Chapter 11
Regeneration of Blood Vessels

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11.1 Introduction

Blood vessels transport blood to deliver oxygen and nutrients. Vascular diseases such as atherosclerosis may result in obstruction of blood vessels and tissue ischemia [1]. Vascular graft has proven to be an effective strategy for the treatment of these vascular diseases [2]. In the United States, there are over 500,000 vascular grafts being used for bypass surgery each year, most of which are autologous venous and arterial grafts. However, autologous grafts are limited by availability, need for additional surgeries, donor site morbidity, and 30 % 10-year failure rate [1]. Artificial blood vessels become indispensable and have received increasing attention. Up to now, some products have gained success and have been commercialized, such as expanded polytetrafluoroethylene (ePTFE) and poly(ethylene terephthalate) (Dacron). Dacron and Teflon work well for large-diameter (>6 mm internal diameter) vascular grafts, where the rate of blood flow is high, but these materials yield disappointing clinical results in small-diameter (<6 mm internal diameter) coronary artery grafts [3]. In addition, nondegradation of the polymer graft often leads to the calcification during long-term implantations [4]. In this regard, development of vascular grafts with relatively slow degradation and controlled regenerative process has become a new concept and direction. These grafts provide a favorable environment for the recruitment of autogenous vascular cells. After a full degradation of the polymer scaffold, "neoartery" could be generated [5]. With the recent advancement of knowledge and technologies in the small-diameter vascular grafts, there are still many scientific questions to be addressed. Among these questions, vascular regeneration and their long-term patency and function should be mentioned.

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11.1.1 Endothelialization of Vascular Grafts

The endothelium is not a smooth inert surface that facilitates laminar blood flow through the blood vessel but a dynamic organ with an active role in coagulation homeostasis, the sensing and transduction of the hemodynamic forces of circulation, and the cellular metabolism of the vascular wall [2]. It is understandable that the main focus of vascular graft studies is endothelialization. Within many general functions, the endothelium is equipped with a number of mechanisms that prevents thrombus formation in the circulatory system. It harbors factors that interrupt the coagulation cascade, such as antithrombin III, the protein C receptor thrombomodulin, and tissue factor pathway inhibitor. It prevents platelet activation by the production of nitric oxide and prostacyclin, exonucleotidases, and surface heparan sulfates [6]. All of these functions make the endothelium an important physiological barrier to maintain the patency and homeostasis of blood vessels. However, a vascular graft that can resist thrombosis by forming a confluent luminal endothelium in vivo is still a dream in the field of vascular tissue engineering.

11.1.2 Restenosis of Vascular Grafts

Transplantation of small-diameter vascular grafts is often accompanied by restenosis, including short- and midterm restenosis. The reasons for short-term restenosis include platelet adhesion and aggregation and the resulting thrombus formation. While those for midterm restenosis include over-proliferation of vascular smooth muscle cells (VSMCs) and neointimal hyperplasia. Generally speaking, the main problem could be ascribed to the poor blood compatibility of artificial vascular grafts, which leads to the adhesion of platelets and plasma protein, and subsequent aggregation and thrombus formation [7]. In addition to biomaterial incompatibility, physical forces have been associated with vascular graft intimal hyperplasia [8]. Prominent among these suggestions have been compliance mismatch between the graft and host artery, which result in adverse local hemodynamic effects at the anastomosis with consequent greater intimal thickening and eventual graft failure [9]. There has been a great deal of work recently on developing more compliant sutures, suturing techniques, mechanical clips, biological glue, and laser-based solder techniques [10]. The aim of this has been to improve pulsatile laminar blood flow in arteries propagation across the anastomosis and to reduce damage to the surrounding endothelium [9]. But beyond that, there is evidence that a confluent endothelium is crucial in prevention of the initiation and progression of the process. One aspect of endothelial protection is the physical barrier, which forms to prevent contact with subendothelial components of the arterial wall and activation of the coagulation cascade. In addition, early events in the cascade, such as platelet degranulation following contact with type-I collagen, have been shown to induce mitogenic factors such as transforming growth factor β (TGF-β) [2]. Animal models in which

endothelial injury was induced show that loss of an intact endothelium results in a change of VSMCs phenotype to a proliferative state. It is widely accepted that increased proliferation of terminally differentiated vascular SMCs contribute significantly to lesional neointima formation [11]. Conversely, such a change was inhibited by the presence of endothelial cells [2]. Therefore, how to construct a hemocompatible surface or interface with antithrombogenic properties is the key issue in research on vascular grafts.

11.1.3 Anticoagulation of Vascular Grafts

Small-diameter prosthetic grafts undergo early thrombotic occlusion limiting their clinical utility for coronary or peripheral revascularization procedures. Several different antithrombotic agents have been evaluated experimentally [12]. One pharmacologic agent that has received wide research focus as a means to reduce neointimal hyperplasia is heparin, which is a mucopolysaccharide found in most tissues. Heparin inhibits thrombin and activated factors IX, X, XI, and XII, which is involved in the conversion of prothrombin to thrombin, thereby reducing thrombin formation. In addition, heparin has a potent antiproliferative effect on vascular VSMCs, and this effect is independent of its anticoagulant activity. Its inhibitory effect on VSMCs is mediated in part through interactions with cell receptors, growth factors, adhesion molecules, and protease inhibitors [13]. Unfortunately, systemic administration of effective levels of antithrombotic drugs is expensive and may be associated with serious hemorrhagic complications. An alternative approach is to immobilize an antithrombotic agent at the graft site. This strategy offers the advantage of inhibiting the thrombotic process at a specific site while avoiding systemic side effects [14]. Beyond that, some groups take the advantage of enzyme prodrug therapy (EPT) technique in the fabrication of anticoagulant vascular grafts [15]. EPT is a versatile technique that allows synthesis of drugs at the site of action when prodrugs are systemically administrated. In addition to targeted and localized drug delivery, the advantage of EPT also includes the fine tune of drug dosage, duration, and administration [16].

11.1.4 Calcification of Vascular Grafts

In the field cardiovascular implants, calcification of heart valves and conduits used in congenital cardiac surgery has been extensively described, and their prevention has been a challenge for researchers and industry for decades [17]. The presence of cardiovascular calcification significantly predicts patients' morbidity and mortality. Calcific mineral deposition within the soft cardiovascular tissues disrupts the normal biomechanical function of these tissues, leading to complications such as heart failure, myocardial infarction and stroke. The realization that calcification results

from active cellular processes offers hope that therapeutic intervention may prevent or reverse the disease. To this point, however, no clinically viable therapies have emerged. This may be due to the lack of certainty that remains in the mechanisms by which mineral is deposited in cardiovascular tissues [18].

11.1.5 Animal Models for the Assessment of Vascular Grafts

In order to assess the capacity of the new conduits to maintain physiologic function in the circulatory system and to determine the response of both the host and the conduits to implantation, evaluation of the grafts in preclinical animal studies is required [19]. Preclinical assessment of vascular grafts using appropriate animal models is essential to determine the clinical potential of engineered tissues. With the advancement of knowledge and technologies in the vascular grafts, there are many criteria that are utilized in the assessment of clinical potential. Each of these criteria may be best analyzed in different experimental settings. At first, the selection of an appropriate animal model needs to include criteria relevant to the vascular graft such as implantation site, vascular diameter and length, and time frame of implantation. Equally important are criteria relevant to the animal species selected such as cost, availability, ease of handling, animal response to surgical procedure, target vessel diameter and length, and target physiology. Optimally, an animal model needs to be selected that meets most of these criteria. It is best to match site and diameter to test the hemodynamics and implantability; use longer grafts (>4 cm) to test patency; select the type of anastomosis (end to end, end to side) to test shear stress; or select species that exhibit similar immunogenicity and thrombogenicity mechanisms as those at work in humans. The type of analysis, for example, serial imaging or monitoring versus a single measurement at the end of the experiment – is also important in determining the choice of animal model. Secondly, similarity to human physiology is one major factor that is considered when assessing criteria specifically related to translational studies. For example, ovine and nonhuman primate models show greater similarity to humans in terms of thrombogenicity mechanisms as compared to dogs or pigs. On the other hand, dogs exhibit lack of spontaneous endothelialization of vascular grafts, and they tend to be hyperthrombogenic, similar to humans. These two features make the dog model more stringent for vascular grafts testing. By contrast, lack of similarity in vascular dimensions and hemodynamics makes small animals like mice and rats poor models for long-term grafts evaluation. However, the plethora of transgenic mice presents a very useful resource to dissect molecular mechanisms related to immune response, remodeling, vascular reactivity, and other aspects of graft physiology. Sometimes the age and gender of recipient animals also should be mentioned. The age of recipient animals may affect the microenvironmental factors that are critical for successful grafting and long-term tissue regeneration. As the concerns of animal rights and limitations on the use of nonhuman primate increase, the pig and sheep models have become more widely used in recent years. In addition to the ethical considerations, cost is

also a factor, making the pig and sheep the models of choice. There are no absolute ideal animal models or international consensus on standards associated with the development and testing of vascular grafts. The lack of standardized models makes it difficult to compare results among investigators. In order to best evaluate the implantation of vascular grafts in such a variety of animal models, it requires optimal model selection and use of proper internal controls [20].

In the next section, we will discuss other factors that influence vascular regeneration and their long-term patency and function. Among these factors, we focus on the selection of suitable polymers, their degradation and elasticity, the scaffolds' structure, and necessary functional modification to the scaffolds.

11.2 Selection of Polymers for Vascular Grafts

11.2.1 Synthetic Polymers

ePTFE Initial vascular prostheses used nondegradable polymers like ePTFE or Teflon. Polytetrafluoroethylene (PTFE) was patented by DuPont as Teflon in 1937, and ePTFE was patented by Gore as Gore-Tex in 1969. ePTFE is an expanded polymer which is manufactured by a heating, stretching, and extruding process resulting in a non-textile porous tube [21]. The ePTFE tubes have an electronegative luminal surface that is antithrombotic. A 5-year patency of 91–95 % is achieved when used as arterial substitute with neither transanastomotic nor transmural endothelialization of this graft. Although these substitutes dominat the clinical market of vascular substitutes for large-diameter vessels, vascular regeneration is nil, and lack of patency limits the use of these as small vessel substitutes and further regeneration. Decreased patency (45 %) is observed when it is used in femoropopliteal bypass surgery [22]. Several attempts have been adopted to improve the patency of ePTFE. Studies showed that seeding ePTFE graft with autologous endothelial cells (ECs) can give adequate ECs coverage. Patency was improved considerably in dogs when compared to unseeded ePTFE grafts [23]. Compared with degradable materials, long immune response can be induced due to its nondegradable properties. Last but not the least, ePTFE is much stiffer than arteries or veins. Those problems limit their use in current practice.

