Gustav Steinhoff *Editor*

Regenerative Medicine - from Protocol to Patient

3. Tissue Engineering, Biomaterials and Nanotechnology

Third Edition

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Foreword: Regenerative Medicine: From Protocol to Patient

Third Edition

The vision to unravel and develop biological healing mechanisms based on evolving molecular and cellular technologies has led to a worldwide scientific endeavour to establish *regenerative medicine*. This field involves interdisciplinary basic and (pre)clinical research and development on the repair, replacement, regrowth or regeneration of cells, tissues or organs in congenital or acquired diseases. Stem cell science and regenerative biology is prompting the most fascinating and controversial medical development of the twenty-first century. It can be envisaged that this development will establish completely new molecular and cellular techniques for medical diagnosis and therapy. The early rush of scientific development was initiated more than one hundred years ago by the physiology of blood regeneration (Hall and Eubanks 1896) and successful vascular surgical techniques for organ transplantation (Carrel and Guthrie 1905). However, the clinical realization of allogenic blood transfusion lasted until the discovery of the blood group antigens (Landsteiner and Levine 1928) and successful routine allogenic organ and bone marrow transplantation towards the end of the last century.

Similar to the field of allogenic cell and organ transplantation, it seems that *regenerative medicine* again condenses mankind's visions, hopes and fears regarding medicine: Hopes of eternal life and effective treatment of incurable disease, as well as fears of the misuse of technology and uncontrolled modifications of life are polarizing the scientific field. The development and public acceptance of new ethical and regulatory guidelines is a necessary process to support further clinical development. Nevertheless, the vision of a new medicine using the regenerative power of biology to treat disease and restructure the organism is setting the aims for scientific, technological and medical development. Viewing the great expectations to restructure and regenerate tissues, organs or even organisms, the current attempts of both scientists and physicians are still in an early phase of development.

The field of *regenerative medicine* has developed rapidly over the last 20 years with the advent of molecular and cellular techniques. This collection of volumes on *Regenerative Medicine: From Protocol to Patient* aims to explain the scientific knowledge and emerging technology, as well as the clinical application in different organ systems and diseases. The international leading experts from four continents describe the latest scientific and clinical knowledge in the field of *regenerative medicine*. The process of translating the science of laboratory protocols into therapies is explained in sections on basic science, technology development and clinical translation including regulatory, ethical and industrial issues.

This collection is organized into five volumes: (1) *Biology of Tissue Regeneration*; (2) *Stem Cell Science and Technology*, (3) *Tissue Engineering, Biomaterials and Nanotechnology*, (4) *Regenerative Therapies I.;* and (5) *Regenerative Therapies II. Biology of Tissue Regeneration (Volume 1)* focuses on regenerative biology with chapters on the extracellular matrix, asymmetric stem cell division, stem cell niche regulation, (epi)genetics, immune signalling, and regenerative biology in organ systems and model species such as axolotl and zebrafish.

Stem Cell Science and Technology (Volume 2) provides an overview of the classification of stem cells and describes techniques for their derivation, programming and culture. Basic properties of differentiation states, as well as their function are illustrated, and areas of stem cell pathologies in cancer and therapeutic applications for these cells are discussed with the emphasis on their possible use in *regenerative medicine*.

Tissue Engineering, Biomaterials and Nanotechnology (Volume 3) focuses on the development of technologies, which enable an efficient transfer of therapeutic genes and drugs exclusively to target cells and potential bioactive materials for clinical use. The principles of tissue engineering, vector technology, multifunctionalized nanoparticles and nanostructured biomaterials are described with regards to the technological development of new clinical cell technologies. Imaging and targeting technologies, as well as the biological aspects of tissue and organ engineering are described.

Regenerative Therapies I (Volume 4) gives a survey of the history of regenerative medicine and clinical translation including regulation, ethics and preclinical development. Clinical state-of-the-art, disease-specific approaches of new therapies, application technologies, clinical achievements and limitations are described for the central nervous system, head and respiratory systems. Finally, *Regenerative Therapies II (Volume 5)* contains state-of-the-art knowledge and clinical translation of regenerative medicine in the cardiovascular, visceral and musculoskeletal systems.

These volumes aim to provide the student, the researcher, the healthcare professional, the physician and the patient with a complete account of the current scientific basis, therapeutical protocols, clinical translation and practised therapies in *regenerative medicine*. On behalf of the sincere commitment of the international experts, we hope to increase your knowledge, understanding, interest and support by reading the book.

After the successful introduction of the first edition in 2011, this publication has been developed and expanded for the third edition into five volumes.

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Chapter 1
Novel Concepts in Design and Fabrication of 'Living' Bioprosthetic Heart Valves: From Cell Mechanosensing to Advanced Tissue Engineering Applications

Maurizio Pesce and Rosaria Santoro

Abstract Despite the use of bio-valve prostheses to replace diseased heart valves dates more than 50 years ago, and the large and increasing need of this type of implants for heart surgery worldwide, definitive solutions to manufacture 'lifetime-long' valve replacements are not yet available. In fact, although various problems in the manufacturing process of these implants have been circumvented compared with the beginnings, these solutions have not yet led to a full biological compatibility in the human system due to long term inflammation, calcification and ultimately structural valve deterioration. Importantly, the more limited duration of the valve bio-prostheses occur in pediatric patients and adults under the age of 65. These are the patients who more often need prosthesis replacement and therefore new invasive surgical interventions with a compromised quality of life.

The present contribution is centred onto the dissection of the valve cells response to mechanical stimuli regulated by the extracellular matrix, and new engineering systems that have been set up to mimic the tissue mechanics in the heart valve leaflets and manufacture the 'living bioprosthetic' valves. This latter goal is being pursued intensely worldwide by exploiting the most advanced technologies in material science and scaffolds design.

Keywords Valve bioimplant • Tissue engineering • Valve interstitial cell • Mechanosensing

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1.1 Introduction – The Current Limitations in Biological/ Bio-Prosthetic Valve Implants Design

Diseased and dysfunctional heart valves are routinely repaired or replaced by surgical interventions. If damage is too severe to enable valve repair, the native valve is replaced by a prosthetic valve. About 300,000 heart valve procedures are performed every year worldwide, and this number is expected to triple by 2050 consequently to the trend of the lifespan to increase. Two types of commercially available heart valve prostheses are used at present: mechanical or biological (David 2013; Kheradvar et al. 2015). Despite having excellent durability and a non-modifiable mechanical performance, the mechanical prostheses are prone to thromboembolic complications requiring lifelong anti-coagulation therapy. Biological valves undergo structural deterioration, and this is still the principal cause of prosthetic valve failure in the mid/long term, affecting significant portions of the patients populations, especially in the young (Forcillo et al. 2013).

The technology employed to produce the commercial bio-valve implants is based on tissues of animal origin. Pericardial membrane and valve leaflets, from bovine and porcine are the most commonly used. In order to increase mechanical resistance, the animal-derived tissues are normally treated with low concentration aldehydes (e.g. glutaraldehyde, GA). This generates covalent bonds between components of the extracellular matrix and prevents acute host immune rejection (Carpentier et al. 1969). Treatment with aldehydes has also major drawbacks concerning pericardial or valve tissue long-term durability. In fact, clinical data from long term follow up of patients receiving pericardium-made bio-prosthetic implants, have indicated severe structural valve deterioration (SVD) and calcification. SVD is primarily caused by a chronic inflammatory condition resulting from a non-complete detoxification of the fixative remnants from the xenograft tissue (Grabenwoger et al. 1996; Siddiqui et al. 2009), and/or by the failure of the fixation protocols to remove major xeno-antigens such as the 1, 3 α-Galactose (Konakci et al. 2005; Naso et al. 2012, 2013; Galili 2005; Hülsmann et al. 2012) (α-Gal). In addition, although it has been demonstrated that bioprosthetic valves are liable to undergo an *in vivo* recellularization process by recruitment of circulating cells (De Visscher et al. 2007), the clinically employed biological implants are not designed to contain living cells, making them prone to infiltration by inflammatory elements of the recipient (Rieder et al. 2005), causing chronic inflammation.

New pericardium and valve tissues decellularization strategies have been therefore proposed, based on treatment with ionic/non-ionic detergents and enzymes that remove the genetic content and xenoantigens (Mirsadraee et al. 2006, 2007). While these methods have been found to reduce immunogenicity of the pericardial tissue for xenotransplantation (Vinci et al. 2013), and to favor post-graft recellularization by host cells (Iop et al. 2014), the decellularized tissues employed to manufacture valve prostheses are still devoid of valve competent cells, which may contribute to renew the extracellular matrix and thus repair the tissue over time increasing its longevity (CardioPulse Articles 2015).

Tissue engineering methods to produce 'off the shelf' living valve substitutes have been set up. This approach consists in combining three-dimensional (3D) biodegradable scaffolds produced with different materials and manufacturing methods or decellularized tissues (e.g. porcine-derived aortic valves; pericardial membrane) with cells of various origin and level of potency (Jana et al. 2014; Kheradvar et al. 2015). Apart from advantages and drawbacks depending on the bio-compatibility of the scaffold materials and the cell types which may have a variable, and in several cases still unknown, adaptability to the high degree of mechanical stimulation acting in cardiac valves, there are two major limitations in this approach: ***(i)*** the insufficient structural stability of the tissue-engineered valve leaflets that undergo retraction and thickening thus causing regurgitation and insufficiency in the mid long term (van Vlimmeren et al. 2011), and ***(ii)*** the limited access of cells in the decellularized tissues (Dainese et al. 2012) that probably requires advanced cell seeding methods to achieve good recellularization efficiency. Finally, in this respect it is surprising that the employment of valve-derived cells, and in particular the so called 'valve interstitial cells' (VICs), has been introduced to this aim only recently in the valve tissue engineering scenario, while other cells such as bone marrow-derived mesenchymal progenitors or endothelial cells have been used for longer time.

1.2 Designing the Right Mechanosensing Environment – Anisotropic Structure and Valve Resistance to Mechanical Load

When observed at an ultrastructural level, the valve tissue has a very complex and well-organized structure (Fig. 1.1). A crucial feature is, for example, the specific arrangement of the extracellular matrix components (namely collagen, glicosaminoglycans and elastin), whose specific orientation and prevalent distribution in the small leaflet thickness and width has evolved to make the tissue very resistant to mechanical stress at valve closure during diastole, and soft and pliable to allow the blood flow through the valve at opening during systole (Breuer et al. 2004; Balguid et al. 2007, 2008; Hammer et al. 2014). In three leaflet valves (the aortic and the pulmonary) for example, it comprises three specialized layer (the *Fibrosa*, the *Spongiosa* and the *Ventricularis*; Fig. 1.1) whose distinct cellular and extracellular matrix composition ensures correct absorption of mechanical stress, confined in a thickness of around 500 μm. The presence of non-uniformly arranged collagen bundles in the *Fibrosa* (the layer exposed to the aortic outflow segment; Fig. 1.2a) is, for example, the crucial structural component determining the anisotropic mechanical behavior of the leaflets. This is adapted to ensure a maximal resistance to stress at the leaflets commissures and at the 'belly' portions, where the largest mechanical stresses are normally acting when valve closes (Fig. 1.2b). The presence of elastin bundles in the *Ventricularis* (the part of the tissue located on the ventricular side of the valve) has specifically evolved to support the recoil of the leaflets to their

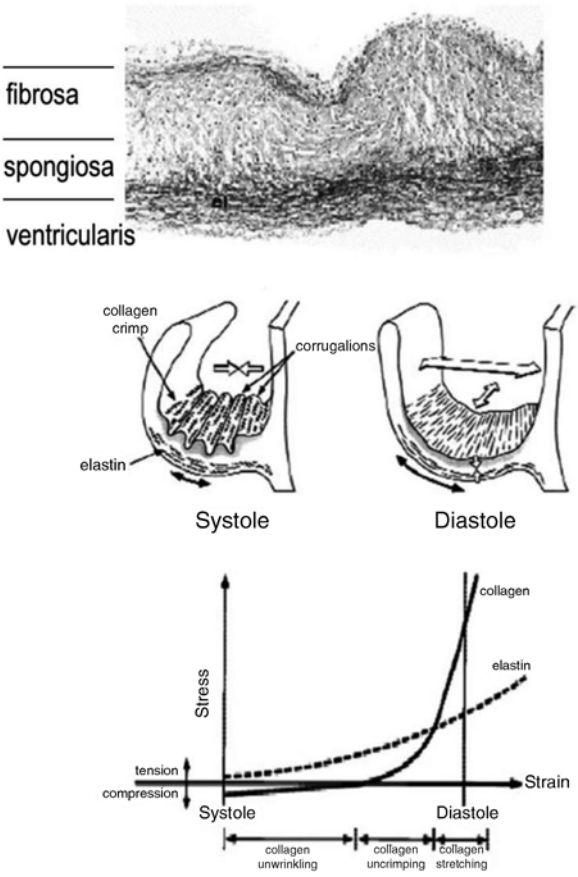

Fig. 1.1 Structure and mechanical behaviour of the aortic valve tissue throughout the cardiac cycle (Adapted from van Vlimmeren et al. 2011). The picture on *top* shows the three layered structure of the leaflet (split in *fibrosa*, *spongiosa* and *fibrosa* layers from the aortic through to the ventricular side). The drawing in the *middle* describes the modifications of the leaflet structure in the diastole/systole transition and the main changes in the strain of the collagen and elastin fibres in the *fibrosa* and *ventricularis*. The *graph* on the *bottom* shows the strain/stress relationship in the transition from systole (valve closed) to diastole (valve closed) and the main structural components in the leaflet layers that are involved (From Breuer et al. (2004)

crimped initial state after diastolic loading (Breuer et al. 2004) (Fig. 1.1). Finally, the spongiosa layer (located in the middle portion between the two other has a lower structural organization, it is mainly composed of Glicosaminoglycans (GAGs) that function as a 'cushion' absorbing mechanical solicitations caused by leaflet motion. Leaflet complexity is increased by a non-homogeneous cell composition and distribution in the valve. This consists in valve endothelial cells (VECs), which line the inflow and outflow valve surfaces, and valve interstitial cells (VICs), a plastic fibroblast/myofibroblast phenotype, that provide the necessary ECM components renewal into a tissue undergoing, in its average lifetime, three billion load/unload cycles (Sacks et al. 2009). Mechanical forces, acting especially during embryonic shaping of the heart valves, give a primary contribution to differentially align and determine different shapes of VECs on the two leaflet surfaces, and are crucial to induce differential strain-dependent maturation of the valve fibrillar matrix structure by modulating the function/phenotype of VICs in the three presumptive layers (reviewed in (MacGrogan et al. 2014)).

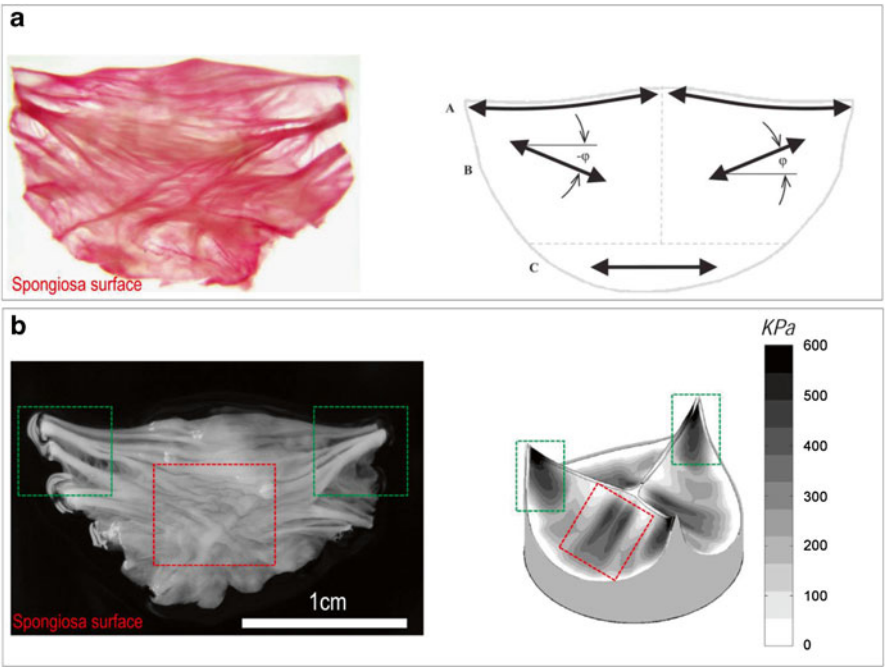

Fig. 1.2 Structure of the aortic valve *fibrosa* (Adapted from Dainese et al. 2012; Breuer et al. 2004; Balguid 2008). (**a**) Sirius Red staining of the aortic valve spongiosa reveals a high degree of anisotropy in collagen fibres deposition. The scheme on the *right* indicates the main direction of tensile strength oriented along the main fibre deposition pattern, resulting in an anisotropic loading distribution. (**b**) The image on the *left* indicates a polarized light picture of the collagen bundles in the *fibrosa. Green squares* indicate the two commissural areas and the red square shows the 'belly' portion. These areas are those where the maximal loading stress is applied at valve closure according to mathematical modelling of stress/strain patterns (From Hammer et al. 2014 and Balguid et al. (2007, 2008)

1.3 Mechanical Load, Mechanosensing and Cellular Responses

The extracellular matrix (ECM) not only provides a passive scaffold to maintain cells in a confined architecture, but it is also an active source of stimuli to the cells that are not just limited to a humoral control of tissue homeostasis. In fact, the ECM is also deputed to transduce mechanical cues that translate into geometric-, positioning- and motion-sensing information by the cells. These powerful forces are thought to be particularly active in the cardiovascular system, including heart valves, where motion is part of the physiologic functions, and positioning information is likely a crucial feedback signal orchestrating correct heart/heart valve patterning. The ability of the cells to sense the environment through mechanical activation of intracellular pathways is intrinsic in the developmental process of multicellular

organisms. For example, the recent implication of the mechanosensing-activated pathway Hippo in the pre-implantation development, has allowed to connect the old concept of cellular 'polarity' acquired by blastomeres undergoing the first embryonic divisions (Johnson and Ziomek 1981) to the events segregating the embryonic (pluripotent) cells from the extraembryonic (trophectoderm) lineages depending on cell positioning (Biggins et al. 2015; Nishioka et al. 2009). This applies also to adult-derived stem cells, e.g. mesenchymal stem cells, in which cellular lineage identity and basic features such as proliferation and differentiation are affected by discrete geometric patterning into cell colonies (Vunjak-Novakovic 2008; Nelson et al. 2005) and intracellular signalling eliciting by shape-dependent discrete cytoskeleton tensioning (Kilian et al. 2010). Finally while geometrical modelling of the environment is strictly associated to normal (stem) cell fate and functions into tissues, alterations of positioning cues can also lead, at least theoretically, to abnormalities in stem cells proliferation and telomeric size (Blagoev 2011).

The ECM composition is complex and highly adapted to regulate the tissue functions. For example, the variable content of adhesive proteins, fibrous proteins and proteoglycans is crucial to generate anchorage and modulate mechanical stimuli that are known to have potent inductive functions on tissue-resident cells. In this regard, one of the most important characteristics of the ECM is its stiffness, described by the Young's elastic modulus (E[Pa]). ECM stiffness is relevant either for passive loading to the cells, or as a stimulus regulating critical cellular activities, such as induction of cytoskeleton tensioning and the associated intracellular signalling pathway. The physiological stiffness changes significantly between different tissues, ranging from the very soft brain tissue (0.1–1 KPa), to the very stiff precalcified bone (25–40 KPa), while intermediate ranges (8–17 KPa) are typical of the skeletal muscle (Engler et al. 2006).

A simple method to investigate the stiffness effects on cell behaviour is to manufacture culture surfaces with a defined elastic modulus. These substrates can be easily generated using various technologies and materials such as the polydimethilsiloxane (Gray et al. 2003), polyethilenglicol (Khatiwala et al. 2009), or polyacrylamide (Pelham and Wang 1997). In these bi-dimensional (2D) culturing environments, whose biophysical features can be accurately detected by nanoindentation methods or atomic force microscopy (Engler et al. 2007), cell attachment is mediated by coating the surfaces with specific ECM components such as, for example, collagen (Engler et al. 2006; Quinlan and Billiar 2012), fibronectin (Peyton and Putnam 2005) or specific integrin-binding adhesion peptides (Wang et al. 2012; Gould et al. 2012). This enables the manufacturing of adhesion surfaces transducing discrete mechanical information to the cells *via* the activation of specific receptor signalling (Balaoing et al. 2015; Ramos et al. 2010). Culturing cells onto substrates of known and controlled stiffness has offered the chance to describe for the first time the correlation between the intracellular machinery regulating cell rigidity (e.g. that dependent on the small-GTP binding protein RhoA and its downstream target ROCK (Zhou et al. 2011)) and progenitor cells differentiation. When cultured on high stiffness substrates, various cell types, such as embryonic fibroblasts (Kim et al. 2012), cardiac fibroblasts (Xie et al. 2014), and mesenchymal

progenitors (Engler et al. 2006) were reported to show increased substrate contact areas and focal adhesions, and this correlated with an induction of genes involved in osteogenic commitment.

How does the matrix rigidity affect the biology of the heart valves? As shown in Fig. 1.1, the valve tissue is a composite structure in which cells are embedded into extracellular matrix layers with distinct elasticity. For example, the fibrosa layer contains an anisotropic arrangement of Collagen fibres whose mechanical compliance is higher compared with that of the amorphous-structured spongiosa, in which Glycosamino-Glycans (GAGs) are the most represented matrix components (Schoen 2008). Although a comprehensive dissection of the VICs phenotype with respect to the mechanical compliance of the three valve tissue layers has not yet been performed, the susceptibility of these cells to differentiate into pro-osteogenic cells (Chen et al. 2009), especially when cultured onto surfaces with high stiffness (Yip et al. 2009), along with their mechanical adaptability to the compliance of the surrounding environment (Wyss et al. 2012; Liu et al. 2013), makes them likely potent mechanical 'sensors' in the valve. These cells in fact, are not only able to fulfil the leaflets tissue turnover and to adapt to the leaflets mechanical complexity, but evolve toward pathologic phenotypes depending on local perturbations of the valve matrix compliance. In keeping with this hypothesis, the regions in the Aortic Valve leaflets that are most subject to mechanical load, i.e. the commissures and the 'belly' portions (regions highlighted in red and green, respectively in Fig. 1.2b), are those into which preferentially calcific lesions are first detected (Hinton and Yutzey 2011), probably as a result of stiffness-dependent calcific nodules deposition by VICs (Bouchareb et al. 2014; Bertazzo et al. 2013). The above considerations demonstrate the ability of the cells in general to 'feel' the mechanical environment and, restricted to valve biology, may have implications for tissue pathologic evolution. This suggests that VICs mechanosensing ability will have to be taken into consideration in future tissue engineering approaches aiming at reconstructing durable valve bioprostheses architecture using these cells or other mechanosensing-susceptible cells.

1.4 From Cell and Tissue Mechanics to Tissue Engineering

As well highlighted in other contributions available in the literature (e.g. reference (Kheradvar et al. 2015)), the design concept of off-the-shelf tissue engineered heart valve bioprostheses is based on the ability of *ex vivo* cultured cells (Weber et al. 2012) to colonize 3D scaffolds manufactured with various biologically compatible polymers, and induce maturation of leaflet-like tissues by *in vitro* mechanical loading, before implantation into suitable animal models (e.g. sheep). In early attempts, the adopted engineering strategy was to employ preformed three-leaflet tubular constructs manufactured with non-woven scaffolds made of biodegradable materials (e.g. Poly-Glycolic-Acid, PGA and Poly-L-Lactic-Acid, PLLA) as 3D seeding substrates for cells (Hoerstrup et al. 2002; Schmidt et al. 2010). Unfortunately, despite the initial success of cell seeding and *in vitro* maturation of the tissue constructs,

in several of these attempts, ‘compaction’ and ‘retraction’ of tissue engineered leaflets were observed. This effect altered the geometry, the compliance and, ultimately, compromised the mechanical performance of the tissue engineered valve prostheses. These effects occurred especially at long term after implantation in animals due to a non-optimized arrangement of the structural elements associated with the cells and inflammatory cells infiltration. In addition, while at least in principles, the presence of a three-dimensioned environment should confine the cells to stay into an ‘instructive’ leaflet geometry, the employment of biodegradable materials does not support a native-like structural and mechanical maturation of the engineered valve tissue, thereby causing loss of structural and mechanical coherence. Models of tissue compaction and retraction have been set to explain this limitation in TEHV design (van Vlimmeren et al. 2011, 2012; van Loosdregt et al. 2014). These investigations have clearly indicated that passive and cell-mediated forces are involved in leaflets retraction. The passive shrinkage is essentially caused by failure of the newly formed tissue to withstand cellular traction forces as the scaffold degrades; a further active retraction is then caused by cellular traction forces that compensate for the hemodynamic loading as well as by cell-mediated remodelling of the ECM components (van Loosdregt et al. 2014; van Vlimmeren et al. 2012).

While the introduction of an anisotropic design in TEHV scaffolds manufacturing may reduce the impact of the cell-mediated shrinkage (Loerakker et al. 2013), the problem of passive compaction due to the rapid reabsorption of the scaffold remains essentially unaddressed. One possibility to circumvent this problem may be in the future to invest into novel ‘hybrid’ approaches, which may take advantage of the physical properties of non-degradable materials to manufacture scaffolds with a specific anisotropic design and mechanical behaviour, and of cell-seeding/depositing techniques to cellularize the anisotropic 3D environment and achieve maturation of the tissues through mechanical stimulation. Another approach, as recently suggested (Kural and Billiar 2014; Hjortnaes et al. 2015), may be to modulate the cell-mediated tensile strength of the tissue directly in the 3D environment by employing materials with defined stiffness to reduce the propensity of VICs to evolve towards myofibroblasts/osteogenic cells and humoral signals (e.g. treatment with TGF-β), and/or to induce VICs to deposit ECM components (e.g. Collagen) with an anisotropic deposition pattern, resembling that present in the native tissue (MacGrogan et al. 2014).

Evolved scaffold fabrication criteria have been finally introduced to achieve a more complex bio-artificial leaflets design with the aim at reproducing the architecture and the mechanical behaviour of the native valve tissues. Examples of these new techniques are electrospinning (Masoumi et al. 2014a), 3D printing (Mosadegh et al. 2015) and stereolithography (Morsi 2014). These methods have been employed with different classes of biocompatible artificial materials (Morsi 2014) and, in some instances, have been combined together (Masoumi et al. 2014b) in order to generate complex scaffolds reproducing the natural layering of the valve leaflets tissue with a specific degree of structural anisotropy. These fabrication methods can be interfaced with computer-added-design (CAD) tools that make possible to

include in the fabrication process the mechanical parameters of the tissue during its motion and loading, thus empowering the manufacture of bio-artificial leaflets with a pre-determined resistance to stress (Lueders et al. 2014).

1.5 Conclusions

Compared with the early and inefficient approaches stemming from application of basic principles in materials/cells interactions, the engineering of 'off-the-shelf' valve substitutes has become a sophisticated process involving an interdisciplinary integration of various techniques and manufacturing strategies. Although advancements in material science allows the employment of various polymers with different chemical composition, different degrees of reabsorption and biophysical properties, the evolution of tools to operate tailored scaffold fabrication makes possible the manufacture of 3D environments where cells might be placed in the right mechanical environment. This will lead to the necessary improvements of the valve bioartificial leaflets manufacturing process to achieve a realistic translation.

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Chapter 2
Recent Progress in Strategies for Adenovirus Mediated Therapeutic Cell Targeting

Ottmar Herchenröder, Julia Reetz, and Brigitte M. Pützer

Abstract Increasing numbers of therapeutic genes and cellular targets are available for gene therapy. Many clinical trials using virus-derived delivery systems are devoted to combat cancer, to correct single-gene malfunctions or to regenerate tissues. To develop gene delivery vectors with high efficiency through target cell selectivity, in particular under in situ conditions, remains a major challenge. The most widely used vector systems to transduce cells are based on adenoviruses. Recent approaches to develop selective adenoviral vectors (Ad) that exclusively target cells or tissues of interest without interfering with all others have focused on the modification of the broad natural tropism of adenoviruses. A popular way of Ad targeting is attained by directing vector particles towards distinct cellular receptors. Retargeting can be accomplished by linking custom-made peptides with unique specificity and reasonable affinity to cellular surface protein moieties via genetic alteration, chemical coupling or bridging with dual-specific adaptor molecules. Ideally, target-specific vectors are incapable of entering cells via their native receptors. Such altered vectors offer new opportunities to delineate functional genomics in the native environment and should enable efficient systemic therapeutic approaches. This review provides a summary of current state-of-the-art techniques to target specifically adenovirus-derived gene delivery vector systems.

Keywords Gene therapy • Vector targeting • Vector tropism • Adaptor molecules

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2.1 Introduction

2.1.1 The Concept of Gene Therapy

Somatic gene therapy is defined as the transfer of nucleic acids into a patient's cells to achieve a lasting therapeutic effect without influencing the germline. A myriad of conditions has been tackled where a cure is envisaged by intervening at the genetic level rather than via conventional application of drugs. Thereby, influence on or correction of inherited or acquired malfunctioning genes should be lasting. The best known tools to affect a cell's genetic makeup are virus-based vectors as the vehicles of choice to intervene on the gene expression profile of cells and ideally, to correct a malfunction, to suppress the expression of an undesired cellular gene or to aim at quantitative elimination of a population of cells out of control. Still, therapeutic approaches to alter inherited or acquired genetic disorders as well as those to eliminate neoplastic cells once disseminated in the patient are still far from regular clinical application.

Effective in vivo transfer of a gene using an adenoviral vector was shown in 1991 by Ronald Crystal's group (Rosenfeld et al. 1991), and the first clinical application of a vector delivering an intact cystic fibrosis transmembrane conductance regulator gene was done in 1993 as reviewed by Crystal (2014). In his review, the author reiterates in retrospect the success and feasible future applications for adenovirus-based vectors in human gene therapy. Due to the strong immune response against vector proteins, Crystal concludes that Ads may serve best in temporary applications to build new structures, to selectively destroy cells or as vehicles to induce immunity against a transgene. The authors of this chapter put the focus on the concepts of targeted adenoviral gene transfer by ablating the native tropism and reroute Ad vectors by altering their physical composititon or by covering the native particle with moieties directed against selected cell plasma surface structures. At the time of assembling this article, cumulatively over 2200 gene therapy trials in different clinical phases were completed or are ongoing with more than half of these clinical trials being dedicated to combat cancer. Amongst those, Ads are the most widely used gene transfer vehicles (Wirth et al. 2013; Das et al. 2015).

2.1.2 The Tools for Gene Therapy

The isolation of adenoviruses dates back to 1953 (Rowe et al. 1953). The family of Adenoviridae consists of four accepted genera (Davison et al. 2003; Cupelli and Stehle 2011) with some species awaiting assignment. Adenoviruses infect numerous vertebrates including man. Nowadays, about 57 human adenoviruses have been identified and were subclassified on the basis of parameters regarding classical standard methods, and more recently, by comparison of the adenoviral genomes (Seto et al. 2011). In the genus Mastadenovirus, the human pathogenic species are

further divided into the subgroups A to G (Russel 2000; Berk 2013). The virions are built up of an icosahedral capsid that carries a single molecule of double-stranded DNA as the viral genome. The particles are studded with 12 fibers consisting of trimeric rigid structural elements linked to the icosahedron's vertices. The capsid components forming the vertices are called penton bases and the fibers are non-covalently linked to these structures. Most virus serotypes bind to their target cells via the interaction of the fiber knob, the most distal part of the fiber with their counterpart, a protein structure on the cell plasma membrane. This moiety, the coxsackie adenovirus receptor (CAR) represents the main high-affinity receptor for many adenoviruses. CAR is a 46-kDa transmembrane protein in the superfamily of immunoglobulins (Bergelson et al. 1997; Roelvink et al. 1998; Bewley et al. 1999). Internalization of the virus depends on the subsequent secondary interaction of an Arginine-Glycine-Aspartate (RGD) protein structure on the penton base with αVβ3 or αVβ5 integrins also present on the cell plasma surface (Wickham et al. 1993). CAR is highly abundant in many tissues and therefore, adenoviruses display a wide tropism and infect a broad spectrum of cells (Wickham 2000; Arnberg 2009; Chailertvanitkul and Pouton 2010).

