became adherent on the copolymer surface and developed confluent cell layers [142]. Also, in the chorioallantois membrane assay, no negative influence of the copolymer samples on the vascularization was detectable [142, 143]. A controlled release of angiogenic factors from vesicles on the polymer surface according to the principles of drug delivery to support angiogenesis is a scientific topic of currently ongoing investigations.

At present an adequate vascularization of the cellular colonized scaffolds *in vivo* is one of the most critical points for tissue engineering of complex and metabolic challenging organs like heart or liver. In case of parenchymal organs, the tissue-engineered microcirculation has to be connected to the recipients' circulation. The currently available techniques for the vascularization of tissue-engineered constructs can be classified in "*in vitro*" and "*in vivo*" methods. In the last years, considerable progress was made to solve the problems of building microcirculatory networks for comprehensive 3D constructs. Kunz-Schughart *et al.* developed a 3D cell culture system with cocultivation of human skin fibroblasts and ECs of the umbilical cord. They found a support of migration, vitality, and development of tubular structures of the ECs by fibroblasts. Based on such models, knowledge about the integration of capillary structures in engineered tissues can be gained [144]. Au *et al.* approached the vascularization of tissue-engineered scaffolds by cocultivation of constructs with blood vessel cells like endothelial and perivascular cells. The authors demonstrated that the co-implantation of the scaffolds with tissue-specific cells and endothelial and perivascular cells led to the development of vascular structures *in vivo*, connecting the scaffolds and recipient's circulation. The stability and adequate functionality of these vascular structures have been shown for more than one year. Based on these results, the authors assumed that this technique of co-implantation is a promising approach for the vascularization of tissue-engineered constructs [145].

On the other side, there are still numerous unsolved problems with and beyond the connection of scaffolds to the recipient's vascularization, like the maintenance or increase of vascular density with an increase of tissue or organ mass or activity, the maturation of functionally inadequate vessels, as well as the unwanted regression of vascular structures. One of the answers to these problems might be gained in future through a comprehensive knowledge about the regulation of the heterogeneous ECs in different organs. Furthermore, an extensive knowledge about the mechanisms of the molecular processes of cellular interactions between ECs, pericytes, and smooth muscle cells and between blood vessels and parenchymal cells are needed. Beyond that, the mechanical characteristics of blood vessels like permeability, elasticity, and compressibility have to be analyzed and the design of nonthrombogenic surfaces of implant materials have to be devised. A review about the current knowledge of microcirculation engineering as a basic requirement for a successful tissue engineering of parenchymal organs is given by Lokmic *et al.* [146].

13.5 Application of Stem Cells in Regenerative Medicine

Stem cells have the capacity for self-renewal and capability of differentiation to various cell lineages. Thus, they represent an important building block for regenerative medicine and tissue engineering. These cells can be broadly classified into embryonic stem cells and nonembryonic stem cells comprising adult stem cells.

Embryonic stem cells are called pluripotent and can differentiate in all cell types of the three embryonic germ layers. The adult stem cells are multipotent and the differentiation of these cells is committed to only one of the germ layers. Embryonic stem cells have a great potential but their use is limited by several ethical and scientific considerations. Limiting factors for the use of embryonic and adult stem cells next to ethical considerations [147, 148] are problems associated with extensive cell expansion *in vitro* [149], problems with *in vitro* cultivation on implant materials [150, 151], cell apoptosis following implantation [152], as well as vascularization [153].

Stem cells were already studied by Becker *et al.* in 1963 who injected bone marrow cells into irradiated mice and noticed that nodules developed in the spleens of the mice in proportion to the number of bone marrow cells injected [154, 155]. They concluded that each nodule arose from a single marrow cell. Later on, they found evidence that these cells were capable of infinite self-renewal, one of the main characteristics of stem cells.

Stem cells have been used successfully in experimental and clinical studies for bone, cartilage, spinal cord, cardiac, and bladder regeneration. A current review about the application of stem cells in the field of regenerative medicine is given by Bajada *et al.* [5].

In 2001, Vacanti *et al.* reported the successful tissue engineering of the distal phalanx and the replacement of this bone in a 36-year-old patient who suffered partial avulsion of the thumb [156]. However, only 25% of the normal strength was obtained. Quarto *et al.* reported on the use of autologous culture-expanded bone marrow stromal cells combined with porous hydroxyapatite for the reconstruction of critical sized defects (bone segmental defects 4–7 cm long) of tibia, ulna, and humerus. The results were encouraging, with good graft integration and return to functionality [157]. In 2006, Hibi *et al.* published the use of tissue engineering to augment bone formation in humans in combination with vertical distraction osteogenesis (DO) by an osteocutaneous fibular transplant for the reconstruction of the mandible after irradiation. DO is a method for the elongation of the bone that is used, among others, in the surgical reconstruction of facial skull to bridge bony defects of different genesis. To promote 3D bone formation and shorten the consolidation period, the authors applied tissue-engineered osteogenic material ("injectable bone") in a patient who was treated with vertical DO and an osteocutaneous fibular flap to reconstruct the mandible. The material which comprised autologous mesenchymal stem cells was culture-expanded and then induced to be osteogenic in character. Platelet-rich plasma was activated with thrombin and calcium chloride and infiltrated into the distracted tissue at the end of distraction and injected into a space created labially with a titanium mesh at implant placement. The reconstructed mandible was expanded from 10 to 25 mm in height despite a lacerated and opened labial periosteum in the distracted area. The authors assumed that DO assisted by tissue engineering could be the therapy of choice in future for the surgical reconstruction of bony defects [158]. Furthermore, the authors used this technique of tissue-engineered osteogenic material ("injectable bone") successfully for the osteoplastic reconstruction in cleft palate in a 9-year-old girl [159].

While stem cells are successfully in clinical use for the regeneration of articular cartilage since several years [160], the complete reconstruction of the auricle by tissue engineering is still a great challenge in head and neck surgery. The reasons are complex and especially related to the unsolved problems of scaffold design and of the differentiation induction of stem cells to produce elastic ear cartilage [161]. There are numerous other less-attended fields of research in head and neck surgery needing stem cell technology, for example, the mucosal reconstruction in the upper ADT. A first approach is the development of ciliated epithelium by cocultivation of stem cells with site-specific cells [78].

While these technologies are already in use for the reconstruction of the mucosa of the urinary tract [162] and the cornea [163] and for teeth regeneration [164], the development of mucosal reconstruction in head and neck surgery except for the salivary gland tissue [165–167] is still at the relative beginning.

13.6 Conclusion

The clinical applicability and quality of an implant material are exclusively shown in successful clinical use. The profile of demands on a material is therefore determined by the conditions *in vivo*. The chemical, enzymatical, bacterial, and mechanical conditions of the upper ADT make high demands on an implant material for the mucosal reconstruction in this area. In reconstructive surgery of the trachea, none of the different implant materials investigated by versatile methodical approaches was successfully introduced into the clinical use. For the reconstruction of pharyngeal defects based on the principles of regenerative medicine, there exist neither animal models nor a clinical application in humans until now. Based on the progress in polymer chemistry, multifunctional implant materials are available nowadays, which can selectively initiate biological processes in a physiological environment and/or change their physicochemical characteristics in reaction to external stimuli. The availability of such multifunctional implant materials and the progress in tissue engineering resulted in the establishment of novel therapeutical options in different medical fields. Applying stem cell technology, further progress is expected for the reconstruction of different tissues based on the principles of tissue engineering. To benefit from the potential of such technologies for the development and the establishment of novel therapeutical options in head and neck surgery, clinicians have to be involved in these interdisciplinary scientific projects of regenerative medicine.