PCL Poly (ε-caprolactone) (PCL) is a promising polymer for the construction of small-diameter vascular grafts due to its good biocompatibility, suitable mechanical strength, and slow biodegradation rate. Pektok et al. [24] compared the electrospun PCL grafts and the ePTFE grafts (2 mm diameter) by implanting them in rat abdominal aorta for 24 weeks. They found that PCL grafts showed better healing characteristics than ePTFE grafts. Fast endothelialization and extracellular matrix (ECM) formation, accompanied by degradation of graft fiber, seem to be the major advantages of PCL vascular graft. Walpoth's group [4] implanted electrospun PCL

vascular grafts into rat abdominal aorta. Results showed no aneurysmal dilation, perfect patency, excellent structural integrity, and limited intimal hyperplasia throughout the study. Endothelialization, cell invasion, and neovascularization of the graft wall rapidly increased until 6 months. However, from 6 to 18 months, regression of cell number and capillary density and severe calcification were observed within the graft wall. The calcification may be linked to the hypoxic conditions and oxidative stress due to the graft dense fibrous structure or the local low compliance. Kong's group [5] fabricated a macroporous electrospun PCL grafts with thicker fibers (5–6 μm) and larger pores (~30 μm). They demonstrated that thicker-fiber electrospun PCL vascular grafts could enhance vascular regeneration and remodeling process by mediating macrophage polarization into M2 phenotype.

PLCL Poly(l-lactide-co-ε-caprolactone) (PLCL) copolymers have been applied as a biomaterial for the construction of vascular grafts due to the high elastic properties. In previous reports, PLCL vascular grafts were fabricated by an extrusion-particulate leaching technique, but there were a few problems for extruded PLCL grafts in cell seeding efficiency, cell ingrowth, and mechanical strength. Sang-Heon et al. [25] fabricated and characterized a new tubular, macroporous, fibrous PLCL (5:5) graft using gel spinning. Compared to extruded PLCL scaffolds, the fibrous PLCL scaffold showed improved biological activities, such as cell seeding efficiency and proliferation, and improved mechanical properties, such as tensile strength and viscoelastic properties. Shafiq et al. [26] fabricated scaffolds by mixing appropriate proportions of linear PLCL and substance P (SP)-immobilized PLCL, using electrospinning to develop vascular grafts. PLCL-SP showed significantly higher host cell infiltration, blood vessel formation, and mesenchymal stem cells (MSCs) recruitment in vivo. Mun et al. [27] seeded VCMSs onto electrospun PLCL scaffolds to construct a three-dimensional network. The vascular grafts constructed using cell-matrix engineering were similar to the native vessels in their mechanical properties, such as tensile strength, tensile strain, and e-modulus.

PGA Poly(glycolic acid) (PGA) is a polyester obtained by the ring-opening polymerization of glycolide. Cho et al. [28] fabricated a hybrid biodegradable polymer scaffold from PLCL copolymer reinforced with PGA fibers. The PGA/PLCL vascular patches were seeded with ECs and VSMCs differentiated from bone marrow stromal cells (BMSCs) and implanted in the inferior vena cava (IVC) of bone marrow donor dogs. Compared with PLCL scaffolds, PGA/PLCL scaffolds exhibited tensile mechanical properties more similar to those of dog inferior vena cava. Eight weeks after implantation, the vascular patches remained patent with no sign of thrombosis, stenosis, or dilatation. Kobayashi et al. [29] produced the composite nanofiber composed of PGA and collagen to accomplish the recruitment of host cells and peripheral blood vessels without the bio-derived matter-like growth factors. Structural analysis revealed that the fiber has the sheath-core-like structure in which the surface region is abundant in PGA and the core region is abundant in collagen. The results of the animal experiment demonstrated that the PGA-collagen nanofiber sponge was entirely populated and vascularized within 5 days after the implantation. Rapoport

et al. [30] utilized electrospinning technique to form tubular scaffold composites with structural features reminiscent of the corrugated laminae seen in blood vessels. This tubular scaffold was fabricated with complex "J"-shaped behavior through the use of elastic polyurethane and reinforcing polyglycolic acid (PGA) woven mesh. The mechanical behavior of this tubular scaffold achieved from a low-stiffness highly elastic zone giving rise to a high-stiffness zone, and the value of burst pressures and toughness was 3095 ± 1016 mmHg and 6.3 ± 1.9 MJ/m^3, respectively.

PLA Poly(lactic acid) (PLA) is a biodegradable thermoplastic polyester that can be produced through ring-opening polymerization of lactic acid. Since lactic acid is a chiral molecule, it exists in two forms, D-PLA and L-PLA. Poly(l-lactic acid) (PLLA) is the result of L-PLA polymerization. Zhu et al. [31] reported novel scaffolds which were fabricated by co-electrospinning collagen/chitosan and PLA. The scaffolds had a more biomimetic structure than PLA, as the fiber diameters approached the size of the ECM. Axially aligned nanofibrous matrices were evaluated as small-diameter cardiovascular grafts. Sankaran et al. [32] fabricated a graft using the PLA and PCL physical blends in the ratios of 75:25 and 25:75 by electrospinning. Hydrophobicity and tensile stress were significantly higher in PLA-PCL (75:25), whereas tensile strain and fiber density were significantly higher in PLA-PCL (25:75). Human umbilical vein endothelial cells (HUVECs) adhesion experiment showed cell viability and proliferation were rationally influenced by the aligned nanofibers, and gene expression revealed the grafts' thromboresistivity, elasticity, and aided neovascularization. A graft comprised of a polyetherurethane scaffold and sealed with polyethylene glycol (PEG)/PLA copolymer exhibited good compliance, and the compliance increased with the degradation of the PEG/PLA components in vitro. Yet, when implanted in vivo, the compliance reduced 20 % after 12 weeks [33].

PGS Polycondensation of glycerol and sebacic acid forms the elastomeric poly(glycerol sebacate) (PGS). PGS shows appreciable mechanical properties and biocompatibility and degrades within 2 months in vivo [34]. In vitro hemocompatibility evaluation of PGS-based biphasic scaffolds were shown to be nonthrombogenic compared to other synthetic grafts [35]. Single-layered three-dimensional microfluidic PGS scaffolds also achieved biomimetic fluid properties [36]. Wang's group investigated the effect of pore size in PGS porous scaffold on VSMCs organization. They found that pores of 25–32 μm increased VSMCs alignment, elastin, and collagen production [37]. Subsequently, Wang's group fabricated a PGS porous tube with an average pore size of 21.2 ± 0.79 μm enveloped by a thin PCL fiber sheath [38]. After implanting the cell-free biodegradable elastomeric grafts into rat abdominal aorta, they found that the grafts degraded rapidly to yield neoarteries nearly free of foreign materials at 3 months. Based on this success, Khosravi et al. [39] developed a novel method for electrospinning smaller grafts composed of a PGS microfibrous core enveloped by a thin PCL outer sheath. Electrospun PGS-PCL composites were implanted as infrarenal aortic interposition grafts in mice and remained patent up to the 12-month endpoint without thrombosis or stenosis.

PU Polyurethane (PU) exhibits good biocompatible, optimum tensile strength and high elasticity, which make it an appealing choice for construction of small-diameter vascular conduits [40]. Recently, results of a study indicated intravascular implant patency of 95 % when an electrospun PU scaffold was implanted into the abdominal aorta of rats. Luminal surface was covered with intact endothelial cell lining, and there was no evidence of neointima formation [41]. He et al. [42] investigated the performance of small-caliber PU vascular prosthesis generated using the electrospinning technique. The results showed that cell proliferation was not inhibited as the small-caliber PU synthetic vascular grafts showed little cytotoxicity. The endothelial cells had faster adherence to the PU scaffolds than to the PTFE surface during the initial contact. Punnakitikashem et al. [43] prepared a biodegradable fibrous scaffold that contained biodegradable elastic polyurethane urea (BPU) and the drug dipyridamole (DPA) by electrospinning. The resulting scaffolds had tensile strengths and strains comparable with human coronary artery. It confirmed that the DPA-loaded BPU scaffolds could extend human blood clotting time and reduce human platelet deposition and hemolysis. Furthermore, the DPA-loaded BPU scaffolds had no adverse effect on human aortic endothelial cell growth, yet it improved their proliferation. Thermoplastic polyurethane (TPU) as a class of PU has linear segmented molecular chains, good processability, high elongation, and excellent biocompatibility. Bergmeister et al. [44] investigated the biocompatibility of TPU grafts in vitro and in vivo. TPU grafts showed significantly increased endothelial cell proliferation in vitro. Population by host cells increased significantly in the TPU conduits within 1 month of implantation. After long-term implantation, TPU implants showed 100 % patency (ePTFE: 93 %) with no signs of aneurysmal dilatation. Enayati et al. [45] also evaluated biodegradable TPU vascular graft by implanting into the infrarenal aorta of rats. After 1 month the results showed that the porous structure of the TPU graft allowed cell migration and proliferation, resulting in a highly cellular graft.

PHA Polyhydroxyalkanoate (PHA) is a polyester produced by microbes like bacteria under nutrient-limited conditions with excess carbon supply [46]. PHA shows good biocompatibility and a wide range of mechanical properties and biodegradation rates. These make it attractive for vascular conduit construction. A 7-mm-long PHA-PGA tubular scaffold seeded with ovine carotid artery cells, implanted into a lamb aorta, showed patency for 5 months and stress-strain behavior similar to a native vessel [47].