All of these characteristics drew an early focus on this virus family as masterpieces for gene therapy approaches. Unlike other viral vector systems, Ad virions have a high packaging capacity and easily replicate to high titers. Moreover, Ad-derived vectors maintain high stability in vivo and transduce both, dividing and non-dividing cells (Douglas 2004). Once internalized into the cell, the genome predominantly persists as episomal DNA with an extremely low frequency of integration into the host genome (Rauschhuber et al. 2012), and thereby, insertional mutagenesis does not occur. Considering this safety aspect, adenovirus based vectors are particularly attractive for gene therapy applications, where temporary gene expression is desired or preferred over permanent genetic modifications. Due to their apathogenicity and non-oncogenic properties, the most commonly utilized Ad vectors for gene therapy are derivatives from adenovirus Serotype 5 (Ad5) in the subgroup C of human adenoviruses (Arnberg 2009; Armendariz-Borunda et al. 2011). What ultimately converts the replicating agent into a one way delivery system is the purposeful removal of a part of the viral genome (Haj-Ahmad and Graham 1986).

Tissue tropism of adenoviral vectors is greatly influenced by the viral serotype and receptor presence and density, depending on a cell type's provenance (Wickham et al. 1993; Hashimoto et al. 1997; Takayama et al. 1998; Havenga et al. 2002; Arnberg 2009). Ad5 are best explored and most widely used in preclinical studies as well as clinical trials. Ad5 particles bind exclusively to CAR. However, in the case of Ads made from Serotype 41, a subgroup F member, only one of its two distinct types of fibers can recognize CAR (Russel 2000; Zhang and Bergelson 2005). Some serotypes enter host cells via other receptors such as CD46, desmoglein 2, CD80 or CD86 or the sialic acid moiety (reviewed by Sharma et al. 2009). Altogether, Ad5 vectors are efficient vehicles for delivering foreign genes into target cells in vitro and in vivo (Haj-Ahmad and Graham 1986; Pützer et al. 1997). Based on the favourable attributes, Ad5 remains the vector of choice in human gene therapy trials due to their proven safety profile (Hedley et al. 2006; Das et al. 2015).

Whenever the utilization of adenoviral vectors as targeted gene delivery systems is desired, the Ad5 vector's broad tropism for a wide range of cells and tissues is a challenging obstacle (Coughlan et al. 2010). If a gene transfer system is required that exclusively alters a single cellular compartment or a particular kind of tissue that spares all other cells and tissues from transduction, alteration of vector particles becomes essential. Another major obstacle after systemic adenoviral vector administration is that 80 % of circulating particles are sequestered in the liver after an interaction with coagulation factors (Shayakhmetov et al. 2005; Stone et al. 2007), and thereby, most particles may not reach the tissue to be addressed. A number of researchers demonstrated that after the virus is recognized by the coagulation system, the immune system is activated, and in turn, an acute inflammatory response is initiated (Reynolds et al. 2001; Shayakhmetov et al. 2004; Khare et al. 2011; Doronin et al. 2012; Xu et al. 2013). In addition, regarding Ad transport in the bloodstream, Duffy and colleagues (2013) identified a number of small molecules capable of efficiently blocking the intracellular virus transport independently of factor X-associated inactivation. Considering these hurdles, a selective gene transfer by wild-type adenoviral vectors imposes an increased risk of toxicity due to vector dissemination to non-targeted cells, even if the particles are administered close to or directly into the tissue of interest. Other undesired side effects of systemic virus administration are virus-associated immunogenic toxicity, thrombocytopenia, intense periportal polymorphonuclear lymphocyte infiltration and elevated liver enzyme secretion (Morral et al. 2002; Thacker et al. 2009a; Coughlan et al. 2012).

The reverse obstacle is the question of how to reach cell types refractory to adenoviral infection, due to their lack of or insufficient CAR expression. Such cells include, for instance, many cancer cells, as well as hematopoietic and neural stem cells (McConnell and Imperiale 2004; Schmidt et al. 2005). To achieve gene transfer into those cell types and to ensure efficient integrin receptor-mediated virus uptake, extremely high vector doses are required. High vector doses in turn increase inadvertent side effects, like viral sequestering in Kupffer cells in the liver (Haisma et al. 2009), and once vectors surpass the latter's binding capacity, hepatocytes will absorb the remaining vector particles.

The restrictions outlined above can be overcome by strategies to modify the vector's cellular tropism, as reviewed by Beatty and Curiel (2012). Redirecting vectors towards cells of interest can also enhance the therapeutic potential with increased safety by reduction of immune responses, since simultaneous re- and de-targeting allows lower vector doses to be administered systemically (Schmidt et al. 2007; Dorer und Nettelbeck 2009; Haisma et al. 2010; Schmidt et al. 2011). In this review, we present and discuss three different methods to alter the natural Ad vector tropism. We describe genetic integration of peptide sequences into the fiber, peptide conjugation via chemical modification using polyethylene glycol (PEG) and bridging the vector and cell of choice with bispecific adaptor molecules. The advantages and benefits, as well as restrictions and limitations of these technological approaches are reported and debated. The initial considerations towards targeting, however,

relate to the identification of suitable moieties on the plasma membrane of the cells or the tissue to be addressed that fulfil the following characteristics: singularity, abundance, and affinity.

2.2 Screening Methods for Cell-Specific Ligands

The ultimate gene "taxi" for systemic gene therapy purposes should exclusively recognize the cells to be treated and leave all others unaffected. Directed gene delivery can be achieved by addressing selective moieties on the cells of interest. Specifically selected peptides possess appropriate properties to serve as targeting agents and are valid alternatives to antibody-based targeting approaches, since unique cellular receptors are often unknown.

The simplest way to design a specific binding peptide for a receptor is to analyse structural data and the expression profile of cells to identify candidate peptides. Often however, structural data are not available or incomplete. To solve this, the phage display method is a frequently used technique to determine specific binding peptides. We and others have used the phage display technology to screen for and to identify tissue- or cell-specific ligands in cell culture systems and animal models (Böckmann et al. 2005a, b; Vives et al. 2008, Reetz et al. 2013). Already in 1990, researchers constructed an epitope library that yielded a mixture of filamentous phage clones with each one displaying a single peptide sequence on the virion's surface (Scott and Smith 1990). After the interaction of the phage with the specific binding partner, the expansion of the phage comprehends several rounds of infection followed by selection. The display of polypeptide repertoires on the surface of phages, together with the efficient enrichment and amplification of the desired binding specificities was shown to be a valuable route towards isolation of unique peptides suitable to serve as vehicles for targeting applications (Arap et al. 1998; Nicklin et al. 2000; Essler and Ruoslahti 2002; Dias-Neto et al. 2009; Chen et al. 2010; Kügler et al. 2013). Taken together, the phage display technique identifies peptides in a range from 7 to 12 amino acids (Vives et al. 2008).

Phage display was successfully employed to acquire peptides that specifically recognize human embryonic progenitor cells (Bignone et al. 2013) and bind normal or aberrant tissues, like vascular endothelium (Pasqualini and Ruoslahti 1996, 2000; White et al. 2001), lymphatic vessels (Laakkonen et al. 2002), kidney tubules (Odermatt et al. 2001), hepatocytes (Piccolo et al. 2014), and several others (Barry et al. 1996; Ravera et al. 1998; Ivanenkov et al. 1999; Mazzucchelli et al. 1999; Cheung et al. 2013; Sclavons et al. 2013). Furthermore, the lack of gene transfer systems that are potent in selectively targeting tumor tissue prompted the search for cancer-specific peptide molecules for yet unknown tumor-associated receptors (Araki et al. 2010; Deutscher et al. 2010; Ahmadvand et al. 2011; Nishimoto et al. 2012). Many novel peptides homing to angiogenic vessels showed cross-affinity with several tumor types (Liu and Wu 2008). In this regard, we conducted biopanning on

human medullary thyroid carcinoma (MTC) cells in vitro and transplanted tumor xenografts in vivo (Böckmann et al. 2005a). MTC, which is caused by dominant activating mutations in the RET proto-oncogene encoding a transmembrane tyrosine kinase receptor, is characterized by aggressive growth and early metastasis and therefore, provides a perfect model for targeting disseminated cancer cells (Drosten and Pützer 2006). The selected phages bound with highest specificity to and were internalized by these tumor cells in culture as well as after systemic injection into nude mice (Böckmann et al. 2005a). The same 7-mer cyclic phage peptide library was injected into the tail vein of RET oncogene transgenic mice carrying bilateral orthotopic tumors in their thyroid glands (Böckmann et al. 2005b). This ligand that also binds efficiently to human MTC cells was covalently linked to adenoviral capsids carrying a RET oncogene inhibitor as therapeutic gene. Systemic delivery of this peptide-tagged adenovirus led to a substantial growth reduction of orthotopic and disseminated xenograft tumors, while the interaction with other organs, such as the liver, was abolished (Schmidt et al. 2011). This precedent opens a promising new road towards using peptide-mediated adenoviral gene transfer to achieve an efficient and selective therapeutic response also against circulating and metastasis initiating tumor cells detected in an advanced stage of metastatic disease that possess features of cancer stem cells. Beyond that, other researchers took the first step in developing a molecular map of the human vasculature by screening a peptide library in patients (Arap et al. 2002; Chang et al. 2009; Seung-Min et al. 2009). Rangel and co-workers developed a novel technique that enables receptor-independent phage particle entry into mammalian cells. Phage particles provide a unique discovery platform for combinatorial intracellular targeting of organelle ligands along with their corresponding receptors and for fingerprinting functional protein domains in living cells (Rangel et al. 2012, 2013).

An alternative approach that aims at detecting molecules with high affinity, adequate specificity and suitable pharmacokinetic properties for in vivo applications is represented by single-stranded nucleic acid ligands, termed aptamers. Aptamers are isolated by the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technology. Applying this technology against whole-living cells in culture or in vivo allows direct selection of aptamers even against rare antigens without prior purification of membrane-bound targets, access to membrane proteins in their native conformation and identification of targets related to a specific phenotype. Their thermal stability, low cost, unlimited applications and high binding affinity to disease-associated proteins or non-protein targets (Song et al. 2012; Wang et al. 2014; Mahlknecht et al. 2015) make them attractive, even in clinical trials for the treatment of distinct medical conditions, as reviewed extensively elsewhere (Sundaram et al. 2013; Tan et al. 2013; Zimbres et al. 2013). The potential of aptamers as tools for tumor targeting gene delivery systems with high transduction efficiency was summarized by others (Zhu et al. 2012; Hu and Zhang 2013). In this perspective, an innovative step towards targeted therapies would certainly be a combination of both technologies, cell-specific aptamers and adenoviral vectors.

2.3 Methods to Specifically Target Adenovirus-Derived Vectors

2.3.1 Genetic Fiber Engineering

To increase the selectivity of adenovirus for target tissues, novel approaches in Ad vector design exploit the concept of tissue-specific expression of therapeutic transgenes or virus replication. With the fiber being the major determinant of adenovirus tropism, the development of genetically targeted vectors has, consequently, focused on this distant protruding entity of the particle. The fiber is a rigid homotrimeric protein structure characterized by a domain organization with an N-terminal tail domain anchoring it in the Ad capsid. A C-terminal globular domain, termed the knob, mediates binding to CAR and a central shaft domain extends the knob away from the particle. Any manipulations of the fiber knob per se significantly reduce the transduction efficiency of CAR-positive cells by Ad vectors (Wickham et al. 1997; Krasnykh et al. 1998; Einfeld et al. 2001). Different strategies of adenovirus fiber modification have been employed, like genetic replacement of the fiber or ligand incorporation into the fiber knob. In this regard, the development of a fiber phage display system (Nishimoto et al. 2012) or a fiber-shuttle library for the adenoviral knobs (Wu et al. 2010) provide tools to alter Ad vector binding specificity.

Several studies narrowed down the insertion positions for targeting peptides within the fiber knob to two locations where the vector system as such tolerates the genetic alterations without structural impairment. The sites of choice for targeting ligand incorporation are the fiber knob's HI loop, which connects the ß-strands H and I, and the C-terminus of the protein (Dmitriev et al. 1998; Krasnykh et al. 1998; Mizuguchi et al. 2001; Koizumi et al. 2003; Glasgow et al. 2004; Nettelbeck et al. 2004; Coughlan 2009; Wang et al. 2011). These findings indicated that ligands whose sizes exceed 25 to 30 amino acid residues cannot be configured into the carboxy-terminus of the fiber, as they destabilize the fiber structure (Wickham et al. 1997) and thus, limit the range of potential ligand candidates to short peptides. The structural properties of the HI loop of the Ad fiber, however, favor the insertion of larger ligands and expand the size of potential targeting moieties. Meanwhile Behr and colleagues (2014) elaborated on insertion of a peptide ligand to target the receptor tyrosine kinase EphA2 by introducing a previously selected peptide into different positions of a chimeric fiber derived from Ad5 and Ad 41 with striking permissiveness concerning the insertion site into several loops of the fibers.

When testing the resilience of fiber modification, Belousova and co-workers (2002) incrementally increased the size of the peptides integrated in the HI loop and generated Ad vectors with fiber inserts ranging from 13 to 83 amino acid residues. The authors concluded that the incorporation of heterologous sequences in the examined size ranges was essentially tolerated without a negative impact or compromising the production yield or transductional properties of the vectors. HI-loop incorporation of rather short 7- and 9-mer peptides was performed to transduce CAR-deficient primary tumor cells, such as ovarian cancer cells, vascular endothelia,

vascular smooth muscle cells, and brain microcapillary endothelia in culture (Dmitriev et al. 1998; Nicklin et al. 2000, 2003; Xia et al. 2000; Work et al. 2004). The last step of Ad entry into target cells depends on the interaction between RGD motif at the penton base protein and the host cell integrins (Cao et al. 2012). Ad vectors containing this RGD peptide in the HI loop of the fiber showed higher yields of gene transfer than vectors containing the identical peptide attached at the fiber's C-terminus, due to the easy access to the receptor (Kurachi et al. 2007; Tanaka et al. 2007; Schmidt et al. 2011). Several researchers efficiently transduced different types of tumor cells by inserting this RGD motif into the HI loop of the Ad fiber in vitro (Terao et al. 2009) and in vivo (Bayo-Puxan et al. 2009; Katayama et al. 2011). Rojas et al. (2012) improved systemic antitumor therapy with oncolytic adenoviruses by replacing the fiber shaft heparin sulfate glycosaminoglycan-binding domain with RGD in order to achieve simultaneously liver de- and tumor re-targeting.

Each fiber knob monomer forms an eight-stranded antiparallel ß-sandwich structure. The ß-strands are connected with turns and loops (Bewley et al. 1999). To further reduce the transduction efficiency of Ad vectors to CAR-positive cells, elaborate mutation studies on the AB, DE or FG loop of the fiber knob have been reported (Roelvink et al. 1999; Jakubczak et al. 2001; Leissner et al. 2001). In order to completely ablating a vector particle's tropism from CAR binding and eventually entering the cell, genetic fiber modification is not sufficient. Rather, the secondary minor interaction of the RGD motif at the penton base with the αv-integrin receptor should be depleted as well (Mizuguchi et al. 2002), to fully ablate Ad vectors from the native binding moieties. Whereas the dual mutation markedly reduces the retention of the vector in the liver (Einfeld et al. 2001; Koizumi et al. 2003), single mutations in the fiber knob or penton base did not alter the biodistribution of adenoviral vectors injected into mice (Alemany and Curiel 2001; Leissner et al. 2001; Nakamaura 2003; Smith et al. 2003a, b). In order to use Ads in cancer gene therapy, gene transduction to tumor cells is limited by the weak expression of CAR on these cells, as reviewed by (Tanaka et al. 2007). Magnusson and co-workers (2007) efficiently transduced human ovarian and breast cancer cell lines with a vector that carried a tandem repeat of the human epidermal growth factor receptor 2 (HER2/neu) reactive affibody molecule in the HI loop of a CAR ablated fiber knob. Later, the group generated a vector with dual specificity by incorporating the HER2/neu-binding (ZH) and Taq polymerase-binding (ZT) sequences at different positions within the HI-loop. Receptor-binding studies revealed that ZT in the first position and ZH in the second position bound to both receptors, whereas the reverse order of both motifs was devoid of binding to HER2/neu (Myhre et al. 2009). Subsequently, these researchers designed a vector to transduce efficiently HER2/neu-presenting cell lines, by altering the RGD motif to EGD (Glu-Gly-Asp) and substituting the KKTK motif in the third shaft repeat to RKSK (Arg-Lys-Ser-Lys). This new vector at last gained the ability to efficiently infect prostate cancer cells in vitro (Magnusson et al. 2012).

Genetic modification also covers the replacement of the entire fiber or just the knob domain with that derived from other adenovirus serotypes (Wu et al. 2002).

Belousova et al. (2003) targeted an Ad vector with bacteriophage T4 fibritin to the CD40 receptor. The tropism was modified by incorporating into the virion capsid a recombinant protein comprising structural domains of the Ad Serotype 5 fiber, phage T4 fibritin, and the human CD40 ligand. The authors achieved specific gene delivery in monocyte-derived dendritic cells in vivo. In a pilot vaccination study, Thacker and colleagues (2009b) successfully targeted these cells in a canine model by integrating the CD40 ligand into the fiber knob. The same group reported later that Ad vectors bound to the CD40 ligand failed to infect integrin-deficient canine lymphoma cells. This study demonstrates that the lack of virus internalization signals can impair targeting approaches (O'Neill et al. 2011).

Yu and co-workers (2011) modified the Ad5 hexon protein by inserting the protein transduction domain from the HIV-1 Tat protein. The resulting viral vector showed significantly higher transduction efficiency on many tumor cells compared to the parental vector. In the next step, this group increased the infection efficiency of human primary cell types even further after swapping wild-type Ad5 fiber against a Serotype 35 fiber specific for the CD46 receptor, a surface marker often upregulated in malignant tumors. This surface modified Ad vector was developed to transduce cells that are otherwise difficult to transduce in basic, pre-clinical, and clinical research (Yu et al. 2013). Another strategy to reroute adenoviral vectors from healthy towards cancer tissue is the utilization of recombinant adenovirus. The Lieber group constructed a capsid-modified adenovirus that expresses the TNF-related apoptosis-inducing ligand (TRAIL) and specifically replicates in tumor cells (Sova et al. 2004). Their Ad capsid contains the Serotype 35-derived short-shafted fibers, which recognize the CD46 receptor. In combination with TRAIL, expression of this oncolytic vector induces apoptosis in tumor cell lines derived from human colorectal, prostate, lung, and liver cancer. Both, the cell culture and xenograft tumor models tested in these experiments showed efficient intratumoral spread of the virus.

Yet others designed Ad vectors presenting the short fibers of Ad41 as a ligand insertion tool, achieving higher infection efficiency when compared to viruses presenting the same ligand cloned into another part of the fiber (Hesse et al. 2007). Even an enhanced transduction efficiency of recombinant adenovirus Serotype 5 vectors with Serotype 35 fibers was observed by Matsui and co-workers (2011). Using a feasible in vitro ligation, the group incorporated two copies of the RGD peptide in two different loops of the fiber 35 knob and observed high infection efficiencies in CD46-positive cells.

Overall and despite these positive results, genetic modifications on the native Ad5 fiber knob remain a laborious technical challenge and its benefit is hard to calculate. The repertoire of incorporable ligands to yield functional retargeted vectors for gene therapy is restricted to a small number of peptides that do not impair correct folding and assembly of the fiber trimer (Magnusson et al. 2002). A general limitation of this approach is the necessity to tediously re-engineer a given Ad vector for every further target cell. On the other hand, if common motifs were found for instance on the surface of cancer cells, corresponding metastases, and/or circulating cancer stem cells, the workload and more fine-tuning on fiber manipulations may pay off. With oncolytic viruses, the agents engineered from wild-type replicating

pathogens and designed to specifically deplete neoplastic cells, both, in solid tumors and straying through the body, chimeric fibers gain further attention. Oncolytic viruses are by definition restricted to replicate only in neoplastic cells and supposed to deliver a payload that attracts the immune system or restores apoptotic pathways (Dias et al. 2012; Schipper et al. 2014) and many ongoing clinical trials rely on the power of this concept (Pol et al. 2014; Jiang et al. 2015). To target pancreatic cancer, Nishimoto and co-workers (2009) screened a peptide-display adenovirus library. They came up with a selected peptide incorporated in the fiber of a virus ablated for CAR interaction. The technique based on a previous paper of the same group showing the validity of incorporating random sections of genomic DNA and screening of the resulting viruses for specifity for a desired cell type (Miura et al. 2007). The virus yielded potent cancer cell killing than the parental virus only deficient for CAR binding.

More recent setups towards fiber modification came for instance from the Curiel group. In a think out of the box approach, the researchers utilized antibodies from a member or the camelid family, the alpaca. Immunoglobulin of these animals consists of heavy chains only. The group immunized the animals with the human carcinoembryonic antigen and, in brief, replaced the adenovirus knob and part of the shaft with protein fragments directed against the antigen which transduced target cells that carried the original epitope (Kaliberov et al. 2014b). Jose and colleagues (2014) made a tumor selective adenovirus altering the fiber in a way that it gets activated by cancer-specific proteases. This approach can be fruitful for both, vector design and the development of virotherapeutic agents.

2.3.2 Alteration of Vector Tropism by PEGylation

Targeting an adenoviral vector can be achieved without altering the fiber by genetic modification. One option to ablate and redirect an adenoviral vector's tropism is to coat the otherwise native particle via covalently attached small molecules. This can be achieved by means of bispecific non-toxic spacer molecules. Polyethylene glycol (PEG) is a linear hydrophilic polymer and often used in pharmaceutic formulations involving proteins and peptides to prolong their blood persistence (Mizuguchi and Hayakawa 2004). In addition, PEG is, therefore, considered as a bridging entity between the capsid and peptide of choice for a given cell plasma membrane target. Optimized transduction by targeted Ad vectors can be accomplished by linking cell-specific peptides, antibodies or antigens to the particle's surface by a chemical process called PEGylation (Kreppel and Kochanek 2008). PEG forms a covalent bridge between the proteins of the virion's surface and the targeting molecule of choice, resulting in a vector coated all over with the desired ligand. This approach of redirecting viral vectors does not require genetic modification, including the efforts to ablate the native tropism. Additional benefits of PEGylated vectors are reduced immunogenicity (Elkon et al. 1997; Zaiss et al. 2002; Croyle et al. 2004; Mok et al. 2005), fewer hepatotoxic side effects (Gao et al. 2007), less cytokine secretion and prolongation of the vector plasma half-life (Alemany et al. 2000).

Such PEG-driven Ad vector modifications have been adapted in a number of targeting approaches (O'Riordan et al. 1999; Alemany et al. 2000; Croyle et al. 2002; Lee et al. 2005; Hofherr et al. 2008; Eto et al. 2010; Wonagan and Croyle 2010). The success of such approaches might depend on the ligand size. Romanczuk and co-workers (1999) were the first to link biologically selected peptides to Ads surface via PEGylation. For instance, coupling of a short RGD motif to the tip of PEG has shown both, high in vitro transduction efficiency (Lanciotti et al. 2003; Eto et al. 2004, 2005¸ Ogawara et al. 2004) and overall improvement of systemic gene delivery (Xiong et al. 2006; Gao et al. 2007). To target ovary cancer cells, the full-length fibroblast growth factor 2 (FGF2) was linked to an Ad vector by PEGylation. This vector mediated increased transgene expression in tumor tissue and reduced localization of adenovirus to non-target cells when compared to the native vector (Lanciotti et al. 2003). To silence the proinflammatory activation status of endothelial cells, Kuldo and colleagues (2013) demonstrated the potential of an E-selectin targeted adenoviral vector to deliver a therapeutic transgene into microvascular endothelial cells in inflammation and downregulate the endothelial adhesion molecule. Besides ablating the native tropism and to redirect a vector to another cellular receptor, the PEGylation treatment prolongs the circulation time and decreases the formation of neutralizing antibodies as shown by Kim and colleagues (2011). A PEGylated Ad that recognizes Her2/neu receptor-positive cancer cells showed longer circulation times than the unmodified control and decreased the level of neutralizing antibodies. These observations raise positive expectations for future therapeutic applications of PEGylated vectors against late-stage cancer diseases. Exploring the suitability of PEGylated Ad vectors to address metastatic tumors, a dual cancer-specific strategy was described using this technology for transductional targeting with transgene expression under control of the telomere reverse transcriptase promoter for transcriptional targeting (Yao et al. 2009).

With regard to the conclusion that the molecular weight of PEG and the PEG modification ratio significantly affects the characteristics of conjugates (Kaneda et al. 2004), Eto and colleagues (2010) optimized adenovirus PEGylation in a way that after systemic administration of PEGylated adenoviral vector expressing tumor necrosis factor-alpha, an antibody reduction against Ad and an increased therapeutic response was observed against metastatic cancer. In a rather elegant experimental setup, Yao and co-workers demonstrated that the CGKRK (Cys-Gly-Lys-Arg-Lys) peptide conjugated to Ad with PEG was highly selective and yielded good gene expression in tumors and tumor vasculatures after systemic administration (Yao et al. 2011, 2012). Their results indicate an important aspect to consider if working with Ads coated with PEG. The appropriate ratio between PEG and targeting ligand concentration is crucial to achieve specific tissue transduction. As described above, the latest success in the treatment of disseminated tumors in mice was made by injecting a low dose with 10^8 plaque forming units per animal of Ad vector encoding RET oncogene inhibitor coated with MTC-specific 7-mer peptide via PEG into the tail vein, which led to the regression of multiple orthotopic and xenograft tumors in mouse models (Schmidt et al. 2011). The same Ad-PEGylation approach using a short artificial peptide selected by phage display, which in this case, specifically

binds neural stem cells isolated from the hippocampus of adult mice, was highly effective after injecting the vector into the brain (Schmidt et al. 2007). Such tools could eventually serve to exclusively manipulate neural stem cells either by direct injection in the brain or systemic vector application with the potential as a delivery system for therapeutic genes to treat various disorders of the central nervous system.

Another chemical Ad modification using diblock copolypeptides as an alternative for PEG was first described by Jiang and co-workers (2013). Copolypeptides are well-defined polypeptide sequences (Deming 1997) which provide non-covalent Ad vector modification resulting in efficiently altering Ad tropism with an obvious potential to target cancer metastases. PEGylation has also been used to link cell-penetrating peptides to adenoviral particles to overcome obstacles with the CAR ablation (Nigatu et al. 2015). Whether this treatment will have advantages when targeting the particles to specific cells remains to be seen.

2.3.3 Bifunctional Non-Covalently Linked Adaptor Molecules

Another way for re-directing and widening Ad vector tropism is the application of bifunctional adaptor molecules or other bispecific protein fragments often derived from antibodies. Such adaptors recognize with one binding site the vector and the other the desired structure on the cell membrane. Bispecific antibodies are usually composed of an anti-fiber binding portion and a component specific for a cell-specific receptor or secondary antibody conjugated with a peptide moiety against a selected cell surface antigen. Since CAR does not play any role in virus internalization, the Ad fiber knob's CAR binding domain accessibility is dispensable and therefore, the candidate of choice to link heterologous binding sites, for instance a bispecific adaptor molecule. A fully studded Ad vector particle with a bridging molecule prevents any interaction with CAR and thus, ablates Ad's native tropism (Curiel 1999; Everts and Curiel 2004; Glasgow et al. 2006).

In an initial demonstration of CAR-independent targeting, a conjugate consisting of folate and a fragment derived from an antibody directed against the fiber was used as a recombinant protein to bind the Ad fiber as well as the target, the folate receptor, which is widely expressed on the surface of numerous malignant cells (Douglas et al. 1999). In a similar strategy, a conjugated FGF was used to target ovarian carcinoma cells (Rancourt et al. 1998). The approach reached a clinical trial, where FGF2-conjugated Ad vector expressing human herpes simplex virus thymidine kinase was applied in patients (Bauerschmitz et al. 2002). Reynolds and colleagues (2000) succeeded in targeting pulmonary endothelial cells in vivo by i.v. injection of Ad vectors bearing fibers studded with a bispecific antibody directed against the Ad fiber knob and the angiotensin-converting enzyme.

In light of the development of new therapeutic strategies for diseases in which angiogenesis plays an important role and considering that physiological barriers for high molecular weight components prevent the transduction of the majority of

tumor cells, vascular targeting became a worthwhile approach in cancer gene therapy (Griffioen and Molema 2000). Targeting of adenovirus to endothelial cells by a bispecific fusion protein directed against the human endoglin CD105 receptor for antivascular cancer gene therapy was published by Nettelbeck and coworkers (2001). In 2004, the same group designed a single-chain adaptor molecule that binds the fiber protein and the high molecular weight melanoma-associated antigen. This antigen is widely and specifically expressed on the surface of melanoma cells and its expression is associated with tumor development and progression (Nettelbeck et al. 2004). Other bispecific constructs directing Ad fibers to cells were developed for endothelial receptors (Haisma et al. 2010), the epidermal growth factor receptor (Haisma et al. 2000; van Beusechem et al. 2000), and the lymphocyte antigen 6 complex (van Zeeburg et al. 2010). An elegant approach uses a truncated soluble form of CAR as the virus attachment site fused to human epidermal growth factor (EGF) to direct a vector against cancer cells that express the EGF receptor (Dmitriev et al. 2000; Kashentseva et al. 2002; Hemminki et al. 2002). In addition, a number of authors described the adaptor-based strategy to target CAR-deficient dendritic cells as a therapeutic vaccination against cancer or infectious diseases (Tillmann et al. 2000; Pereboev et al. 2004; Kim et al. 2010; Echeverria et al. 2011; Hangalapura et al. 2012; Williams et al. 2012). Figure 2.1 provides a schematic representation of all three strategies used to alter virus tropism described above.