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14
Biodegradable Polymers as Scaffolds for Tissue Engineering

Yoshito Ikada

Abbreviations

bFGF	basic fibroblast growth factor
BMCs	bone marrow cells
BMPs	bone morphogenetic proteins
ECM	extracellular matrix
ES	embryonic stem
FDA	Federal Drug Administration
GTR	guided tissue regeneration
iPS	induced pluripotent stem
IRB	Institutional Review Board
IVC	inferior vena cava
MSCs	mesenchymal stem cells
OA	osteoarthritis
PCBM	particulate cancellous bone and marrow
PCL	poly(ε-caprolactone)
PGA	polyglycolide
PLA	polylactide
PLLA	poly-L-lactide
TCPC	total cavopulmonary connection
TEVAs	tissue-engineered vascular autografts
VEGF	vascular endothelial growth factor
3D	three-dimensional

14.1
Introduction

Tissue engineering is a new paradigm that offers new medical means for clinicians and patients who need new tissues for their defective or lost ones [1]. It has long been recognized that the limb of salamanders and newts are readily regenerated when lost. The ability to regenerate damaged human organs would constitute a

Handbook of Biodegradable Polymers: Synthesis, Characterization and Applications, First Edition. Edited by Andreas Lendlein, Adam Sisson.

medical revolution. However, the regeneration of human tissues is currently almost impossible except for a few tissues including blood cells, epithelia, and bones. The reason may be disclosed when the developmental biology will have much more advanced in the near future.

Notwithstanding, it seems instructive for scientists and engineers of tissue engineering to first learn mechanisms of the natural development progress of human embryo and adults, even if the biological environments for the embryonic organogenesis are substantially different from those for the tissue regeneration in adults.

14.2
Short Overview of Regenerative Biology

Throughout the history of experimental biology, certain organisms have repeatedly attracted the attention of researchers. For instance, we cannot look at the phenomenon of limb regeneration in newts or starfish without wondering why we cannot grow back our own arms and legs [2]. The reactivation of development in postembryonic life to restore missing tissues has been a source of fascination to humans. We are beginning to find answers to the great problem of regeneration, so that we might be able to alter the human body so as to permit our own limbs, nerves, and organs to regenerate. This would mean that severed limbs could be restored, that diseased organs could be removed and regrown, and that nerve cells altered by age, disease, or trauma could once again function normally. To bring these treatments to humanity, we first have to understand how regeneration occurs in those species that have this ability. Gilbert points out three major ways by which regeneration can occur [2]. The first mechanism involves the dedifferentiation of adult structures to form an undifferentiated mass of cells that then become respecified. This type of regeneration is called epimorphosis, characteristic of regenerating limbs. The second mechanism is called morphallaxis. Here, regeneration occurs through the repatterning of existing tissues with little new growth. Such regeneration is seen in hydra. A third intermediate type of regeneration can be thought of as compensatory regeneration. Here, the cells divide, but maintain their differentiated functions. They produce cells similar to themselves and do not form a mass of undifferentiated tissue.

14.2.1
Limb Regeneration of Urodeles

When an adult salamander limb is amputated, the remaining cells are able to reconstruct a complete limb, with all its differentiated cells arranged in the proper order. It is appropriate to begin with the example of the urodele amphibian limb, simply because the adult urodele responds to amputation by regenerating a perfect replica of the original limb.

As a result of decades of research, we have considerable knowledge about the cell- and tissue-level biology of limb regeneration [3]. Of particular significance are those findings that indicate that once the regeneration cascade progresses to blastemal stages, the mechanisms controlling growth and pattern formation are the same as those in developing limbs [4]. Thus, the challenge to understanding what might be needed to induce regeneration in humans becomes focused on the developmental signals controlling the transformation of the differentiated stump into a blastema. In addition, a number of key requirements necessary for a successful regeneration response have been disclosed. These include the formation of a wound epidermis that creates a permissive environment necessary for a regeneration response, the dedifferentiation of cells at the injury site, the requirement for adequate innervation, and the need to reinitiate patterning programs involved in limb outgrowth. The absence of any one of these requirements will result in regenerative failure. If we assume that the successful induction of limb regeneration in higher vertebrates will proceed in a manner similar to urodeles, then we can anticipate that all of these requirements must be satisfied at the amputation site. These requirements for a regeneration response may be potential barriers to regeneration in higher vertebrate limbs. Urodele limb regeneration is characterized by the formation of a blastema composed of undifferentiated mesenchymal cells from which many of the different tissues of the regenerated limb develop. Similarly, regeneration of developing tissues proceeds via a blastema-like stage with the re-expression of developmentally relevant genes.

Despite the value of the urodele limb as a model for a regeneration, research progress in recent years has been relatively slow due to difficulties of bringing the power of functional analysis to bear on urodeles. It seems likely that the critical breakthroughs in regeneration research will come from the identification of the molecules that control the early events, preceding the convergence of the regeneration and development pathways. Given techniques for efficient high-throughput screening and analysis of differentially expressed genes, combined with techniques for identifying interacting molecules, urodeles will provide the opportunity to identify all the candidate genes for the control of limb regeneration. With the ability to test the function of these genes, it will be possible to identify the molecules that regulate the key steps in the process, allowing for the realization of the longed-for goal of human regeneration.

14.2.2
Wound Repair and Morphogenesis in the Embryo

Adult wound healing is notoriously imperfect and generally results in fibrosis and scar contracture with poor reconstitution of epidermal and dermal structures at the site of the healed wound, whereas embryonic wounds heal extremely well, rapidly, efficiently, and perfectly.

Adult wound closure involves active movements of both connective tissue and epidermis. The exposed connective tissue of the wound – the granulation

tissue – contracts to tug the wound edges together and, as this is happening, the epidermis migrates to cover over the exposed connective tissue. The embryo also utilizes a combination of connective tissue contraction and re-epithelialization movements to close a wound, but the cellular mechanisms for both movements are quite different in embryo and adult. Another major difference between adult and embryonic tissue repair concerns the extent of inflammation during healing – at adult wound sites, there is always an extensive inflammatory response, but in the embryo, inflammation is minimal, if not nonexistent. Wound healing is an initial and critical event in any regeneration response. If wound healing occurs perfectly, that is, without scarring, then the skin (epidermal and dermal tissues) can be considered to have regenerated. Indeed, embryonic and fetal wounds heal rapidly without scarring, just as embryonic limb buds and fetal digits are able to respond to amputation by mounting a regenerative response. During a limb regenerative response, wound closure results in the formation of a specialized structure, the wound epidermis, which creates a subepidermal environment essential for regeneration. It seems likely that a similar type of subepidermal environment will be necessary for a regeneration response during healing of the skin. It seems unlikely that successful limb regeneration can occur under healing conditions that results in the deposition of scar tissue. Thus, scar-free wound healing is likely to be a necessary precondition for a successful regeneration response.