11.2.2 Natural Polymers

Collagen is the major component of the ECM in the body. It is a structural protein whose main function is to provide mechanical integrity to different tissues and organs such as tendon, bone, etc. Collagen gels as scaffolds for engineering vascular substitutes were first reported by Weinberg CB in 1986 in the first attempt to

produce a tubular construct in vitro that mimicked the structure of an artery [48]. Building on his early work, Achilli et al. [49] investigated UV-C-treated collagen gels to improve the integrity of collagen-based scaffold by adding cross-links. Schutte et al. [50] used a bioreactor to apply mechanical conditioning through cyclic strain. Mechanical stimulation improved tissue strength through increasing collagen content as well as some radial tissue compaction. Wu et al. [51] cocultured the endothelial and smooth muscle cells on a collagen membrane. This tissue-engineered vascular substitute had not only enough tensile strength and good biocompatibility but also advanced vascular regeneration.

Elastin is a protein normally found in the wall of arteries and in several mammalian tissues. Its main function is to provide elasticity, allowing high strain and efficient elastic energy storage in arteries, to ensure blood flow and perfusion to the rest of the body's tissues. Leach et al. [52] recently detailed a protocol to process elastin with ethylene glycol diglycidyl ether (EGDE), a diepoxy cross-linker, to develop an elastin-based scaffold. In order to imitate the native three-layered architecture, Koens et al. [53] prepared a triple-layered construct consisting of elastin and collagen. The highly purified type-I collagen fibrils and elastin fibers used did not evoke platelet aggregation in vitro. Some researchers also discovered that elastin addition to a porous collagen scaffold played a major role in altering its biological and mechanical response [54]. Smith et al. [55] fabricated cross-linked suture-reinforced polydioxanone (PDO)-elastin tubes. These tubes exhibited a range of compliance values, including those matching native artery. These tubes display many characteristics of the ideal small-diameter grafts.

Fibrin is an insoluble body protein largely involved in blood clotting. It is formed through fibrillogenesis of a monomer (called fibrinogen) that flows in the blood. In 2000, Ye et al. [56] prepared a three-dimensional matrix of fibrin gel for cardiovascular tissue engineering and reported uniform cell growth and collagen deposition into the gel. Syedain et al. [57] fabricated a tissue-engineered arteries based on entrapment of human dermal fibroblasts in fibrin gel. The completely biological vascular grafts that possessed circumferential alignment characteristic of native arteries, which was essential to their mechanical properties. Recently, this group confirmed that hypoxia coupled with insulin supplementation was also shown to improve the strength of fibrin-based tissue-engineered vascular grafts (TEVGs) by enhancing collagen deposition in the entrapped cells [58]. Additionally, Swartz et al. [59] have examined a fibrin-based TEVGs in an ovine model. In this study, fibrin tubes with entrapped vascular VSMCs were implanted as vein interposition grafts in lambs. At 15 weeks of postimplantation, TEVGs exhibited remarkable matrix remodeling with production of collagen and elastin fibers and orientation of VSMCs perpendicular to the direction of blood flow. Implanted vessels gained significant mechanical strength and reactivity that were comparable to those of native veins.

Chitin is, after cellulose, the most common polysaccharide found in nature. Chupa et al. [60] introduced a method to produce a porous scaffold by freezing and lyophilizing chitosan. Ling et al. [61] used a mesh of knitted chitosan fibers, coated in a chitosan and gelatin solution and then freeze-dehydrated. The scaffold possessed proper swelling property, burst strength of almost 4000 mmHg, and high

suture retention strength. An alternative, constructed from cross-linked and freeze-dried chitosan and collagen, had also been shown to support vascular cell adhesion and proliferation, and additionally, exhibited suitable biocompatibility in vivo when implanted in rabbit livers [62]. Chitosan could also be fabricated with PCL by sequential quantity grading co-electrospinning. To prevent thrombosis, researchers used heparinization and immobilization of vascular endothelial growth factor (VEGF) in the gradient CS/PCL [63].

Hyaluronic acid (HA) (or sodium hyaluronate) is a non-sulfated glycosaminoglycan (GAG). It is comprised of linear, unbranching, polyanionic disaccharide units consisting of glucuronic acid and N-acetyl glucosamine. Milella et al. [64] characterized the physicochemical properties of nonwoven hyaluronan benzylic esters (Hyaff 11) as a tissue engineering scaffold. Zhu et al. [65] developed a suitable intimal layer scaffold for endothelialization using novel humanlike collagen/HA. The result showed the scaffold with an interconnected porous network, better mechanical properties, and excellent biocompatibility. Joo et al. [66] reported that bioactive HA could be chemically modified into hyaluronic acid-catechol by a single-step method. This method could enhance endothelialization in vitro.

ECM serves as a biologically active scaffold on which cells can migrate or adhere. Li et al. [67] demonstrated that elastin haploinsufficiency led to compensatory increase in VSMCs number which resulted in profound arterial thickening. It may regulate the phenotype of the cells. It has been demonstrated that VSMCs rapidly lose their contractile apparatus and adopt a synthetic phenotype in culture [68]. The ECM serves as an anchor for many proteins including growth factors and enzymes such as proteases and their inhibitors.

Rothuizen et al. [69] presented a novel approach to generate autologous tissue-engineered blood vessels (TEBVs) in vivo. Polymer rods were engineered and implanted, evoking an inflammatory response that culminates in encapsulation by a fibrocellular capsule. After 4 weeks, rods with tissue capsules grown around it were harvested. Tissue capsules were grafted bilaterally as carotid artery interposition. Patency was 100 % after 1 week and 87.5 % after 4 weeks. Wall thickness and α-smooth muscle actin (α-SMA)-positive area significantly increased. The lumen was largely covered with ECs.

By combining ultrahigh hydrostatic pressure (UHP)-decellularized ostrich carotid arteries with the peptide modifier, Mahara et al. [70] created a tissue-engineered small-caliber long-bypass grafts measuring 20–30 cm in length and having a 2-mm inner diameter. A novel peptide modifier containing a collagen-binding peptide sequence and an endothelial cell-binding sequence improved the affinity of the luminal surface for endothelial cells. The decellularized carotid artery modified with the peptide demonstrated good patency and stable pulsatile blood flow in pig femoral-femoral bypass model.

Small intestinal submucosa (SIS) is a natural biodegradable material derived from the small intestine of vertebrates, usually from swine. Vascular grafts were engineered using SIS-fibrin hybrid scaffold and implanted interpositionally into the arterial circulation of an ovine model. No occlusions or anastomotic complications were observed in 18 animals that received these grafts. Notably, the grafts exhibited unprecedented levels of host cell infiltration [71].

11.2.3 Hybrid Materials: Synthetic and Natural Polymers

Autologous saphenous vein is used as a conduit to bypass atherosclerotic lesions in both the coronary artery (coronary artery bypass graft surgery [CABG]) and in femoral arteries (infrainguinal bypass graft surgery [IIBS]). Despite the undoubted success and benefits of the procedures, graft failure occurs in 50 % of cases within 10 years after surgery. A principal cause of late vein graft failure is intimal and medial hyperplasia caused by medial vascular VSMCs migration, proliferation, and ECM deposition, followed later by superimposed atherosclerosis. These changes directly compromise graft blood flow and provoke thrombosis.

Jeremy et al. [72] studied the effect of external synthetic stents and sheaths in pig models of vein into artery interposition grafting and showed a profound effect on vein graft remodeling and thickening. It has been clearly demonstrated that external synthetic stents or sheaths elicit a complete inhibition of neointima formation, an axiomatic lesion in vein graft disease, and an overall reduction of graft thickening. These effects appear to be mediated by the promotion of angiogenesis which is mediated by the accumulation of a proangiogenic exudate in the space between the graft and the sheath or stent. Macroporosity is also a crucial factor since it allows for a fully integrated and functional microvascular system to develop. Ultimately, they suggest that the profound local accumulation of inflammatory cells, in particular, macrophages and giant cells, play a key role in that they initiate accumulation of ECs and VSMCs in and around the material and promote angiogenesis. Another facet of the external stents or sheaths is that they impose symmetry on the graft.

Decellularized xenografts are commonly used as a tissue engineering substitute for vascular reconstructive procedures. Although acellular allogeneic vascular grafts have good histocompatibility and antithrombotic properties, the decellularization process may damage the biomechanics and accelerate the elastin deformation and degradation, finally resulting in vascular graft expansion and even aneurysm formation. Here, to address these problems, Gong et al. [73] combined synthetic polymers with natural decellularized small-diameter vessels to fabricate hybrid tissue-engineered vascular grafts (HTEV). The donor aortic vessels were decellularized with a combination of different detergents and dehydrated under a vacuum freeze-drying process. PCL nanofibers were electrospun outside the acellular aortic vascular grafts to strengthen the decellularized matrix. The intimal surfaces of the hybrid small-diameter vascular grafts were coated with heparin before the allograft transplantation. Mechanical testing of scaffolds showed that electrospun PCL fibers significantly enhanced the biomechanics of decellularized vessels. Vascular ultrasound and micro-CT angiography showed that all grafts after implantation in a rat model were satisfactorily patent for up to 6 weeks. Electrospun PCL fibers successfully prevented the occurrence of vasodilation and aneurysm formation after transplantation and reduced the cell inflammatory infiltration.

Hemodynamic factors play a major role in the development of intimal hyperplasia and subsequent bypass failure. To evaluate the potential protective effect of

external reinforcement on such a failure, Longchamp et al. [74] developed an ex vivo model for the perfusion of segments of human saphenous veins under arterial shear stress. The data showed that, in an experimental ex vivo setting, an external scaffold decreased intimal hyperplasia and maintained the integrity of veins exposed to arterial pressure, via increase in shear stress and decrease wall tension, that likely contributed to trigger selective molecular and cellular changes. Ex vivo, such reinforcement prevented the dilatation of the vessel, decreased the development of intimal hyperplasia, and preserved the architecture of the media layer by reducing the apoptosis of VSMCs and subsequent fibrosis. At the molecular level, the mesh prevented the upregulation of matrix metalloproteinases (MMP-2, MMP-9) and plasminogen activator type I, which are involved in the remodeling of the ECM.