Watkins and colleagues (1997) utilized a construct that encodes a fusion protein derived from a single-chain neutralizing anti-adenovirus fiber antibody, designated S11, fused to a specific peptide ligand directed against cellular receptors, termed the

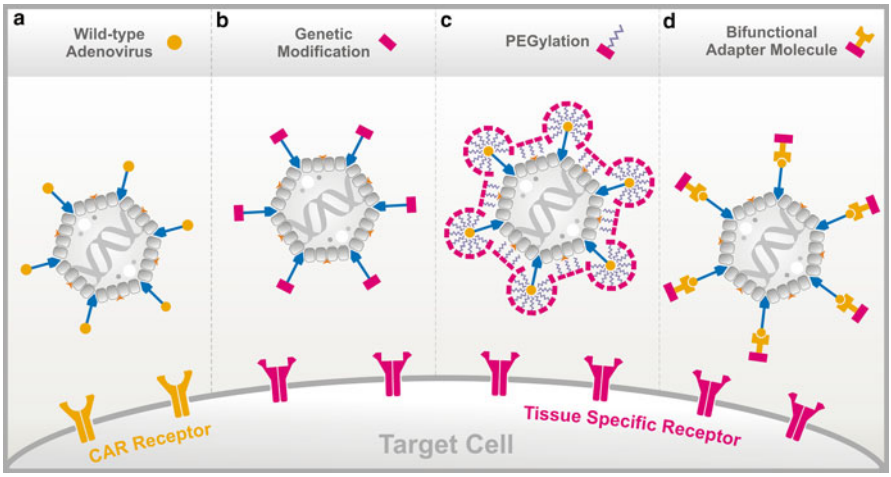

Fig. 2.1 Methods to alter adenovirus tropism. Wild-type adenovirus enters target cells after binding the coxsackie-adenovirus receptor (CAR), an entity present on a wide number of cell types (**a**). Ablation of CAR binding and re-directing adenovirus-derived vectors towards the cells of choice by means of specific peptides can be achieved by genetically integrating the peptide into the fiber knob (**b**), chemically coating the vector particle with bi-specific polyethylene glycol (**c**) or through bridging by means of a bifunctional adapter molecule (**d**)

bispecific adaptor molecule. Coating virus with this adaptor molecule ablates CAR binding and directs the viral particle to the desired cellular receptor. S11 can be produced in eukaryotic, as well as prokaryotic cells. By means of its 6-His-tag, purification and concentration of the fusion protein can be easily performed by nickel-affinity chromatography. This procedure ensures the high yield of pure protein without the loss of activity.

Based on the S11 strategy, we intended to specifically transduce in vitro and in vivo activated hepatic stellate cells (HSCs), whose number is increased in fibrotic livers (Reetz et al. 2013). Therefore, we picked a peptide derived from nerve growth factor (NGFp) with specific affinity for the p75 neurotrophin receptor (p75NTR) present on activated HSCs. Coating the GFP-expressing Ad vector with NGFp was done either via chemical conjugation using bifunctional PEG or, alternatively, by molecular bridging with an S11-based fusion protein specific for viral fiber knob and p75NTR (S11-NGFp; Fig. 2.2). After systemic administration of the targeted viral particles, we observed that Ad.GFP-S11-NGFp transduced activated HSCs better than Ad.GFP-PEG-NGFp. The latter's low transduction potential could be explained either by an improper ratio between PEG and targeting ligand concentration that prevented successful and specific tissue transduction or due to the ablation of the viral internalization signals such as the RGD motif by the chemical procedure. These experiments contributed to the development of a targeted gene transfer system to specifically deliver antifibrotic compounds into activated HSCs by systemically applied adenoviral vectors modified by the NGFp ligand. In our study, we demonstrate that adenoviral-mediated targeting of HSCs via p75NTR, concurrently avoiding its binding to hepatocytes, provides a potentially feasible and effective strategy for therapeutic gene delivery to activated HSCs in the liver in vivo and the technique may be useful to support approaches to regenerate liver tissue (Best et al. 2015; Salazar-Montez et al. 2015). Beyond, Haisma and co-workers (2010) observed a selective targeting of Ad5 to the endothelial receptors in vitro and obtained viral transgene expression only in tumors infected with adenobody retargeted adenovirus from mice bearing subcutaneously colon carcinoma cell derived tumors.

Most recently, we utilized the S11-based Ad targeting method to transduce exclusively neural stem cells in the subventricular zone of adult mice (Reetz et al. 2015). Like in our previous in vivo study on HSC transduction, a relevant peptide to be fused to S11 was beforehand detected with the phage-display and biopanning technique (Schmidt et al. 2007). For easy production of the fusion protein, we established by means of lentiviral transduction, a eukaryotic cell line that permanently secretes the bivalent adaptor. Thinking further, bivalent vector targeting approaches may also allow the creation of so-called mosaic modified vectors for instance for patients suffering from metastatic cancer in analogy to work by Pereboeva and colleagues (2004, 2007). The researchers targeted EGFR with a metabolically biotinylated fiber-mosaic adenovirus and demonstrated enhanced binding of vectors with heterologous fiber trimming. Utilizing heterologous fibers, a recent pancreatic cancer therapy approach improved the effect of conditionally replicating adenovirus-

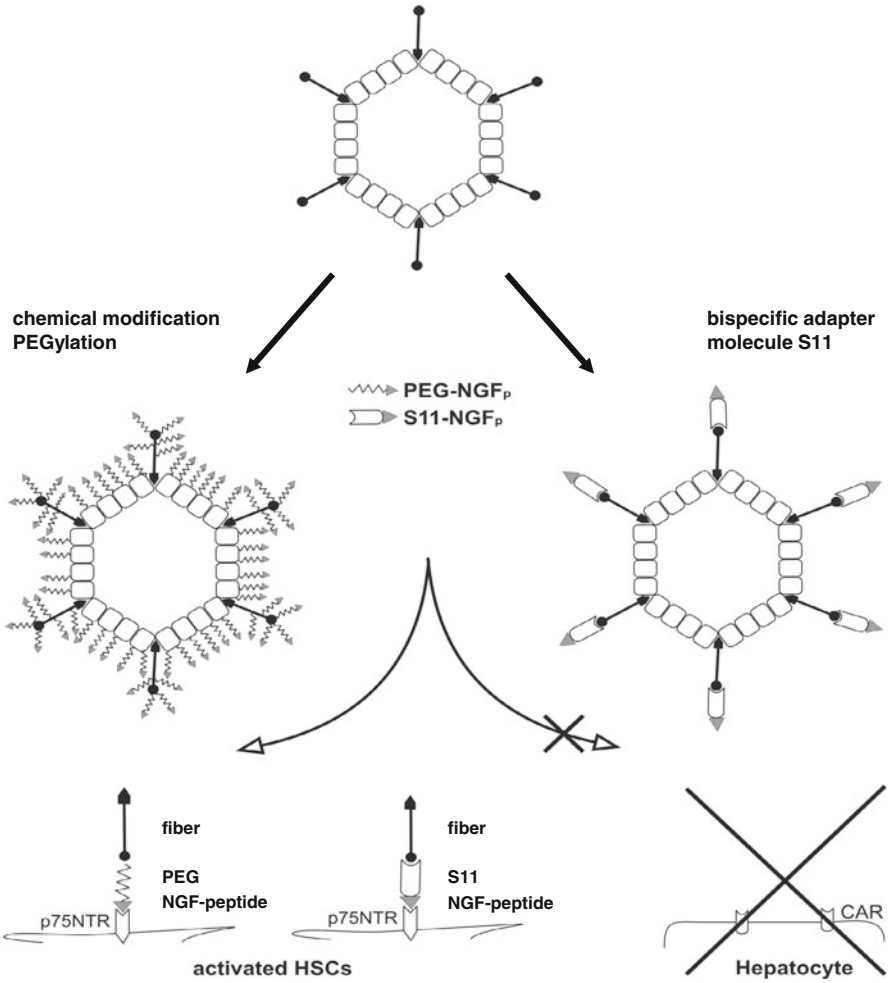

Fig. 2.2 Specific targeting of hepatic stellate cells (HSC). Shown are two different HSC-specific retargeting strategies for adenoviral vectors by linking NGFp. This peptide has specificity for p75NTR, an entity expressed by HSC but not hepatocytes. NGFp was attached to the vector's surface via chemical coupling by PEGylation or as part of the bispecific single-chain immunoglobulin-derived adapter molecule S11. *Top*: Wild-type vectors enters target cells after binding the coxsackie-adenovirus receptor (CAR), the receptor present on a wide range of cell types including hepatocytes and HSC. *Center*, *left*: the edging formed by PEGylation shields the entire vector surface including RGD-binding motifs and exposes NGFp. *Center, right*: the S11 adapter molecule is stoichiometrically attached to the fiber knobs. *Bottom, left*: p75NTR-binding via PEG-NGFp. *Bottom, center*: S11-NGFp-mediated binding of the HSC-specific moiety. *Bottom, right*: both coupling methods ablate the interaction of the vector particle with CAR (Reetz et al. 2013)

based oncolytic virus (Kaliberov et al. 2014a). This approach opens applications adenoviral vectors to target cells where single targeting molecules are sparce. Different bivalent adaptors on the same particle may enhance transduction of such cells.

A potential alternative to targeting approaches by single-chain antibodies are Ad vectors coated with adaptor molecules based on designed ankyrin repeat proteins (DARPins). DARPins differ from antibodies in size, structure, binding pattern, and stability. These properties paired with high-yield, easy production in E. coli makes them promising candidates for targeting purposes. Dreier and colleagues (2011, 2013) designed an adaptor molecule consisting of two DARPin modules fused to each other. One binding site anchors the molecule to the Ad fiber knob and the other enables the particle to attach to tumor cell markers, like the human epidermal growth factor receptor or the epithelial cell adhesion molecule. In their work, the authors convincingly demonstrate that DARPins are high-affinity adaptor molecules that allow efficient gene transfer and are a promising tool to rapidly target Ad vectors to any desired receptor.

To alter the tropism of adenoviral vectors, the recombinant fusion protein technology offers a number of technical advantages compared to the methods of chemical conjugation. The conveniences of rerouting adenoviral tropism using recombinant proteins include simplified production in prokaryotic or, preferably, in eukaryotic expression systems, as well as vector purification. In addition, this approach may allow the application of different fusion proteins suitable for retargeting Ad to other receptors, simply by the substitution of the peptide ligand. This procedure offers, according to our experience, the method of choice to retarget Ad vectors.

2.4 Conclusions

In conclusion, adenoviral vectors have been proven to serve as efficient tools for gene delivery when temporary gene expression is beneficial. The major challenge towards applying the technology remains the development of a target system for specific gene delivery that reaches a high level of efficiency. Genetic approaches to modify the fiber require tediously re-engineering of a given Ad vector, and PEGylation causes decreased transduction efficiencies due to improper PEG to ligand ratios as well as RGD ablation, bifunctional adaptor molecules seem to be the most favorable targeting approach. An expeditious and simple production followed by a broad portfolio of different fusion proteins suitable to retarget Ad by substitution peptide ligands offers a standardized method to retarget vectors for both in vitro and/or in vivo applications. Moreover, engineering of bifunctional adaptors may be customized more easily than fiber modifications and chemical treatment of vector preparations. Increased knowledge of adenovirus biology and powerful techniques such as the phage display method to identify new cell or tissue specific targets as well as unique receptors on neoplastic cells provide the opportunity to develop innovative strategies for gene therapy. Custom-made gene shuttles that

exclusively deliver into cells to be addressed a therapeutic nucleic acid or a potent inhibitor of pathogenic genes may, in future, allow success in the treatment of patients with systemic disease first and foremost with metastasized cancer.

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Chapter 3
Regenerative Chimerism Bioengineered Through Stem Cell Reprogramming

Timothy J. Nelson, Almudena Martinez-Fernandez, Satsuki Yamada, and Andre Terzic

Abstract Regenerative medicine aims to restore damaged tissues in order to reverse disease progression and provide a sustainable solution that cures the root cause of the disease process. Although natural mechanisms of repair are ubiquitous, disruption of the homeostatic balance affects the equilibrium between health and disease due to insufficient tissue renewal in chronic degenerative conditions. Augmentation of the diseased tissue repair capacity through chimerism offers a strategy that spans all fields of medicine and surgery from natural chimerism for tissue rejuvenation, to surgical chimerism for organ replacement, to bioengineered chimerism for targeted regeneration. Technological breakthroughs in nuclear reprogramming now provide a platform to advance a broad range of solutions for regenerative medicine built on the foundation of pluripotent autologous stem cells. By optimizing the safety and effectiveness for stem cell production and ensuring tissue-specific differentiation of progenitors, induced pluripotent stem cells (iPS) offer an unprecedented opportunity to accelerate personalized applications with cell-based products to bioengineer health from disease.

Keywords Rejuvenation • Chimerism • Regeneration • Cardiac repair

3.1 Introduction: Regenerative Medicine

Regenerative medicine is primed by recent progress made in transplant medicine, stem cell biology and biomedical engineering to expand the therapeutic armamentarium available for the future of clinical practice. By providing patients with tissue-based products or biologics, regenerative medicine aims to ameliorate disease outcome while reducing the dependency on long-term palliative options. Advances in the science of regenerative medicine offer a transformative paradigm with

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curative objectives to address patient management demands unmet by non-curative approaches. Notably, the magnitude of chronic diseases increasingly challenges the sustainability of global health care systems (Cortese 2007; Waldman and Terzic 2007). In part, the success of modern medical care has allowed patients to survive initial presentation of acute disease processes, such as the pandemic of myocardial infarction, giving way to a prolonged course of disease management that relies primarily on palliative strategies to mitigate overt symptoms. Furthermore, the aging population is increasingly susceptible to degenerative diseases, which additionally escalates the growing burden on the health care system (Jahangir et al. 2007). Thus, the scope of chronic degenerative diseases will require interventions targeted towards the root cause of disease typically linked to progressive cellular destruction and irreversible loss of tissue function.

The concept of therapeutic repair (Fig. 3.1) encompasses the converging triad of *rejuvenation, replacement, and regeneration* as an overall goal to provide a sustainable cure (Nelson et al. 2008a). Although frequently under appreciated, rejuvenation provides the basis for self-healing from simple injuries of the skin to complex disease within tissues of the heart, liver, kidney, or brain. The unreliability of innate healing and the limited ability to augment inherent stem cell pools required to promote tissue homeostasis defines the unique challenges and opportunities of regenerative medicine. Likewise, transplant medicine exploits a replacement strategy as a valuable option to recycle used parts, and restore failing organ function by means of exogenous substitutes – it is however limited by donor shortage. Ultimately, stem cell-based regeneration has revealed the next frontier of medical therapy through delivery of essentially unlimited pools of autologous or allogeneic, naive or modified, natural or bioengineered progenitor cells to achieve structural and functional repair of all lineages. Collectivley, stem cell-based regenerative medicine designed to supplement natural progenitors and facilitate chimeric healing of damaged tissues is poised to drive the evolution of medical sciences from traditional palliation towards curative therapy (Rosenthal 2003; Daley and Scadden 2008).

3.1.1 Rejuvenation

The rejuvenation strategy refers to self-renewal of tissues from endogenous stem cells within the individual body to promote tissue healing. This innate process of tissue refreshing enables the body to heal itself with younger cells through *de novo* biogenesis (Surani and McLaren 2006). Daughter cells can also be derived from reactivation of the cell cycle within mature cell types in response to stress or injury. This strategy replenishes tissue structure with endogenous stem cells, which generates natural chimeric tissue composed of cells distinguishable only by their birth dates. Although rejuvenation ensures continuous production of renewable tissue required for long-term stress tolerance, most tissues are only partially able to

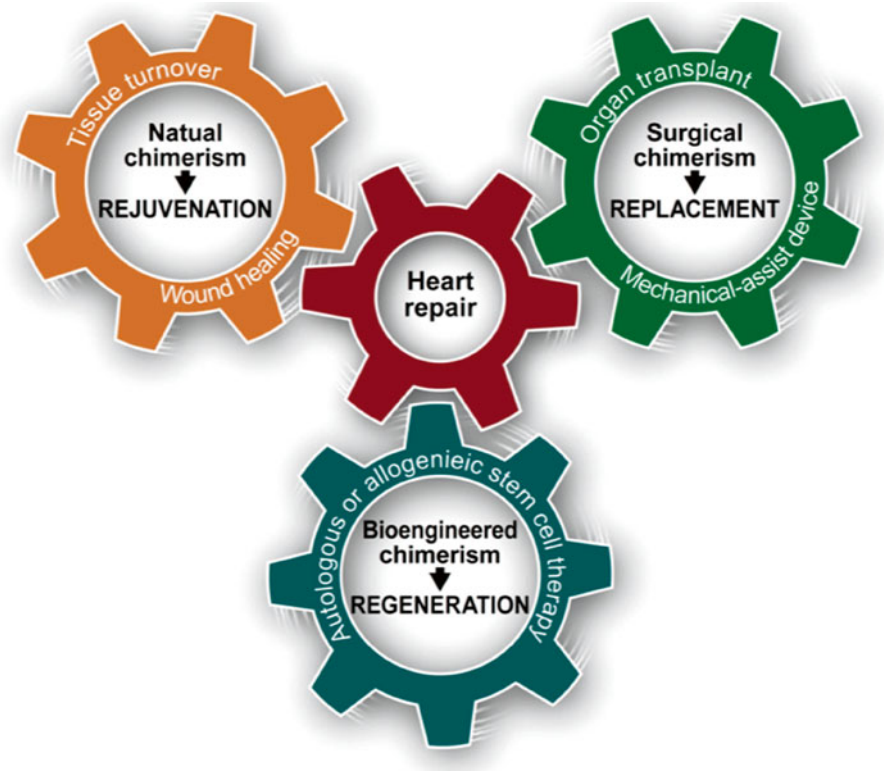

Fig. 3.1 Heart repair is the central goal of cardiovascular regenerative medicine that encompasses the strategic triad: rejuvenation, replacement, and regeneration illustrated here in the context of heart repair. Rejuvenation is defined as the repair of damaged tissue through activation of endogenous mechanisms that can stimulate natural tissue turnover and wound healing in order to replenish tissue function through "natural chimerism". Replacement is defined as repair of damaged tissue by recycling used parts through cardiac organ transplantation and now includes mechanical assist devices to achieve "surgical chimerism". Regeneration is defined as repair of damaged tissue through application of stem cells to generate new tissue and restore function following autologous or allogeneic cell-based "bioengineered chimerism". Collectively, these therapeutic strategies are recognized as an integrated approach for heart repair and could be applicable to damaged tissues and organs throughout the body

self-renew. Moreover in the context of massive acute injury, such as myocardial infarction, inherent repair strategies are frequently inadequate (Anversa and Nadal-Ginard 2002). A boost in healing processes is likely required to stimulate adaptive response and promote adequate biogenesis of functional tissue to abrogate the progression of chronic heart diseases.

3.1.2 Replacement

The replacement strategy refers to transplantation of a donor tissue/organ that maintains functional integrity and re-establishes homeostasis within the host (Atala 2008). The field of surgery pioneered the concept of total replacement with the advent of solid organ transplantation. If the heart was irreversibly damaged, then replacing the diseased tissue with a functioning donor organ has remained a viable option. Cell-based replacement is also exemplified with routine use of donated red blood cells to replace circulating blood volume and treat life-threatening blood loss or anemia. This strategy recycles used cells, tissues, or organs to restore physiologic function for the recipient of the transplant upon establishment of a surgical chimera between donor tissue and host environment. A significant limitation of the replacement strategy remains the shortage of appropriate donors, and the difficulty to match the immunological criteria for safe and effective clinical applications.

3.1.3 Regeneration

The regenerative strategy refers to engraftment of progenitor cells that require *in vivo* growth and differentiation to establish repair within the host environment. Advances in hematology gave rise to the concept of regenerative cytotherapy with the identification of bone marrow-derived stem cells that could be harvested and transplanted in small quantities in order to reconstitute the entire hematopoietic stem cell pool naturally residing within the bone marrow (Kørbling and Estrov 2003). Success of stem cell transplantation was facilitated by engraftment into host bone marrow, which provides a protective environment to nurture the long-term survival of self-renewing stem cell properties. This strategy replenishes functional progenitor cells to allow on-demand differentiation of all hematopoietic lineages and sustained production of bioengineered chimeric tissue from donor stem cells within the host environment. Tissue-specific, non-hematopoietic stem cells have, furthermore, the capacity to re-establish lost function when ectopically transplanted into a wide range of diseased tissues as evident in diabetes, heart disease, and degenerative neurological conditions expanding regenerative applications.

3.2 Natural Chimerism: Heart Rejuvenation

Endogenous stem cells and self-repair mechanisms have been increasingly recognized as a natural process for tissue homeostasis (Laird et al. 2008). Fundamental to cardiac tissue rejuvenation is cardiomyocyte renewal through recruitment of endogenous progenitor pools within the body (Anversa et al. 2006; Torella et al. 2006). Notably, stem cell contribution to postnatal heart formation has been validated by

the self/non-self chimerism characteristic of patients following allogeneic transplantation (Quaini et al. 2002; Kajstura et al. 2008a; Deb et al. 2003). Furthermore, innate stem cell loads increase in failing hearts and contribute to a regenerative response, involving ongoing derivation of cardiomyocytes from circulating or resident progenitors (Kubo et al. 2008; Rupp et al. 2008). However, in the context of large-scale destruction following ischemic injury, the regenerative response required for tissue homeostasis is limited in its ability to salvage a deteriorating myocardium (Urbanek et al. 2005).

The magnitude of the natural process of cardiac tissue self-renewal is likely dependent on multiple factors such as patient age, disease status, co-morbidities, patient-specific medications, as well as genetic predispositions, epigenetics or ecogenetic influences. Utilizing quantification of radio-isotopes, introduced at high levels into the atmosphere during above ground nuclear bomb testing between the years 1955 and 1963 leading to subsequent DNA incorporation within living material, the birth date of individual cardiomyocytes was recently calculated (Bergmann et al. 2009). Based on these data, it has been estimated that cardiomyocytes can renew at <1 % annually to achieve on average a renewal approaching upto 50 % of the total heart mass over a lifespan (Bergmann et al. 2009). Although the magnitude is generally thought to be insufficient to compensate for severe tissue loss in acute disease states, the natural chimerism that is produced as a result of rejuvenation may gradually contribute to the prevention of heart disease and provide a significant protective mechanism of self-renewal to the heart, as originally suggested in transplant patients (Anversa et al. 2006; Torella et al. 2006). The precise mechanism of autologous self-renewal remains only partially addressed, but mounting evidence confirms the presence of *in situ* cardiogenic differentiation (Kajstura et al. 2008b; Hsieh et al. 2007). Direct evidence of allogeneic circulating stem cell contribution to the heart has been demonstrated in multiple patient-derived samples (Anversa et al. 2006; Torella et al. 2006; Quaini et al. 2002; Kajstura et al. 2008a). Importantly, this data from chromosomal mismatch does not preclude an active participation of resident stem cells in cardiac tissue renewal as calculated by the spectrum of de-identified cardiomyocyte birth dates. Therefore, based on the established paradigm of heart rejuvenation it is appropriate to surmise that augmentation of natural chimerism either by reactivation of endogenous or transplantation of exogenous progenitor cells, offers a legitimate target to ameliorate the burden of chronic, degenerative heart disease presented herein as a disease paradigm (Fig. 3.1).

3.3 Surgical Chimerism: Heart Replacement

In response to end-stage heart failure in which the heart was damaged beyond reasonable probability for recovery, cardiac transplantation was pioneered over the past century to engineer a therapeutic option. Pre-clinical breakthroughs originated from the innovative efforts of Alexis Carrel along with Charles Guthrie who

together succeeded to transplant the first heterotopic canine heart in 1905 (Carrel and Guthrie 1905). Over the next 60 years, significant discoveries in cardiac transplant biology and surgical techniques laid the fundamental groundwork for clinical translation that resulted in the first successful human-to-human cardiac transplantation performed by Christiaan Barnard (Lower and Shumway 1960; Hardy et al. 1964; Barnard 1967). The proceeding decade witnessed decreased enthusiasm for the experimental procedure with poor survival rates and enivitable post-procedural complication due to allogeneic immune status. However, with the introduction of effective immunosuppression in the early 1980s (Oyer et al. 1983), technical improvements accelerated clinical practice of cardiac transplantation towards not only a viable strategy but into the standard of care for end-stage heart failure (Fig. 3.1). Today, there are more then 2500 heart transplants done annually in the United States (Hunt et al. 2009); however, the numbers have not changed in the past decade despite a focused effort to address the organ shortage (Taylor et al. 2008). This has presented a significant clinical challenge for the estimated 100,000 patients in the United States alone that would be a potential candidate for this life-saving procedure (Rosamond et al. 2008).

Due to the magnitude of the unmet need, alternative strategies such as mechanical assist devices have gained significant attention throughout this period (Fig. 3.1). The use of mechanical circulatory assist devices in refractory heart failure has been investigated in clinical trials for more than a decade. Extracorporeal devices have been used for short-term circulatory support in selected patients who are expected to have a reversible pathology and a transient need. Of note, advances in mechanical assist technology have produced devices that now offer remarkable hemodynamic support and have been introduced for long-term support of patients even in the ambulatory setting (Goldstein et al. 1998). Success of this technology has led to the concept of not only "bridging" to transplant or recovery, but now is being offered in selected patients not eligible for transplant as a permanent or "destination" therapy. The Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure (REMATCH) trial enrolled 129 patients for a 2-year follow-up period that demonstrated a 23 % survival in mechanical device treated versus 8 % survival with medical therapy alone (Rose et al. 1999). These data established the therapeutic value of mechanical support devices in the treatment algorithm of end-stage heart disease, and justified a role for patients that have a 1-year survival of less than 50 %. Improved outcomes are likely to be reported as patient selection is refined, surgical and post-surgical care evolves, and devices are re-engineered for long-term sustainability. However, these advancements do little to prevent the pandemic of candidate patients with refractory heart failure and only expands palliative approaches that are largely limited to symptomatic management of a progressive disease.

3.4 Bioengineered Chimerism: Heart Regeneration

3.4.1 Recapitulating De Novo Cardiogenesis

Pluripotent stem cells have demonstrated the ability to contribute to chimeric tissues and illustrate the potential for exogenous stem cells to augment structure and function of host cardiac tissue. Chimeric offspring can be bioengineered through multiple techniques that place competent stem cells in direct contact with early stage embryos (Wood et al. 1993b; Wakayama et al. 2001; Nelson et al. 2009c; Stillwell et al. 2009). Two common methods of producing chimeric mice from pluripotent stem cells consist of either microinjection into the blastocoel cavity of a blastocyst-stage host embryo or by the non-coerced aggregation with a morula-stage host embryo (Wood et al. 1993a; Tam and Rossant 2003). Aggregation techniques include diploid aggregation in which host embryo is an 8-cell wild-type morula (Fig. 3.2) and tetraploid aggregation in which host embryo is the product of electrofusion of a two-cell embryo into a single cell containing two copies of the genome. In both diploid aggregation and blastocoel injections, the resulting fetus will be comprised of a mixture of stem cell-derived progeny along with lineages originating from the host embryo. Because host embryo has unaltered differentiation capacity, the transplanted pluripotent stem cells offer an equivalent source for the

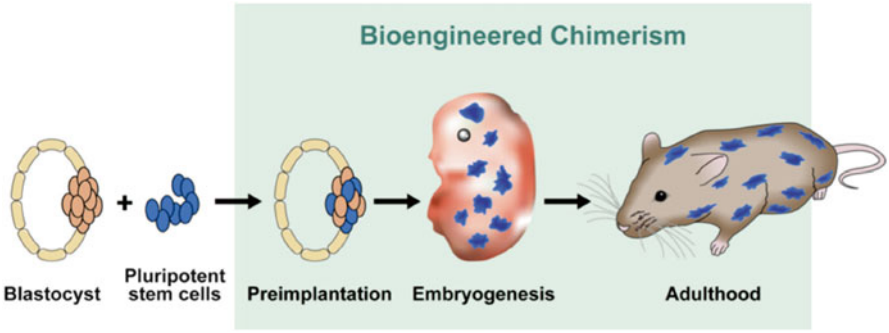

Fig. 3.2 Bioengineered chimerism. Adult chimeric offspring can be generated from pluripotent stem cells by manipulation of the blastocyst prior to implantation within surrogate mother. By injecting 10–20 cells into the cavity of a developing blastocyst or aggregation between compact morula and pluripotent stem cell, the preimplantation blastocyst becomes a random mixture of original blastomeres and transplanted stem cells. The chimeric inner cell mass gives rise to a mosaic pattern of chimeric tissue throughout development and are sufficient to produce adult offspring with random distributions of tissues derived from the transplanted pluripotent stem cells. The bioengineered chimerism offers unique opportunities to study the cell-autonomous defects of stem cells throughout development and into pathophysiological homeostasis of the adult system

differentiating embryo and allow stochastic integration of the chimeric offspring. In contrast to the non-competitive combination of stem cells with similar differentiation capacity, tetraploid aggregation utilizes a partially defective host embryo and requires the transplanted stem cells to compensate for developmental deficiencies inherent within the host embryo (Nagy et al. 1993). In this way, bioengineering has confirmed the ability of chimeric tissues to rescue genetic defects and provide viable offspring from otherwise lethal mutations innate to the host environment. Case in point, the deletion of vascular endothelial growth factor (VEGF) disrupts the vascular development in the placenta and results in a developmental arrest (Carmeliet et al. 1996). However, chimeric complementation with pluripotent stem cells containing wild-type VEGF rescues the definitive pre-natal defects and produces viable offspring (Hirashima et al. 2003). Thus, chimeric tissue has provided a powerful platform for discovery sciences and establishes the potential therapeutic value of bioengineered chimerism.

Beyond cell-autonomous rescue of defective tissues, chimeric tissue reconstruction has also been demonstrated to repair disease-causing mutations through neomorphic (non-cell autonomous or paracrine) mechanisms (Fraidenraich et al. 2004). Tissue formation is dependent on multiple cell types that develop together and rely on the cooperative microenvironment, exemplified in cardiogenesis in which juxtaposition of definitive endoderm to secrete growth factors guides pre-cardiac mesoderm maturation (Lough and Sugi 2000; Foley et al. 2006). Therefore, mapping of defective signaling pathways in individual cardiac disease conditions that depend on paracrine support has provided therapeutic targets for chimeric tissue reconstruction. The translational value of this approach was originally discovered according to dramatic rescue of the embryonic lethal phenotype in Id1/Id3 knockout embryo upon blastocoel injection of wild-type embryonic stem cells (Fraidenraich et al. 2004). Repair of cardiogenesis in the defective embryos has been recapitulated with both indirect transplantation of embryonic stem cells into the peritoneal cavity of mother and the acellular delivery of insulin-like growth factor (IGF-1) as the principle active ingredient (Fraidenraich et al. 2004).

Extending the rational basis of chimeric tissue reconstruction for disease management, preemptive cell-based intervention has more recently been demonstrated for ischemic heart disease (Yamada et al. 2009). Embryonic stem cells delivered into an early stage host embryo were tested to determine whether bioengineered chimeric tissue could impact the tolerance of the adult to ischemic injury. Chimera offspring were generated through microinjection of pluripotent stem cells into pre-implantation embryos, and then examined for cardiac stress tolerance in adulthood. Indeed, bioengineered chimera demonstrated a functional and structural benefit compared to non-chimeric counterparts in the setting of coronary artery occlusion. The proof-of-concept provided the initial evidence of preventive regenerative medicine in the setting of myocardial infarction implemented through prenatal intervention (Yamada et al. 2009). Thus, chimeric incorporation of healthy progenitor cells into host embryos, before and after disease onset, has provided mechanistic insight to a wide range of pathology and increasingly offers a novel therapeutic strategy (Schneider et al. 2009).

3.4.2 *Advancing Stem Cell-Based Cardiac Repair*

Along with the paradigm shift that the heart is a self-healing organ came the design and implementation of clinical trials to test the hypothesis that additional stem cell load would lead to accelerated heart repair following ischemic injury (Leri et al. 2008). Promising pre-clinical data documenting improvement in cardiac performance following stem cell transplantation provided the foundation to test in patients the safety and feasibility of stem cell therapy in cardiac disease (Dimmeler et al. 2005; Segers and Lee 2008).

Autologous skeletal myoblasts were the initial cell type used in clinical trials (Menasché et al. 2001). Approximately 9×10^8 myoblasts were obtained from muscle biopsy, and transplanted into the myocardium during open-heart surgery. Ventricular tachycardia was recognized as a possible side-effect of therapy. However, in individual cases patients reported improvement in symptoms significant enough to decrease their heart failure class score, and had improvement in left ventricular ejection fraction. Subsequent trials have used lower concentrations of myoblasts, and have demonstrated a lower incidence of ventricular arrhythmias (Opie and Dib 2006). Long-term follow-up demonstrated improvement in clinical status and a decrease in hospitalizations for heart failure, while the risk of arrhythmia was appropriately controlled with medical therapy and/or device implantation (Hagège et al. 2006). A placebo controlled, multicenter phase III clinical trial (MAGIC) demonstrated significant decrease in left ventricular diameter after 6 months indicative of improved remodeling in patients with heart failure following myoblast injection directly into the myocardium, despite no significant change in systolic function of the treated heart muscle (Menasché et al. 2008).