14.2.3 Regeneration in Human Fingertips

The transition from urodele limb studies to experimental attempts to induce a regenerative response in higher vertebrates has met with few successes, none resulting in a normal limb. This has led to the general conclusion that a "magic bullet" for regeneration is unlikely, but that the induction of a regeneration response will involve a coordinated effort to overcome multiple barriers to regeneration. While the regenerating urodele limb is the system of choice, alternative approaches are to study the limited regenerative responses that are known to occur in the limbs of higher vertebrates: digit tip regeneration in adult mammals. In fact, human digit tips can regenerate. Digit tip regeneration in adult primates (including humans) and rodents occurs without the formation of a blastema; instead, fibroblastic cells appear to be involved in the regeneration response. Fingertip amputations are among the most common traumas seen in hospital emergency rooms [5]. There are numerous reports that a conservative treatment consisting simply of covering the amputation wound with sterile dressings and allowing it to heal by secondary intention (i.e., without assisted wound closure) will result in the regeneration of the missing distal portion of the finger [6]. The phenomenon of fingertip regeneration in humans was initially described for children, but later shown to extend to adults. For both children and adults, regeneration of the fingertip involves the integrated regeneration of many tissues including nail matrix, nail bed, finger pulp, sensory organs, dermis, and epidermis, all of which reform to a normal or nearnormal cosmetic and physiological state through

healing by secondary intention. Elongation of the distal phalangeal bone during regeneration has only been documented for children [7], but most studies lack radiographic data that allow for the assessment of bone regrowth. Animal models for digit tip regeneration in adults demonstrate distal bone growth associated with a regeneration response. There are several documented instances of regeneration of the distal phalangeal element of the toe following traumatic injury or voluntary resection to relieve hummer toe [8]. Thus, it would appear that the regenerative capabilities in human limbs include the tips of both fingers and toes.

14.2.4
The Development of Bones: Osteogenesis

The skeleton is generated through three lineages: the somites generate the axial skeleton, the lateral plate mesoderm generates the limb skeleton, and the cranial neural crest gives rise to the branchial arch and craniofacial bones and cartilage. There are two major modes of bone formation or osteogenesis, and both involve the transformation of a preexisting mesenchymal tissue into bone tissue. The direct conversion of mesenchymal tissue into bone is called intramembranous ossification. In other cases, the mesenchymal cells differentiate into cartilage, and this cartilage is later replaced by bone. The process by which a cartilage intermediate is formed and replaced by bone cells is called endochondral ossification. The cranial neural crest cells form bones through intramembranous ossification. In the skull, neural crest-derived mesenchymal cells proliferate and condense into compact nodules. As shown in Figure 14.1, some of these cells develop into capillaries; others change their shape to become osteoblasts, committed bone precursor cells. The osteoblasts secrete a collagen–proteoglycan osteoid matrix that is able to bind calcium. Upon embedding in the calcified matrix, osteoblasts become osteocytes. As calcification proceeds, bony spicules radiate out from the region

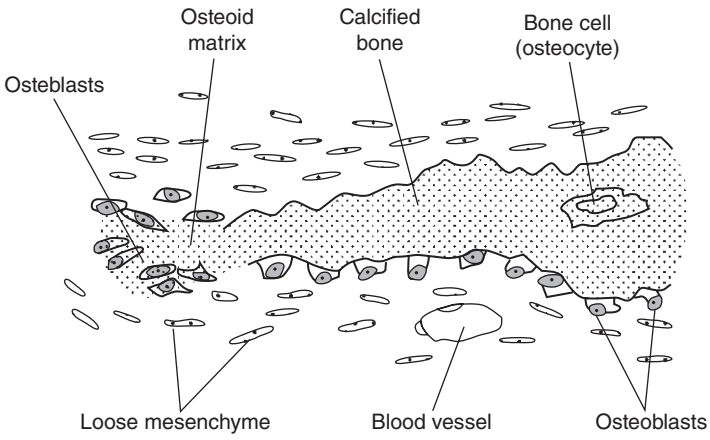

Figure 14.1 Schematic diagram of intramembranous ossification.

where ossification began. Furthermore, the entire region of calcified spicules becomes surrounded by compact mesenchymal cells that form the periosteum. The cells on the inner surface of the periosteum also become osteoblasts and deposit matrix parallel to the existing spicules. The mechanism of intramembranous ossification involves bone morphogenetic proteins (BMPs) and the activation of a transcription factor called Runx2.

Endochondral ossification involves the formation of cartilage tissue from aggregated mesenchymal cells and the subsequent replacement of cartilage tissue by bone [9]. This is the type of bone formation characteristic of the vertebrae, ribs, and limbs. The process of endochondral ossification can be divided into five stages, as shown in Figure 14.2. First, the mesenchymal cells commit to becoming cartilage cells. This commitment is caused by paracrine factors that induce the nearby mesodermal cells to express two transcription factors, which will then activate

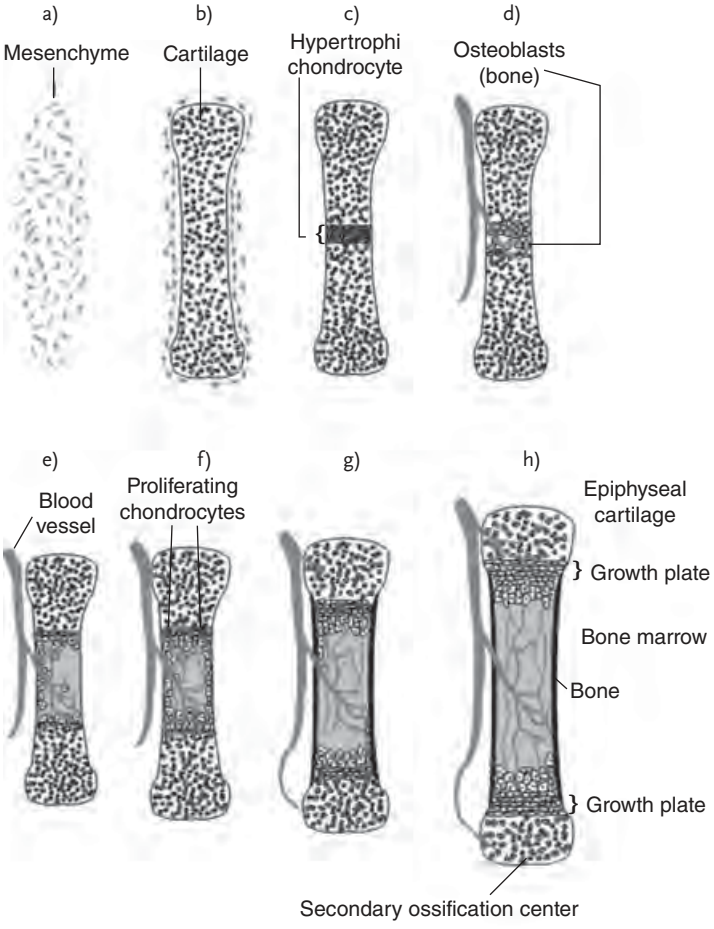

Figure 14.2 Schematic diagram of endochondral ossification.

cartilage-specific genes. During the second phase of endochondral ossification, the committed mesenchymal cells condense into compact nodules and differentiate into chondrocytes.

During the third phase of endochondral ossification, the chondrocytes proliferate rapidly to form the cartilage model for the bone. As they divide, the chondrocytes secrete a cartilage-specific extracellular matrix (ECM). In the fourth phase, the chondrocytes stop dividing and increase their volume dramatically, becoming hypertrophic chondrocytes. These large chondrocytes alter the matrix they produce (by adding collagen X and more fibronectin) to enable it to become mineralized by calcium carbonate. They also secrete the angiogenesis factor, vascular endothelial growth factor (VEGF), which can transform mesodermal mesenchymal cells into blood vessels. A number of events lead to the hypertrophy and mineralization of the chondrocytes, including an initial switch from aerobic to anaerobic respiration, which alters their cell metabolism and mitochondrial energy potential. Hypertrophic chondrocytes secrete numerous small membrane-bound vesicles into the ECM. These vesicles contain enzymes that are active in the generation of calcium and phosphate ions and initiate the mineralization process within the cartilaginous matrix. The hypertrophic chondrocytes, their metabolism and mitochondrial membranes altered, then die by apoptosis.