11.3 Fabrication of Vascular Grafts

To date, numerous techniques such as electrospinning, particle leaching, melt spinning, and 3D printing have been used to fabricate vascular grafts with synthetic and natural materials. Each of these methods has its own unique advantages and disadvantages. Microstructure of vascular grafts formed by those methods has a great effect on their regeneration and remolding. An important factor in vascular grafts is pore size. Too small pores will hinder cell infiltration, but too large pores can cause problems such as blood leakage. Current studies tend to fabricate vascular grafts using combination of two or more approaches. The following are some typical technique usually applied in fabrication of polymer-based vascular grafts.

Electrospinning is the most commonly used method to fabricate vascular graft. It is a simple and effective way to produce fibers ranging from 50 nm to 10 μm. It consists of a syringe pump, a high-voltage power supply, a grounded iron rod, and a spinneret. Using this technique, fibers are collected on the target and can be modified by regulating parameters such as rod rotating speed, voltage, flow velocity, and solution concentration. As discussed in greater detail above, a number of synthetic and natural polymers have been explored for the fabrication of nanofibers [75]. Walpoth's group fabricated PCL vascular grafts by electrospinning and evaluated the long-term performance of grafts in the rat abdominal aorta replacement model. Results showed excellent structural integrity during 18 months, with no aneurysmal dilation, and perfect patency with no thrombosis and limited intimal hyperplasia. However, calcification took place on the medium term, and a cellular regression was observed at 12 and 18 months [4]. In order to prevent blood leakage and facilitate cell infiltration and applicability of vascular grafts in the clinic, bilayered grafts were prepared by electrospinning a high-porosity layer with a low-porosity layer on external or internal side. In vitro blood leakage through these bilayered grafts was significantly reduced compared with a high-porosity graft. The study showed that most of the cellular infiltration came from the graft surrounding tissues and not from the blood flow [76]. This phenomenon owns important directive significance for fabrication of biodegradable vascular grafts.

Limited cell infiltration into the grafts will hamper the regeneration and remodeling of the grafts into neoarteries. To overcome this problem, macroporous electrospun PCL grafts with thicker fibers (5–6 μm) and larger pores (30 μm) were fabricated in Kong's lab. In vivo implantation by replacing rat abdominal aorta demonstrated that the macroporous grafts markedly improved cell infiltration and ECM secretion. The regenerated arteries demonstrated contractile response to adrenaline and acetylcholine-induced relaxation. Analysis of the cellularization process revealed that the thicker-fiber scaffolds induced a large number of M2 macrophages to infiltrate into the graft wall, which further promoted cellular infiltration and vascularization [5] (Fig. 11.1). Regeneration of VSMCs with circumferential orientation within the grafts is crucial for functional vascular reconstruction in vivo. Thus, Kong's lab designed a bilayered vascular graft, of which the internal layer was composed of circumferentially aligned microfibers prepared by wet spinning and an external layer was composed of random nanofibers prepared by electrospinning. The internal circumferentially aligned microfibers provided topographic guidance for in vivo regeneration of circumferentially aligned VSMCs, and the external random nanofibers could offer enhanced mechanical property and prevent bleeding during and after graft implantation (Fig. 11.2). The results demonstrated that the circumferentially oriented VSMCs and longitudinally aligned ECs were successfully regener-

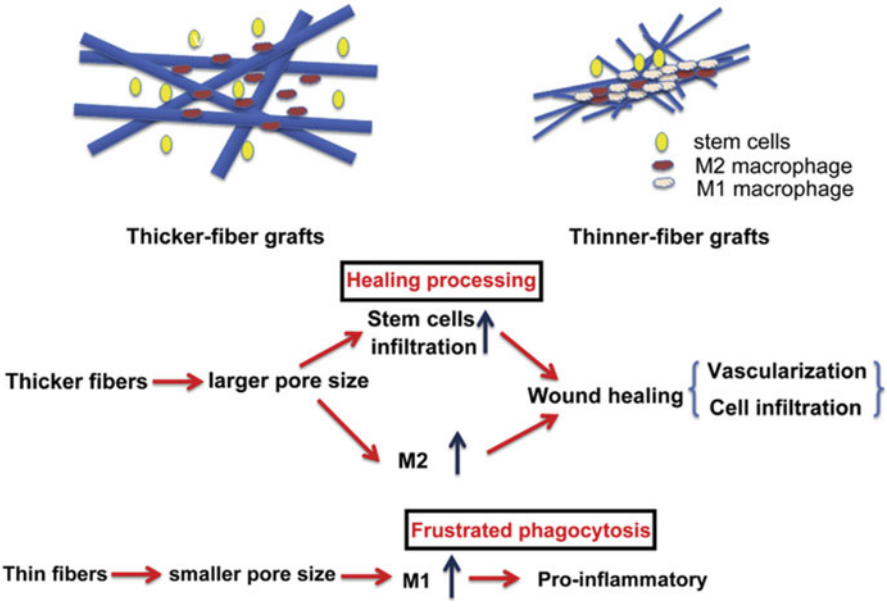

Fig. 11.1 Schematic illustrates that the pore size of electrospun PCL grafts may modulate the polarization of macrophages phenotype (Reprinted from Ref. [5] with permission, Copyright 2014 Elsevier Ltd.)

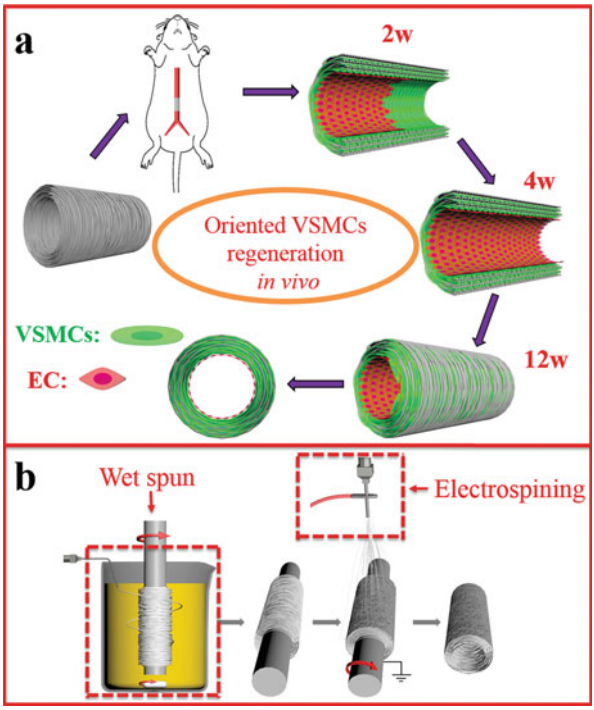

Fig. 11.2 (**a** Schematic illustration shows the circumferentially aligned microfibers of the grafts which guide VSMCs' regeneration in circumferential orientation. (**b**) The bilayered grafts are prepared by wet spinning and electrospinning method (Reprinted from Ref. [77] with permission. Copyright 2015 Elsevier Ltd.)

ated in vivo after the bilayered vascular grafts were implanted in rat abdominal aorta, and the regenerated neoartery exhibited contraction and relaxation property in response to vasoactive agents [77].

Despite great progress has been made over recent decades and various techniques have been developed to fabricate a TEVGs, only one kind of polymer-based graft has been successfully executed in clinical application, which was described by Hibino et al. in 2001 in a high-flow, low-pressure pulmonary venous system. A hybrid biodegradable polymer vascular graft degrade within a certain period of time was fabricated by pouring a solution of the copolymer of PCL-PLA (50:50) onto the PGA woven fabric sheet, followed by freeze-drying [78]. Twenty five grafts were implanted (median patient age, 5.5 years) and underwent a Fontan procedure under informed consent and institutional review board approval. Six of these 25 patients died, although no graft-related mortality occurred. There was no evidence of aneurysm formation, graft infection, graft rupture, or ectopic calcification [79, 80].

Particle leaching method also has been developed for fabrication of vascular grafts. Wang's lab developed bilayered vascular grafts composed of PGS layer fabricated by salt leaching method and PCL sheath generated by electrospinning which increased graft strength and prevented bleeding. Three months' postimplantation in rat abdominal aorta, the neoarteries resembled native arteries in several aspects: a

confluent endothelium and contractile smooth muscle layers and regular, strong, and synchronous pulsation [38]. The long-term study showed that the neoarteries contained nerves and had the same amount of mature elastin as native arteries and responded to vasomotor agents, although with smaller magnitude than native aortas [81]. Also, Wu's study confirmed that the thickness and density of PCL sheath in bilayered grafts could affect the vascular remodeling and regeneration [82].

Sugar spheres can also be used as porogen to produce highly interconnected vascular graft by several steps. Polymer solution was firstly cast into an assembled sugar template under a mild vacuum. The polymer-sugar composite was phase separated at low temperature overnight and then immersed into cyclohexane to exchange Tetrahydrofuran (THF). The consequential composites were freeze-dried, and the sugar spheres were leached out in distilled water and freeze-dried again. In vivo subcutaneous implantation studies indicated VSMCs differentiation and host tissue infiltration in the scaffolds [83]. A thin dense layer needed to prevent leakage of blood after this kind of macroporous vascular graft was implanted in vivo.

The technique of phase separation can generate macroporous scaffolds which can increase cell migration and cell seeding efficiency. Polymer dissolution is processed by liquid-liquid phase separation and polymer gelation to generate a nanofibrous sponge. Then the solvent is extracted and the foam is freeze-dried. Many parameters such as gelation temperature, polymer concentration, solvent characteristics, and thermal treatment can affect scaffolds morphology, Young's modulus, and tensile strength. Ma's lab developed a porous vascular grafts with biodegradable PLLA through thermally induced phase-separation (TIPS) techniques. The grafts with oriented gradient microtubular structures in the axial or radial direction can be produced by utilizing different thermal conductivities of the mold materials and using benzene as the solvent. The porosity, tubular size, and the orientational direction of the microtubules can be regulated by the TIPS temperature, the polymer concentration, and by utilizing materials of different thermal conductivities [84].

Bilayered vascular grafts of poly(ester urethane) urea (PEUU) were fabricated by electrospinning and TIPS and implanted in vivo after seeded with pericytes. Cell-seeded TEVGs showed extremely higher patency rate than the unseeded control. The remodeled vascular grafts consisted of multiple layers of α-SMA- and calponin-positive cells and a von Willebrand factor-positive monolayer in the lumen [85, 86].