The TOPCARE-AMI trial was designed to test the safety and feasibility of stem cell transplantation after acute myocardial infarction using circulating or bone marrow-derived progenitor cells (Schächinger et al. 2004). Initial studies demonstrate a safe clinical profile without ventricular arrhythmia, thrombus formation, distal embolization or dissection of coronary artery throughout a 1-year follow-up period. Furthermore, serial MRI imaging of the left ventricle demonstrated improved ejection fraction of ~8 % as early as 4 months, and up to 12 months after transplantation. Safety and feasibility was independently confirmed using bone marrow cells transplanted into patients with a large ST-elevation myocardial infarction (Sánchez et al. 2006). The first randomized clinical trial, BOOST, examined patients after having an ST-elevation myocardial infarction that involved successful treatment by percutaneous stent placement into a single coronary artery (Drexler et al. 2006). Five days after optimal management according to standard medical practice, patients were treated with autologous bone marrow cell therapy and demonstrated a 6.7 % improvement in ejection fraction compared to <1 % improvement in the medically managed control cohort at 6-months follow-up. However, the significance of left ventricular function improvement in stem cell treated patients was not sustained at 18-month follow-up. The ASTAMI trial limited the inclusion criteria to acute myocardial infarction involving the left anterior descending coronary artery

and randomized patients to receive bone marrow mononuclear cells via coronary artery delivery (Lunde et al. 2007). After a 6-month follow-up period, no significant difference between groups was detected in global left ventricular function. This randomized open-labeled study involving 100 patients confirmed the low risk of cell transplantation without increased risk of thrombosis, re-stenosis, or arrhythmia, and importantly showed significant improvement in exercise time and heart rate responses (Schächinger et al. 2006). Two phase II randomized clinical trials consisting of 200 patients in a multi-institutional study, REPAIR-AMI (Janssens et al. 2006), and 67 patients in the STEMI study (Perin et al. 2004) not only randomized patients, but also performed placebo injections in the control group via a similar coronary artery catheter approach. The primary outcome of functional improvement as measured by ejection fraction was significantly increased by 5.5 % with cell transplantation in the REPAIR-AMI study compared to 3.0 % in placebo group at 4 months. In a sub-group analysis it was surmised that initial ejection fraction of less than 49 % had a significant benefit to cellular transplantation when compared to patients with baseline ejection fraction of greater than 49 %. The STEMI study demonstrated significant decrease in left ventricular infarct size as measured by MRI but was unable to demonstrate any significant increased in left ventricular function between the placebo control and cell transplantation groups. Moreover, transendocardial injection of autologous bone marrow mononuclear cells in patients with end-stage ischemic heart disease has also been demonstrated to produce a durable therapeutic effect and improve myocardial perfusion and exercise capacity (Perin et al. 2004).

To date, over 3000 patients with ischemic heart disease have received stem cell therapy in a clinical trial setting worldwide (Bartunek et al. 2007). Meta-analysis collectively indicates the safety profile of stem cell-based therapy, with modest improvements in functional parameters and apparent benefit in structural remodeling (Abdel-Latif et al. 2007). Ongoing optimization of the most appropriate cell type, selection of patient populations amenable to cell-based therapy, timing of intervention, and route of administration are the areas of focus to determine the clinical utility of cell-based therapy in cardiovascular disease.

3.5 Induced Pluripotent Stem Cells: A Platform for Unlimited Cardiac Repair

Beyond natural sources of stem cells that are limited by availability, immune intolerance, and lineage specification, bioengineered stem cell platforms are rapidly being developed for regenerative medicine applications. Converted from parental somatic cell types, bioengineered stem cells have acquired the ability to give rise to all cell types of the adult body, previously only possible from embryonic stem cells. Furthermore, recent studies have demonstrated that chimeric tissue from bioengineered stem cells is able to produce significant functional and structural repair in disease models.

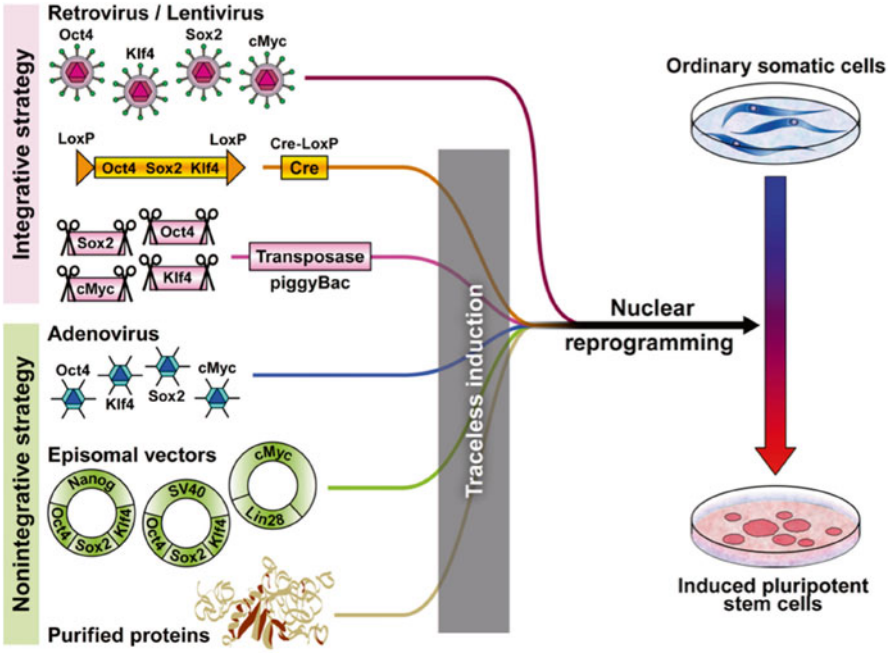

Fig. 3.3 Strategies for nuclear reprogramming of induced pluripotent stem cells. Ordinary somatic cell types can provide the parental source for nuclear reprogramming, including patient-specific tissue samples. Integrative strategies based on both retrovirus and lentivirus provided the initial successful model system to reprogram ordinary cells into iPS cells. The next-generation of this platform included the second step of removing the ectopic transgenes with either Cre recombinase or transposon-transposase systems, thus allowing a truly traceless approach. Alternatively, non-integrative strategies became feasible with both viral transduction of adenovirus and episomal vectors or plasmid-based constructs. These strategies delivered the same ectopic stemness-related factors without the risk of insertional mutagenesis, although at lower overall efficiency of nuclear reprogramming. Furthermore, non-integrative strategies also include protein-based bioengineering to deliver the transient levels of stemness-related factors needed for successful nuclear reprogramming

3.5.1 Principles of Nuclear Reprogramming

Bioengineered stem cells offer the ability to provide unlimited supply of progenitor cells at any time point for virtually all cell types and tissues of the adult body starting from ordinary self-derived tissues (Fig. 3.3). By exploiting epigenetics and the microenvironment of somatic nuclei, reprogramming platforms aim to reverse cell fate of common cell types that are readily available in order to achieve conversion of a mature cell type back to the embryonic ground state (Jaenisch and Young 2008). Advancement of bioengineered platforms was realized through the pioneering work of somatic cell nuclear transfer technology that demonstrated the efficacy of trans-acting factors present within the mammalian oocytes, conserved across species, to

reprogram somatic cell nuclei to an undifferentiated state (Yang et al. 2007; Beyhan et al. 2007). Thus, therapeutic cloning refers to somatic cell nuclear transfer (SCNT) in which the nuclear content of a somatic cell from an individual is transferred into an enucleated donor egg to derive blastocysts that contain pluripotent embryonic-like stem cells. In this way, SCNT has produced cloned embryonic stem cells from multiple mammalian somatic cell biopsies (Hall and Stojkovic 2006; Sha et al. 2009; Byrne et al. 2007). The pluripotency of derived cells has been confirmed through germline transmission, and reproductive cloning. However, due to technological limitations cloned human blastocytes have only recently been achieved albeit in low efficiency (French et al. 2008), and successful isolation of embryonic stem cells from the inner cell mass has yet to be demonstrated with human protocols.

Nuclear reprogramming of adult somatic cells through ectopic introduction of a small number of pluripotency-associated transcription factors is a streamline approach to induce an embryonic stem cell-like phenotype (Takahashi and Yamanaka 2006; Yamanaka 2008, 2009a, b, c). Transcription factors sets, Oct4, Sox2, c-Myc and Klf4 or alternatively Oct4, Sox2, Nanog and Lin28 (Yu et al. 2007), are sufficient to reprogram human somatic cells by inducing a sequential reversal into a pluripotent phenotype (Fig. 3.3). The process of nuclear reprogramming requires controlled expression of specific stemness factors in the proper stochiometry for a defined period of time (Meissner et al. 2007; Maherali et al. 2007; Takahashi et al. 2007b; Yamanaka 2007; Park et al. 2008a, b; Papapetrou et al. 2009). Multiple source tissue has been successfully reprogrammed such as fibroblasts (Takahashi et al. 2007a), keratinocytes (Aasen et al. 2008), blood (Loh et al. 2009), or adipose tissue (Sun et al. 2009). The balanced exposure of ectopic factors is sufficient to induce telomere elongation (Marion et al. 2009b), histone modifications (Deng et al. 2009), secondary gene expression profiles (Mikkelsen et al. 2008), and cellular metamorphosis that collectively re-establish a self-stabilizing phenotype of pluripotency (Silva et al. 2009). Reprogramming occurs typically within weeks of coerced equilibrium of the trans-acting factors that can be delivered to the nucleus either by plasmids, viruses, or recombinant proteins (Fig. 3.3). Thereby, ectopic transgene expression initiates a sequence of stochastic events that eventually transforms a small fraction of cells (<0.5 %) to acquire this imposed pluripotent state characterized by a stable epigenetic environment indistinguishable from the blastocyst–derived natural stem cell *milieu*. The acquired pluripotent ground state culminates in the maintenance of the unique developmental potential to differentiate into all germ layers. In this way, induced pluripotent stem cells (iPS) with the ability to derive patient specific progenitor cells should largely eliminate the concern of stem cell shortage, immune rejection of non-autologous sources, and inadequate capacity for lineage specification (Nishikawa et al. 2008; Nakagawa et al. 2008; Park et al. 2008c). Moreover, iPS based technology will facilitate the production of cell line panels that closely reflect the genetic diversity of a population enabling the discovery, development and validation of diagnostics and therapeutics tailored for each individual (Waldman and Terzic 2008).

3.5.2 Autologous Pluripotent Stem Cells

Bioengineered platforms bypass the need for embryo extraction to generate true pluripotent stem cell phenotypes from autologous sources. In the mouse, bioengineering has yielded iPS clones sufficient for complete *de novo* embryogenesis as the highest evidence of pluripotent stringency (Zhao et al. 2009; Boland et al. 2009) and in humans, by giving rise to all three germ layers, has ensured comprehensive multilineage tissue differentiation. Self-derived iPS cells will be recognized within the transplanted hosts as native tissue due to their autologous status, but will also require new level of protection from dysregulated growth. The next generation of bioengineered stem cells will likely include specialized properties to improve stress tolerance, streamline differentiation capacity, and increase engraftment/survival to improve regenerative potential.

3.5.2.1 First-Generation Technology

Retroviral and lentiviral approaches offered the initial methodology that launched the field, and established the technological basis of nuclear reprogramming with rapid confirmation across integrating vector systems (Takahashi and Yamanaka 2006; Yu et al. 2007; Meissner et al. 2007; Aasen et al. 2008; Okita et al. 2007; Aoi et al. 2008; Huangfu et al. 2008; Eminli et al. 2008; Kim et al. 2008; Hanna et al. 2008; Feng et al. 2009). The risk of oncogenic genes and insertional mutagenesis inherent to stable genomic integration has been recognized as potential limitations from the onset of this technology. However, distinct advantages of the retroviral-based vector systems enabled critical insight to the mechanisms of reprogramming. Retroviral and lentiviral systems have built-in sequences that silence the process of transcription upon pluripotent induction, thus persistent exposure to ectopic gene expression was temporally restricted at the time of re-induction of pluripotency. This allows an essential observation to be made in that successful self-maintenance of the pluripotent state was possible without long-term transgene expression. Thereby, systems were envisioned for transient production of stemness-related genes without integration into the genome to improve the safety and efficacy of nuclear reprogramming. The first proof-of-principle was achieved by non-integrating viral vector systems, such as adenovirus (Stadtfeld et al. 2008), and confirmed by repeated exposure to extra-chromosomal plasmid-based transgenes (Okita et al. 2008). Importantly, these reports demonstrated that expression of stemness-related factors was required for only a limited timeframe until progeny developed autonomous self-renewal, establishing nuclear reprogramming as a bioengineered process that resets a sustainable pluripotent cell fate independent of permanent genomic modifications. The inherent inefficiency of non-integrated technologies has however hindered broader applicability and stimulated the search for more efficient methodology.

3.5.2.2 Second-Generation Technology

The latest innovation that advances iPS-based technology towards clinical applications has most recently been reported in which non-viral approaches are capable of high-efficiency production (Kaji et al. 2009; Woltjen et al. 2009). These approaches are dependent on short sequences of mobile genetic elements that can be used to integrate transgenes into host cell genomes and provide a genetic tag to "cut and paste" flanked genomic DNA sequences (Nelson and Terzic 2009). The piggyBac (PB) system couples enzymatic cleavage with sequence specific recognition using a transposon/transposase interaction to ensure high efficiency removal of flanked DNA without residual footprint. Importantly, this technology achieves a traceless transgenic approach in which non-native genomic sequences that are transiently required for nuclear reprogramming can be removed upon induction of pluripotency. Specifically, using the PB transposition system with randomly integrated stemness-related transgenes, recent studies have demonstrated that disposal of ectopic genes could be efficiently regulated upon induction of self-maintaining pluripotency according to expression of the transposase enzyme without infringement on genomic stability (Woltjen et al. 2009). This state-of-the-art system is qualified to allow safe integration and removal of ectopic transgenes, improving the efficiency of iPS production and facilitating a minimally invasive methodology without permanent modifications to the progeny. Alternatively, the security of unmodified genomic intervention can be achieved with non-integrating episomal vectors (Yu et al. 2009). Collectively, these recent strategies allow genetically unmodified progenitor cells to acquire the capacity of pluripotency.

Alternatively, high-stringency iPS cells have also been produced with proteins in the absence of genetic material (Zhou et al. 2009; Kim et al. 2009). The protein-only approach has successfully induced reprogramming with either whole cell extract enriched in four stemness factors used in combination with pharmacological induction of cell permeability or with stemness factors modified by cell-permeating polyarginine tag (Zhou et al. 2009). Although the reprogramming efficiency compared to genetic methodology is reduced, there are emerging strategies that complement the influence of stemness factors exposure within somatic cells. Namely, small molecules targeting histone modifications have increased reprogramming efficiencies (Shi et al. 2008) along with the latest discovery that tumor suppressor gene, p53, is responsible for inhibiting the reprogramming process (Banito et al. 2009; Hong et al. 2009; Utikal et al. 2009; Marion et al. 2009a; Li et al. 2009; Kawamura et al. 2009). Thereby, transient knockdown of p53 according to siRNA strategies targeting the breakdown of mRNA or overexpression of MDM-2 to increase p53 protein degregation has proven to successfully increase the overall efficiency 1–2 orders of magnitude with up to 20 % of selected cells undergoing *bona fide* reprogramming (Banito et al. 2009; Hong et al. 2009; Utikal et al. 2009; Marion et al. 2009a; Li et al. 2009; Kawamura et al. 2009). Together, the rapid advancements in nuclear reprogramming have accelerated bioengineered pluripotent stem cells closer to the milestones required for possible clinical translation.

3.5.3 *Regeneration of Diseased Tissues*

To date, therapeutic benefit of iPS-based technology has been tested in four disease models, namely sickle cell anemia (Hanna et al. 2007), Parkinson's disease (Wernig et al. 2008), hemophilia A (Xu et al. 2009), and ischemic heart disease (Nelson et al. 2009d). As differentiation protocols are refined to produce "on-demand" tissue-specific progeny, additional pre-clinical disease models will be screened to address the full regenerative value of iPS technology.

In regards to cardiogenesis, embryonic stem cells have a spontaneous propensity for cardiac differentiation that fulfills an early requirement for heart formation during embryonic development. Compared to the gold standard of embryonic stem cells, iPS have demonstrated a similar capacity for *in vitro* cardiac differentiation. Using methodology established for embryonic stem cell-derived cardiogenesis, iPS differentiating in embryoid bodies or aggregates of tissue starting with 400–500 cells systematically produce mesoderm lineages and pre-cardiac cytotypes according to established gene expression profiles (Schenke-Layland et al. 2008). Within appropriate time frames, mouse and human tissue give rise to early cardiomyocytes with spontaneous beating activity (Fig. 3.4). This tissue expresses contractile proteins, such as troponin and actinin. Furthermore, the cardiac-like tissue is regulated according to excitable inputs through gap junctions, and calcium from extracellular and intracellular sources. As the cardiac tissue matures *in vitro*, specialized heart muscle cells become evident with assembly of characteristic ion channel sets responsible for physiological regulation of cardiac contraction and electrical conductance within ventricular, atrial, and pacemaker cell types (Schenke-Layland et al. 2008; Narazaki et al. 2008; Mauritz et al. 2008; Zhang et al. 2009; Yokoo et al. 2009).

The therapeutic value of iPS in cardiovascular medicine was recently documented in a model of acute myocardial infarction [121]. Post-ischemic cardiac performance was compared in randomized cohorts transplanted with parental fibroblasts versus bioengineered iPS (Nelson et al. 2009d). As quantified by echocardiography, occlusion of anterior epicardial coronary blood flow permanently impaired regional wall motion and cardiac function. Treatment with parental fibroblasts was unable to improve performance of post-ischemic hearts (Fig. 3.4). Yet, iPS intervention in the acute stages of myocardial infarction improved cardiac contractility by 4 weeks post-transplantation. Functional benefit in response to iPS therapy was verified by the improvement in fractional shortening and regional septal wall thickness during contraction that demonstrate coordinated concentric contractions visualized by long-axis and short-axis 2-D imaging (Nelson et al. 2009d). Beyond functional deterioration, maladaptive remodeling with detrimental structural changes prognosticates poor outcome following ischemic injury to the heart. In contrast to non-reparative fibroblasts, iPS-based intervention attenuated global left ventricular diastolic diameter predictive of decompensated heart disease. A consequence of pathologic structural remodeling is evident by prolongation of the QT interval,

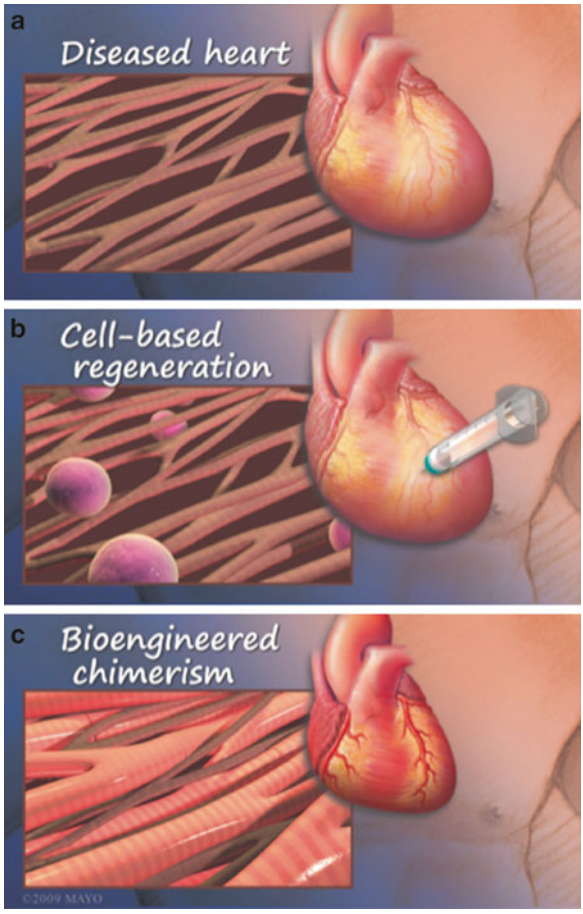

Fig. 3.4 Induced pluripotent stem cell-based repair of heart disease. (**a**) Patients with heart failure develop progressive disease that results in weakened and dilated heart muscle, unable to function normally. (**b**) Direct intramyocardial delivery of stem cells leads to iPS engraftment within disease heart. (**c**) Bioengineered chimerism according to iPS-based therapy has demonstrated functional benefit to the diseased heart muscle with direct evidence for stable engraftment and *in vivo* cardiovascular regeneration of new tissue

which increases risk of life-threatening arrhythmias (Nelson et al. 2009d). Successful iPS treatment prevented structural remodeling to avoid deleterious effects on electrical conductivity.

These real-time surrogates for tissue remodeling have been confirmed by gross inspection of specimens. Autopsy allowed histological analysis to determine the extent of scar tissue formation within the post-ischemic region of the anterior circulation distal to the coronary ligation (Nelson et al. 2009d). In contrast to parental fibroblasts, iPS treatment halted structural deterioration with decreased fibrotic scarring and induction of remuscularization with *de novo* heart muscle tissue along with evidence for angiogenesis according to vascular endothelial markers. Surgical dissection verified absence of tumor infiltration or dysregulated cell expansion following iPS transplantation in the myocardium itself, as well as in organs with high metastatic risk such as the liver, lung and spleen. Collectively, iPS-derived regeneration of the ischemic heart has been demonstrated at multiple levels of stringency

that include cellular, tissue, structural, functional, and metabolic levels, providing a foundation for development of this novel platform towards clinical applicability (Nelson et al. 2009d).

With ongoing understanding of priciples of myocardial regeneration (Srinivas et al. 2009), clinical translation of iPS technology faces similar challenges that have in part been addressed by natural stem cell applications, including embryonic stem cells approved early in 2009 by the Food and Drug Administration in the United States for trials involving patients with incurable spinal cord injuries. The first universal obstacle for clinical translation of pluripotent stem cell technology is unregulated tumor formation (Li et al. 2008). Even a limited contamination of undifferentiated cells can, in theory, result in the formation of dysregulated tumors. Therefore, a critical milestone is to secure differentiation of iPS into the required cell type, purifying them away from residual undifferentiated precursors prior to transplantation (Yamanaka 2009a; Li et al. 2008). This becomes a unique challenge for iPS technology when the immune system is no longer involved in the elimination process of dysregulated foreign tissue, active with embryonic stem cell applications. The second issue that is unique to iPS is the accuracy of complete reprogramming of ordinary cells into pluripotent progeny. Inadequate conversion according to nuclear reprogramming strategies could result in impaired differentiation of iPS cells into target tissues required for specific applications (Yamanaka 2009a). Third, the issue of persistent transgene expression in iPS progeny requires careful consideration. Generally, iPS cells have been produced by transduction of ordinary cells with retroviruses or lentiviruses carrying ectopic transgenes in order to efficiently transfer stable expression into the host nucleus. This creates the risk of not only continuous expression of transgenes that are known to promote dysregulated tumor growth, but also involves permanent genomic modifications that raise the concern for insertion mutagenesis of endogenous loci.

Cardiac tissue specificity from stem cells has been investigated for more than a decade and as of yet no single gene or cluster of genes has been identified to secure cardiac differentiation. However, recent studies have significantly enriched the cardiac propensity with either exogenous growth factors (Behfar et al. 2008), cell sorting of cardiac progenitors (Nelson et al. 2008c; Moretti et al. 2006; Kattman et al. 2006; Yang et al. 2008), or genetically engineering pre-cardiac pathways all to encourage cardiogenesis from primitive stem cell pools (Takeuchi and Bruneau 2009). Collectively, these technologies offer the rational basis to design strategies to ensure cardiogenic specification and avoidance of undifferentiated subpopulations prior to transplantation. The crucial balance between lineage specification and progenitor cell proliferation (Martinez-Fernandez et al. 2009) will be essential to develop a robust manufacturing process that can be scaled and applied to clinical grade production of a cardiac stem cell-based product.

In order to translate iPS technology into clinical reality for heart disease, additional milestones will need to be considered. First, the target patient population will need to be identified based on disease-severity and lack of alternative options to justify inclusion into a first-in-man study. Many patients are too severely deconditioned or have significant co-morbidities to allow consideration for heart transplant,

thus limiting treatment strategies to palliative medicines and procedures. This category of patients needs to be considered a priority in terms of experimental cell-based interventions. An advantage with autologous iPS technology is that no toxic immunosuppression is required, yet provides a unique strategy to overcome poor natural stem cell pools in elderly patients, limiting more traditional regenerative approaches. Thus, iPS-based products should be considered in patients with no other options to decrease not only symptoms but also the need for hospitalization along with expensive yet invasive palliative management strategies such as destination left ventricular assist devices. Next, a good-manufacturing-practice production process and facility will need to be developed and implemented to ensure clinical-grade production of patient-derived iPS cells, as well as tissue-specific differentiation for targeted applications. Finally, regulatory agencies will require evidence of proper engraftment, survival, and safety of transplanted iPS-derived progeny (Nelson et al. 2008b). This will require proof-of-principle studies using clinical grade cell products in disease model systems encompassing comparative effectiveness for optimized outcomes (Nelson et al. 2008b, 2009b).

3.6 Clinical Perspective

Built on emerging discoveries in stem cell biology (Nelson et al. 2008a), regenerative medicine has begun to define the scope of future clinical practice (Nelson et al. 2009b; Waldman et al. 2007). Regenerative medicine and stem cell biology cross all disciplines of medicine/surgery, and provide a universal paradigm of curative goals based on scientific discovery and clinical translation. The challenges to realize the full potential of stem cell biology remain substantial, and thus requires integration of multidisciplinary teams with expertise to form a dedicated regenerative medicine community of practice (Nelson et al. 2009a). Building on the foundation of transplant medicine, regenerative medicine will continue to expand and implement technologies to treat new diseases at earlier stages with safer and more effective outcomes, not achievable with current standards of care. Individualized treatment algorithms for regenerative medicine will require quantification of the inherent reparative potential to determine patients that would benefit from stem cell therapy in order to target personalized regenerative medicine solutions.

Induced nuclear reprogramming through ectopic transgene expression of stemness factors offers a revolutionary strategy for embryo-independent derivation of autologous pluripotent stem cells from an ordinary adult source (Yamanaka 2009b). In such, iPS have attained functions previously demonstrated only by natural embryonic stem cells to independently produce all tissues types and develop the complete organism within an embryonic environment. To date with regard to cardiovascular applications, the reprogrammed iPS progeny have established the therapeutic value for cardiac tissue regeneration in a setting of experimental ischemic heart disease (Nelson et al. 2009d). Specifically, transplantation of iPS in the acutely ischemic myocardium yielded structural and functional repair to secure

performance recovery as qualified clones contributed to *in vivo* tissue reconstruction with "on-demand" cardiovasculogenesis. Moreover, Parkinson's disease, sickle cell anemia, and hemophilia A are early examples of successful iPS applications in disease models (Nelson et al. 2010). Furthermore, patient-specific iPS cells have been generated from individuals with diabetes, amyotrophic lateral sclerosis, Fanconi anemia, and myeloproliferative disorders (Yamanaka 2007; Park et al. 2008a; Maehr et al. 2009; Dimos et al. 2008; Ye et al. 2009; Raya et al. 2009). Therefore, converting self-derived fibroblasts into reparative progenitors can now be considered as a goal of regenerative medicine to individualize treatment algorithms for multilineage repair. In this way, clinical-grade, pluripotent, autologous stem cells offer a unique bioengineered tool to repair disease tissue through chimeric integration.

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Chapter 4
Biodegradable Polymeric Materials

Michael Schroeter, Britt Wildemann, and Andreas Lendlein

Abstract The ability of polymers to be degraded in physiological environments makes them interesting candidates for various medical applications. Degradation and metabolisation or excretion of polymeric implants can avoid a second surgery for the removal of an implant. They follow a distinct pathway for degradation, depending on their structure. Biodegradable materials can serve as a temporary substitute of the extracellular matrix or as matrix in controlled drug release systems, which both can be utilized in Regenerative Therapies.

This chapter gives an overview about polymeric materials established in clinical use such as polyesters, polyurethanes, polyanhydrides, or carbohydrates. It describes further their synthesis and exemplary applications such as surgical sutures. Finally the importance of a continuing development of novel materials for future applications is pointed out, since the number of potential applications in the medical field is expanding rapidly.

Keywords Biomaterial • Biodegradation • Surface erosion • Biodegradability of polymers • Biocompatibility

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4.1 Introduction

Many people experience biomaterials in the form of dental fillings, contact lenses or suture materials. Further applications are artificial joints, blood vessel substitutes or drug delivery systems. A biomaterial is defined as any material intended to interact with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body (The European Society for Biomaterials 1991). Biomaterials can be inorganic materials like bioactive glasses, ceramics or metal alloys as well as polymers including natural polymers (e.g. collagen), synthetic polymers and combinations of both. Biomaterials can be applied in permanent or temporary implants, depending on the particular indication. While all biomaterials must be biocompatible, their permanent application requires long term stability in physiological environments. An example for long term application is for the acetabular cup in artificial hip joints. These materials need to be integrated into the surrounding tissue after implantation and retain their function for a long time. For temporary applications, biodegradable materials are demanded, being degraded and eliminated or metabolized by the organism in the course of time (Lendlein 1999). Biodegradation is defined as the gradual breakdown of a material mediated in or by a biological system. The advantage of the bodies' capability for self-healing can be utilized by the use of degradable materials. A temporary implant is completely substituted by natural tissue in the best case. An overview about biodegradable polymers and natural materials, their synthesis and approved clinical applications will be given in this chapter.

The degradation of polymeric materials depends on their molecular structure and architecture. In copolymers the sequence structure (see Fig. 4.1) can substantially influence the degradation rate.

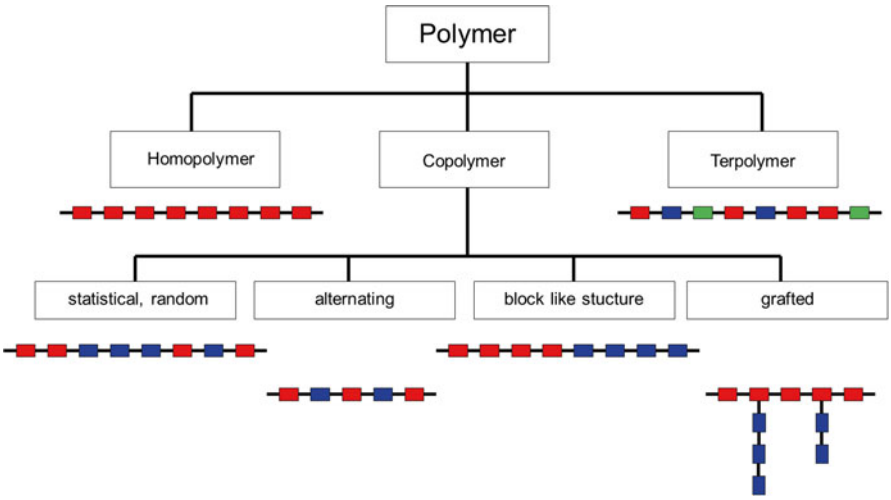


Fig. 4.1 Polymer architecture with different sequences of the monomers or building blocks. Each *rectangle* represents a repeating unit; different *colors* represent different types of repeating units which can form building blocks

Using different synthetic methods, such as polymerization, polycondensation or polymer analogous reactions, sequences and functional groups of the polymers can be systematically varied. In this way the properties can be adjusted e.g. from hydrophobic to hydrophilic.