In the fifth phase, the blood vessels induced by VEGF invade the cartilage model. As the hypertrophic chondrocytes die, the cells that surround the cartilage model differentiate into osteoblasts. These cells express the Runx2 transcription factor, which is necessary for the development of both intramembranous and endochondral bone. The replacement of chondrocytes by bone cells is dependent on the mineralization of the ECM. This remodeling releases VEGF, and more blood vessels are made around the dying cartilage. These blood vessels bring in both osteoblasts and chondroclasts (which eat the debris of the apoptotic chondrocytes). Eventually, all the cartilage is replaced by bone. Thus, the cartilage tissue serves as a model for the bone that follows.

14.2.5
Regeneration in Liver: Compensatory Regeneration

Today, the standard assay for liver regeneration is to remove specific lobes of the liver (i.e., a partial hepatectomy), leaving the others intact. The removed lobe does not grow back, but the remaining lobes enlarge to compensate for the loss of the missing liver tissue. The amount of liver regenerated is equivalent to the amount of liver removed. The liver regenerates by the proliferation of the existing tissues. The regenerating liver cells do not fully dedifferentiate when they reenter the cell cycle. No regeneration blastema is formed. Rather, the five types of liver cells – hepatocytes, duct cells, fat-storing (Ito) cells, endothelial cells, and Kupffer macrophages – each begin dividing to produce more of themselves. Each type of cell retains its cellular identity, and the liver retains its ability to synthesize the liver-specific enzymes necessary for glucose regulation, toxin degradation, bile synthesis, albumin production, and other hepatic functions. As in the regenerating

salamander limb, there is a return to some embryonic conditions in the regenerating liver. Fetal transcription factors and products are made, as are the cyclins that control cell division. But the return to the embryonic state is not as complete as in the amphibian limb.

14.3 Minimum Requirements for Tissue Engineering

14.3.1 Cells and Growth Factors

The leading player in tissue engineering is cells because it is only this living microsystem that is able to regenerate living tissues. This is different from the conventional artificial organs and tissues, where biomaterials play a pivotal role. Very recently, pluripotent stem cells such as embryonic stem (ES) and induced pluripotent stem (iPS) cells have attracted extraordinarily much attention, but these cells cannot be applied directly to tissue engineering. The cells applicable to tissue engineering should be differentiated to regenerate target tissues or will be readily differentiated depending on the environment surrounding the predifferentiated cells. The cells closely associated with tissue engineering include fibroblast, osteoblast, chondrocyte, epithelial cell, and smooth muscle cell. In addition, it should be mentioned that numerous organs contain multipotent stem cells, even in the adult. Multipotent stem cells can give rise to a limited set of adult tissue types. However, they are not as easy to use as pluripotent ES cells. First, they appear to have a relatively low rate of cell division and do not proliferate readily. Second, they are difficult to isolate, and are often fewer than one of every thousand cells in an organ. The mesenchymal stem cells (MSCs) from the bone marrow are still relatively undifferentiated, but commited to a certain lineage, and have the capability to readily differentiate based on the circumstance.

To produce a clinically applicable size of tissues by tissue engineering, we need a large number of cells, but the amount of cells that can be harvested from patients is limited. Therefore, attempts have been made to multiply the harvested cells retaining the ability to generate tissues. One of the unsolved problems in tissue engineering is to multiply the MSC keeping the undifferentiated state. If this is achieved in culture, the benefit is potentially enormous. An addition of high concentrations of basic fibroblast growth factor (bFGF) in culture has been claimed to facilitate the MSC multiplication [10], but the positive effect has not always been reported.

Cytokines greatly affect tissue engineering in terms of cell multiplication, cell differentiation, and neovascularization. A well-known example is BMPs that are able to induce ectopic bone formation without any cell addition. Similarly, bFGF encourages capillary formation without exogenous cell addition. Such vascularization is critical for nutrient supply to cells in the regeneration site. An important

strategy associated with growth factors in tissue engineering is not to use a bolus dose of growth factors but to maintain the growth factor concentration at an optimal level for a certain period. For the sustained delivery of biologically active agents, carriers or delivery vehicles are generally employed, but there are few reports that have explored carriers effective in the sustained release of growth factors. Much more efforts are required to enhance the beneficial effects of growth factors on tissue engineering.

14.3.2 Favorable Environments for Tissue Regeneration

There are two modes of tissue engineering for tissue construction. One is *in vitro* (*ex vivo*) tissue engineering and the other *in vivo* (*in situ*) tissue engineering. In the beginning of tissue engineering research, many people attempted to construct living tissues outside the human body, that is, *in vitro* or *ex vivo*. Although a number of joint ventures were established to this end, most of them failed in the *in vitro* production of clinically applicable tissues on large scales. It may imply that it is difficult for us to create the artificial environment that is effective for cells to generate tissues outside the human body. Generally, a substrate to which cells attach is required for cells to survive, proliferate, and differentiate. It will be not difficult to prepare such substrates from biomaterials, but continuous supply of oxygen and nutrients to cells producing tissues is a hard task, because the supply is often disturbed by the tissues produced. The ideal route for oxygen and nutrient supply to cells is through capillaries, but sufficient capillary formation is impossible in the *in vitro* tissue engineering. This may be the reason for very limited applications of *in vitro* tissue engineering mostly to epidermal production. Tissue engineering below means the *in vivo* tissue engineering unless specified.

An essential requirement for tissue engineering is to provide cells with a favorable environment for tissue regeneration. In the case of *in vitro* tissue engineering, we, researchers, should create the environment that is the most effective for the cells in terms of tissue regeneration including cell proliferation, migration, and differentiation. In contrast to the *in vitro* tissue engineering, we do not need to create the optimal environment by ourself in the *in vivo* tissue engineering. The patient body will produce the most effective environment for the tissue regeneration by itself, if we could effectively support it.

What tissue engineers can help cells is to offer a good substrate for cell attachment, an effective barrier for preventing undesirable cells from invasion into the regeneration site, and a facility for promoting capillary formation, in other words, neovascularization. When a permissive environment optimal for cells to regenerate tissues is formed expectedly by these supplies, tissue regeneration will smoothly proceed by itself. However, a very large number of current studies on tissue engineering but a very small number of clinical trials so far imply that such an environment optimal for the *in vivo* tissue engineering can be produced only with great difficulty.

14.3.3
Need for Scaffolds

It should be noted that the natural ECM, a major component of connective tissues, is not a template or scaffold in organogenesis of embryo, but simply a product accompanying the embryogenesis. This suggests that it is not reasonable to regard a scaffold as an artificial ECM, although the current major topic in scaffold research is to mimic the natural ECM.