Sugiura et al. developed a novel bioresorbable vascular graft with a porous PLCL sponge-type scaffold reinforced by PLA nanofibers which is fabricated by phase-separation method and electrospinning. The animal experiments show that it has potential to be applied as small-diameter arterial grafts [87].

Despite these promising results from the preclinical studies, it is too early to evaluate the full potential of these grafts in clinical applications. Future studies should include long-term implantation of grafts in large animals as well as more rigorous functional evaluations.

11.4 Functional Modification of Vascular Grafts

11.4.1 Nitric Oxide-Releasing Materials

Commercialized Dacron, ePTFE, and microporous PU vascular prostheses have been widely used in clinical practice as large-diameter (>6 mm) vascular grafts. However, these polymers have been proven inadequate when used as small-diameter (<6 mm) grafts due to occlusion and restenosis. To solve this problem, researchers used different nitric oxide (NO)-based strategies to modify these polymer materials. NO-releasing materials were firstly reported by Smith et al. in 1996 [88]. Covalent attachment of NO donors directly to the polymer backbone has been recognized as a further route for fabrication of ePTFE bypass grafts (W.L. Gore & Associates, Inc.). NONOate formed by using gaseous NO demonstrates potent antiplatelet activity to predict the inhibition of thrombosis. Many researchers incorporated NO donors into PU to prepare novel NO-releasing materials for promoting graft endothelialization while preventing thrombus formation and intimal hyperplasia. Among those studies, Fleser et al. [89] implanted small-diameter (5 mm) PU vascular grafts coated with a polymer containing the NO donors (dialkyl hexanediamine diazeniumdiolate) in a sheep arteriovenous bridge-graft model for 21 days. Approximately 80 % of the NO-eluting grafts were found to remain patent in vivo compared with only a 50 % patency rate with control grafts. Although the improvement in patency between NO-releasing grafts and control grafts was not statistically significant, control grafts contained significant adherent thrombus and fibrin matrix with inflammatory and red blood cells, an observation not found in NO-releasing grafts.

There has been substantial effort directed to fabricating materials that mimic endothelial properties. Kushwaha et al. [90] developed a nanofibrous matrix, which is formed by self-assembly of peptide amphiphiles (PAs), containing NO donating residues and Tyr-Ile-Gly-Ser-Arg (YIGSR) peptide sequence, a laminin-derived cell-adhesive peptide sequence. The NO-releasing nanofibrous matrix demonstrated a significantly enhanced proliferation of ECs but reduced VSMCs proliferation and platelet attachment. Andukuri et al. [91] reported a similar design in which electrospun PCL nanofibers were coated with NO-releasing PAs containing cell-adhesive ligands (YIGSR and KKKKK) by a solvent evaporation technique. The presence of YIGSR ligands and release of NO promoted the adhesion and proliferation of ECs while simultaneously limiting the adhesion and proliferation of VSMCs and the adhesion and activation of platelets.

Besides immobilization of NO donors on polymer matrix, there are many studies on modification of polymers by catalysts for promoting the transfer of NO from NO donors in blood. Wang et al. [15] constructed a functional vascular graft by immobilization of β-galactosidase on vascular graft surface for catalyzing prodrug to release NO locally and sustainably (Fig. 11.3). The functional vascular grafts were implanted into the rat abdominal aorta with a 1-month monitoring period. Results in vivo showed effective inhibition of thrombus formation and enhancement of vascular tissue regeneration and remodeling on the grafts. Lewis and coworkers

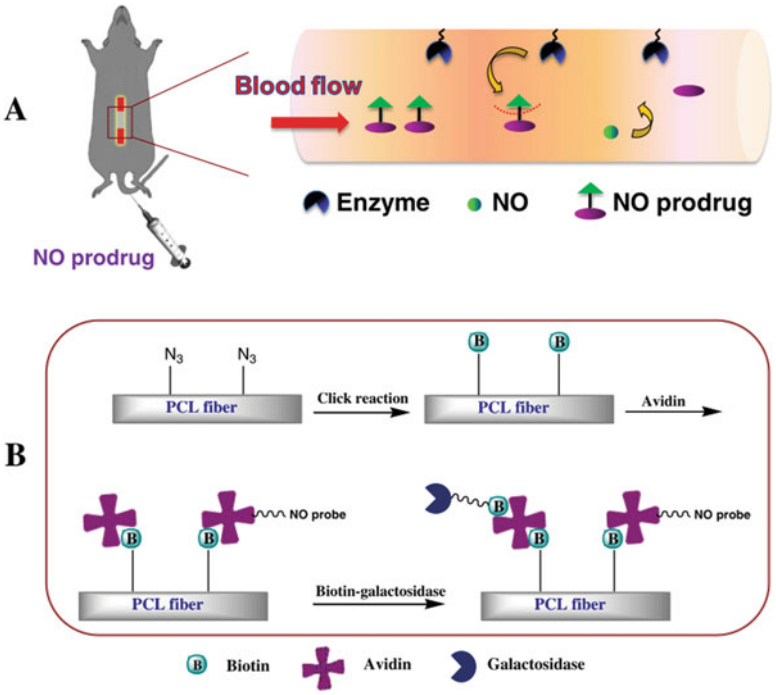

Fig. 11.3 Illustration for the enzyme immobilized on the vascular grafts to catalyze the decomposition of exogenously administrated NO prodrug to release NO. (**a**) Pathway for the surface enzyme functionalization of PCL vascular grafts (**b**) (Reprinted from Ref. [15] with permission. Copyright 2015 Elsevier B.V.)

modified polyethylene terephthalate (PET) and PU with thiol groups that could promote the release of NO from S-nitrosothiol compounds (RSNO) naturally found in plasma and whole blood solutions and thus inhibited platelet adhesion [92, 93]. Chen et al. prepared a novel vascular graft in situ with catalytic generation of NO [94]. PGA and poly α-lysine were deposited alternately onto the surface of electrospun PCL matrices, and selenocystamine (SeCA) was loaded as a catalyst. Cellular compatibility of the new material was shown to be good in the fibroblast proliferation experiment. In biological function evaluations, the catalyst-loaded material could inhibit the spread of VSMCs in the presence of NO donors and prevent acute thrombosis when in contact with blood. An et al. developed an electrospun PCL matrix loaded with organoselenium-immobilized polyethyleneimine (SePEI) as NO donor catalyst via electrostatic layer-by-layer self-assembly [95]. This material revealed significant NO generation when contacting NO donor S-nitrosoglutathione (GSNO). Modified material could inhibit adhesion and spreading of VSMCs and promote proliferation of ECs. In addition, antithrombotic properties of these NO-generating materials were proven by reduced platelet adhesion and prevention of acute thrombosis.

Other studies demonstrated the immobilization of NO-releasing catalysts on the surface of stents for localized delivery of NO. Huang's group coated metallic stents with titanium dioxide (TiO_2) films, followed by immobilization of cystamine and selenocystamine (Se) on TiO_2 film surface for catalytic generation of NO by decomposing endogenous S-nitrosothiols (RSNO) [96, 97]. Results in vitro showed that both modifications reduced collagen-induced platelet activation. In addition, the Se-modified stents inhibited neointimal hyperplasia in dog femoral artery model. Recently, the group developed a NO-catalytic bioactive coating that obtained by covalent conjugation of 3,3-diselenodipropionic acid (SeDPA) with glutathione peroxidase (GPx)-like catalytic activity to generate NO from RSNO via specific catalytic reaction [98]. Results in vivo showed SeDPA-PPAam-coated stents remarkably reduced neointimal stenosis compared with bare stent by promoting re-endothelialization and reducing proliferation and migration of VSMCs.

11.4.2 Polypeptide Modification

In general, biological recognition between cell-surface receptors and their ligands is the key switch to mediate cell migration and adhesion in physiological environments. Verhaar's group reported that stromal cell-derived factor 1α (SDF1α)-derived peptides can be chemically modified with a supramolecular fourfold hydrogen bonding ureido-pyrimidinone (UPy) moiety that allowed for the convenient incorporation of the UPy-SDF1α-derived peptides into a UPy-modified polymer scaffold [99]. The chain-extended UPy-poly(l-lactic acid caprolactone) (CE-UPy-PLLCL) polymer was obtained by replacing the poly(2-methyle1, 3-propylene adipate) diol of chain-extended UPy-poly(2-methyle1, 3-propylene adipate) with poly(l-lactic acid caprolactone) diol. The CE-UPy-PLLCL and either the UPy-SDF1a(R) peptide or the UPy-SDF1a(NR) peptide were dissolved in a mixture of chloroform and hexafluoroisopropanol and then produced fibrous tubular graft by electrospinning. This kind of graft can retain and stimulate leukocyte populations in an anti-inflammatory, pro-tissue formation signaling environment.

Mahara et al. demonstrated the excellent patency of tissue-engineered small-caliber long-bypass grafts [70]. The inner surface of an acellular ostrich carotid artery was modified with a novel heterobifunctional peptide composed of a collagen-binding region $(Pro\text{-}Hyp\text{-}Gly)_n$, $(POG)_n$, and the integrin α4β1 ligand, Arg-Glu-Asp-Val (REDV), which was expressed on ECs and circulating endothelial progenitor cells (EPCs). The $(Pro\text{-}Hyp\text{-}Gly)_7$ (POG_7) peptide was conjugated to Arg-GluAsp-Val (REDV) through Gly-Gly-Gly (G_3) spacer domain, which was termed $(Pro\text{-}Hyp\text{-}Gly)_7$-Gly-Gly-Gly-Arg-Glu-Asp-Val (POG_7G_3REDV). Decellularized carotid arteries were immersed in this peptide solution and incubated at 60 °C for 1 h and then cooled down to room temperature to accomplish peptide modification. The modified graft was transplanted into Gottingen minipig femoral arteries and observed for 20 days and received no anticoagulant medica-

tion. Five cases were patent and no thrombogenesis was observed on the luminal surface. In contrast, all unmodified grafts became occluded, and severe thrombosis was observed. This vascular graft is the first successful demonstration of short-term patency at clinically applicable sizes.