In general, there are three main principles for degradable or removable materials (Fig. 4.2). Principle a demonstrates the degradation of crosslinks between mostly hydrophilic (water soluble) polymer chains to restore their solubility and ability to be removed from the implantation site. Principle b shows the transformation of a former hydrophobic side chain in a polymer into a hydrophilic group and makes the polymer water soluble and excretable via the renal or hepatic system. Principle c is used in the majority of cases for degradable polymers. The covalent bonds in the functional groups of the polymer backbone are degraded by hydrolysis or enzymatic degradation and finally yield monomers, which can be metabolized.

Degradable biomaterials must include linkages, cleavable under physiological conditions (see Fig. 4.2). One possibility is the incorporation of hydrolytically degradable bonds (see Fig. 4.3).

Hydrolytic degradation has the advantage that water is generally available in the body. Therefore degradation should occur at different locations of application / implantation. In contrast, concentrations of enzymes can differ locally. As the degradation rate of hydrolytically cleavable bonds can be increased by enzymes substantially, the degradation rate can differ significantly in different body parts or individuals. Chemical bonds whose cleavage is accelerated by enzymes can be used

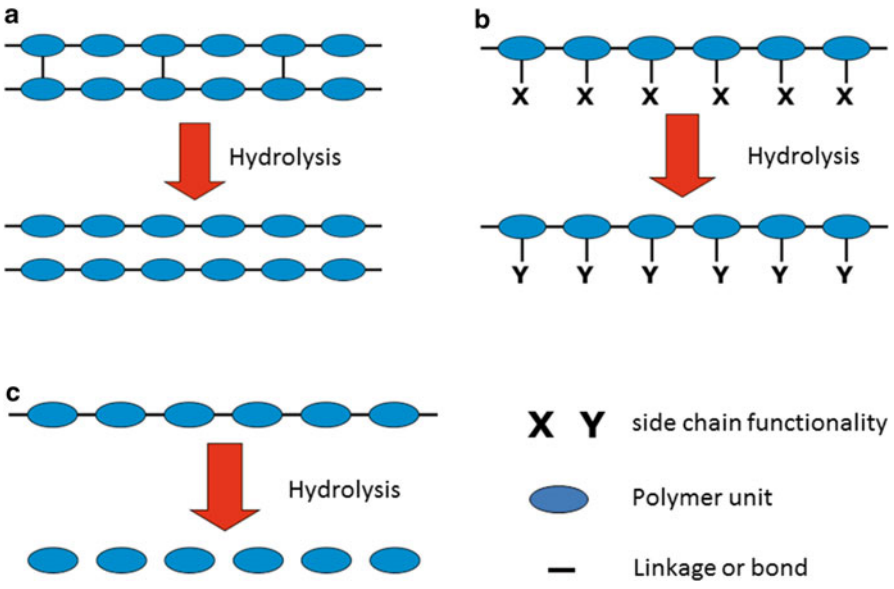


Fig. 4.2 Three general principles for hydrolytic degradation of polymers. *Blue circles* represent a repeating unit, *black lines* a covalent connection between the repeating units (**a**) Cross-linked material, where cross-links are degraded, (**b**) Transformation of side chains by hydrolytic or enzymatic degradation, (**c**) Degradation of the polymer backbone

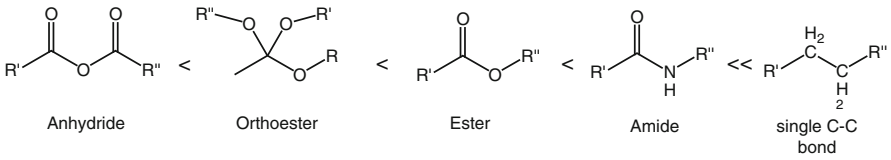


Fig. 4.3 Hydrolytically cleavable bonds in comparison to carbon-carbon bond in order of their hydrolytic stability

for the generation of local effects, such as the specific targeting of drugs or organ-specific processes. In general, the advantage of devices made from degradable polymers is that a second surgery for explantation can be avoided.

Two mechanisms for the hydrolytic biodegradation of polymers are discussed (i) bulk degradation and (ii) surface erosion. In case of bulk degradation the diffusion of water into a polymer matrix is faster than the hydrolysis rate. The hydrolytically cleavable bonds in the amorphous parts of the matrix can be degraded as water molecules are available because of fast diffusion (Brannon-Peppas 1997). Therefore, the average molecular weight of the matrix polymers decreases. In case of surface erosion the diffusion of water into the polymer is (much) slower than the degradation rate of the macromolecule. Hydrolysis is limited to a thin layer on the surface, while the molecular weight of the polymer in the bulk remains unchanged. In surface erosion the velocity of degradation depends on the shape of the sample. The higher the surface area is, the higher is the rate of degradation. The number of hydrolytically cleavable bonds in a macromolecule affects the hydrolysis rate (see Fig. 4.3).

Macromolecules containing orthoester or anhydride bonds as examples for easily hydrolysable bonds show a high tendency for surface erosion (Wu 1995). Many other parameters related to the polymer, the device (shaped body) or the environmental conditions can influence or determine the degradation behavior of polymers (see Table 4.1).

The biodegradability of polymers can be determined in vitro and in vivo. For in vitro experiments, the materials are exposed to an aqueous (buffer) solution which may contain ions or to cell culture medium, which may contain amino acids, sugar as well as serum. PH-value and temperature can be varied to mimic specific situations and environments. The partially degraded materials as well as the degradation products can be isolated and characterized. The addition of specific enzymes is also possible (Kulkarni et al. 2007). In vivo experiments are performed with different species e.g. mice or rats to investigate the biodegradation and biocompatibility.

Standards exist for biocompatibility testing of materials used in the human body. The American Food and Drug Administration (FDA), the Health and Welfare Canada, and Health and Social Services UK introduced 1986 the “Tripartite Biocompatibility Guidance for Medical Devices”. The guidance was developed to help FDA reviewers, but also manufacturers of medical devices, in judging and

Table 4.1 Parameters influencing the degradation rate of polymers

Structural parameters of macromolecules	Shaped body (device)	Environmental influence
Chemical composition	Processing conditions	Location of implantation
Sequence structure in copolymers	Shape of the sample	Adsorbed or absorbed molecules
Presence of ionic groups	Sterilization	Ion exchange, –strength, pH-value
Branches/chain defects	Thermomechanical "history" of the polymer	Changes of diffusion coefficient
Average molecular weight and distribution	Material inhomogenities and internal stress	Mechanism of hydrolytic degradation (H_2O, Enzymes)
	Surface roughness	Cracks due to hydrolytic degradation or mechanical tension

selecting appropriate tests to evaluate the biological responses to medical devices. Four different device categories of biomaterials were defined: Non-Contact Devices, External Devices, Externally Communicating Devices, Internal Devices. The biological test of the materials include: Sensitization Assay, Irritation Tests, Cytotoxicity Tests, Acute Systemic Toxicity Tests, Hemocompatibility Tests, Hemolysis Tests, Implantation Tests, Mutagenicity (Genotoxicity) Tests, Chronic Toxicity Tests, Carcinogenesis Bioassay, Pharmacokinetics, Reproductive and Developmental Toxicity Tests. To harmonize the biocompatibility testing, the International Standards Organization (ISO) developed a standard for biological evaluation of medical devices (ISO 10993). Until today, this standard consists of 20 parts and the first part "Biological Evaluation of Medical Devices: Part 1: Evaluation and Testing" provides guidance for selecting the tests to evaluate the biological response to medical devices. The appropriate methods to conduct the biological tests are described in most of the other parts. The ISO 10993 is under permanent actualization and covers aspects of biomaterial testing.

4.2 Polymer-Based Biomaterials

Biodegradable polymers can be divided in two main groups: materials based on natural polymers, and purely synthetic polymers, designed to meet different demands. Important groups of degradable polymers used in medical applications are: Polyesters, Polyesteramides, Poly(ortho ester)s, Polyurethanes, Polyanhydrides, Cyanoacrylates, Hydrogels (e.g. based on poly(ethylene glycol)). Carbohydrates and proteins form the basis for many biomaterials based on natural polymers. Synthesis or isolation and exemplary applications of such materials are presented in the following.

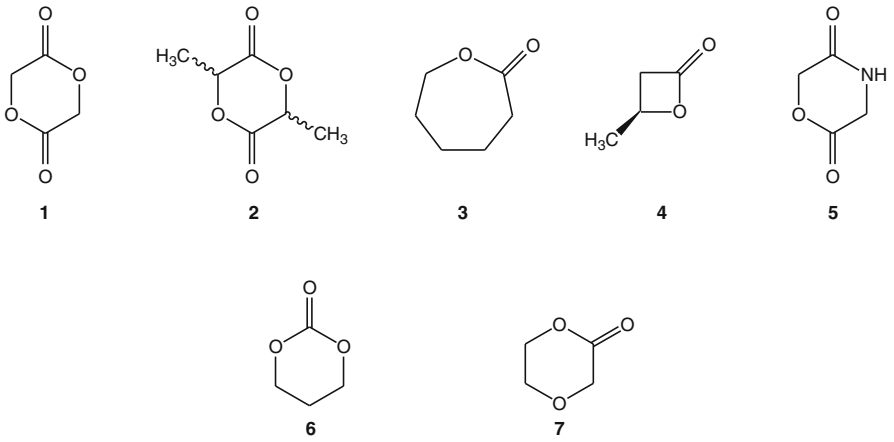


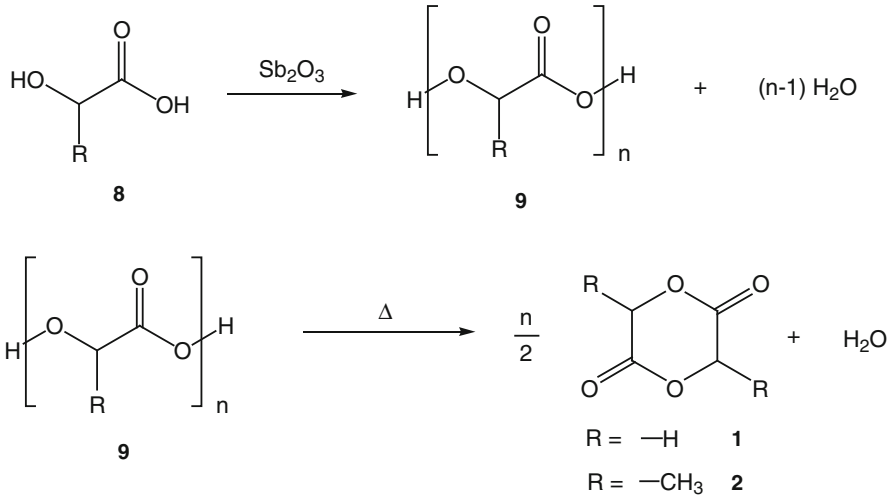
Fig. 4.4 Chemical structures of cyclic diesters and lactones used as (co)monomers for the synthesis of degradable (co)poly(ether)esters

4.2.1 Polyesters

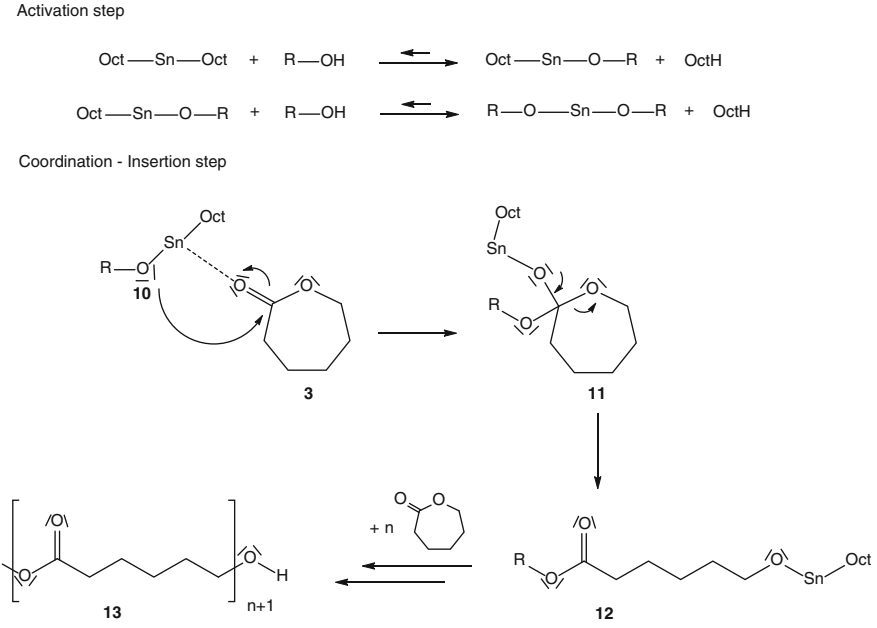
An important group of biodegradable biomaterials are (co)-polyesters used in surgical sutures. The degradation of ester bonds occurs under hydrolysis of the bond, forming a carboxylic acid and an alcohol. The rate of hydrolysis depends on the neighboring groups to the ester. They are typically prepared by ring-opening polymerization of lactones or cyclic diesters (Deasy et al. 1989; Piskin 1995; Vert 1986). A ring-opening polymerization proceeds in an anionic, cationic or coordination polymerization mechanism in the presence of catalysts and is started by initiators. Monomers like the cyclic diesters digylolide **1**, and dilactide **2**, as well as the lactones ε-caprolactone **3**, and β-butyrolactone **4** are frequently used. Further cyclic compounds, which can be polymerized in an analogue way, are cyclic carbonates (e.g. trimetylene carbonate **6**) dioxanone-compounds (e.g. *p*-dioxanone **7**), and compounds based on morpholino-2,5-dione **5** (see Fig. 4.4). Due to two stereo centres in dilactide **2** three different isomers exist: *L,L*-dilactide, *D,D*-dilactide and *meso*-dilactide.

Cyclic diesters are generated from the corresponding hydroxyl carboxylic acids (Scheme 4.1). Oligoesters are formed by elimination of water in the presence of catalysts (e.g. Sb_2O_3). In the case of poly(*L*-lactic acid) molecular weights of >100 kDa can be obtained by a solution condensation process with water removal by application of vacuum or by azeotropic distillation using diphenyl ether (Ajioka et al. 1995; Södergård and Stolt 2010).

The ring-opening polymerization of cyclic diesters can be performed as anionic polymerization or as coordination-insertion polymerization (Scheme 4.2). Sn (II) compounds like Sn-dioctanoate are used as coordinative catalysts for the bulk polymerization (Leenslag and Pennings 1987). It has to be considered that such compounds catalyze transesterification reactions as well. Therefore side reactions



Scheme 4.1 Synthesis of cyclic diesters by depolymerization of oligoesters at elevated temperatures



Scheme 4.2 Mechanism of coordination insertion polymerization of ε-caprolactone

like inter- and intramolecular transesterification as well as depolymerization may occur. Heating of a mixture of two polyesters at 140 °C will change the sequence structure of both polymers (Kricheldorf and Serra 1985; Nieuwenhuis 1992). As alternative catalysts Zn (II) ethylhexanoate (Leenslag et al. 1984), and Zn-powder (Chabot et al. 1983) have been studied. The application of these catalysts leads to polyesters of high molecular weight. Catalysts based on Magnesium and other metals are under development with the aim to decrease toxicity of the catalyst or facilitate its removal from the reaction mixture (Kricheldorf and Stricker 2000). According to the actual literature the catalyst is first activated by the nucleophilic initiator and coordinates in the following to the carbonyl function of the lactone (Masutani and Kimura 2015). Several active catalytic species have been discovered depending on the conditions during polymerization reaction.

In addition to bulk polymerization, polymerization processes in solution and in suspension are possible. The lower viscosity of the reaction mixture compared to a bulk process enables a better heat transfer and by that a better control of the reaction temperature.

Homopolymers like poly(glycolic acid) (PGA), poly(*L*-lactic acid) (PLLA), or poly(*D,L*-lactic acid) (PDLLA) as well as copolymers of them with different amounts such as poly[(*rac*-lactid)-*co*-glycolide] (PLGA) were prepared by ring-opening polymerization. PGA and PLLA are semicrystalline polymers, whereas PDLLA is amorphous. PLGA has a glass transition temperature (T_g) close to body temperature ($T_g = 36$ °C), whereas PLLA and PDLLA have T_g values between 57–60 °C and 50–54 °C, respectively (Vert 1989). PLLA has been studied as degradable biomaterial extensively (Tsuji et al. 2003; Ye et al. 2008).

The discovery of so called stereocomplexes of poly(L-lactide) with its enantiomeric counterpart poly(D-lactide) in polymeric structures enables a better thermoplastic processing by increasing the melting point and glass transition temperature, higher degree of crystallinity, and increased resistance towards hydrolytic degradation (Ikada et al. 1987). The enantiomers are forming strong complementary interactions between the chain structures; they build crystallites in a 3_1 helix form. This results in stereocomplex crystallites which will act as cross-links. The melting temperature of PLLA can be increased by 40–50 °C by physically blending the polymer with PDLA due to stereocomplex formation. The temperature stability is maximised when a 1:1 blend is used, but even lower concentrations of 3–10 wt% PDLA improve the properties substantially (Bao et al. 2012; Tsuji et al. 2012). Applications of the polymer e.g. in 3 D – printing applications with high resolution of PLA-based scaffolds become possible (Serra et al. 2013). Due to the higher crystallinity the hydrolytic resistance of the polymer is higher (Tsuji 2003). The 3D – printing or rapid prototyping technology developed into a tool in the field of biomaterials that enables fabrication of three dimensional structures with well-defined and reproducible geometries and architectures. The use of degradable polymers in electrospinning for tissue engineering applications (Pham et al. 2006) and membranes (Ahmed et al. 2015) is a further improvement for processing and tailoring polymers for biomedical application. The degradation speed of electrospun fibres can be controlled by their crystallinity between 23 and 46 % via their spinning parameters (Ero-Phillips et al. 2012).

Poly(ε-caprolactone) (PCL), prepared from ε-caprolactone **3**, is a semi-crystalline degradable polymer with sufficient mechanical strength and thermal stability for application as scaffold material or matrix material for drug delivery. Its melting point is in the range of 59–64 °C and its T_g is around −60 °C. The homopolymer is slowly degradable, due to high hydrophobicity and relatively low hydrolysis rate (Little et al. 2009).

Poly(p-dioxanone) (PPDO), synthesized from *p*-dioxanone **7** (Fig. 4.4), is a semi-crystalline degradable polymer with a melting point of 115 °C and a T_g in the range of −10 – 0 °C. Above its melting point this polymer depolymerizes to *p*-dioxanone **7** (Shalaby and Johnson 1994).

Several medical devices based on (co)polyesters are applied in the clinic. The main products made out of polyester are sutures, orthopaedic implants and scaffolds (Nair and Laurencin 2007; Weigel et al. 2006). The FDA approved a suture called DEXON®, based on polyglycolide (Table 4.2) in 1969.

(Co)polyesters degrade mainly by bulk erosion. Due to water uptake random scission of polymer chains occurs in the amorphous domains. Oligomers and hydroxy acids are obtained as water soluble degradation products. The generated carboxy groups may induce an autocatalytic process.

Despite the good results of the resorbable suture materials, concerns exist regarding the use of (co)polyesters in ligament reconstruction surgery. A review article published in 2009 by Konan and Haddad summarized adverse reactions due to the use of resorbable screws in anterior cruciate ligament reconstruction surgery (Konan and Haddad 2009). They concluded that the resorbable materials offer advantages compared to metal screws, but also possible disadvantages, such as potential adverse biological responses resulting in the worst scenario, in a failure of the surgery. Further long term studies and the improvement of the material are necessary.

If polyesters are used as matrix material for drug delivery the bulk erosion must also be considered (Li and Jastri 2006). The water uptake into the bulk material and the acidification might potentially interact with the drug. Several drug releasing implants have been developed (Table 4.2).

Injectable local drug delivery systems were developed based on in situ forming implants. For this method a biodegradable, water insoluble polymer and the drug are dissolved in a non-toxic organic solvent. After injection the solvent dissipates into the tissue and water permeates into the polymer solution resulting in a precipitation and consequently the polymer forms an implant with the enclosed drug (Li and Jastri 2006). Atrigel uses this principle and two products are FDA approved: Eligard® (leuprolide acetate for injectable suspension) as a prostate cancer product that provides systemic release of leuprolide acetate for 1–4 month, and Atridox® (8.5 % doxycyline) for localized subgingival delivery of doxycycline.

A just recently published meta-analysis including over 18,000 patients showed better results regarding very late in-stent late loss and stent thrombosis when using degradable polymer drug-eluting stents compared to permanent polymer drug-eluting stents. No difference between the drug-eluting stents was seen for target-lesion revascularization, myocardial infarction or death (Wang et al. 2014).

Table 4.2 Examples for devices – approved or in clinical trials – made of polyester

Implant material	Polymer	Name	Application
Sutures	PGA	Dexon®	All kinds of sutures (Gunatillake et al. 2006)
	PLGA	Vicryl®, Polysorb®	
	PDO	PDS®	
	PGATMC	Maxon®	
	PHA	TephaFLEX®	(Shrivastav et al. 2013)
Screws	PLLA	Absolute®	ACL reconstruction (Purcell et al. 2004)
	ß-TCP/PLA	Biocryl®, Intrafix®	
	PLLA	Sheathed Femora® BioScrew® Bio-Cortical® Bioabsorbable Wedge®	
	PLA	BioRCI®	
	PLLA/PGA	Gentle Threads®	
Plates and screws	PLGA	RapidSorb®	Craniomaxillofacial surgery (Peltoniemi et al. 2002; Pietrzak and Eppley 2000)
	SR-PLDLA	Biosorb®	
	Copolymers of L-lactide, D,L-lactide, glycolide and TMC	Inion CPS® Fixation	
Skin and cartilage	PLGA (Vicryl mesh)	Dermagraft®	Artificial skin
		NeoCyte®	Engineered cartilage (Ueda and Tabata 2003)
Anastomotic ring	PGA and barium sulphate	Valtrac®	Anastomoses in the gastrointestinal tract surgery (Kaidar-Person et al. 2008)
Membranes/ meshes	PLGA	ResoIut® Vicryl Mesh®	Guided tissue regeneration, wound support
	PHA	TephaFLEX® Absorbable Monofilament Mesh, Surgical Film	(Greenstein and Caton 1993; Wolff and Mullally 2000)
Facial surgery	PLLA	Sculptura®	Correction of facial fat loss
Nerve conduits	PGA	Neurotube®	(Meek and Coert 2008)
	PDLACL	Neurolac®	

(continued)

Table 4.2 (continued)

Implant material	Polymer	Name	Application
Stents	PLLA	Igaki-Tamai® stent	Peripheral artery (Commandeur et al. 2006)
Drug delivery:			
Drug eluting stents	PLLA plus PDLLA coating and Everolimus	BVS everolimus-eluting stent	Coronary artery disease (Ormiston and Serruys 2009)
	Metal stent with degradable coating releasing specific drug	Axxess BioMatrix® Cardiomind ELIXIR JACTAX MAHOROBA NEVO OSIRO® Supralimus SYNERGYTM	Coronary artery disease or acute coronary syndromes (Commandeur et al. 2006; Sun et al. 2014)
Drug delivery	PLGA	LUPRON DEPOT® Eligard® Zoladex® Decapeptyl® Telstar® Pamorelin® Profact Depot®	Release of gonadotropin releasing hormone agonist or goserelin acetate, benign gynaecological disorders; prostate/breast cancer (Sinha and Trehan 2003)
		Posurdex®	Release of dexamethasone, retinal vein occlusion (Kuppermann et al. 2007)
		Risperdal Consta®	Schizophrenia
		Sandostatin® LAR®	Acromegaly
		Bydureon®	Type 2 Diabetes
	PLGA plus triclosan	Vicryl®plus	Antimicrobial sutures (Leaper et al. 2011)
	Titanium coated with PDLLA and Gentamicin	Expert Tibial Nail PROtect	Prevention of bacterial colonization on the implant (Schmidmaier et al. 2006)

PGA poly(glycolic acid), *PLA* poly(lactide acid), *PLLA* poly(L-lactide acid), *PDLLA* poly(D,L-lactic acid), *PLGA* poly[(rac-lactid)-co-glycolide], *PCL* poly(ε-caprolactone), *PDLACL* Poly[(rac-lactide)-*co*-(ε-caprolactone)], *TMC* trimethylene carbonate, *PDO* polydioxanone, *β-TCP* beta-tricalciumphosphate, *PGATMC* poly(glycolide-co-trimethylcarbonate)

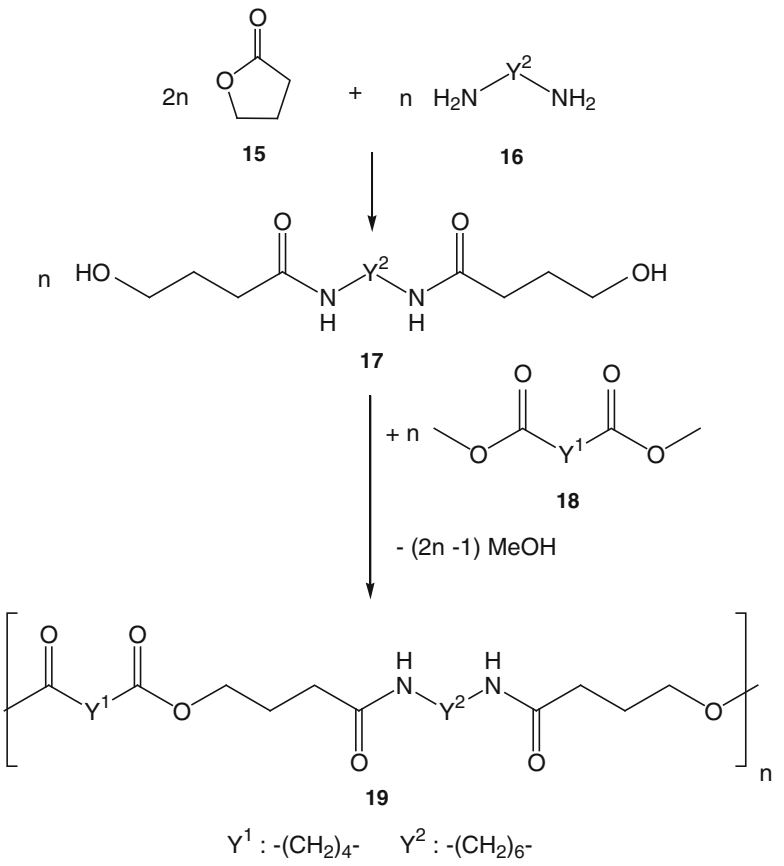
A multi-center randomized controlled trial in maxillofacial surgery showed disappointing results on the use of degradable implants. In contrast to the expectations, the 2-year follow up of 230 patients either treated with a titanium or a degradable plate showed a higher rate of plate removal in the group treated with the degradable plate comparted to the titanium implant (van Bakelen et al. 2013). Therefore, further research is necessary towards a higher biofunctionality of degradable implants.

Very successful are degradable synthetic suture materials, which are in clinical use since the early 1970s (Dexon®) with a huge market exceeding 1.3 billion dollar annually (Pillai and Sharma 2010). In general, degradable suture materials should be easy to handle, evoke only minimal tissue reaction, must not support bacterial growth, degrade after serving its function, and have an appropriate mechanical strength also during degradation. A challenge in the development of suture materials is tailoring of the degradation time period and the change of the mechanical strength as well as suture elasticity during degradation, which needs to be adjusted to the clinical needs. Newly developments of suture materials are focusing on self-knotting actions by usage of shape-memory polymers (Lendlein and Kelch 2002) and bioactive materials, which have e.g. an antimicrobial activity (Vicryl Plus, approved material) or inhibit matrix degradation (Pasternak et al. 2008).

4.2.2 Poly(Ester Amide)s

Poly(ester amides) (PEAs) can be prepared by different synthetic routes, which yield polymers with segmented, statistical distribution of chain segments. A random copolymer can be derived from 1,4-butanediol, adipic acid and ε-caprolactame (Grigat et al. 1998). The mechanical properties of segmented PEAs (and also of polyurethanes) are interesting because of the microphase separation of their hard and soft segments. In PEA soft domains were formed by the ester-rich domains, and hard domains are formed by the amide-rich domains acting as physical crosslinkers determining the shape of a sample body. A segmented PEA could be synthesized by reaction of an alternating ester-amide oligomer, obtained from the reaction of adipic acid with a bisamide diol derived from 1,6-diaminohexane (**16** with Y^2=C6) and γ-butyrolactone (**15**), with an oligoester prepared from 1,2-ethanediol and dimethyl adipate (see Scheme 4.3) (Bera and Jedlinski 1993).

There are four types of biodegradable PEAs: (a) Polydepsipeptides, which combine properties of poly(α-hydroxy acids) and poly(α-amino acids). These polymers can be prepared by ring-opening polymerization of morpholine-2,5-diones (see **5** in Fig. 4.4) (Feng and Guo 2009). (b) Derivatives of α-hydroxy acids obtained by reaction of an acid dichloride with a bisamide diol prepared from glycolic acid and diaminoalkanes. The polymers showed promising results in mechanical properties, degradability, and biocompatibility (Horton et al. 1988); (c) Derivatives from α-amino acids: Poly(ester amides) containing α-amino acid units have been developed and extensively studied (Guo and Chu 2007). These polymers can be obtained by polymerization of an acid dichloride and the p-toluenesulfonic salt of a bis (α-amino acid) α,ω-alkylene diester (Paredes et al. 1998). This polymer type has the



Scheme 4.3 Synthesis of a PEA

disadvantage of relatively high production costs, insolubility in common organic solvents, and thermal instability (Vera et al. 2006); (d) Polymers made from carbohydrate derivatives: carbohydrates like arabinose, xylose and tartaric acid have been used for the formation of polymers by reaction with amines and esters and their degradation properties were investigated (Martinez et al. 1997). The degradation is strongly influenced by the chain microstructure of the resulting polymer. The ester moieties degrade faster than the amide moieties, so the degradation rate is adjustable by the amount of ester moieties used.

4.2.3 Poly(ortho ester)s

Poly(ortho ester)s (POEs) were developed for drug delivery applications. Four types of POEs have been developed, which are shown in Fig. 4.5.