In discussing the rational design of scaffolds, it is necessary and pertinent to divide scaffolds into two groups (Scaffold type I and type II) on the basis of the cells to be seeded in scaffolds. Scaffold type I is used for differentiated cells including fibroblast, osteoblast, and chondrocyte, as represented in Figure 14.3. Figure 14.4 demonstrates Scaffold type II that is used for not yet fully differentiated pro-

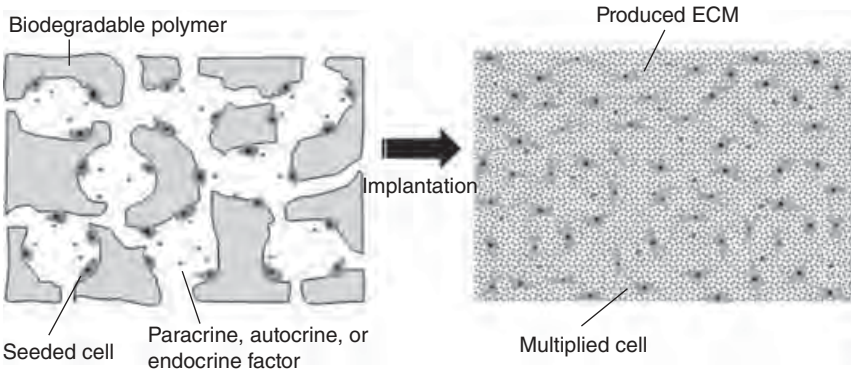

Figure 14.3 Scaffold type I for differentiated cells.

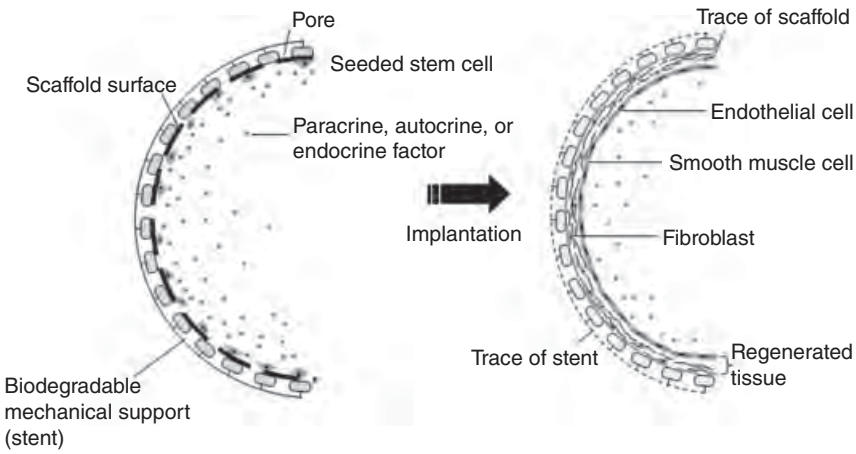

Figure 14.4 Scaffold type II for progenitor cells.

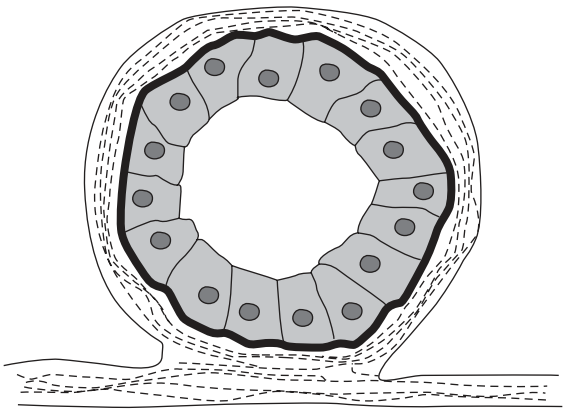

Figure 14.5 Cells assembling during organogenesis of embryo.

genitor or stem cells such as MSCs. The cells seeded in Scaffold type I produce mostly the ECM consisting of fibrous proteins, proteoglycans, and glycoproteins, constructing a connective tissue, combined with differentiated but still active cells. In this case, the 3D structure of the regenerated tissue may be regulated by the 3D structure of the scaffold.

In contrast to Scaffold type I, Scaffold type II primarily provides a perforated surface for progenitors to proliferate and differentiate into the target cells. The wall tissue of large-calibered blood vessels is exemplified in Figure 14.4. The perforated structure acts to allow oxygen and nutrient supply from the surrounding. Generally, the scaffold surface may be fabricated with a thin porous material to accommodate cells as many as possible. In the beginning of embryonic organogenesis, cells assemble into a characteristic form, as demonstrated in Figure 14.5. This cell assembling must be definitely affected by biological signaling in addition to cell–cell interactions. The biological signaling will be conveyed by endocrine factors migrated into the regeneration site from the adjacent environment as well as the autocrine and paracrine factors secreted by the seeded cells. These cytokines transform recruited precursor cells from the host into the cells producing target tissues. Similarly, the factors will dictate the fate of the cells attaching to the surface of Scaffold type II, finally resulting in regeneration of the tissue with the shape different from the scaffold.

When a large-sized, lost tissue is to be replaced by a neo tissue regenerated *in situ*, a mechanical support will be tempolarily necessary until to the full regeneration of the tissue. The tissues requiring such mechanical supports include large tubular tissues such as large-calibered blood vessels, trachea, and large tubular bones. For instance, when a partially lost aorta is to be replaced with a regenerated tissue, a tubular template should be placed in the lost site. If the tubular material is too weak in mechanical strength, it will undergo rupture before the formation of a new tissue. Loss of large bones also requires a mechanical support until to bone regeneration. A problem accompanying these events is the disturbance of

tissue regeneration by the supporting materials. Such a trouble would not arise if small experimental animal models like rat are used for tissue engineering studies. Only the use of bioabsorbable materials that will be resorbed, matching with the neo tissue formation, would circumvent this problem.

14.4 Structure of Scaffolds

When a scaffold is defined as any biomaterials used to encourage tissue regeneration, it may include the substrate for cell attachment, the barrier for cells to retain the site for tissue regeneration, the guide for cells to create a tissue giving the contour of the regenerated tissue, the carrier for the sustained release of growth factors, and the mechanical support until to tissue regeneration. It is unlikely that a single biomaterial can address all these requirements, although such a simple case is often seen in studies using rats as animal model. It should be emphasized that the scaffold that is clinically applicable is practically different from that for small animals, mostly because of difference in mechanical strength. The material property necessary and common to all scaffolds is temporally controlled biodegradability.

14.4.1 Surface Structure

The most important role of scaffolds in tissue engineering is to provide an attachment site for the cells responsible to the tissue regeneration. Similar to embryonic development, multiple cells should assemble to a specified form for tissue formation. To this end, cells would bind each other through the cell–cell interactions and, in addition, cells attach to a substrate for their survival, proliferation, and differentiation. If tissue regenerates by the help of growth factors alone, a carrier, not a substrate, will be required for their sustained delivery.

Fibronectin is well known as a cell-adhesive protein and has been very often attempted to immobilize on the scaffold surface. However, chemical modification of synthetic polymer materials with entire ECM molecules or relevant peptide fragments is not always necessary for scaffolds used in tissue engineering, because fibronectin molecules are more or less present in both serum and body liquids and adsorb to the scaffold surface unless it is too hydrophilic like nonionic hydrogels or too hydrophobic like fluorinated polymers. As the fibronectin adsorption needs a certain period, scaffolds lacking immobilized fibronectin would take a longer time for cell attachment than those with immobilized fibronectin. Collagen is also cell-adhesive and hence frequently employed for the enhancement of cell attachment.

Because of poor cell adhesion, hydrogel scaffolds need surface modification, when applied in tissue engineering, but they are basically not appropriate for scaffolds, since their mechanical strength is too low to retain the environment necessary for tissue regeneration.

The surface of Scaffold type II has mostly curvature that guides the regeneration of complicatedly shaped tissues. It is this surface contour of scaffolds that determines the 3D structure of tissues with a complicated contour.