The tripeptide sequence of Arg-Gly-Asp (RGD) was identified by Pierschbacher and Ruoslahti in 1984 as a minimal essential cell adhesion peptide sequence in fibronectin. Kong's lab modified electrospun PCL scaffold with RGD containing molecule (Nap-FFRGD) using a surface coating method. The Nap-FFRGD molecule contains both the RGD peptide and hydrophobic naphthalene group, which can self-assemble onto hydrophobic PCL surfaces to form an RGD coating layer. The modified surface was shown to improve hydrophilicity and enhance cell adhesion and spread in vitro [100]. And then, the electrospun PCL grafts were functionally modified by Nap-FFGRGD molecule through self-resembling. The potential of the grafts as small-diameter vascular grafts was investigated in a rabbit carotid arterial implantation model. The modified grafts exhibited an improved inhibition of platelet adhesion, enhanced cell infiltration, endothelium formation, smooth muscle regeneration, and patency, which suggested that the as-prepared PCL-RGD graft may be a promising candidate for the small-diameter vascular grafts [101].

The tetrapeptide, REDV, is a fibronectin-derived peptide that can specifically bind to the α4β1 integrin, which is abundant on ECs, whereas scarce on VSMCs. Wang et al. constructed an NO and REDV dual modificated vascular graft and examined its effect on enhancing rapid in situ endothelialization. [102]. A macroporous electrospun PCL graft was prepared and modified via layer-by-layer self-assembly. Assembly of PCL grafts was performed with constant-flow pumps driving the solutions through the grafts at a very slow rate. To prepare catalyst-loaded material, deposition was continued until ten SePEI/hyaluronic acid (SePEI/HA) bilayers were obtained. To get REDV peptide functionalized material, a SePEI/HA-REDV bilayer was deposited after nine bilayers of SePEI/HA, and the sample was marked as PCL-$(S/H)_9$-S/H-R. The cooperation of NO and REDV promoted ECs adhesion with an increased ECs/SMCs ratio in a coculture system. Grafts built by this method exhibited rapid endothelialization and a well-organized ECs pattern. The dual modification proposed in this study may be a promising approach to improve the endothelialization and long-term performance of small-diameter vascular grafts.

Larsen et al. reported a peptide fluorosurfactant polymer (FSP) biomimetic construct that promoted ECs selective attachment, growth, shear stability, and function on ePTFE [103]. The peptide FSP consisted of a flexible poly(vinyl amine) backbone with ECs-selective peptide ligands (primary sequence: Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys, CRRETAWAC) and pendant fluorocarbon branches for stable anchorage to underlying ePTFE. This peptide has demonstrated high binding affinity for the α5β1 integrin found on ECs, which was attributed to Arg-Arg-Glu (RRE) motif interaction with the β1 subunit and Trp interaction with a Trp residue in the α5 integrin subunit. They demonstrated low affinity of CRRETAWAC for platelets and platelet integrins, thus providing it with EC selectivity. This EC

selectivity could potentially facilitate rapid in vivo endothelialization and healing without thrombosis for small-diameter ePTFE vascular grafts.

Zhou et al. reported that a short peptide REDV was linked to trimethyl chitosan (TMC) via a bifunctional (Polyethylene Glycol) PEG linker for the targeted delivery of microRNA-126 to vascular endothelial cells [104]. TMC was reacted with a bifunctional PEG, ω-2-pyridyldithio polyethylene glycol α-succinimidylester (OPSS-PEG-SCM), in distilled water for 6 h, allowing the primary amino groups on TMC to react specifically with the SCM group of the heterobifunctional PEG. After lyophilized, the obtained TMC-g-PEG-OPSS reacted with REDV-Cys to link the REDV peptide by means of a specific reaction between the OPSS group in TMC-g-PEG-OPSS and the thiol group in the REDV-Cys peptide. By REDV modification, the TMC-g-PEG-REDV/miRNA complex showed negligible cytotoxicity, increased expression of miRNA-126, and enhanced ECs proliferation compared with the TMC/miRNA and TMC-g-PEG/miRNA complexes. It was suggested that the REDV peptide-modified TMC-g-PEG polyplex could be potentially used as a miRNA carrier in artificial blood vessels for rapid endothelialization.

Ji et al. developed a method for the dual functionalization of a PCL surface through the supramolecular assembly technology [105]. Functionalization of PCL-cyclodextrin (PCL-CD) through host-guest inclusion complexation was performed in aqueous medium. PEG can decrease protein adsorption, and TPSLEQRTVYAK (TPS) peptide can specifically bind EPCs. The two kinds of functional molecular were immobilized on the PCL surface through host-guest inclusion complexation (Fig. 11.4). Aqueous solution of adamantine (AD) conjugated guest compounds (PEG-AD and TPS-AD) alone or in combination was prepared. Typically, the total concentration of guest compounds was kept at 1 mg/mL irrespective of the composition. Then PCL-CD films were put into the solutions, incubated and dried. The relative composition of the PEG and TPS could be further fine-tuned by adjusting the feeding ratio. The PEG functionation significantly inhibited the adsorption of fibrinogens and the adhesion of platelets, thus reducing the possibility of thrombus formation. Moreover, the TPS-functionalized surface showed enhanced attachment toward EPCs compared with the one without TPS functionalization. The dual functions provided by the corresponding functional molecules were well preserved, which indicated that the host-guest supramolecular assembly technology is particularly useful for covalent immobilization of bioactive molecules onto polymeric scaffolds.

Hydrophobin HGFI is a member of amphiphilic proteins, which can form a self-assembly layer on the surface of hydrophobic polymer scaffolds and convert their wettability. A fusion protein, TPS-linker-HGFI (TLH), which was composed of HGFI that originated from *Grifola frondosa* and functional peptide TPS, was expressed by *Pichia pastoris* expression system. PCL scaffolds were incubated overnight in a sterilized aqueous TLH solution, followed by blowing off the excess protein solution and drying in a super clean bench. Cell adhesion test showed that the TLH-modified PCL could specially enhance the adhesion of ECs and EPCs [106, 107]. This work presented a new perspective to apply hydrophobin in tissue

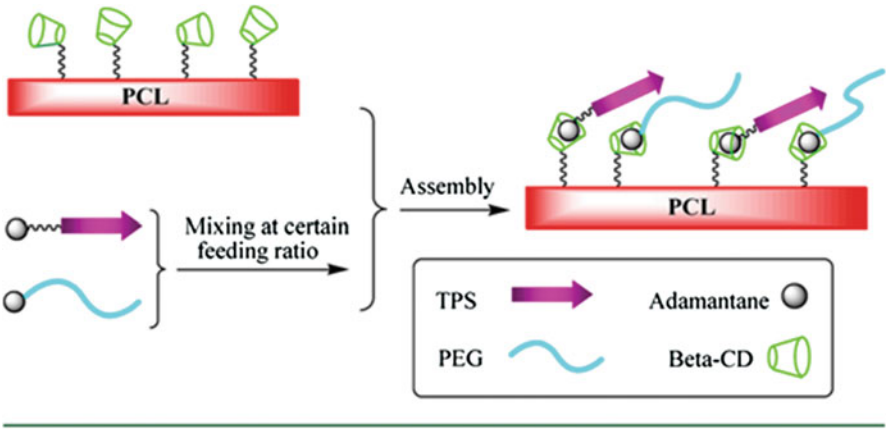

Fig. 11.4 Schematic illustration for the supramolecular assembly of functional molecules on the surface of PCL-CD film (Reprinted from Ref. [105] with permission. Copyright 2013 American Chemical Society)

engineering and regenerative medicine and provided an alternative approach in surface modification.

11.4.3 Incorporation of Growth Factors

Endothelialization is a complex process that mainly relate to ECs adhesion, migration, proliferation and differentiation. Numerous growth factors are involved in the regulation of ECs activities and new blood vessel formation. So there are many researchers committed to investigate how to modify the artificial vascular graft with growth factors and other bioactive molecules.

Fibroblast growth factors (FGFs) can promote fibroblast mitosis, ECs migration, and VSMCs proliferation. Brewster et al. [108] engineered a thrombin-resistant mutant of FGF-1 through a lysine (K) for arginine (R) base substitution at residue 136 (termed R136K), the primary thrombin-induced cleavage site Then R136K was bound to a collagen-binding domain (CBD) to generate R136K-CBD. Comparing with control group, the R136K-CBD has significantly better angiogenic activity, ECs sprout lengthening, and mitogenic activity.

Researchers found that the regeneration potential of endogenous cells could be contributed to blood vessel regeneration. Since adult vascular cells lack expansion capability, it will be highly desirable to develop vascular grafts that can recruit endogenous stem cells or progenitor cells of both ECs and VSMCs. Stromal cell-derived factor-1a (SDF-1α) is considered to be a potent factor for EPCs homing and neovascularization. Yu et al. [109] use di-NH-PEG as a linker molecule and Sulfo-n-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as cross-linking agents, to make heparin connect to PLLA scaffold surface.

Following heparin conjugation, SDF-1α in PBS was incubated with the scaffolds over night at 4 °C to allow it to bind to heparin immobilized on the scaffolds. They implanted the heparin-SDF-1α-treated PLLA scaffold into the left common carotid artery of rats. The result demonstrated that heparin could stabilize the immobilized SDF-1α on microfibers, which enhanced the self-regeneration capability of the grafts by recruiting EPCs and smooth muscle progenitor cells, contributing to endothelialization and the remodeling of the vascular wall.

In small animals, transanastomotic cell ingrowth can trigger vascular regeneration. That is very rare in humans. To prove that functional blood vessels can be formed in situ through the host inflammatory response solely by circulating cells, Talacua et al. [110] utilize Gore-Tex sheet and masterly constructed a novel rat model. In addition, this group modified PCL conduit with MCP-1 through impregnating PCL conduit into fibrin gel added monocyte chemoattractant protein–1 (MCP-1). Then the conduit was implanted into the abdominal aorta of rats, and an end-to-end anastomosis was made to a 4 × 10 mm^2 impenetrable Gore-Tex strip using 10-0 interrupted sutures distally and proximally of the electrospun tube. Then Gore-Tex was wrapped around the PCL in samples, creating an impenetrable outer layer. The results demonstrated that Gore-Tex shielding led to a significant reduction of cell ingrowth from neighboring tissues and MCP-1 was beneficial to endothelialization and tissue formation.