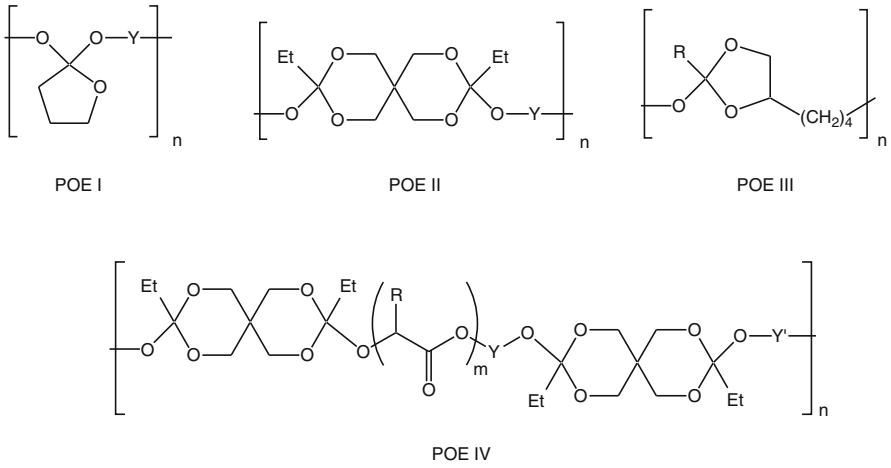
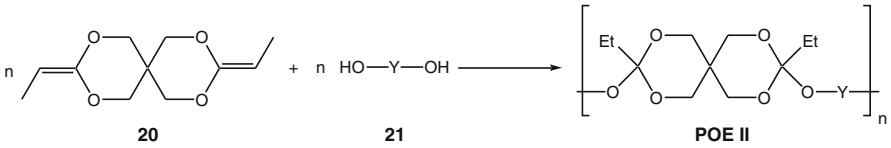


Fig. 4.5 Chemical structures of the four types of poly(ortho ester)s



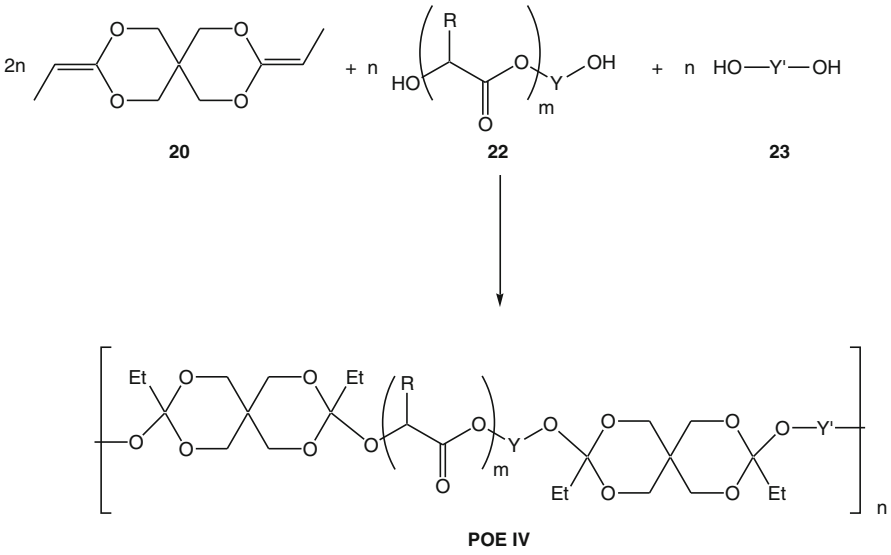
Scheme 4.4 Synthesis of POE type II

POE II is prepared by reaction of diketene acetal **20** (DETOSU) with an appropriate diol **21** (see Scheme 4.4) (Heller et al. 1992). DETOSU was synthesized from the corresponding ketene acetals (Crivello et al. 1996).

This type of poly(ortho ester) was investigated as a drug delivery system for ivermectin containing strands to prevent heartworm infestation in dogs using a cross-linked matrix containing a trivalent alcohol as cross-linker (Shih et al. 1993). The degradation behavior was not sufficiently predictable.

POE IV is prepared by the reaction of DETOSU (**20**), a mixture of diols (**23**) and a poly α-hydroxyacid (**22**) as shown in Scheme 4.5 (Ng et al. 1997). The concentration of the α-hydroxyacid segments in the polymer chains controls the degradation rate.

The mechanical properties of the polymers (POE II and IV) can be influenced by using rigid diols such as trans-cyclohexandimethanol and flexible diols like 1,6 hexanediol. The glass transition temperature (T_g) is determined by the ratio of such diols in the polymer (Heller et al. 1983; Heller et al. 1995). For drug delivery systems the drug has to be uniformly distributed over the polymer matrix. POE IV can be processed by melt extrusion at 100 °C without significant change in molecular weight. POE II and IV are soluble in solvents like methylene chloride, ethyl acetate, or THF, enabling formation of microspheres by conventional procedures.



Scheme 4.5 Synthesis of POE type IV

Poly(ortho ester)s are stable when stored under water-free conditions at room temperature and can be sterilized (Heller et al. 2002).

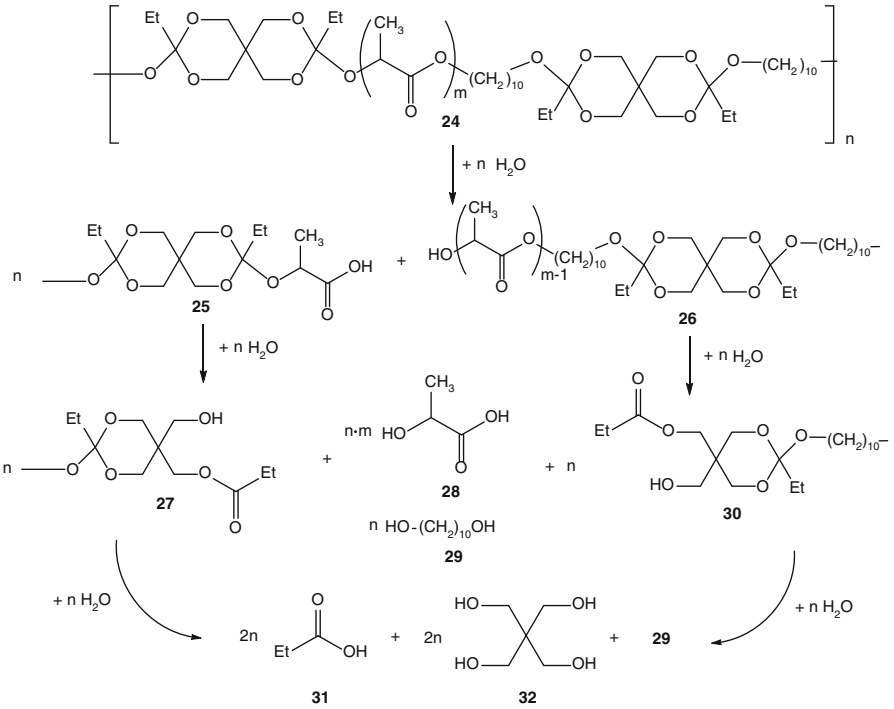
The hydrolysis of the POE IV proceeds in three consecutive steps (see Scheme 4.6).

The weight loss during degradation is linear for POE Type IV. First the ester bonds were cleaved in the polylactide moiety of the polymer (**25**, **26**) and as a second step the orthoester moiety degraded. This resulted finally in the release of lactic acid (**28**), propionic acid (**31**), pentaerytritol (**32**) and decandiol (**29**). The process is predominatly confined to the surface layers of the polymer matrix (surface erosion). Only a small amount of bulk erosion occurs, which is in contrast to the poly(lactide-*co*-glycolide) copolymers or poly(lactic acids) (Vaccaro et al. 2002).

Hydrolytically labile poly(ortho ester amide) (POEA) copolymers were developed to overcome the drawbacks of the traditional methods of POE synthesis by solution polycondensation between an acid labile diamine with a build-in ortho ester bond and fatty diacid esters of different chain-length (Tang et al. 2009).

4.2.4 Polyurethanes

Polyurethanes (PURs) are used for industrial applications since the 1940s, but development of biocompatible polymers based on urethanes started in the 1960s. These polymers are often used for long-term applications because of their beneficial characteristics like toughness, durability and biostability. Also polyurethanes with a



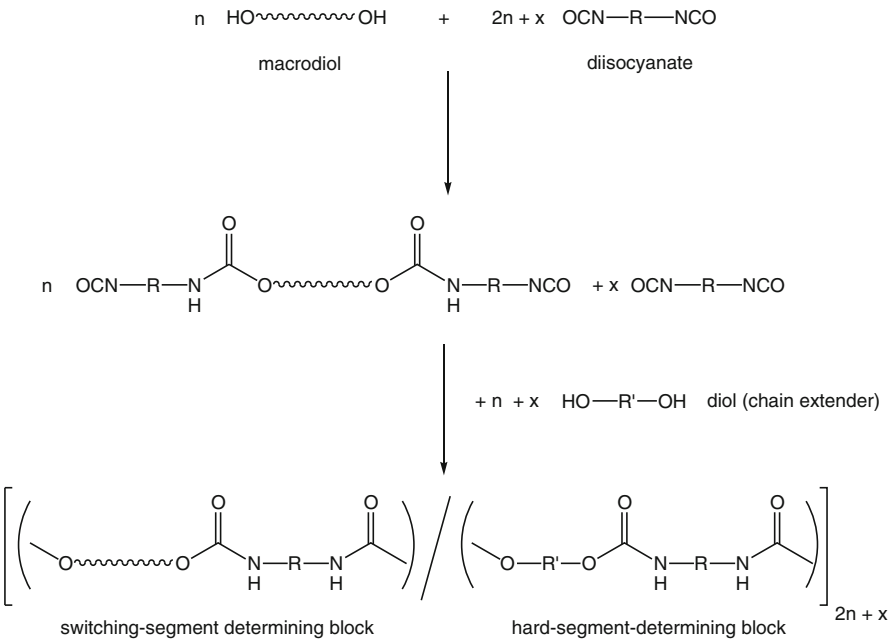
Scheme 4.6 Degradation mechanism of POE type IV in the presence of water

controlled degradation rate have been developed due to a high demand on degradable biomaterials (Lendlein et al. 1998).

PURs are basically synthesized using a diisocyanate, a diol and a chain-extender as main components (Syzcher 1999). In these cases aromatic diisocyanates were substituted by an aliphatic compound such as 1,6-hexamethylene diisocyanate (HDI), 1,4-butylene diisocyanate (BDI), lysine methylester diisocyanate (LDI), or trimethyl hexamethylene diisocyanate (TMDI) (Cardy 1979).

The diol in degradable PURs is commonly an oligomer with hydroxyl end groups, so called macrodiol, with a backbone corresponding to polyester or polycarbonate. Polyester urethanes are the most common degradable polymers of this type. The macrodiols can be prepared by ring-opening polymerization of a cyclic lactone (see under polyester). The reaction between the diol and the isocyanate is carried out with an excess of diisocyanate to obtain a reactive prepolymer with isocyanate end groups. To obtain a thermoplastic PUR with a segmented architecture the prepolymer is further reacted with a chain extender, which is a short chain diol (see Scheme 4.7).

PURs are multi-block copolymers, which show microphase separation. This phase separation comparable to PEAs allows another functionality beside degradability in the materials: these polymers show shape-memory properties. Using poly(ε-caprolactone)diol and poly(p-dioxanone) together with TMDI a degradable



Scheme 4.7 Synthesis of a polyurethane using a macrodiol, a diisocyanate and a short chain diol as a chain extender

shape-memory polymer can be generated (Lendlein and Kelch 2002; Lendlein and Langer 2002; Spaans et al. 1998). Shape-memory polymers are materials which can be deformed and fixed in a temporary shape, from which they recover their original shape only when exposed to an appropriate stimulus (Behl and Lendlein 2007). They show at least two separated phases. The phase with the highest thermal transition acts as a physical cross-link and is responsible for the so called permanent shape of the polymer. A second phase serves as a molecular switch and enables the fixation of a temporary shape. The transition temperature T_{trans} for the fixation of the switching segments can either be a glass transition (T_g) or a melting temperature (T_m). After deforming the material above the switching temperature, the temporary shape can be fixed by cooling the polymer below the switching temperature. Heating up the material above T_{trans} again cleaves the physical cross-links in the switching phase. As a result of its entropy elasticity the material is forced back to its permanent shape. Potential applications are intelligent degradable sutures and degradable shape-memory-stents. Degradation is controlled by the amount of degradable bonds (e.g. ester bonds) in the used macrodiols for synthesis.

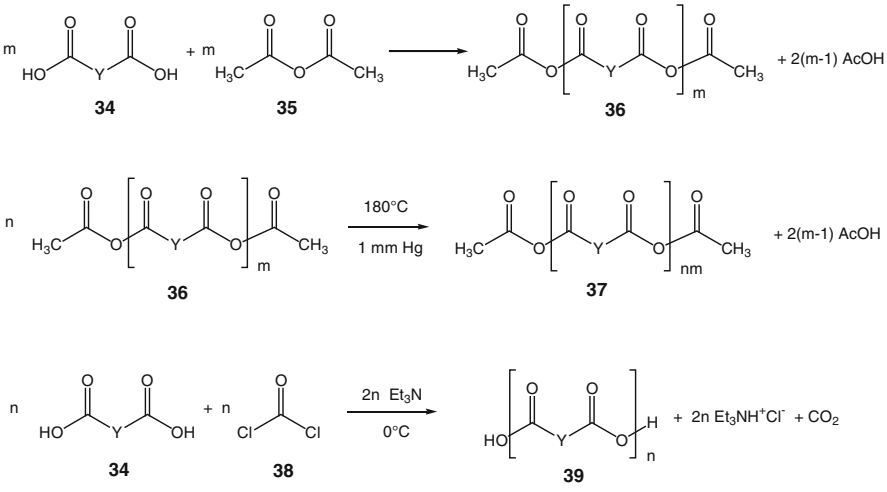
Degradable PURs are of interest in the design of scaffolds for in vivo tissue engineering as well as for cardiovascular applications. Elastic materials are required for soft tissue engineering due to mechanical conditions during the development of the new tissue. For cardiovascular tissue engineering the material should have sufficient elasticity and high tensile strength.

4.2.5 *Polyanhydrides*

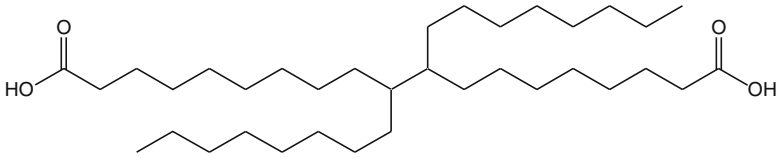
A group of polymers showing surface erosion are the polyanhydrides (Bucher and Slade 1909; Carothers and Hill 1932; Domb et al. 1994; Laurencin et al. 1995). Since beginning of the 1980s polyanhydrides are developed for biomedical applications. The easily cleavable anhydride bond is introduced into a hydrophobic polymer, such as aliphatic long chain diacids (such as **34**). For variation of the mechanical properties by adjusting the crystallinity, sebacinic acid is often used as a comonomer.

Aliphatic diacids can be polycondensated to polyanhydrides by reaction with acetic acid anhydride (**35**, Scheme 4.8). The reaction proceeds in two steps. First, oligomeric polyanhydrides with terminal acetate groups are received (**36**), further reaction to a high molecular product occurs at elevated temperatures under vacuum. When glutaric acid (y = C10) and succinic acid instead of sebacic acid are used under the same conditions, they form cyclic compounds. The reaction between dicarboxylic acids and dicarboxylic diacidchlorides results in low molecular weight products. To gain higher molecular weight products phosgene (**38**) is used as condensation agent. Et_3N is used as a proton acceptor and precipitates the evolving hydrochloride.

While polysebacinic acid is semi-crystalline (T_m = 82 °C) the homopolymer of the oleic acid dimer (**40**) is liquid. Copolymers of them are partly crystalline with melting points between 30 °C and 78 °C having average molecular weights between 24.000 and 280.000 g·mol^{-1}. Sebacinic acid (**41**) can be condensed with benzoic acid derivative (**42**) to form the drug delivery matrix Septacin® for curing chronic bone infections (Fig. 4.6, see applications).



Scheme 4.8 Synthetic routes for the synthesis of polyanhydrides



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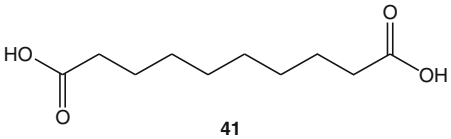

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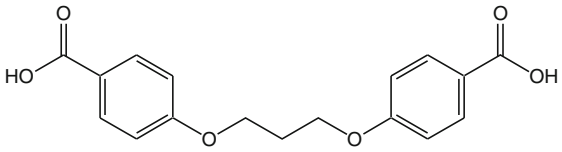

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Fig. 4.6 Different diacids used as monomers in the preparation of polyanhydrides

It is assumed that polyanhydrides degrade by surface erosion mainly driven by two processes: (a) the easily hydrolysable anhydride bonds at the surface, and (b) the restriction of water permeability into the bulk due to hydrophobicity (Jain et al. 2005). These two processes allow a control of the release and a protection of the drug within the bulk material until release. In addition, the release of the drug is timely correlated to the material degradation. The duration of the polymer degradation can be controlled by varying the type of monomer and the comonomer ratio. Various polyanhydrides have been used experimentally as drug delivery systems (Table 4.3). As a localized drug delivery system for chemotherapeutic agents GLIADEL® is used in brain cancer treatment. The first approval in 1996 was for its limited use as an additive therapy in patient with recurrent Glioblastoma multiforme (GBM) for whom surgical resection is indicated. In 2003, the approval was expanded for use of GLIADEL® in patients with newly diagnosed high-grade malignant gliomas, as an adjunct to surgery and radiation. SEPTACIN® is a Gentamicin delivering product for osteomyelitis treatment (Li et al. 2002).

4.2.6 Polycyanoacrylates

Cyanoacrylate is a generic name for fast acting glues based on various cyanoacrylates such as methyl-2-cyanoacrylate, ethyl-2-cyanoacrylate, n-butyl-2-cyanoacrylate, and octyl-2-cyanoacrylate (see **44**, Fig. 4.7). These polymers are suitable for bonding tissue, and have been exploited for the benefit of suture-less surgery.

Table 4.3 Experimentally used polyanhydrides for drug delivery

Delivery System	Polyanhydride	Drug	Disease
Matrix	Ricinoleic acid based	Methotrexate	Cancer
	P(RA- SA)	Cisplatinum	Cancer
	P(FAD-SA)	Cisplatin, 5-FU, methotrexate, paclitaxel	Cancer
	P(FAD-SA)	Bupivacaine HCL	Local anesthesia
	P(OA/LAD-SA)	Gentamicin	Osteomyelits
	P(DDDA-TA)	Ciprofloxacine hydrochloride	Local infection
Implant	P(CPP-SA)	BrdU & N-(phosphonacetyl)-l-aspartic acid; 5-fluorouracil or Camptothecin	Cancer
	P(CPP-SA)	Dibucaine, bupivacaine	Local anaesthesia
	P(CPP-SA)	Etoposide	Glaucoma
	P(FAD-SA)	Taxol	Cancer
	P(EAD-SA)	Heparin	Restenosis
Injectable paste	P(RA-SA)	Paclitaxel	Cancer
Microspheres	Poly(anhydride-esters)	Aminosalicylates	Inflammatory bowel disease
	PLA-PSA-PLA	Triamcinolone	Inflammation
	P(FAD-SA)	GnRHa	Hormone therapy
	SA copolymers	Bethanechol	Alzheimer disease

Taken from Jain et al. Copyright 2005, with permission from Elsevier
P(BA-PA) Poly(brassylic acidpentadecandioic acid), *P(CPP-SA)* Poly[1,3-bis(p-carboxyphenoxy) propane-co-sebacic anhydride], *P(DDDA-TA)* Poly(dodecane dioic acid-*co*-tetradecanedioic acid), *P(EAD-SA)* Poly(erucic acid-*co*-dimersebacic acid), *P(FAD-SA)* Poly(fatty acid dimer-*co*-sebacic acid), *PLA-PSA-PLA* Poly(lactic acid)-poly(sebacic acid)-poly(lactic acid), *P(OA/LAD-SA)* Poly(Oleic acid/linoleic acid dimer-*co*-sebacic acid), *P(RA-SA)* Poly(ricinoleic acid-*co*-sebacic acid), *SA* Sebacic acid

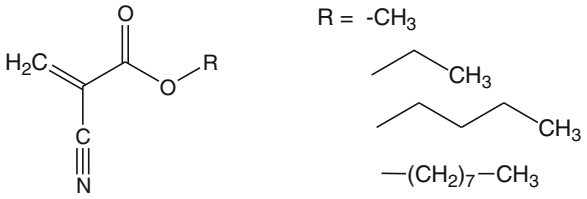

Fig. 4.7 Chemical structure of cyanoacrylates

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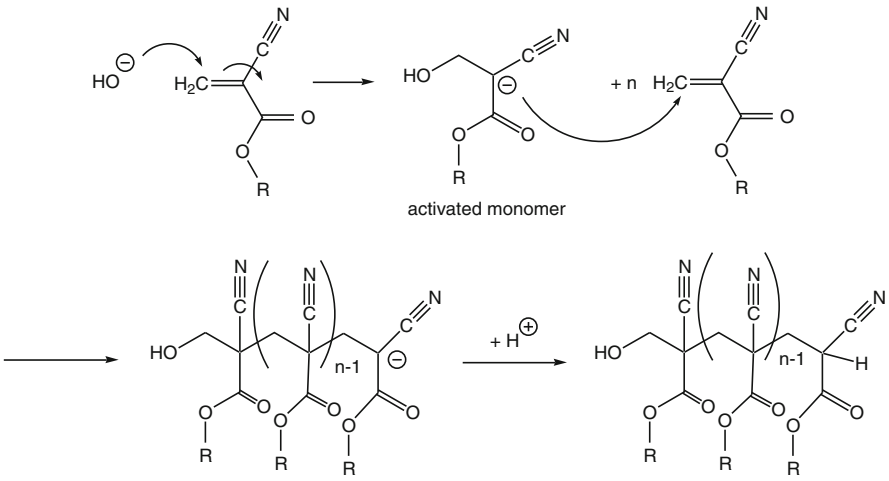
Cyanoacrylates rapidly polymerize in the presence of traces of water (specifically hydroxyl ions), forming polymers with chain length sufficient for the demanded physical properties (see Scheme 4.9). Such polymers are able to join surfaces of different roughness.



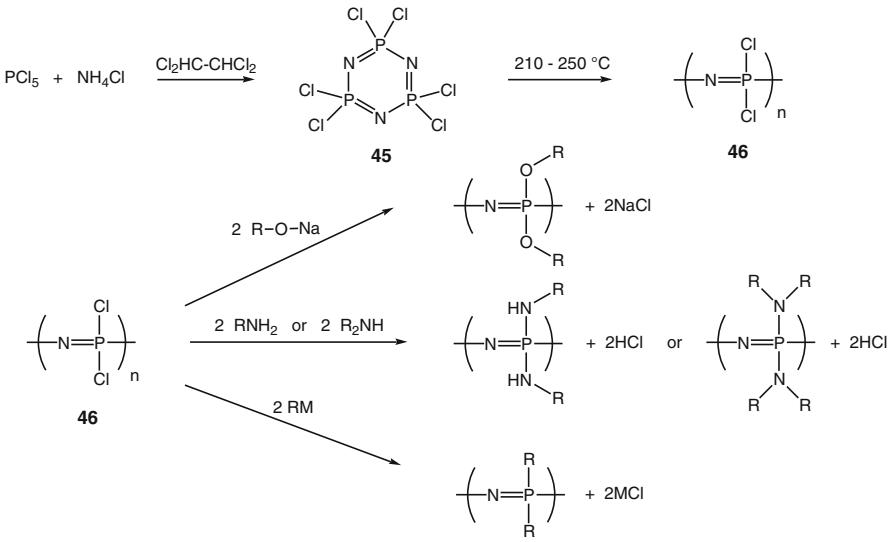
Scheme 4.9 Polymerization mechanism of cyanoacrylates in the presence of water

N-butyl, isobutyl, and octyl ester derivatives of the cyanoacrylates were used In medical and veterinary applications. They are considered bacteriostatic. Polymers made from n-butyl monomers are rigid; octyl ester containing polymers provide more flexible materials. The polymer generated from octyl-2-cyanoacrylate degrades more slowly compared to formulations from shorter alkyl ester chains. Degradation products remain below the threshold of tissue toxicity, if the polymers degrade slowly. The degradation of the cyanoacrylates happens via an unzipping mechanism of the polymer, which proceeds by a retro Knoevenagel reaction after elimination of the hydroxyl group. The ester bonds in the structure can be cleaved under acetic or basic pH (Han et al. 2008).

Cyanoacrylate based products are: Dermabond®, LiquiBand®, SurgiSeal™, or Nexaband® (all 2-octyl cyanoacrylate) and Indermil® and Histoacryl® (both are *n*-butyl-cyanoacrylates). All products are approved for topical use only. They serve as adjuncts to closure of skin incisions and Dermabond® and Indermil® are also indicated as barrier to bacterial skin penetration (Spotnitz and Burks 2008).

4.2.7 Polyphosphazenes

Polyphosphazenes are a class of biocompatible polymers (Andrianov 2009), which are prepared by reaction of phosphorus pentachloride with ammonium chloride in tetrachloroethane forming hexachlorocyclotriphosphazene (**45**) in a first step. After heating to 210–250 °C the chlorine substituted polymer (**46**) forms by thermal ring-opening polymerization (Allen 1981). In a second step the polymer is functionalized by nucleophilic attack on the phosphor (see Scheme 4.10) in solutions with



Scheme 4.10 Synthesis and chemical functionalization of polyphosphazenes

benzene, toluene or tetrahydrofurane. A high variety of functional groups can be introduced such as amines, amino acids, poly(ethylene glycol)s or aliphatic chains. The hydrophilic substituted polymers are able to degrade to phosphate, ammonia and an organic residue depending on the functionalization of the backbone. Phosphate and ammonia create a pH buffer system during degradation.

The properties of the polymers depend on the nature of the side groups. With side groups derived from trifluoroethanol ($-O-CH_2-CF_3$) the polymers show high flexibility and a low glass transition temperature, in this respect the polymers resemble the commercial significant siloxanes.

Polyphosphazenes are explored as degradable scaffolds for bone regeneration in tissue engineering and drug delivery (Lakshmi et al. 2003). Here, the polymers are functionalized with amino acid ethyl esters and can be electrospun to generate a non-woven scaffold. Introduction of carboxylic groups enable ionic cross linking with calcium ions. They are also intended as temporary substrates that accommodate moderate cell infiltration and tissue in-growth in regenerative medicine. The 3D scaffold built from polyphosphazene with glycylglycine dipeptide and 4-phenylphenoxy group as side chains and blended with a polyester (PLAGA) allows the formation of an interconnected porous structure out of a solid coherent film to an assemblage of microspheres (Deng et al. 2010).

The degradation products are aside of phosphate and ammonia, the amino acid ethyl esters. The same type of polyphosphazene can be used as a drug delivery device in the form of nano- or microparticles (Sethuraman et al. 2011). They show a long blood circulating life time and are PEG coated. Furthermore bioerodable blends of the polyphosphazenes with e.g. PLGA have been developed (Krogman et al. 2007).

4.2.8 Hydrogels

Hydrogels are three dimensional networks from hydrophilic polymer chains, which are able to take up high amounts of water under retention of their shape. The networks can be based on physical or covalent cross-links. Potential applications include matrices for cell culturing or drug delivery systems (Hoffman 2002; Peppas 1987). Hydrogels suitable for long term applications are approved as soft contact lenses, made from 2-hydroxyethylmethacrylate and a cross-linker or poly(ethylene glycol) with reactive end groups. Hydrogels can be designed to be stimuli-sensitive by introduction of specific functional groups or segments (Qiu and Park 2001).

Degradability can be established by introduction of degradable blocks like poly(lactic acid) in the main chain of the hydrogel.

Examples for approved products are CoSeal™, Baxter, Fremont, CA (for vascular sealing) and DuraSeal™, Covidien, Waltham, MA (for dural sealing). Both of these sealants are synthetic and form hydrogels that seal tissues. It is indicated as an adjunct to blood vessel hemostasis by mechanically sealing areas of leakage. DuraSeal™ consists of two solutions. The first is a PEG ester and the second contains trilisine amine with a blue dye for visualization. Following normal dura suturing the use of DuraSeal™ allows a true watertight closure.

4.3 Biomaterials Based on Natural Products

Carbohydrates are isolated from different natural sources. Hyaluronic acid, a non-sulfated glycosaminoglycan and one of the main components of the extracellular matrix, was isolated e.g. from cock's combs (Boas 1949), or isolated by gel filtration on agarose together with the protein complex (Barker and Young 1966) and is nowadays isolated from various sources. The repeating unit is a disaccharide composed of D-glucuronic acid and D-N-acetylglucosamine, linked together via alternating β-1,4 and β-1,3 glycosidic bonds (4GlcUAβ1-3GlcNAcβ1) (see Fig. 4.8). Hyaluronic acid is degraded enzymatically by hyaluronidases. In humans, there are at least seven types of hyaluronidase-like enzymes.

In the human body the polysaccharide hyaluronic acid (HA) is found in almost every tissue and half of the total HA content in the body is present in the skin. The main clinical application is the use of esterified HA as a wound dressing (HYAFF®)

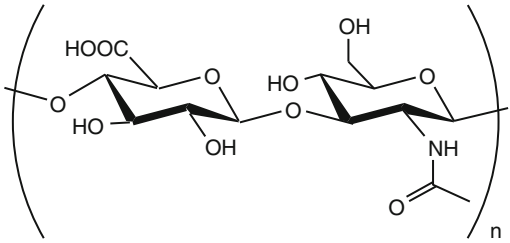


Fig. 4.8 Chemical structure of hyaluronic acid

(Little et al. 2009). In orthopaedic surgery, hyaluronic acid scaffolds are used experimentally and in clinical trials as carrier for stimulating factors and cells (HYAFF®11, HYALOGRAFT C®). Viscous HA is used as a synovial substitute in osteoarthritis patient for pain relief and to improve joint mobility (SYNVISC® ORTHOVISC®).

Chitin and Chitosan are carbohydrates, which are FDA approved as food additives. Two other products are approved as medical device: CHITOSKIN® is a wound dressing and CHITOSTYPE® is used to reduce bleeding.

Polysaccharide spheres are the basis of the absorbable haemostat Vitasure®. Natural haemostasis is enhanced by the spheres that act as hydrophilic molecular sieves concentrating blood solids (platelets, red blood cells, and blood proteins) on the particle surfaces to form a gelled matrix. This gel matrix reduces further blood loss and is formed regardless of the patient's coagulation status.

Alginic acid is a polysaccharide of brown algae. As an alternative bone grafting material, ALGISORB™ is available. After cleaning and manufacturing, the algae is transformed into calcium phosphate a major inorganic component of bone.

4.3.1 Other Natural Materials

Natural materials have been used for centuries and have a broad range of applications in human medicine. In addition to autologous (patient's own) material, allogenic (human donor) and xenogenic (animal donor) transplant material, including various collagen products are currently used (Table 4.4). Furthermore non-animal materials, for example from algae, are also approved for human application (see carbohydrates).

4.3.2 Examples for Approved Natural Materials

Collagen is the main protein of connective tissue and the most abundant protein in mammals (Di Lullo et al. 2002). 29 types of collagen have thus far been identified and described in literature. Over 90 % of the collagens in the body are of so called type I, II, III, and IV. Different methods are used to isolate collagen from tissues (for example see Xiong et al. 2007).

A large number of collagen products are on the marked. Collagen is clinically used, for example as a nerve conduit NeuraGen® and NeuroMatrix™ (bovine) and received FDA approval in 2001 (Purcell et al. 2004).

In combinations with osteoinductive growth factors (BMP-2 or BMP-7), bovine collagen is used as a carrier in the form of a sponge (InductOs®) or as granules with a particle size of 75–425 μm (Osig raft®) (Friedlaender et al. 2001; Govender et al. 2002).