14.4.2 Porous Structure

Most of the scaffolds studied in tissue engineering have porous structure which can be created by salt leaching, freeze-drying, sintering, or other much sophisticated technologies. The pores are not independent with each other, but interconnected. There are several reasons for the porous structure. One is to make route for the transport of oxygen and nutrients to the cells in the scaffolds and of the cell waste products to the outside. For the oxygen and nutrient supply, the pore size is less important than the porosity. Another reason, especially for Scaffold type II, is to fill cells as many as possible in the limited space of scaffold. Clearly, the scaffold with interconnected pores has a higher accommodation capacity for cells than nonporous materials. In addition, it is also critical for the new ECM produced by the seeded cells to have space.

The scaffolds (type I) prepared by electrospinning have many interstices, but they are too small for cells to infiltrate, although sufficiently large for nutrient supply. To facilitate neovascularization inside a porous scaffold, the pore size should be much larger than the capillary diameter with recommended size around 200–300 μm.

14.4.3 Architecture of Scaffold

The marked difference between Scaffold type I and type II is the dimension; type I is 3D, while type II is 2D. The surface is characteristic to type II, but the thickness or depth is important for type I. The thickness of the target tissue is influenced by the thickness of Scaffold type I, while the surface of type II regulates the regeneration of tissue. Probably, the cells evenly distributed on the scaffold surface will produce a new tissue toward the outside of the scaffold or into the new environment. New tissue ingrowth into the scaffold inside will be prevented by the scaffold material still remaining without being resorbed. Even if Scaffold type II has thickness, it has nothing to do with the thickness of the regenerated tissue. In this case, the scaffold is not the mold for the tissue to be regenerated. Scaffold type II is very thin with low mechanical strength, but it is not recommended to increase the mechanical strength by making the scaffold thicker, because the presence of a large mass of biomaterial at the location of tissue regeneration will disturb the proceeding of tissue regeneration, even if the biomaterials are biodegradable. This is because it is extremely difficult to match the biodegradation rate with the tissue regeneration. An effective method for strengthening scaffolds is to make use of stenting or reinforcement. Composites made from a porous sheet and a stent or a reinforcement material are often overlooked, but they will yield a

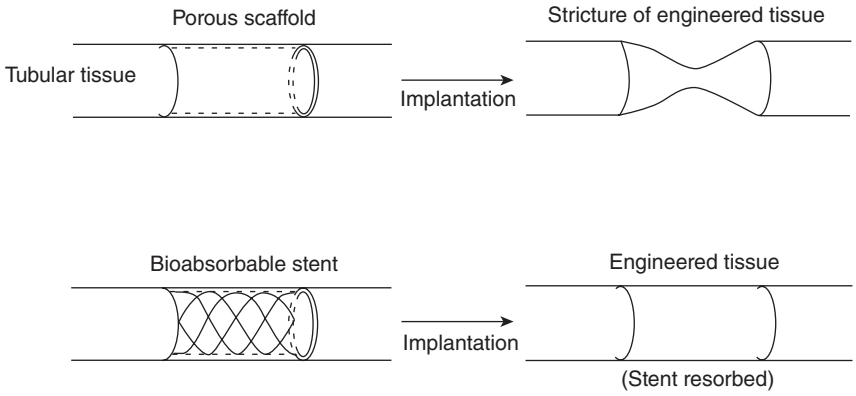

Figure 14.6 Protection of a tubular tissue by a biodegradable stent.

beneficial surface of Scaffold type II for tissue engineering. Moreover, stenting is very effective in preventing a tubular scaffold from stenosis (narrowing), as demonstrated in Figure 14.6.

14.4.4
Barrier and Guidance Structure

Any scaffolds for tissue engineering should retain and protect the environment where the tissue regeneration proceeds. The protection is generally performed by placing a barrier membrane around the permissive environment for the tissue regeneration. Well-known examples are the barrier membrane clinically used as a sheet for the guided tissue regeneration of periodontal tissues and as a tube for the regeneration of peripheral nerves. A long tube will guide the end of the extending peripheral nerve to reach the destination. The protection against invading cells and overloading from the outside will be also achieved by bulky 3D scaffolds to a certain extent.

In addition, a barrier membrane will act as a container for the bone marrow harvested from patients. The MSCs present in the bone marrow will initiate tissue regeneration in the protected environment where the bone marrow has been filled.

14.5
Biodegradable Polymers for Tissue Engineering

To fulfill the diverse needs in tissue engineering, various biodegradable materials have been exploited as scaffolds for tissue regeneration. Strictly speaking, biodegradable polymers are not identical to bioabsorbable (absorbable or resorbable) polymers, because the mechanism of polymer disappearance from the implanted site varies. Biodegradation is defined as chain scission of polymers constructing a biomaterial to shorter chains, finally to the monomer or oligomers in biological

environments which contain a variety of hydrolytic enzymes, while bioabsorption simply means that the biomaterial has disappeared from the implanted location by any means. They include both enzymatic biodegradation and no chain scission of polymers. The polymer disappearance without any chain scission occurs due to the dissolution of polymer chains into the body fluid as a result of release of crosslinks that have made the polymer water-insoluble. Such crosslinking is mostly of physical type such as salt bridging with calcium ion, electrostatic interaction between cationic and anionic charges, and hydrogen bonding.

An example of salt bridging is alginate crosslinked with Ca^{2+}. If polyethylene glycol is a component of a biodegradable block copolymer, the water-soluble polyethylene glycol portion will be absorbed into the body fluid upon biodegradation of the other component. Such absorbable polymers are here included in biodegradable polymers.

A broad variety of biodegradable polymers have been studied, but those which are clinically applicable as scaffolds for tissue engineering are not many and very limited.

14.5.1 Synthetic Polymers

Traditional aliphatic poly(α-hydroxy acid)s alone have been virtually used as scaffold biomaterials for large animal experiments among numerous synthetic, biodegradable polymers. This may be due to the ease of the polyesters for fabricating porous scaffolds with different porosities and pore sizes, mechanically strong or elastomeic scaffolds, biodegradable scaffolds with different biodegradation rates, and nontoxic scaffolds with proved biosafety. Additionally, the Federal Drug Administration (FDA)/CE mark approval of several of these polyesters has motivated the application of those polymers in the tissue engineering field.

Polyglycolide (PGA) that includes here both glycolide homopolymer and glycolide-L-lactide (90:10) copolymer has the largest medical use among all commercially available, biodegradable polymers. The medical application of PGA is almost as sutures. Nonwoven fabrics fabricated from PGA fibers are also commercially available and have been used as scaffolds for tissue engineering, greatly contributing to the rapid progress of tissue engineering during the initial phase of tissue engineering research.

It is extremely difficult to prepare porous scaffolds starting from PGA powders in academic laboratories, because PGA of high molecular weights is soluble only in specific solvents like 1,1,3,3-hexafluoro-2-propanol. In contrast, copolymers of glycolide and lactide around equal monomer ratios (PGLA) are soluble in conventional organic solvents and hence have been widely used for scaffold fabrication, although the tensile strength of PGLA scaffolds is much less than PGA nonwoven fabric scaffolds. The property characteristic to PGA and PGLA is their high biodegradation rates; the mechanical strength decreases to the half within one month in the presence of water. It follows that the scaffolds prepared from these aliphatic polyesters can be applied only to the tissue engineering where tissue regeneration proceeds at relatively high rates.

In contrast to these glycolide polymers, polylactide (PLA) synthesized from L-lactide or D,L-lactide monomer undergo hydrolysis at much lower rates than glycolide polymers. It has been observed that a part or all the parts of PLA mass still remain without resorption when implanted up to one year. This implies that the application of PLA to tissue engineering may be limited to the tissues which require relatively long periods of time for regeneration.