VEGFs can induce enhancement of the endothelial functions that mediate the inhibition of VSMCs proliferation, suppression of thrombosis, and anti-inflammatory effects. Thus, there are many approaches to immobilize VEGF on scaffold surface. Koobatian et al. [111] used NHS and EDC as cross-linking agents to make heparin bind to SIS surface, and the heparin-bound SIS was immersed in PBS with recombinant human VEGF-165 to make VEGF immobilize on the heparin surface by utilizing the heparin-binding domain. This acellular tissue-engineered vessel was implanted into the carotid artery of an ovine model. The result showed that the grafts possessed of high patency rates, fast endothelialization, high elastin and collagen content, as well as impressive mechanical properties and vascular contractility comparable to native arteries. Wang's group also used heparin to immobilize VEGF to obtain the same effect [112]. PCL scaffolds were prepared by electrospinning, followed by treatment with ammoniating ethylenediamine (EDA). Heparin was covalently cross-linked through EDC and NHS. Finally, heparin-loaded PCL was incubated with VEGF solution. Shin et al. [113] dissolved PLCL in chloroform and poured it onto a glass plate walled with aluminum tape and covered with aluminum foil. The polydopamine formed by oxidizing and cross-linking dopamine was coated on the PLCL film. Subsequently, the polydopamine-deposited films were immersed and shaken in solutions containing growth factors for immobilization of VEGF. And they found that VEGF-immobilized substrate could effect on adhesion of HUVECs, significantly enhance proliferation of HUVECs, and improve their migration. This modification method could act as a versatile tool to modify surface characteristics of vascular grafts potentially for accelerated endothelialization.

An ideal vascular graft should be noncytotoxic, nonthrombogenic, and infection resistant; have appropriate mechanical properties; minimize intimal hyperplasia; and support the regeneration of vascular tissue. In order to achieve these purposes, Han et al. [114] prepared a multilayered small-diameter vascular scaffold dual loaded with VEGF and platelet-derived growth factor-bb (PDGF). The multilayered grafts including inner, middle and outer layer were prepared by dual-source and dual-power electrospinning. The inner layer consisited of poly(ethylene glycol)-b-poly(Llactide-co-ε-caprolactone) (PELCL) and gelatin fibers. The PELCL fibers containing chitosan hydrogel loaded with VEGF were fabricated by suspension electrospinning. The middle layer consisted of PLGA and gelatin fibers. The PLGA fibers loading PDGF were prepared through emulsion electrospinning. The outer layer consisited of PCL and gelatin fibers. The PCL fibers were obtained via electrospinning of PCL solution in chloroform with N,N-Dimethylformamide. All gelatin fibers were fabricated by dissolving gelatin in tetrafluoroethylene and electrospinning. The results suggested that the small-diameter vascular scaffold dual-loading VEGF and PDGF could enhance vascular regeneration maintain patency in rabbit left common carotid artery for 8 weeks.

Besides growth factors, researchers also adopted other bioactive molecules to modify the scaffolds to accelerate endothelialization. Lu et al. [115] used anti-CD133 antibody to coat the ePTFE grafts. After adsorption of PEI, dipped alternately in heparin solution and collagen solution, synthetic ePTFE grafts coated with five bilayers of heparin/collagen were prepared. Then the multilayers were immersed in glutaraldehyde to promote cross-linking and to immobilize the anti-CD133 antibody. Finally, they implanted the graft into the carotid artery of pig. The results demonstrated that these synthetic ePTFE grafts coated with anti-CD133 antibody-functionalized heparin/collagen multilayer may achieve rapid endothelialization.

11.4.4 Gene Modification

Dannowski et al. [116] developed a nonviral gene carrier based on liposomes and transfected plasmids coding for the acidic fibroblast growth factor (aFGF) and enhanced green fluorescent protein (eGFP) into human corneal ECs. The transfection efficiency, toxicity of the gene carriers, and the proliferation of ECs were investigated. The liposome gene carrier showed high transfection efficiency.

Vein graft failure caused by narrowing and occlusion after coronary artery bypass grafting with saphenous veins is a major clinical problem and is an important target for gene therapy. Akowuah et al. [117] engineered vascular grafts with genetically modified BMSCs on poly(propylene carbonate) graft, which delivered the tissue inhibitor of metalloproteinase 3 (TIMP-3) plasmid to the saphenous vein graft in vitro by ultrasound exposure (USE) in the presence of echo contrast microbubbles (ECM). Then the transgened vein graft was tested by the model of Yorkshire White pigs' carotid model. At 28 days post-grafting, lumen and total vessel area were significantly greater in the TIMP-3 group compared to the untransfected or con-

trol group. Meng et al. [118] developed a receptor-targeted nanocomplex (RTN) vector system. The RTN vector was composed of cationic liposome lipofectin, a peptide (Peptide-Y: K16GACYGLPHKFCG), and plasmid DNA. Vein grafts were transgened by RTN with a plasmid-encoding inducible nitric oxide synthase (iNOS) and then engrafted into the rabbit's carotid artery to inhibit the neointimal hyperplasia. Fluorescent immunohistochemistry analysis of samples from rabbits killed at 7 days after surgery showed that mostly ECs and macrophages were transfected. Morphometric analysis of vein graft samples from the 28-day groups showed approximately a 50 % reduction of neointimal thickness and 64 % reduction of neointimal area in the iNOS-treated group compared with the surgery control group. Zhong et al. [119] developed a novel recombinant lentivirus for the delivery of hepatocyte growth factor (HGF) and Bax in a rabbit vein graft model of bypass grafting. Rabbit vein segments were dissected and transgened by the lentivirus vector harboring HGF and Bax cDNAs (Lenti-HGF-Bax) and then were interposed into the rabbit's carotid arteries. HGF and Bax expression in vein grafts was detected by immunohistochemical and western blot analysis. The result showed that vein graft thickening was remarkably reduced in Lenti-HGF-Bax-treated rabbits compared to controls.

Zhang et al. [120] created small-diameter vessels by seeding and culture of genetically modified MSCs onto a synthetic polymer scaffold produced by an electrospinning technique. Rat MSCs were modified with nitric oxide synthase (eNOS). The results showed that the seeded cells integrated with the microfibers of the scaffold to form a three-dimensional cellular network, indicating a favorable interaction between this synthetic scaffold with MSCs. High transduction efficiency was obtained with the use of concentrated retrovirus in the gene transfection of MSCs. The use of MSCs and therapeutic genes in tissue engineering of blood vessels could be helpful in improving vessel regeneration and patency.

11.5 The Mechanical Properties of Vascular Grafts

11.5.1 Synthetic Polymers

Human mammary arteries and saphenous veins have burst pressures between 2031 and 4225 mmHg [121] and 1250 ± 500 mmHg [85], respectively. Mechanical characteristics of vascular grafts, such as compliance and Young's modulus, have significant potential to influence their long-term patency [122].

Surgeons have used nondegradable synthetic PTFE or Dacron medium-to-large-diameter grafts which provide 10 years of symptom-free lifestyle. However, they have extremely poor performance in replacement of small-diameter (<6 mm) blood vessels due to neointimal hyperplasia and thrombosis [123]. The commercial grafts such as woven and knitted Dacron and ePTFE usually show a low radial compliance [8]. A high-porosity ePTFE graft demonstrated no significant deterioration in suture

retention strength, radial tensile strength, or longitudinal tensile strength for periods ranging from 2 to 80 weeks compared to preimplantation grafts [124].

PCL has excellent mechanical characteristics, and it does not undergo plastic deformation and failure when exposed to long cyclic strain. Therefore, it can be a critical component in vascular graft application [125]. Sang et al. [126] indicated that the PCL/collagen composite graftss, with fiber diameters of approximately 520 nm, possessed appropriate tensile strength (4.0 ± 0.4 MPa) and adequate elasticity (2.7 ± 1.2 MPa). The burst pressure of the composite grafts was 4912 ± 155 mmHg, which was much greater than that of the pure PCL grafts (914 ± 130 mmHg) and native vessels. Ángel et al. [127] synthesized biocompatible and biodegradable PCL urethane macromers to fabricate hollow fiber membranes of different sizes as small-diameter vascular graft candidates. Their tensile stiffness ranged from 0.09 to 0.11 N/mm and their maximum tensile force from 0.86 to 1.03 N, with minimum failure strains of approximately 130 % and burst pressures from 1158 to 1468 mmHg.

The polyethylene terephthalate (PET)/PGA graft was woven to be partially degradable with a double-layered fiber (core; PET and sheath; PGA). The PGA component had degraded and been replaced by host tissue that contained a mixture of α-SMA positive cells and other host cells. The graft was a unified structure with the aorta. The adhesion strength between the graft and aortic wall was significantly enhanced in the PET/PGA group. The vascular graft demonstrated histologic and mechanical integration with the native aorta [128].

Porous elastomeric grafts made of PGS enforced with PCL nanofibers degraded rapidly and yielded neoarteries nearly free of foreign materials in rat abdominal aorta. 3 to 12 months after implantation in rat abdominal aortas, the PGS grafts with rationally strengthened sheath were remodeled into neoarteries that resembled native arteries in the following aspects: high patency rate and even vessel wall thickness; a confluent endothelium and contractile smooth muscle layers; expression of elastin, collagen, and GAG; and tough and compliant mechanical properties. This study confirmed that adequate density of PCL sheath in PGS grafts could initiate stable and high-quality muscular remodeling, which contributed to long-term success in arterial circulation before clinical translation [82]. Similarly, Ramak et al. [39] developed a novel method for electrospinning smaller grafts composed of a PGS microfibrous core enveloped by a thin PCL outer sheath. Electrospun PGS-PCL composites were implanted as infrarenal aortic interposition grafts in mice and remained patent up to the 12-month endpoint without thrombosis or stenosis. Lack of rupture over 12 months confirmed sufficient long-term strength, due primarily to the persistent PCL sheath.

PU can potentially provide a greater degree of graft-artery compliance match than ePTFE prostheses [129]. Finer tensile strength and elastic property facilitates the usage of PU as a vascular scaffold, but it is shown to have less compliance and a biologically unstable nature. The compliance values of poly(carbonate)polyurethane (CPU) and artery (mean over the pressure range) were 8.1(0.4) and 8.0(5.9)

percent per mmHg × 10^{-2}, respectively, although the elastic behavior of artery was anisotropic unlike CPU, which was isotropic [130]. Uttayarat et al. [131] combined electrospinning with the spin casting method to pattern microfibers and microgrooves on the electrospun PU tubular scaffold. The elastic modulus of the grafts in the axial direction was also in a range similar to those of native vascular grafts.