Table 4.4 Overview on tissue grafts

	Source/organ	Application	
Autograft	Bone	Defect filling	
	Mesenchymal stem cells	Various applications	For detailed information see Part II
	Tendon	Tendon repair	
	Skin	Wound repair	
	Cartilage	Cartilage repair	
	Vessels aorta; coronary artery	Vessel replacement	
Allograft	Bone: spinal fusion grafts, cortical and dense cancellous bone, demineralized bone matrix	Defect filling, Spinal fusion, Periodontal Surgery	From tissue banks, processed materials
	Tendon	Tendon repair	
	Split Thickness Skin	Wound repair	
	Acellular dermis	Hernia repair and abdominal wall reconstruction.	
	Liver	Liver transplantation (TX)	Live donations, unprocessed
	Kidney	TX	
	Heart	TX	
	Lung	TX	
	Pancreas	TX	
	Skin, cornea	TX	
	Bone Marrow, stem cells	Leukemia	
Xenograft	Cardiac valve	Heart surgery	Mainly porcine, processed
	Collagen	Various applications	Bovine, porcine, equine, processed
	Bone	Defect filling	Mainly bovine, processed
Mixed	Fibrin human fibrinogen and human thrombin	Tissue sealant	
	Gelatine	Haemostatic	

KOLLAGEN-resorb®, GENTA-COLL resorb®, GentaFleece® and Septocoll® for example have several indications in surgery and are used for hemostasis, as a wound dressing, defect filler, and for bone regeneration. The supplementation of the collagen by adding gentamicin allows for protection against infections.

A product group based on natural porcine small intestinal submucosa (SIS) is marketed under the name Surgisis (SIS®) (Hodde 2006) for the treatment in: congenial diaphragmatic hernias (CDH), colon and rectal surgery, gastroenterology, general surgery, obstetrics & gynaecology, otolaryngology, plastic surgery, thoracic surgery, urology, vascular surgery. Surgisis is an acellularized matrix mesh composed of collagen, proteins, and glycosaminoglycans proteoglycans.

A gelatinous protein mixture secreted by Engelbreth-Holm-Swarm sarcomosa cells (EHS) is commercialized under the name BD Matrigel™. This mixture resembles the complex extra cellular environment found in many tissues and contains laminin, entactin, and collagen. These proteins self-assemble to a structure which enables coating of glassware and 3D scaffolds for tissue engineering (Hughes et al. 2010).

Open porous collagen scaffolds under the name Optimax® are available for a drop in or drop on cell seeding. They are stable in cell culture for several weeks and are used for drug production in bioreactors, cell expansion, and tissue engineering application in preclinical development. Their oriented pore structure in the scaffold enables directed growth of e.g. muscle cells in cell cultures (Kroehne et al. 2008). The pores are generated from controlled freezing of a water suspension of collagen, where the collagen molecules orient on the surface of finger like ice crystals. In the final step the ice is removed by freeze drying.

Remaix® is a resorbable dental barrier membrane for applications in guided bone regeneration (GBR) and guided tissue regeneration (GTR). It is used to cover the space filled with bone graft material. This secluded space assists bone regeneration by protecting the slowly growing bone from infiltration with cells from the surrounding soft tissue. The membrane is composed of a network of purified porcine collagen fibers intermingled with purified porcine elastin fibers, by providing a barrier against migration of unwanted cells from the soft tissue and allows the ingrowth of osteogenic cells in the space of the bone defect.

Several materials of natural origin are available as hemostats to reduce or stop bleeding due to surgery. Sponges or meshes made from porcine gelatine (Gelfoam sponge®, Surgifoam sponge/powder®) or bovine collagen (Avitine sponge/flour®, Helistat® & Helitene®, Instat®) function as a mechanical barrier. To actively stop bleeding, active substances based on thrombin are approved. These materials can be of bovine origin (Thrombin JMI®), made from human thrombin (Evithorm®) or be recombinant (Recothrom®) (Han et al. 2008).

Fibrin is also used as a hemostat and sealant (Tisseel®, Evicel®). It is a combination of plasma fibrinogen and thrombin from human or bovine origin. A dual chamber syringe separates the thrombin and the fibrinogen and after mixture of thrombin with the fibrinogen, a fibrin clot forms. Cryoseal® Fibrin Sealant System is a semi-automated product designed to produce an autologous fibrin sealant during surgery. Vitagel® is a combination product of microfibrillar collagen and thrombin in combination with the autologous plasma (fibrinogen and platelets).

A further very interesting biomaterial is spider silk. The product (SERI®) is approved as a surgical scaffold for plastic, reconstructive and supportive treatment of soft tissue. An interim report from 2015 documents the first results of a prospective single-arm study using SERI® scaffold for breast reconstruction with a high satisfaction of the patients and investigators but no results regarding the performance of the scaffold (Fine et al. 2015).

4.4 Conclusion and Outlook

For each application of degradable polymers a specific set of properties e.g. mechanical properties such as degradability or a certain modulus is demanded. With increasing number of potential applications a greater extend of various materials with different property combinations is required. It is still only a limited amount of materials in clinical application, which are not able to fulfil all the new requirements. Therefore a substantial need of novel degradable biomaterials with tailored properties exists. Additional to the purely synthetic materials, biomimetic approaches are integrated into material design.

Emerging fields of modern medicine, e.g. regenerative medicine require materials with a variety of several functionalities (e.g. shape-memory effect or other stimuli sensitive functions) combined in one material. Hence multifunctional materials are an important research topic. One example is a degradable shape-memory polymer with the additional ability of controlled drug release.

Multifunctional materials will be designed to mimic the microenvironment of cells, e.g. of mesenchymal stem cells, which is of high significance for regenerative therapies.

For a successful development of new materials a solid knowledge of existing applied polymers in clinical use it is necessary to avoid old pitfalls and enable new combinations.

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Chapter 5
Biomaterials-Enabled Regenerative Medicine in Corneal Applications

Naresh Polisetti, Geeta K. Vemuganti, and May Griffith

Abstract The human cornea is the transparent surface of the eye, which serves as the main refractive element of the visual system. Its function depends upon its optical clarity so irreversible loss of transparency due to disease or damage results in permanent vision loss or blindness, necessitating corneal transplantation (keratoplasty) in entirety or in part. While keratoplasty is considered as one of the most successful forms of transplantation, lack of availability of donor tissues and rejection are major limiting factors. Advances in knowledge of biomaterials and stem cell biology have paved the way for tissue engineering of various organs including cornea. An ideal biomimetic for corneal tissue replacement would be the one which is transparent, provides mechanical support, promotes epithelial resurfacing, corneal innervation, and integrates into the surrounding corneo-scleral tissues and combats infection when challenged. This chapter reviews several of the advances made in development of biomaterials for promoting regeneration of the human cornea, with or without exogenous cells.

Keywords Cornea transplantation • Acellular and decellularised scaffolds • Keratoprostheses • Corneal cell sheets • Biomimetic implants

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5.1 Introduction

5.1.1 The Cornea

The cornea is the transparent, dome-shape front surface of the eye, and the main structure that focuses light entering the eye to the retina for vision. Hence, its transparency is critical. It is comprised of five layers, three cellular (epithelium, stroma, and endothelium), and two acellular (Bowman's layer or membrane, and Descemet's membrane) (Fig. 5.1).

The outermost epithelial layer comprises 5–6 layers of stratified, non-keratinizing cells, representing about 10 % of the total corneal thickness. It is responsible for protecting the eye against foreign material, including pathogens, as well as absorbing oxygen and nutrients from the tear film. Corneal integrity and function are dependent upon the self-renewing properties of the corneal epithelium, which is maintained by the presence of stem cells located in the limbus region, at the cornea-conjunctiva interface (Fig. 5.1b). These stem cells, known as limbal stem cells (LSC) or limbal epithelial stem cells (LESC) can divide both symmetrically to self-renew, and asymmetrically to produce daughter transiently amplifying cells (TAC) that migrate centripetally to populate the basal layer of the corneal epithelium (Kinoshita et al. 1981). The TAC divides and migrates superficially, progressively becoming more differentiated, and eventually becoming post-mitotic terminally differentiated cells.

Underlying the epithelium is the stroma. In adults, the stroma is approximately 500 μm thick and is composed of flatterned, interconnected cells called keratocytes embedded in an extracellular matrix (ECM) (Poole et al. 1996) of hydrated type I/V heterotypic collagen fibrils (15 % wet weight) of uniform diameter (32 nm) in humans (Meek and Leonard 1993). Interspersed amongst the collagen fibrils are; glycosoaminoglycans (GAGs) keratin sulfate and dermatan sulfate (1 % wet weight (Anseth 1961); various proteoglycan core proteins (Axelsson and Heinegard 1975); and other protein constituents including fibronectin, laminin, and type VI collagen. The collagen fibrils are packed in 300–500 parallel arrays (lamellae) tangential to the corneal surface (Hamada et al. 1972), that are principally responsible for the tensile mechanical properties of the cornea. The proteoglycans and their associated GAGs contribute to the corneas compressive and swelling material properties (Hedblom 1961), and to the uniform spacing of the collagen fibrils. The stromal cells or keratocytes are relatively quiescent in the healthy, uninjured cornea.

The innermost layer comprises a single layered endothelium, which is essential for the maintenance of appropriate stromal hydration for corneal transparency. Corneal endothelial cells contain Na^+/K^+ ATPase pumps that circulate aqueous humor between the anterior chamber and stroma.

Bowman's layer (BL) is a cell-free, non-regenerating layer located between the epithelial basement membrane and the anterior corneal stroma of human corneas (Kenyon 1983). It is approximately 8–14 μm thick, and is comprised mainly of randomly oriented collagen fibrils (Hogan et al. 1971). It has a smooth anterior

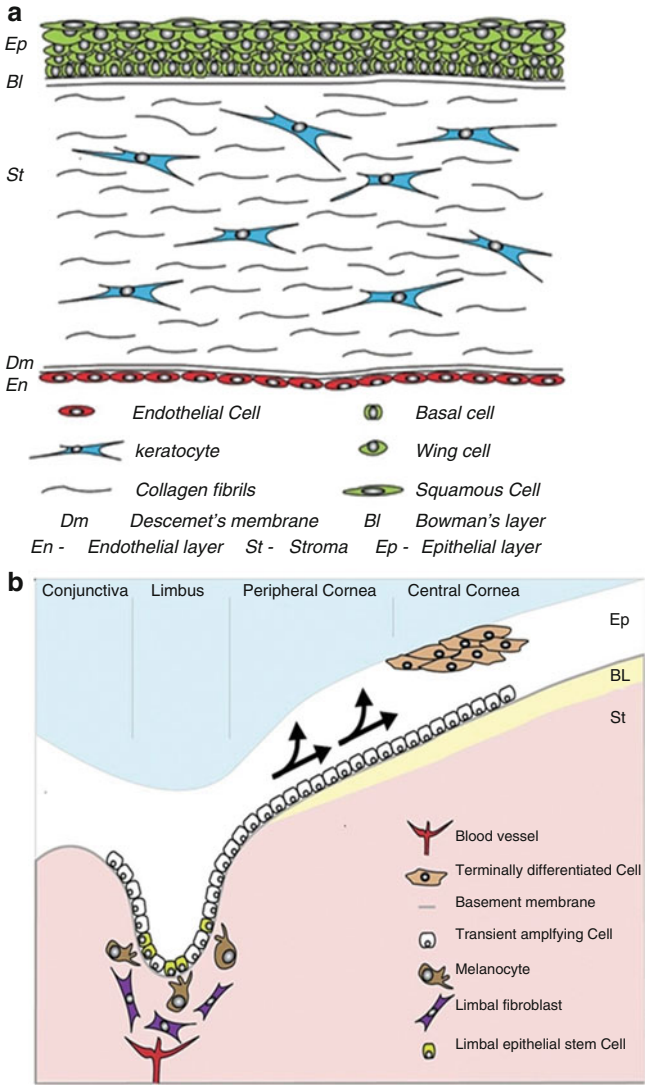

Fig. 5.1 (**a**) The human cornea in cross-section. The outer surface comprises an epithelial layer that rests on a basement membrane, which in turn overlies a cell-free layer: Bowman's membrane. The middle cellular stromal layer contains a mainly collagenous extracellular matrix sparsely populated with keratocytes. The final layer consists of a single sheet of endothelial cells, which is physically separated from the stroma by the acellular Descemet's membrane. (**b**) Diagram of the human limbus. Limbal epithelial stem cells reside in the basal layer of the epithelium (Ep), at the limbus where the peripheral cornea meets the conjunctivum. Daughter transient amplifying cells divide and migrate towards the central cornea (*arrows*) to replenish the epithelium, which rests on Bowman's layer (BL). The stroma (St), of the limbal epithelial stem cell niche, is populated with fibroblasts and melanocytes and also has a blood supply (From: Secker and Daniels 2009)

surface that underlies the epithelial basement membrane, and a posterior surface that merges with the less dense, but ordered, collagen lamellae of the corneal stroma proper (Kenyon 1983). Unmyelinated nerve axons penetrate BL to terminate within the epithelium (Klyce and Beuerman 1988). The functional role of BL is unknown, but it has been suggested that it may be superfluous to human corneal function (Wilson and Hong 2000).

Descemet's membrane is the second acellular layer, and is essentially a thickened basement membrane, with a unique structure that lies between stroma and the endothelial layer of cornea. It contains Type IV collagen and a significant amount of Type VIII collagen.

5.1.2 The Need for Alternatives to Donated Corneas for Transplantation

Diseases affecting the cornea are considered the major cause of blindness all over the world, ranked second only to cataracts in overall importance (Whitcher et al. 2001). Using the World Health Organizations (WHO; Geneva, Switzerland) definition of blindness, it is estimated that number of people with visual impairment is 285 million, with 65 % of individuals aged over 50 years. Of these, 246 million have low vision (63 % over 50 years old) and 39 million are estimated to be blind (82 % over 50 years old). Infectious conditions, trachoma and corneal ulcer, are common causes of vision loss in the developing world, whereas non-infectious entities like corneal dystrophies and pseudophakic bullous keratopathy are more common in developed countries (Cosar et al. 2002; Edwards et al. 2002; Dada et al. 1999; Gupta et al. 2001).

Cornea donation like other solid organ donations suffers from a serious worldwide shortage of donated organs. In developed countries like the UK, the estimated annual need for corneas is approximately 5000, with a shortfall of 1500 (30 % of patients remain untransplanted) (Gaum et al. 2012). The situation is particularly dire in developing countries, where about 200,000 donor corneas per year are needed but only 30–40,000 are collected (Sangwan et al. 2010). However, even if available, donor cornea transplantation is contraindicated or carries a high risk of immune rejection in a number of situations despite advances in anti-inflammatory therapy, surgical technique and post-operative care.[1] Examples of such high risk transplantation cases include corneas with chemical burns, autoimmune diseases, a history of previously rejected grafts and vascularised corneas.

Many corneal diseases are treatable by transplantation with donated corneal tissue, by penetrating keratoplasty, lamellar keratoplasty or endothelial keratoplasty.

[1] Regenerative Medicine in Corneal Applications.

Corneal transplants are not always possible in many parts of the world including developed countries (Muraine et al. 2002) due to limitations in the storage and distribution of corneal tissue, or because of cultural or religious barriers. Even with available donor tissues, the success rate for transplantation beyond the first few years is moderately low. For example, long term survival is about 60 % at 10 years (Coster and Williams 2005). Moreover, the supply of human corneal tissue is expected to diminish further due to aging population and corresponding need for transplantation, and the increasing popularity of refractive surgery such as laser-assisted *in situ* keratomileusis (LASIK), which renders these corneas unacceptable for donation and with the increasing incidence of infectious diseases, including Human Immunodeficient Virus, hepatitis and Herpes Simplex Keratitis (Trinkaus-Randall et al. 2000; Khan et al. 2001).

In addition to the short supply of donor corneas, an additional serious disadvantage of cornea allograft transplantation is the potential possibility for transmission of infection. Hence, all donated corneas are screened at very high costs, as person-to-person transmission of the rabies virus (Houff et al. 1979) and at least one case of Creutzfeldt-Jakob disease (Duffy et al. 1974) have been reported. Even though very rare, another concern is that transmission of as yet unknown pathogens could also occur.

There are also conditions that are not amenable to donor allograft transplantation. These include autoimmune conditions or cases where the ocular surface is badly damaged by disease or injury. For example, pathologies that chronically disrupt the ocular surface mucosa (ocular cicatricial pemphigoid, Stevens-Johnson syndrome, etc) or disrupt tear production (Sjogren's) or injuries (severe chemical or alkali burns) that destroy the limbal stem cell niches (Limbal Stem Cell Deficiency; LSCD) are contraindications for donor grafts because the ocular surface will not re-epithelize properly and is prone to continuous de-epithelization.

Hence, there have been significant efforts in the development of both biomaterials and stem cell based methods and combinations of both, to replace part or the full thickness of damaged or diseased corneas. The best known alternatives to human allograft tissue are the "Artificial Corneas" or keratoprostheses (KPro's). The classical KPro's were developed using plastic-based materials and were designed to restore minimal light transmission and protective functions of the cornea. These devices have now been used clinically but only as last resorts to save corneal vision (Avadhanam and Liu 2014), as they are still associated with *in vivo* complications like retroprosthetic membrane formation, infection and glaucoma. There are a several reviews that cover the traditional KPro's (Myung et al. 2008; Gomaa et al. 2010,, Rafat et al. 2010; Chang et al. 2015, Avadhanam et al. 2014).

There are now numerous approaches for cornea regeneration ranging from decellularisation of corneas (both human and xenogeneic sources) to self-assembled cell based techniques to biomaterials based methods. In this chapter, we focus on a selected number of biomaterials-enabled corneal regeneration technologies that have been developed or are under development.

5.2 Natural Acellular and Decellularised Scaffolds

The concept of using natural acellular and decellularized extracellular matrices (ECM) is a logical one. Acellular human amniotic membranes are known for their anti-inflammatory properties and have now been used as a substrate for expansion of corneal therapeutic stem cells. The ECM scaffold of the target organ which had supported the growth and development of the organ should also be ideal for promoting regeneration. Hence, decellularisation of human and xenogeneic corneas have been gaining in popularity as corneal ECM replacements.

5.2.1 Human Amniotic Membrane

The amniotic membrane is the innermost avascular layer of placenta and protects the embryo during gestation (Shortt et al. 2009). The first application of human amniotic membrane (HAM) in ophthalmology was in the successful treatment of a chemical burn of the ocular surface (de Rotth 1940). After that there are various reports for the ocular use of HAM highlighting novel increasing indications and therapeutic applications. The structural integrity, transparency and elasticity of basement membrane of HAM make it the most widely accepted substrate for ocular surface reconstruction. Essentially, when stripped of its cell population, HAM acts as a decellularized scaffold. Various groups have shown HAM has additional anti-inflammatory, anti-scarring and anti-angiogenic properties (Gomes et al. 2005; Hao et al. 2000; Tseng et al. 1999). HAM also produce growth factors EGF, transforming growth factor α (TGF α), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and TGF-β that can stimulates epithelialization (Tosi et al. 2005; Koizumi et al. 2000). HAM can act as a basement membrane that enables the migration of cells through the presence of laminin isoforms (Dua et al. 2004) and is extensively used in ophthalmic surgery for corneal and conjunctival reconstruction (Gomes et al. 2005; Sangwan et al. 2014; Ramachandran et al. 2014; Nakumura et al. 2011; Satake et al. 2011) (Fig. 5.2).

Despite the successful use of HAM, however, caution is required as the risk of disease transmission through the use of donated human tissue require careful screening, sterilization and preservation, and, thus, is sub-optimal for cell therapy Good Manufacturing Practice (GMP) compliance (Levis et al. 2015).

5.2.2 Decellularised Corneas

The drawbacks of allografts, keratoprostheses, xenografts and tissue engineering corneal constructs have led to the development of human decellularized corneas. In general, detergents and enzymes are used to remove the cells while preserving as

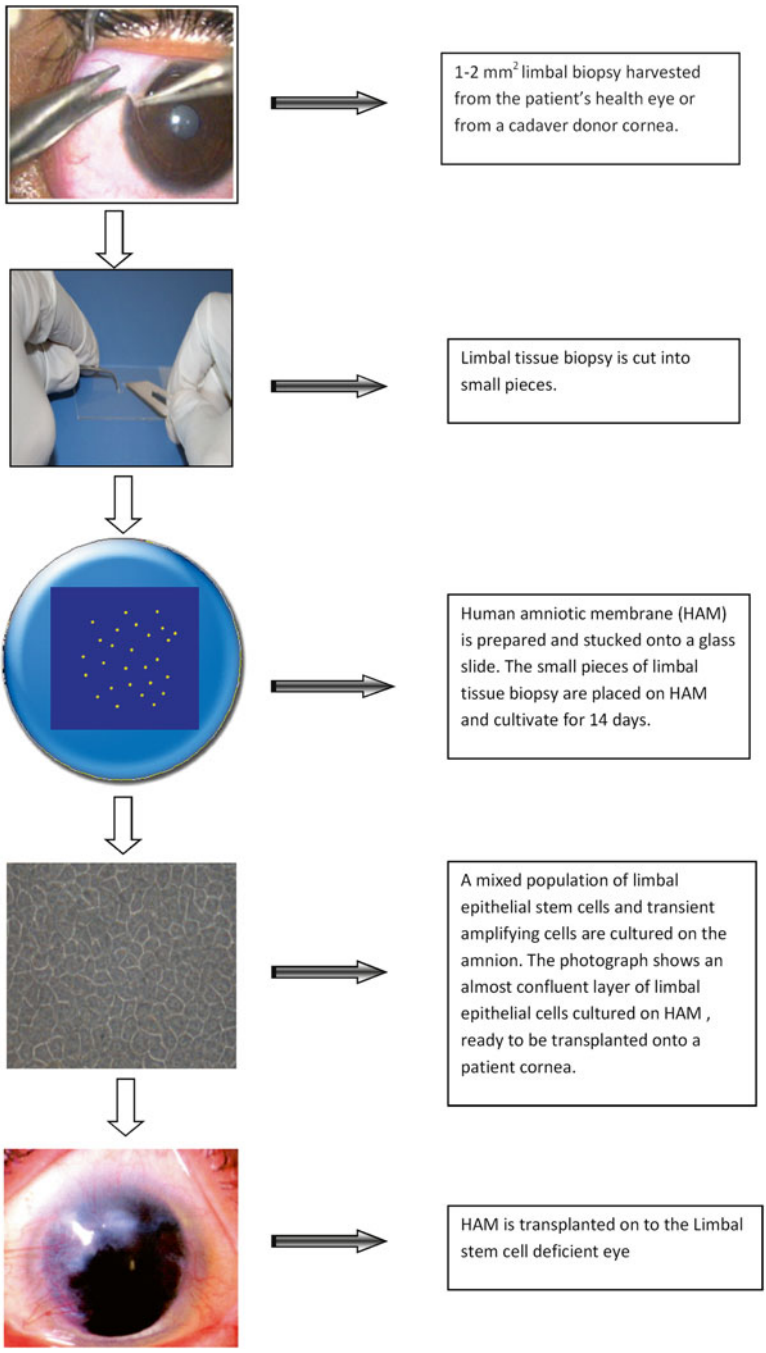

Fig. 5.2 Cornea limbal epithelial stem cells are explanted onto Human Amniotic Membranes for expansion and subsequent transplantation to patients with limbal stem cell deficiency

much of the native ECM found in the native cornea (Ponce Márquez et al. 2009; Choi et al. 2010; Gonzalez-Andrades et al. 2011). To decellularize the cornea and to eliminate the toxic effect of chemicals, Hashimoto and co-workers have used high-hydrostatic pressurization (HHP) to decellularize porcine corneas and later transplanted these into rabbit corneas (Hashimoto et al. 2010). In the transplanted animals, no immune reaction occurred and turbid corneas became clear which showed that cornea obtained through HHP could be useful as a corneal scaffold for tissue regeneration. Lee and co-workers have demonstrated another method for decellularization of porcine cornea by freeze/thaw centrifugation with preservation of the corneal stroma (Lee et al. 2011).

Treatment of porcine corneas with 1.5 M sodium chloride treatment could generate an acellular corneal stroma with adequate histologic and optical properties and human keratocytes were able to penetrate and spread within this scaffold with proper levels of cell differentiation (Gonzalez-Andrades et al. 2011). Various method have been developed for decellularization of cornea including biological (enzymatic agents, chelating agents), chemical (Acid and alkali treatment, Alcohols, detergents, hyper-and hypo-tonic solutions), physical (freezing, pressure, sonication, mechanical agitation)_ (Wilson et al. 2013). Daoud and co-workers study reported the result of 150 patients who received anterior lamellar keratoplasty with human allogenic sterile cornea by decellularizing with gamma radiation. They showed high levels of epithelialization within a few days and no postoperative infection or rejection in all but four cases, all of which occurred in patients with pre-existing corneal melting (Daoud et al. 2011).

Recently, Antonio Campos' group developed a detergent based protocol for decellularization of corneas with an intact sclerocorneal limbus for future translational potential for the treatment of corneal diseases (Gonzalez-Andrades et al. 2015). Recently, Zhang et al. (2015) reported the implantation of aceelular porcine corneal stroma (APCS), which are decellularized porcine corneas, into the corneas of 47 patients who had corneal fungal ulcers. The reported that 41 of the implants became transparent over time and 34 (72 %) showed vision improvement.

While these are promising results, the use of xenogeneic transplantation or use of poor quality cadaveric human corneas that cannot be used as living allografts will still require confirmation of safety, as immunogenicity and risk of disease transmissions are still considerations that remain.

5.3 Self-Assembled Corneas

In contrast to decellularization, researchers have also used corneal cells in culture to generate their own ECM. Various reports have shown the reconstruction of corneas in vitro using all three corneal cell types including immortalized cell lines (Vrana et al. 2008; Griffith et al. 2009; Doillon et al. 2003; Zieske et al.

1994), animal cells (Reichl and Müller-Goymann 2003; Minami et al. 1993; Werner et al. 2008). Recently, Proulx and co-workers used human corneal cells for the reconstruction of cornea and stromal cells secreted their own ECM and formed sheets that were superposed to reconstruct a stromal tissue and epithelial and endothelial cells adhered to the reconstructed stroma. After 10 days of air-liquid interface, the epithelial cells stratified (4–5 cell layers) and differentiated into well-defined basal and wing cells and reconstructed epithelium secreted a well-defined basement membrane rich in laminin V and collagen VII (Proulx et al. 2010). A recent study by Karamichos and co-workers reported the use of ascorbic acid to induce more secretion of ECM by human umbilical cord mesenchymal stem cells compared with human corneal fibroblasts (Karamichos et al. 2011). With further development, this approach may be used to engineer full thickness corneal substitute.

5.4 Keratoprostheses Enhanced for Biointeraction and Regeneration

The KPro's are the original "artificial corneas". While conventional KPro's are literally prostheses that are designed to restore minimal function, it has been argued that epithelization of the devices is important for the reduction of post-operative infection, in particular by restoring the eyes' natural cellular barrier to external contaminants. Therefore, new KPro's being designed include the capacity to support epithelial cell growth.

For epithelization to occur, two innovations are required: (1) a surface that supports the adhesion of cells, and (2) sufficient capacity for transporting solutes by bulk diffusion to adherent cells. It is not sufficient to have a surface that is just adhesive to cells; permeability to nutrients, primarily glucose, is required to maintain the health of an overlying epithelium (Myung et al. 2008). Permeability has been made possible by development materials include ionic copolymers of poly(2-hydroxyethyl methacrylate) (PHEMA) (Wallace et al. 2005; Jacob et al. 2005), intrinsically higher water content homopolymers like poly(vinyl alcohol) (PVA) (Miyashita et al. 2006; Shimmura et al. 2003; Uchino et al. 2007) and hydrophilic double polymer networks of polyethylene glycol (PEG) and poly(acrylic acid) (PAA) (Myung et al. 2007, 2008). Myung and co-workers reported collagen-coupled poly(ethylene glycol)/poly (acrylic acid) (PEG/PAA) interpenetrating polymer networks allowed for epithelial coverage in wound healing models both *in vitro* and *in vivo* in rabbits (Myung et al. 2009). Recently, Karkhaneh and co-workers used mixtures of 2-hydroxy methacrylate acid polydimethylsiloxane (PDMS) films were modified with two-step oxygen plasma treatment, and then type I collagen was immobilized onto this modified surface (Karkhaneh et al. 2011). The authors showed the attachment and proliferation of epithelial cells onto modified PDMS. Wang and co-workers reported polymethyl methacrylate (PMMA) discs,

the principal component of Boston KPro's, coated with hydroxyapatite greatly improved cell viability, implant adhesion to tissue, and biocompatibility compared with unmodified PMMA (Wang et al. 2011).

The adhesion of epithelial cell can be enhanced by modifying these materials using extracellular matrix proteins, cell adhesion peptides and various growth factors.

5.4.1 Extracellular Matrix Proteins and Cell Adhesion Peptides

It has been shown that coating polymers with extracellular matrix (ECM) proteins, including collagen, laminin, and fibronectin, which mimic the epithelial basement membrane and promote epithelial cell adhesion and migration such as in the wound healing process (Griffith et al. 2002; Sweeney et al. 2003; Evans et al. 2000). It is thought that matrix proteins present on an implant surface may trigger migrating cells to reform a basement membrane by ECM protein secretion and formation of adhesion complexes at the surfaces (Sweeney et al. 2003). Other studies have reported the use of cell adhesion peptides such as IKVAV, YIGSR & RGD, (Kobayashi and Ikada 1991; Merrett et al. 2001; Aucoin et al. 2002) and/ or covalent tethers (Kobayashi and Ikada 1991; Wallace et al. 2005; Jacob et al. 2005) to bind the proteins or peptides to the surface may further improve the epithelial cell adhesion versus simple adsorption of ECM proteins. Tanihara group have shown using collagen like polypeptide poly(Pro-Hyp-Gly) conjugated with GRGDS and PHSRN peptides enhances cell adhesion, migration, stratification and proposed it may be useful scaffold for tissue regeneration (Shibasaki et al. 2011).

5.4.2 Growth Factors

Using of growth factor is another strategy to improve epithelialization. Epidermal growth factor (EGF) is a potent stimulator of corneal epithelial cell proliferation, migration, and is active in the wound healing process. Covalent grafting of EGF on various supports such as glass (Kuhl and Griffith-Cima 1996) or PDMS (Klenker et al. 2005) substrates through the use of PEG linkers has been reported to have clear effects on cell growth, while adsorbed EGF showed no biological activity (Kuhl and Griffith-Cima. 1996). Nonetheless, covalent grafting combined with the use of PEG linker presents several caveats. Boucher and co-workers tethered EGF via coiled coil interactions shown enhanced adhesion, spreading and proliferation of human corneal epithelial cells compared to EGF that was either physically adsorbed or present in solution (Boucher et al. 2010).

5.4.3 *Biomaterials with Anti-Microbial Properties*

Along with biocompatibility and biointegration, there is also a great need for keratoprostheses and other implantable medical devices that inherently resist bacterial infections long-term. Recently, non-leaching, long chained hydrophobic polycations that can be attached covalently to the material surface and render them strongly antimicrobial have been developed (Lewis and Kilbanov 2005; Klibanov 2007). Specifically, immobilized N,N-hexyl, methyl-polyethylenimine (HMPEI) has broad antibacterial, antifungal, and antiviral properties (Milovic et al. 2005; Lin et al. 2002; Halder et al. 2006). Behlau and co-workers covalently attached HMPEI to Boston KPros and showed that these were biocompatible and were able to show inhibitory effect on biofilm formation by *Staphylococcus aureu*s clinical isolates (Behlau et al. 2011). They have also shown no toxicity or adverse effect with HMPEI-derivatized materials after intrastromal or anterior chamber implantation in rabbits *in vivo*. A recent study by Tan and co-workers have shown the immobilization of SESB2C antimicrobial peptide on Titanium osteo-odonto keratoprostheses successfully functionalized in a rabbit keratitis model to prevent perioperative bacterial corneal infection (Tan et al. 2014).