Poly(ε-caprolactone) (PCL) has often been used for scaffold studies, probably because of its high processability (low melting point, many available organic solvents, and high strength product). However, PCL scaffolds would not be applicable to clinical tissue engineering, simply because PCL materials with high mechanical strength is virtually non-biodegradable *in vivo*. Clearly, only biodegradable polymers can be used for preclinical and clinical trials of tissue engineering.

In marked contrast to PLA and PCL, copolymers from P[LA/CL] are partially crystalline and produce strong, elastomeric scaffolds that have biodegradation rates ranging between those of PGA and PLA.

14.5.2 Biopolymers

Biodegradable biopolymers or biomacromolecules include polysaccharides, proteins (polypeptides), and nucleic acids. The most frequently used polymer for scaffold fabrication among these biopolymers is collagen, whereas nucleic acids have been scarcely used in tissue engineering studies. As collagen is ubiquitously distributed in our body, it is no wonder that many researchers have chosen collagen as a candidate material for scaffold fabrication, although mad cow disease has greatly hampered the use of collagen. Porous collagen sheet can be prepared by freeze-drying of aqueous collagen solution, followed by crosslinking with glutaraldehyde or dehydrothermal treatment. The porous structure can be controlled by changing the freeze-drying temperature. Lower freezing temperature yields scaffolds with smaller pores. Porous collagen sheets have been clinically applied to the skin tissue engineering from the 1980s, often making composite with glycosaminoglycan. Freeze-drying of aqueous solution of gelatin, denatured collagen, also produces porous gelatin sheets, but they have been applied to tissue engineering much less frequently than collagen sheets. As collagen and gelatin are able to serve as carrier of growth factors, the scaffolds fabricated from these polypeptides may have features different from others.

The low mechanical strength and high rate of degradation of biopolymers can be improved by chemical crosslinking. Fibrin glue which quickly forms upon mixing fibrinogen with thrombin has been used as scaffold preferably by surgeons. This biomedical hydrogel has low mechanical strength, but is capable of holding a large number of cells. In addition, this gel can seal another scaffold, if it has large interstices.

Chitin and chitosan are biomaterials that have been common choices for scaffold studies among polysaccharides. Scaffolds of high mechanical strength can be prepared from these crystalline biopolymers, but it should be mentioned

that chitosan undergoes no appreciable biodegradation *in vivo*, at least, in rat model [11].

14.5.3
Calcium Phosphates

In contrast to organic biomaterials, inorganic biomaterials or minerals have been used for scaffolds only to a limited extent due to poor processability into highly porous structures and brittleness despite their good osteoconductivity. Among them are calcium phosphate compounds, because they are more or less biodegradable. Tetracalcium phosphate [$Ca_4O(PO_4)_2$] is resorbed more quickly than tricalcium phosphate [$Ca_3(PO_4)_2$] which has wide application as scaffold with interconnected pores. Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] has been most widely used as biomaterials in orthopedic and oral surgery, but this calcium phosphate has been applied less frequently to tissue engineering than tricalcium phosphate, because hydroxyapatite is resorbed at much lower rates. However, the low resorption rate may not matter if we take it into consideration that hydroxyapatite is virtually identical to the mineral part of natural bones.

14.6
Some Examples for Clinical Application of Scaffold

14.6.1
Skin

Bilayered biomaterials composed of an inner porous collagen sheet, with or without chondroitin-6-sulfate, and an outer silicone layer have been clinically applied to skin tissue engineering [12, 13]. When placed on wounds even without any cell seeding, the collagen scaffold is replaced by a regenerated dermis-like tissue, while the silicone layer can be readily peeled off.

14.6.2
Articular Cartilage

Wakitani *et al*. applied tissue engineering to the repair of human articular cartilage defects in osteoarthritis (OA) knee joints [14]. The study group comprised 24 knees of 24 patients with osteoarthritis knee, who underwent a high tibial osteotomy. Adherent cells in bone-marrow aspirates were expanded by culture, embedded in a collagen gel scaffold, transplanted into the articular cartilage defect in the medial femoral condyle, and covered with autologous periosteum at the time of 12 high tibial osteotomies. The other 12 subjects served as cell-free controls. In the cell-transplanted group, as early as 6.3 weeks after transplantation, the defects were covered with white to pink soft tissue, in which metachromasia was partially observed. Forty-two weeks after transplantation, the defects were covered with

white soft tissue, in which metachromasia was partially observed for almost all the area of the sampled tissue and hyaline cartilage-like tissue was partially observed.

14.6.3 Mandible

Autologous particulate cancellous bone and marrow (PCBM) that is rich in osteogenic progenitor cells and bone matrices has excellent properties as bone graft because it has full bone formation ability. However, PCBM does not have structural strength and the ability to hold its desired shape by itself. Kinoshita *et al.* developed a mesh manufactured from PLA that can sustain high mechanical strength for a long period of time [15]. PLA monofilaments with diameter of 0.3 or 0.6 mm were woven into a mesh. Figure 14.7 shows the PLA mesh and tray scaffold used for mandible regeneration. After preclinical studies with dogs, they started clinical studies using the PLA sheet/tray and autologous PCBM as illustrated in Figure 14.8. In eight hospitals in Japan, 62 cases underwent mandibular reconstruction between 1995 and 2001 [16]. Mesh trays were used in 28 cases and mesh sheets in six cases. The PCBM was harvested from the iliac bone of patients

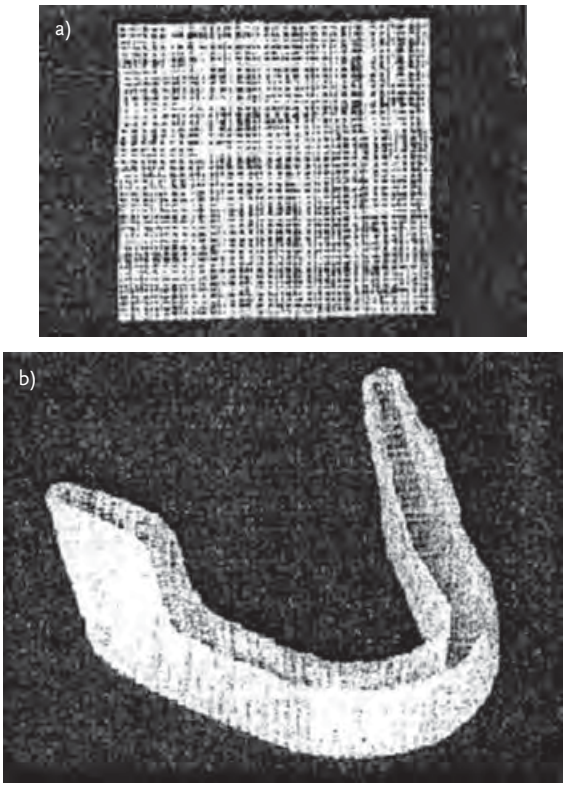

Figure 14.7 (a) PLLA mesh sheet and (b) mandibular mesh tray.