Poliglecaprone (PGC) in the form of monocryl monofilament sutures displayed excellent tensile properties and 20–30 % reduction in strength after 2 weeks in vivo [132]. In another study, blends of PGC and PCL of varying compositions were electrospun into tubular conduits and their mechanical, morphological, thermal, and in vitro degradation properties were evaluated under simulated physiological conditions. Generally, mechanical strength, modulus, and hydrophilic nature were enhanced by the addition of PGC to PCL. A 3:1 PCL/PGC blend was concluded to be a judicious blend composition for tubular grafts based on overall results on the mechanical properties and performance after a 1-month in vitro degradation study [133].

Electrospun PLLA scaffolds with NaCl-made pores had a lower elastic modulus (8.05 MPa) and yield stress (349 kPa) and a higher yield strain (0.04) compared to their traditional counterparts (40.36 MPa, 676 kPa, and 0.0188) [134]. Grafts were prepared using the PLA and PCL physical blends in the ratios of 75:25 and 25:75 with the dimension of (40 × 0.2 × 4) millimeter by electrospinning. Hydrophobicity and tensile stress were significantly higher in PLA-PCL (75:25), whereas tensile strain and fiber density were significantly higher in PLA-PCL (25:75). Cell viability and proliferation were rationally influenced by the aligned nanofibers. Gene expression revealed the grafts' thromboresistivity, elasticity, and aided neovascularization. Thus, these scaffolds could be an ideal candidate for small-diameter blood vessel engineering [32].

11.5.2 Natural Polymers

Collagen-based scaffolds have been the platform for a number of exploratory clinical trials that shows the true potential of tissue engineering for repairing significant tissue damage. Hirai et al. [135] specifically worked on the original development of a collagen-based construct for a low-pressure-loaded venous system. By pouring a solution of bovine aortic VSMCs and type-I collagen into a tubular glass mold and using a Dacron mesh support, a vascular construct that could tolerate luminal pressures as great as 100 mmHg was obtained in 24 weeks of culture [136]. Vivek et al. [137] proposed a design strategy for the fabrication of tubular conduits comprising collagen fiber networks and elastin-like protein polymers to mimic native tissue structure and function. Comparing favorably to an ultimate tensile strength (UTS) and a Young's modulus for native blood vessels of 1.4–11.1 MPa and 1.5 ± 0.3 MPa, dense fibrillar collagen networks exhibited an UTS of 0.71 ± 0.06 MPa, strain to failure of 37.1 ± 2.2 %, and Young's modulus of 2.09 ± 0.42 MPa, respectively.

Elastin is found to be very brittle and shows mechanical properties lower than those of native elastin. As such, the material was suggested as a provisional matrix for in vivo remodeling. Buijtenhuijs et al. [138] used a freeze-drying method with insoluble type-I collagen and insoluble elastin to produce a porous scaffold with fibers of collagen and elastin interspersed together. Elastin exhibited less stress relaxation than intact or decellularized aorta. The rate of stress relaxation of intact and decellularized aorta was linearly dependent on the initial stress levels. The rate of stress relaxation for elastin increased linearly at stress levels below about 60 kPa [139]. McKenna et al. [140] fabricated a tubular construct using electrospun recombinant human tropoelastin (rTE). The electrospun scaffolds were formed by using 1,1,1,3,3,3-hexafluro-2-propanol (HFP) as the solvent and disuccinimidyl suberate (DSS) as the cross-linking agent to stabilize the fibers in aqueous solution. The fabricated scaffold had an elastic modulus in the range of 0.15–0.91 MPa and an ultimate tensile strength of 0.36 MPa. The results of in vitro studies demonstrated that the fabricated electrospun rTE scaffolds supported ECs growth with typical ECs cobblestone morphology following 48 h in culture, which confirmed the suitability of this biomaterial for application in vascular grafts. Vaibhav et al. [141] used biaxial force-controlled experiments to quantify regional variations in the anisotropy and nonlinearity of elastin isolated from bovine aortic tissues proximal and distal to the heart. Results from this study showed that tissue nonlinearity significantly increased distal to the heart as compared to proximally located regions. Their studies suggested that it was important to consider elastin fiber orientations in investigations that used microstructure-based models to describe the contributions of elastin and collagen to arterial mechanics.

Esterified hyaluronic acid (HYAFF) is routinely used for clinical tissue engineering applications. The problems that are generally associated with hyaluronic acid (HA)-based biopolymers are their poor mechanical properties and their rapid degradation rate. In order to increase their mechanical properties, Arrigoni et al. [142] added sodium ascorbate (SA) to a culture of VSMCs seeded in HYAFF nonwoven sheets, and SA improved mechanical properties of the vascular construct lowering material stiffness and increasing tensile strength. Amaliris et al. [143] demonstrated the ability of PU containing HA in its backbone structure to reduce protein adsorption, platelet and bacterial adhesion, and fibroblast and macrophage proliferation while allowing the retention of both ECs and vascular-appropriate mechanics. The molecular weight of the HA oligosaccharides influenced the mechanical properties of the PU-HA copolymers. In another study, PU materials modified with HA were more effective than either PEG- or heparin-grafted materials with respect to limiting protein adsorption and platelet adhesion. These modifications in PU chemistry were performed while retaining material mechanics in the range of native vascular tissue. Thus, this study described the generation of materials that possessed the unique ability to display excellent hemocompatibility while simultaneously supporting extensive endothelialization and retaining vascular-appropriate mechanics [144].

It is increasingly recognized that biomimetic, natural polymers mimicking the ECM have low thrombogenicity and functional motifs that regulate cell-matrix interactions, with these factors being critical for TEVGs, especially small diameter grafts. Elsayed et al. [145] devised the fabrication of novel, electrospun, multilayer, gelatin fiber scaffolds, with controlled fiber layer orientation and optimized gelatin cross-linking to achieve not only compliance equivalent to that of coronary artery but also for the first time strength of the wet tubular acellular scaffold (swollen with absorbed water) same as that of the tunica media of coronary artery in both circumferential and axial directions. Most importantly, the suture retention strength of gelatin scaffolds firstly achieved in the range of 1.8–1.94 N for wet acellular scaffolds, and was same or better than that for fresh saphenous vein.

11.5.3 Hybrid Materials: Synthetic and Natural Polymers

A synthetic polymer will provide mechanical integrity, and natural proteins provide biocompatibility and ECM-mimicking environment for better cell attachment and proliferation. Ju et al. [146] used a co-electrospinning technique to fabricate PCL/collagen bilayer scaffolds with an outer layer containing large pores that enhanced VSMCs infiltration and an inner layer with smaller pore sizes that facilitated ECs attachment. Thus, the microstructure and mechanical properties of the resultant scaffolds could be controlled by the fiber diameter. Increasing the fiber diameter from 0.27 to 4.45 μm enhanced the scaffold's porosity and reduced its Young's modulus from 2.03 to 0.26 MPa. Stitzel et al. [147] discovered that controlling the ratio of collagen, elastin, and PLGA could improve electrospinning characteristics and physical strength of the scaffolds, which resisted bursting at nearly 12 times normal systolic pressure.

As for chitosan, its poor mechanical properties represent a major limit to its development. PCL can increase the mechanical properties. A mixture of 1:1 chitosan and PCL demonstrated an ultimate strength twice than that of chitosan alone [148]. In another study, Chen et al. [149] fabricated electrospun collagen/chitosan/TPU nanofibrous scaffolds with functional and structural properties resembling the native ECM. The tensile strength of scaffolds with random and aligned fibers was 4.6 MPa and 10.3 MPa, respectively. In addition, the mechanical properties of resultant scaffolds were significantly improved by cross-linking the fibers. The tensile strength of glutaraldehyde cross-linked scaffold with random fibers was twofold higher than uncross-linked samples. The fabricated scaffolds could support the growth and alignment of ECs and were used to form a vascular graft in vitro.

11.6 The Degradation of Vascular Grafts

The degradation rate of these polymers is determined by initial molecular weight, exposed surface area, crystallinity, and ratio of monomers. ePTFE is very stable in vivo with no reported failures due to degradation of the graft. PGA is a biodegradable macromolecular material that degrades through hydrolysis of its ester bonds in vivo, loses 100 % of its strength within 4 weeks and is completely absorbed within 6 months [150].

Both PCL and PLLA are slowly degrading polymers. PLA is less crystalline than PGA but more hydrophobic, making it less susceptible to hydrolysis. Surgical sutures made of PLA require more than a year to lose their tensile strength. Walpoth's group [4] reported that electrospun PCL grafts were degraded to 20 % of original molecular weight at 18 months of postimplantation. But the grafts neither dilated nor had a significant increase in compliance. Tara et al. [151] have shown that the PLCL coating completely degrades 4 months after implantation in a mouse model.

Random copolymerization of PLA (both L- and D,L-lactide forms) and PGA, known as PLGA, is the most investigated degradable polymer for biomedical applications and has been used in sutures, drug delivery devices, and tissue engineering scaffolds. With a number of commercial manufacturers and easy polymer processability, researchers do not have to be polymer synthesis experts to utilize PLGA in their work. Because PLA and PGA have significantly different properties, careful choice of copolymer composition allows for the optimization of PLGA for intended applications. Property modulation is even more significant for PLGA copolymers because with 25–75 % lactide composition, PLGA forms amorphous polymers, which are very hydrolytically unstable compared with the more stable homopolymers. This is evident in the degradation times of 50:50 PLGA, 75:25 PLGA, and 85:15 PLGA being 1–2 months, 4–5 months, and 5–6 months, respectively [152].

Ye et al. [153] described that PGS elastomer was used to construct the microvessel framework. In vivo studies of scaffolds implanted subcutaneously and intraperitoneally, without or with exogenous cells, into nude rats demonstrated biodegradation of the membrane interface and host blood cell infiltration of the microvessels. This modular, implantable scaffold could serve as a basis for building tissue constructs of increasing scale and clinical relevance.

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