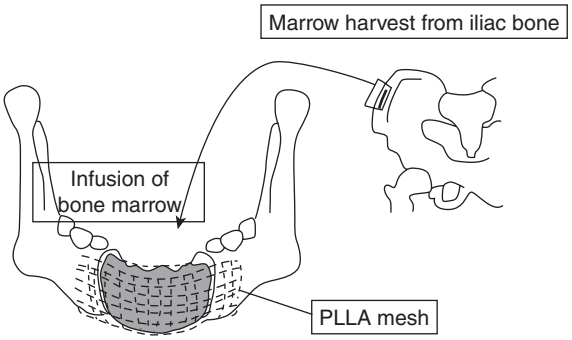

Figure 14.8 Schematic drawing of mandibular reconstruction by means of PLLA mesh and PCBM. The PLLA mesh tray was adjusted to the shape and size of the bone defect, cutting with scissors and warming at about 70 °C. The PLLA mesh tray was fixed to the residual bone with stainless steel wires and filled with PCBM taken from the iliac bone.

and 10–40 g of PCBM was transplanted to each patient. The clinical results were evaluated as *excellent* when the area of osteogenesis was over two-thirds in comparison to right after operation, based on X-ray films 6 months after surgery. Results were evaluated as *good* when osteogenesis was less than two-thirds with no reconstruction required. All other results were graded as *poor*. Forty cases were judged as *excellent*, 17 cases as *good*, and 10 cases as *poor*.

14.6.4
Vascular Tissue

Shin'oka *et al*. reconstructed peripheral pulmonary artery in a 4-year-old girl with the patient's own venous cells [17]. After that, three patients underwent tissue-engineered graft implantation with cultured autologous venous cells. However, since cell culturing was time-consuming and xenoserum had to be used, they began to use bone marrow cells (BMCs), readily available on the day of surgery, as a cell source. Matsumura *et al*. evaluated the endothelial function and mechanical strength of tissue-engineered vascular autografts (TEVAs) constructed with autologous mononuclear BMCs and a P(LA/CL) scaffold using a canine inferior vena cava (IVC) model. The mechanical strength change *in vitro* with time is shown in Figure 14.9 [18]. Figure 14.10 indicates no statistical differences in strength among IVCs of dog (shown as a control) and 6- and 12-month TEVAs. Encouraged by this successful result of the supplementary examination in the dog IVC replacement model, a 5-mL/kg specimen of bone marrow was aspirated from patients under general anesthesia before skin incision. The P(LA/CL) tube serving as a scaffold for the cells was the same as used for dogs. Twenty-three tissue-engineered conduits for extracardiac total cavopulmonary connection (TCPC) and 19 tissue-engineered patches were used for the repair of congenital heart defects. Mean follow-up after surgery was 490 ± 276 days [19]. There were no complications such as thrombosis, stenosis, or obstruction of the tissue-engineered autografts. The maximal trans-sectional area was calculated and compared with the implanted

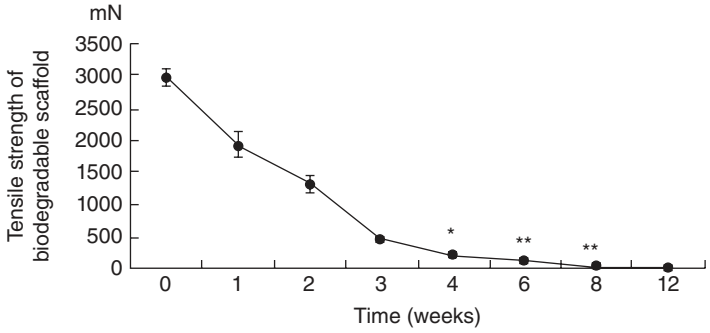

Figure 14.9 Tensile strength of biodegradable scaffold *in vitro*. The biodegradable scaffold used in this study diminished continuously within 1 month. Data represent the mean ± standard error of five samples at each time point. *$P < 0.01$, **$P < 0.001$ vs. week 0.

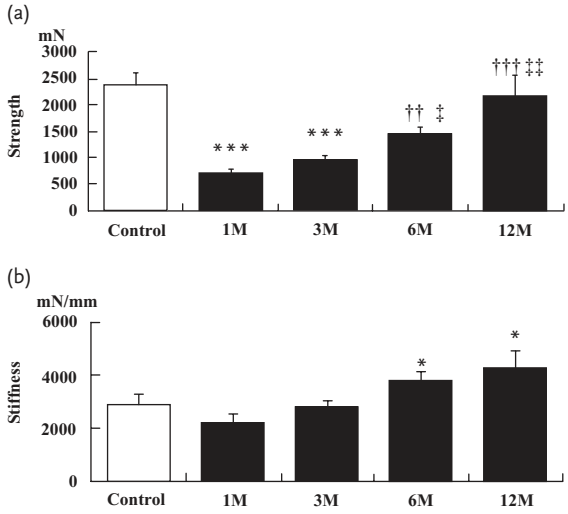

Figure 14.10 Increase in mechanical properties of TEVAs. (a and b) Tensile strength and stiffness of TEVAs. $P < 0.001$. ***$P < 0.001$ vs. inferior vena cava, $^{\dagger\dagger}P < 0.01$ vs. TEVAs at 1 month, $^{\dagger\dagger\dagger}P < 0.001$ vs. TEVAs at 1 month, $^{\ddagger}P < 0.05$ vs. TEVAs at 3 months, $^{\ddagger\ddagger}P < 0.01$ vs. TEVAs at 3 months. (b) Stiffness/width was calculated as stiffness (mN/mm) = elastic modulus (mN/mm^2) × wall thickness (mm). $P < 0.01$. *$P < 00.05$ vs. TEVAs at 1 month. Data from dog inferior vena cava are shown as control. Data represent mean ± standard error.

size in the TCPC group. There was no evidence of aneurysm formation or calcification on cineangiography or computed tomography. All tube grafts were patent and the diameter of the tube graft increased with time (110 ± 7% of the implanted size), suggesting that these vascular structure may have the potential for growth, repair, and remodeling, and provide an important alternative to the use of prosthetic materials in the field of pediatric cardiovascular surgery.

14.7 Conclusions

It is likely that recent studies on scaffolds have focused on the novel synthesis of well-defined, biodegradable polymers, fabrication of porous structures with tailored architecture using sophisticated tools such as computer-assisted manufacture techniques, chemical modifications of scaffold surface by emulating advantageous features of the natural ECM, and genetic engineering [20–22]. Despite these continuous efforts for creating a variety of new scaffolds, the number of reports representing the results of clinical trials directly associated with tissue engineering is not increasing but rather decreasing in recent years. This tendency is similar to preclinical trials using large animal models, although it seems that small animals like rat have increasingly been used for tissue engineering studies. This trend might be potentially inevitable because the preferred motivation of scientists these days is to publish their experimental results in international journals with high impact factors, rather than to contribute to their society. Unfortunately, it is doubtful that the explosively growing life science, nanoscience, and biomedical technology will certainly encourage in warranting the clinical applications of current tissue engineering research to patients.

The engagement of biomedical industries on tissue engineering is crucial in transferring the accomplishments of tissue engineering studies to clinical applications. It is too difficult for academic people to fabricate by themselves a number of large-sized scaffolds applicable to patients. However, even if tissue engineers have developed a "promising" scaffold, companies would not be interested in manufacturing the scaffold on a large scale unless the nontoxicity of the scaffold material has already been proved by authorized procedures. It should be always kept in mind that the proved nontoxicity of biomaterials is a prerequisite in their clinical applications in preference of any other attributes of biomaterials. Recently, the regulatory organization of Japan has strictly inhibited biomedical companies to provide a surgeon with scaffolds manufactured by companies that are not yet approved, even if the project, which the surgeon attempts to apply to patients, has been approved by the IRB of his or her facility.

In this respect, close communications among tissue engineers, surgeons, biomedical manufacturers, and regulatory organizations are absolutely needed for promoting clinical trials in the tissue engineering field.

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