5.5 Temperature Responsive Substrates for Corneal Cell Sheets

Thermoresponsive culture substrates based on poly-isopropylacrylamide (PNIPAAm) and its derivates have been used to establish cell sheets that are carrier free for transplantation (Nishida et al. 2004a). The progenitor cells seeded upon thin films of theramoresponsive polymer, which with media supplementation elaborate the ECM that they require to form a sheet and to stratify at 37 °C. Once the cell sheets are deemed ready for transplantation, lowering of the temperature to below 30 °C alter the hydration properties of the polymers to a sol state promoting complete detachment of the sheet of adherent cells without the use of proteolytic enzymes or treatment with EDTA.

Mebiol gel, a copolymer comprised of thermoresponsive polymer poly(N-isopropylacrylamide-co-n-butyl methacrylate) (poly-NIPAAm-co- BMA) and hydrophilic polymer polyethylene glycol (PEG), is hydrophilic below 20 °C and hydrophobic above 20 °C forming cross-linking points and a homogenous three-dimensional network in water (Vemuganti et al. 2009). This hydrogel has increased transparency compared to HAM (Vemuganti et al. 2009). Various studies on Mebiol gel shown, good proliferative capacity and exhibiting limbal and epithelial phenotype (Sudha et al. 2006) without any cytotoxicity (Madhavan et al. 2004). Transplantation of LSCs cultured on Mebiol gel showed that these cells may restore a nearly normal ocular epithelial surface in rabbit eyes with unilateral LSCD (Sitalakshmi et al. 2009).

The Cultured Autologous Oral Mucosal Epithelial Cell-Sheet (CAOMECS) is manufactured using a novel temperature-responsive culture well, UpCell®-Insert (Cellseed Inc, Tokyo, Japan) (Yamato et al. 2001), and is harvested without proteolytic processing retaining cell to cell junctions as well as deposited extra-cellular matrix of the basal membrane of the sheet (Burillon et al. 2011). The transplantation of CAOMECS is proposed for the treatment of total bilateral LSCD patients with moderate or severe symptoms, for whom any other treatments are not applicable. The efficacy of CAOMECS transplantation has been suggested by the presence of epithelium replacement in a clinical study including four patients suffering from total bilateral LSCD with severe loss of vision (<1/10) in Japan (Nishida et al. 2004b). A study found that CAOMECS was considered as a success based on a composite criterion in 16 out of 25 patients assessed at 360 days post-graft (64 %) (Burillon et al. 2011).

5.6 Biomimetic Implants Derived from Extracellular Matrix Components or Analogues

A range of materials derived from or based on analogues of the ECM have also been tested as tissue engineered scaffold for regenerating corneas. Several of these have been tested as substrates for delivery of potential therapeutic for regeneration of the corneal epithelium or endothelium. Others have been tested for their support of regeneration of more than one cell type. Several examples are discussed below.

5.6.1 Fibrin

Fibrin sealant or fibrin glue, used to create a fibrin clot, produced from combining fibrinogen and thrombin. Autologous fibrinogen and thrombin, the precursors of fibrin can be isolated from the patient's own blood to provide scaffolds for delivering stem cells to effect regeneration (Ahmed et al. 2011) and would promote integrin-based adhesion (Ahmed et al. 2015). While autologous sources of fibrin may not appear to have been used, various groups also have been used fibrin sealant as a substrate for LESC growth and is particularly useful in this context as it is a quickly degradable, natural substrate (Higa et al. 2007; Rama et al. 2001; Han et al. 2002). However, the use of fibrin gels may not be appropriate when a population of stem cells must be maintained as it has been shown to affect cells by causing differentiation (Han et al. 2002). Recently, Sheth and co-workers used fibrin gel to culture and characterize the oral mucosal epithelial cells for ocular surface reconstruction (Sheth et al. 2014). The authors have shown oral mucosal epithelial cells derived epithelium displays characteristics similar to that of a human cornea.

At the University of Granada, an on-going clinical study (registered at ClinicalTrials.gov identifier NCT01765244) uses fibrin-agarose to encapsulate stromal fibroblast cells to form a stroma. This is then overlaid with corneal epithelial progenitor cells. These implants are being tested clinical to restore the corneas of patients with epithelial and stromal defects.

5.6.2 Collagen

The major constituent of corneal stroma is collagen; therefore the use of collagen as substrate for corneal repair would be a good choice. Carbodiimide-crosslinked recombinant human collagen type III has been tested as substrates and potential carriers for LESCs (Dravida et al. 2008). The thin hydrogels had a refractive index, transmission and backscatter properties that were similar to that of native cornea and LESCs were able to stratify and express putative stem cell and differentiated cell type markers in a similar fashion to cells on HAM. Even crosslinking enhance the mechanical properties of collagen, major drawbacks include the cytotoxicity of the crosslinker, reduced biomimetic qualities of the scaffold and prevention of cell based surface remodeling (Neel et al. 2006). Highly concentrated (90 mg/ml) collagen matrices have similarities to the native corneal stroma, and human corneal epithelial cells have been shown to successfully colonize the surface of collagen scaffolds and generate an epithelium with characteristics of corneal epithelial cells (i.e. expression of cytokeratin 3 and presence of desmosomes) and maintenance of stemness during culture (i.e expression of ΔNp63α and formation of holoclones in colony formation assay) (Tidu et al. 2015).

5.6.2.1 Vitrigel Membrane

Collagen Vitrigel™, a transparent, thin and biocompatible membrane, could be a promising material for supporting the regeneration of corneal epithelium and stroma. Collagen vitrigel membrane composed of high density collagen fibrils equivalent to connective tissue in vivo and it possesses excellent transparency and permeability of protein with high molecular weight and consequently the various researchers utilizing it as a cell culture substratum (Takezawa et al. 2004). Takezawa and co-workers established a reconstruction method of rabbit corneal epithelium model by culturing normal rabbit corneal epithelial cells on the collagen vitrigel membrane substratum and inducing differentiation to form multilayers of cells (Takezawa et al. 2008). Culturing of human limbal epithelial, bovine fibroblast and fabricated rabbit endothelial cells on the surface of vitrified collagen membranes exhibited both stem and differentiated phenotypes (McInthosh Ambrose et al. 2009). A recent study by Takezawa group has used collagen vitrigel membrane for creating a corneal epithelial model for an ocular irritancy evaluation as an alternative to the Draize eye irritation test (Takezawa et al. 2011). A recent study by Chae

and co-workers have shown collagen vitrigel membrane supports regeneration of corneal epiothelium in rabbit models for corneal stromal wound and limbal stem cell deficiency (Chae et al. 2015). These properties makes vitrigels novel and very promising options for corneal tissue engineering and regeneration.

5.6.2.2 Collagen Fibers

Various groups have produced electrospun collagen fibres from solutions that are combined with synthetic polymers (Buttafoco et al. 2006; Casper et al. 2007; Matthews et al. 2002; Zhong et al. 2006), but many of the polymers or solvents are cytotoxic and so not appropriate for use in cellular applications. Wray and Orwin group have produced collagen type I fibres using a less toxic solvent (Wray and Orwin 2009). They showed that corneal fibroblasts elongated along the axis of fiber alignment, responding changes in microstructure and organization of the matrix environment. This method appears to provide a viable scaffold material for corneal stroma replacement but again, further investigations need to be undertaken to determine how LESCs would react to this material.

5.6.2.3 Compressed Collagen

Collagen hydrogels are composed of a high proportion of water they are intrinsically weak unless modified with chemical crosslinking or blended with other polymers to create collagen composites, preventing direct seeing of cells within the scaffold (Rafat et al. 2008; Liu et al. 2009; Grolik et al. 2012). Brown and coworkers developed a novel method of plastic compression of type I collagen hydrogels by applying simple engineering techniques such as external mechanical loading and capillary fluid flow (Patent Number WO2012004564, Brown et al. 2005; Brown and Mudera 2012).

Daniels and colleagues refined the simple engineering technique of plastic compression of collagen to a process and called it as Real Architecture for 3D tissue (RAFT) (Levis et al. 2015). The authors have shown this method is suitable for the production of both corneal epithelial and endothelial tissue equivalents which may be suitable for transplantation (Levis et al. 2010; Kureshi et al. 2014; Massie et al. 2014). Moreover, the tunable nature of hydrophilic porus absorbers production method allowed to create surface topography in the tissue equivalents to rapidly and reliably recreate LESC niche features, which helps in studying cell-cell and cell-matrix interactions of LESC niche using a more biomimetic in vitro model (Levis et al. 2015).

5.6.2.4 Synthetic Collagen Based Scaffolds

We found that hybrid bio-synthetic hydrogels based on collagen, NIPAAm, acrylic acid and N-acryloxysuccinimide grafted with YIGSR peptide induced epithelial, stromal and nerve regeneration (Li et al. 2003). These implants emulated the

corneal extracellular matrix by allowing for cell-matrix interaction in the restoration of functional structures including the generation of a basement membrane between the implant and overlying epithelium, stromal cell, and nerve axon ingrowth; potentiating differentiated cell state; and integration into the host tissue.

5.6.2.5 Collagen Analogues as Corneal Substitute in Clinical Studies

In 2010, members of our current team tested ethyl(dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) crosslinked recombinant human collagen corneal (RHC) substitutes (in humans in a phase I clinical study as lamellar grafts in ten patients (Fagerholm et al. 2009). At 2 years post implantation, clinical results (Fagerholm et al. 2010) showed that implants are stably retained without adverse immune reactions. Six of the ten patients had improved vision. Nine of the ten experienced corneal tissue, nerve and tear film regeneration, meaning that corneal epithelial cells grew over the implant, while stromal cell and nerves grew into the implant allowing for a tear film to form over the corneal surface. The tear film formation may have allowed the patients who were not able to tolerate contact lenses before to the surgery to be able to now wear contact lenses to improve their eyesight. At 4 years post-operation, the regenerated neo-corneas were stably integrated in all patients (Fig. 5.3). The most surprising result was that while mature immune dendritic cells were found in the control human donor corneas even at 4 years after grafting, the biosynthetic implants did not have the dendritic cells. Like normal, healthy corneas, there were only a few immature cells found (Fig. 5.4).

More recently, the RHC implants were reinforced with a second network of synthetic polymeric phosphorylcholine lipid, 2-methacryloyloxyethyl phosphorylcholine (MPC) crosslinked with poly(ethylene glycol) diacrylate (Liu et al. 2009). The resulting interpenetrating network gave a hydrogel that was able to prevent neovascularization in a rabbit alkali burn cornea model (Hackett et al. 2011) When grafted as tectonic implants into three patients who suffered from corneal ulcers and recurrent erosions that put them at high risk for rejection of conventional human donor corneas, the implants provided relief from pain and discomfort. These patients had pathologic stromas that could not support epithelial growth. They stabilized the corneal surface by promoting endogenous regeneration of corneal tissues, and improved vision in 2 of 3 patients (Buznyk et al. 2015).

5.7 Other Biomaterials and Recent Developments

5.7.1 Silk Fibroin

Silk fibroin membranes can be prepared from fibroin, a protein isolated from the domesticated silkworm (*Bombyx mori*) silk. It is a particularly useful material in corneal bioengineering as it displays a non-immunogenic response on implantation

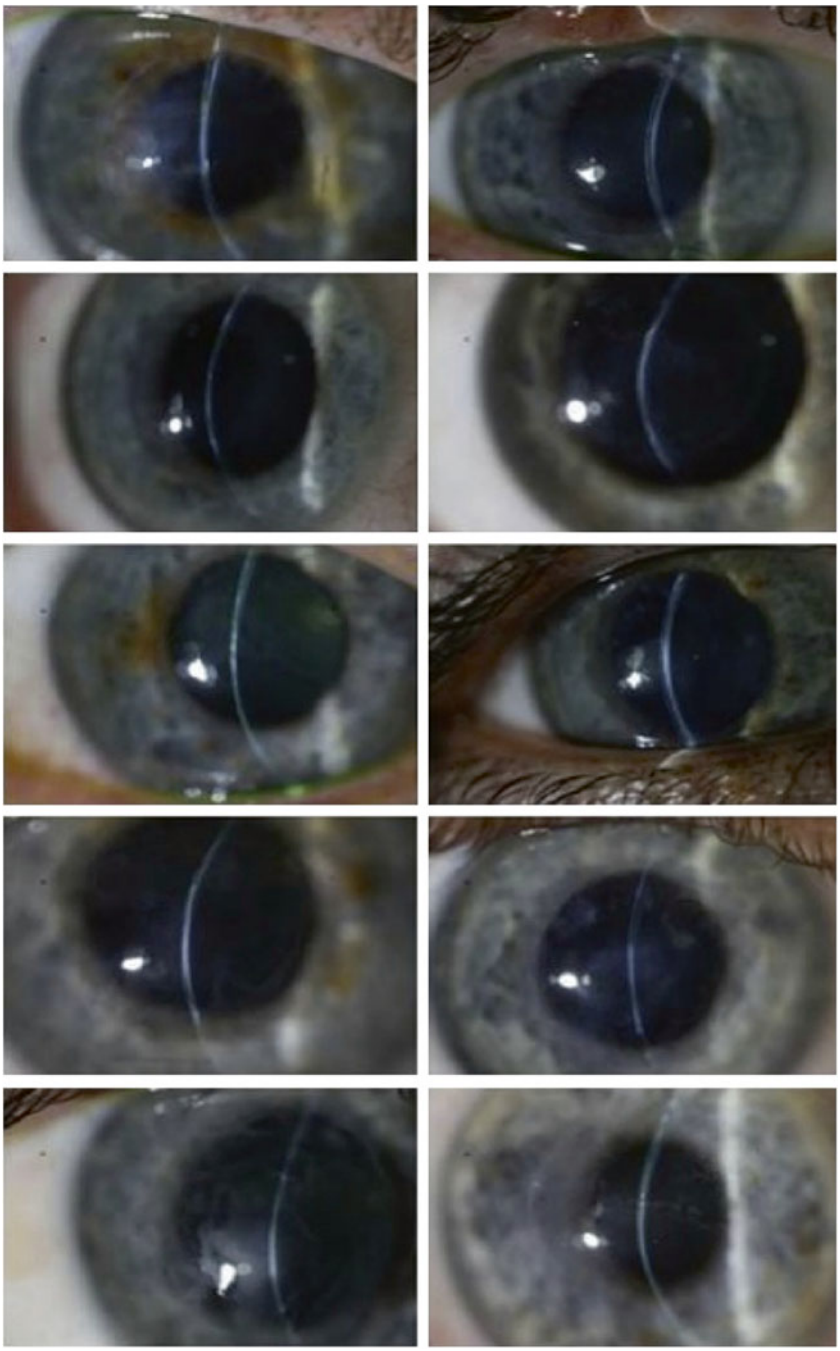

Fig. 5.3 Slit lamp biomicroscopy images of the corneas of all ten patients at 4 years after grafting with a biosynthetic implant (From: Fagerholm et al. 2014)

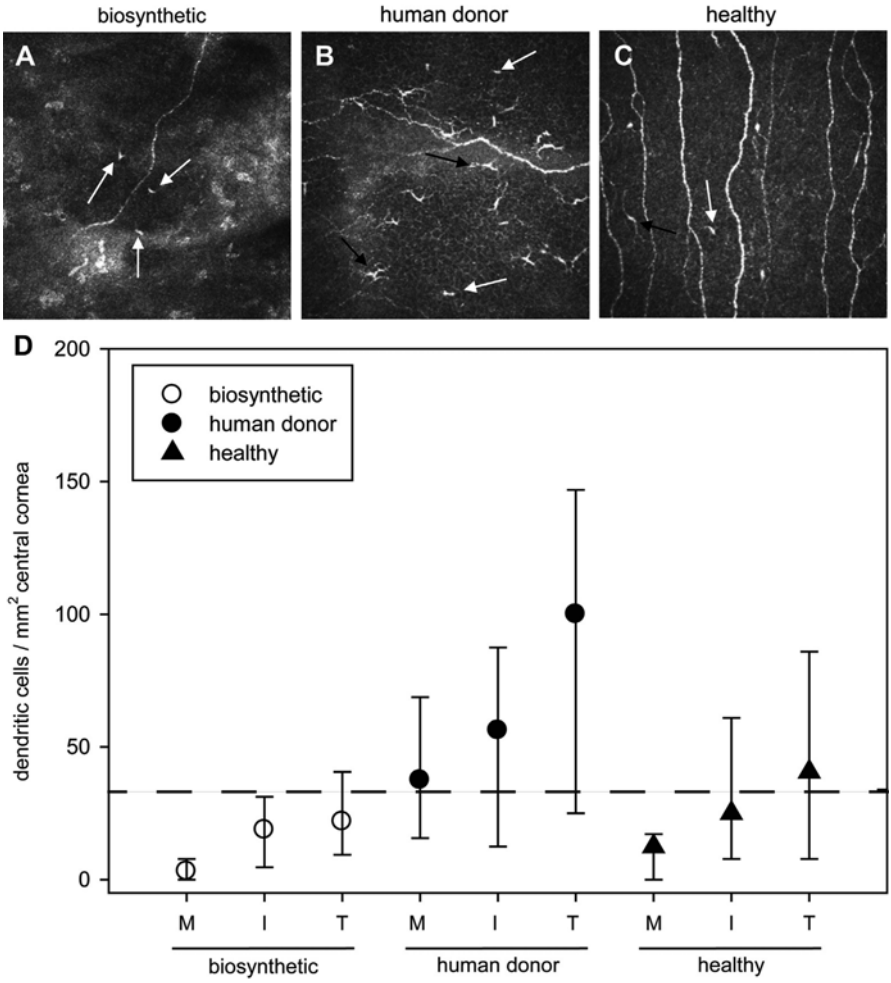

Fig. 5.4 In vivo confocal microscopic images of inflammatory dendritic cells at 4 years post-operation in regenerated neo-corneas compared to donor cornea grafted and normal, healthy eyes. (**a**) Few immature dendritic cells (*white arrows*) are seen in the basal epithelium of a regenerated neo-cornea. (**b**) Mature (*black arrows*) and immature (*white arrows*) dendritic cells in the central basal epithelial region of a cornea grafted with donor tissue, localized to the sub-basal nerve plexus. (**c**) Mature (*black arrow*) and immature (*white arrow*) dendritic cells in the normal corneal basal epithelium. Scale bars, 100 μm. (**d**) Density of dendritic cells in the sub-basal epithelium of the central cornea in healthy and 4-year post-operation groups. Data are given as median, 25th and 75th percentiles. *M* mature dendritic cells (cell bodies with dendrites), *I* immature dendritic cells (cell bodies only), *T* total dendritic cells (mature + immature). The *dashed line* indicates the previously reported mean value of 34±3 cells/mm^2 for the central corneal dendritic cell density in healthy eyes (From: Fagerholm et al. 2014)

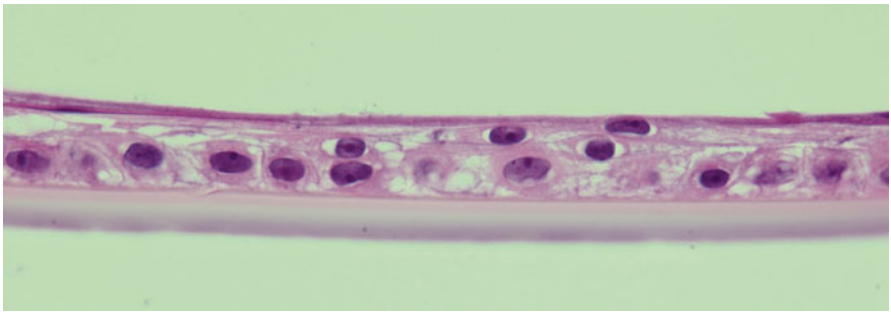

Fig. 5.5 H & E stained image of human cornea limbal epithelial cells cultured on silk fibroin, after 2 weeks in culture (Photo courtesy of Drs. Damien G Harkin, Laura Bray, Karina George and Traian Chirila, Queensland Eye Institute & Queensland University of Technology)

in vivo, is mechanically robust, transparent, easy to handle and has controlled degradation rates (Lawrence et al. 2009). Fibroin based materials support the attachment, growth and differentiation of cells isolated from all three main layers of the cornea (Chirila et al. 2008; Bray et al. 2011; Bray et al. 2012). Lawrence and co-workers have demonstrated that porous ultrathin fibroin films support the growth of primary rabbit corneal fibroblasts (Lawrence et al. 2009). Fibroblast growth on fibroin was found to be less than that observed on tissue culture plastic, but the cells retained production of ECM molecules associated with a normal corneal phenotype. Nanopatterning technology allows surface modification of the silk fibroin with RGD peptide and improved the alignment of corneal stromal cells and their growth (Gil et al. 2010). Bray and co-workers have constructed human corneal epithelial equivalents on silk fibroin membranes maintained evidence of corneal phenotype (K3/K2 expression) and displayed ΔNp63 positive cells (Bray et al. 2011).

Silk fibroin a novel biomaterial, has been shown to support corneal epithelial cell proliferation, differentiation and stratification (Fig. 5.5), while retaining the normal characteristic epithelial phenotype. Compared with amniotic membrane, its unique features that include transparency, ease of handling and transfer, and inherent freedom from disease transmission, makes it a promising substrate for corneal wound repair and tissue engineering (Wang et al. 2013). Madden and co-workers have shown successful growth of human corneal endothelial cells on coated fibroin as a step in evaluating fibroin as a substratum for the transplantation of tissue-constructs for endothelial keratoplasty (Madden et al. 2011). A recent study by Kim and coworkers showed the growth of rabbit corneal endothelial cells on different concentrations of sericin silk fibroin films although the percentage of sericin showed no significant difference (Kim et al. 2015). Basing on these studies, silk fibroin could be a potential biomaterial carrier for all the three corneal cell types in corneal tissue engineering.

5.7.2 Plasma-Treated Contact Lenses

Plasma polymerization can be utilized to deposit functional groups that promote cell growth and allowing the cells to transfer from the surface onto a wound bed (Desphande et al. 2009). Brown and co-workers found that lenses coated with a high percentage of acid functional groups resulted in a higher number of cells transferred onto the corneal wound bed in rabbit models of LSCD. The authors have also shown stratified epithelium at 26 days post transplantation and presence of autologous, transplanted 5-bromo-2′-deoxyuridine-labeled cells (Brown et al. 2014) This evidence proposes the possibility of using plasma polymer-coated contact lenses with high acid functional groups as substrates for the culture and transfer of limbal cells in the treatment of LSCD.

5.7.3 Self-Assembling Peptides

Versatile functional groups, the easy implementation capability and the self-assembly property of peptide amphiphile networks make them promising biomaterials for corneal tissue engineering (Gouveia et al. 2013; Uzunalli et al. 2014). Self-assembling peptides were first described in 1993 (Zhang et al. 1993), comprise short peptide repeats, with hydrophilic and hydrophobic faces that stack to form long, entangled fibers. The bioactive peptide nanofiber scaffolds are formed by self-assembling peptide amphiphile molecules containing laminin and fibronectin inspired sequences (YIGSR and RGD respectively) which mimic the extracellular matrix. Gouveia and co-workers designed self –assembling peptides with RGD, which aided in adhesion, proliferation and alignment of human stromal fibroblasts (Gouveia et al. 2013). Human corneal keratocyte cells cultured on laminin-mimetic peptide nanofibers retained their characteristic morphology, and their proliferation was enhanced. When these nanofibers were used for damaged rabbit corneas, increased keratocyte migration and supported stroma regeneration (Uzunalli et al. 2014).

5.7.4 Molecular Crowding

Macromolecular crowding (MMC), a biophysical phenomenon that regulates the intra- and extracellular milieu of multicellular organisms and increases thermodynamic activities and biological process will facilitate accelerated tissue specific extracellular matrix deposition of human corneal fibroblasts, while maintaining their function *in vitro*. Kumar and co-workers have shown the addition of negatively charged galactose derivative (carrageenan) in human corneal fibroblast culture increases by 12-fold tissue specific matrix deposition, while maintaining physiological cell morphology and protein/gene expression (Kuma et al. 2015). This evidence

supports MMC may be suitable for commercialization of tissue engineering by self-assembly therapies and for the development of *in vitro* pathophysiology models.

5.8 Conclusion

There have been significant developments in regenerative medicine-based approaches to replace partial or the full thickness of damaged or diseased corneas. Biomaterials have been developed to assist in these reparative procedures. They have been designed as interactive scaffolds to promote endogenous stem cell repair and regeneration, and they have also been used as substrates for the implantation of exogenous stem cells. These different approaches may soon be able to supplement the supply of post-mortem human corneas harvested for transplantation, or allow restoration of diseased or damaged corneas that cannot be treated by currently available techniques. It should be noted that purely cell based techniques of injecting stem cells into damaged corneas are also being tested, but are not within the scope of this chapter.

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Chapter 6
Functionalized Nanomaterials

Jie Zhou, Changyou Gao, and Wenzhong Li

Abstract Regenerative medicine aims to repair tissues or organs for restoring normal functions, which represents one of the greatest challenges in modern day science and medicine. Diverse techniques and materials are required to truly understand the process of tissue repairing and build a proper scaffold for cells attachment, proliferation and differentiation. Functionalized nanomaterials with nanotechnologies are the ideal to solve most of the problems of regenerative medicine. Multifunctionalized nanoparticles and nanostructured biomaterials can be powerful tools for cell tracking and matrix-like scaffold rebuilding.

Keywords Nanoparticles • Magnetic nanoparticles • Quantum dots • Cell tracking • Self assembly • Electro spinning

6.1 Introduction

Regenerative medicine is an interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneracy of cells, tissues or organs to restore impaired function resulting from any cause (Daar and Greenwood 2007), which combines diverse techniques to stimulate or support the body's own self-healing capacity. Though numerous implants and engineered tissues have been developed which are based on the current knowledge of the superstructure and the microstructure of tissue in last several decades, the truly regenerative therapies require significant understanding and controlling the underlying nanostructures in cells, the extracellular matrix (ECM) and also the cell behaviors during the tissue regeneracy (Harrison 2008; Zhang and Webster 2009).

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In order to truly understand of the regenerative process during the tissue reconstruction, the knowledge of cell migration, proliferation and/or differentiation after the regenerative therapies is critical. Cell imaging and cell tracking as one of these methods is used to investigate the detail process during tissue regeneracy. The knowledge from cell tracking helps to advance new technologies for improving regenerative medicine in vice versa (Vaccaro et al. 2008; Solanki et al. 2008). An ideal cell tracking technology should be multifunctional (able to be both imaged and quantified), high sensitive, nontoxic, have long life time after labeling, and label cells with high efficiency (Vaccaro et al. 2008).

Furthermore, in a regenerative strategy, a scaffold normally required for promoting new tissue formation by providing adequate space (porosity) and appropriate surface to foster and direct cellular attachment, migration, proliferation, desired differentiation of specific cell phenotypes throughout the scaffold where new tissue formation is needed (Wei and Ma 2008; Chaikof et al. 2002).

Nanomaterials is a prosperous field in materials science based on the nanotechnology which was first defined by Taniguchi (1974). Nanotechnology, as a tool for fabricating nanomaterials, is the study of the control of matter on an atomic and molecular scale, which has the potential to create many new materials and devices with wide-ranging applications, such as in medicine, electronics, and energy production. The scale of nanomaterials made by nanotechnology is usually smaller than 100 nm meter in at least one dimension (Buzea et al. 2007), though sometimes also smaller than 1 μm, especially in the biological area.

Nanotechnology or the use of nanomaterials may have the answers since only these materials can be a powerful tool to track cell and mimic surface properties (including topography, composite, etc.) of natural tissues or delivery growth factors for tissue regeneracy. Nanomaterials are the materials with complex nanostructures, normally are fabricated by bottom-up or top-down methods. At the nanometer scale, where many biological processes operate, for example, the functional structures on the cell membrane, enzyme reactions, protein dynamics and DNA all possess some aspect of nanodimensionality (Harrison 2008). With significant advancements in synthetic and modification methodologies, nanomaterials can be modified to desired sizes, shapes, compositions and properties, which can be used as an ideal cell tracking label the cells without toxicity (Solanki et al. 2008). Furthermore, the ECM that the cells interact with also abounds with nanosized features which not only adjust the behaviors of the cells contacted with, but also influence the other cells and even tissues. These nanosized features, such as the size of fibers, the pores of matrix, and the chemical composition control the mechanical properties, the cell adhesion, proliferation and even differentiation on the matrix (Harrison 2008).

6.2 Principles

Since tissue regeneracy is a complex and precise process, in which cell behaviors, nanosized structures, chemical components of ECM and cytokines play critical roles. Understanding these critical aspects in the tissue repairing process helps to

develop new techniques for fabricating proper materials in the regenerative medicine.

6.2.1 Nanoparticles for Cell Tracking

The importance of tracking cells in regenerative medicine is increasing because of the developing of basic cell therapy science, which is critical for cell delivery optimization and for accurate biodistribution studies (Vaccaro et al. 2008; Solanki et al. 2008).

In general, there are little cells retained in the target site after cell injection, which is found by cell tracking method. Wentworth et al. (2007) labeled skeletal myoblasts, and bone marrow stromal cells with Europium nanoparticles in advance, then the labeled cells were injected in vivo into the rat heart. The results showed that only approximately 15 % of the delivered cells were retained shortly after cell injection and the cells kept losing during the following 5 days. Other groups have reported similar cell retention numbers at the therapeutic site after injection, ranging from 5 to 15 % (Wentworth et al. 2007; Freyman et al. 2006). By immunohistochemical detection, the authors found that the macrophage infiltrate contribute to losses of both cell types (Wentworth et al. 2007).

Nanoparticles, especially iron oxide nanoparticles and quantum dots (QDs), are one of exciting materials for cell labeling, cell tracking and in vivo imaging, because of ease to synthesize in large quantities from various materials using relatively simple methods. The diameter of the nanoparticles can be tuned from several to a few hundred nanometers with controlled size distribution. Among them, QDs are considered as the ideal tool to label cells for tracking the cells, because of broad adsorption spectra, narrow emission spectra, high fluorescent intensity and long fluorescence lifetime (Solanki et al. 2008).

6.2.2 Scaffold for Tissue Regeneracy

As mentioned above, cell lost is a big problem for the cells injection method for tissue repair. It's a really necessary requirement to make cells adhere to the surface of a scaffold which can prevent cell losing, support three-dimensional tissue formation. Furthermore, depending upon the setting, progenitor cells may need to mature into a tissue-specific phenotype, and fully differentiated cells will need to operate with appropriate functional responses (Chaikof et al. 2002).

Actually, the ECM, which is the natural environment for cells growing, is full of nanosized structure. Bone, as an example, is a nanocomposite that consists of a protein network (i.e., collagen, laminin, fibronectin, and vitronectin) and hard inorganic components (hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$) (Zhang and Webster 2009; Webster 2001). Specifically, 70 % of the bone matrix is composed of nanocrystalline HA which is typically 20–80 nm long and 2–5 nm thick (Simon 1994).

Other protein components in the bone ECM same with the other tissues are also nanometer in dimension. This self-assembled nanostructured ECM in all tissues closely surrounds and affects these cell behaviors, for example, cell adhesion, proliferation and differentiation. Apparently, the design of novel nanomaterials which possess not only excellent mechanical properties but that are also biomimetic in terms of their nanostructure, has become quite popular in order to improve the functions of cells in regenerative medicine (Zhang and Webster 2009).

In 2004, Miller et al. (2004) reported endothelial and vascular smooth muscle cells adhesion and proliferation were enhanced comparing a nanostructured PLGA surfaces with smooth one. Later, a series of PLGA surfaces were fabricated by composing of submicron scale spheres on them. The results revealed that surfaces with 200 nm lateral diameter spherical features exhibited highest fibronectin and collagen type IV adsorption comparing the 100 nm or 500 nm lateral diameter spherical surface features. Furthermore, the higher fibronectin and collagen IV adsorption, the more endothelial and vascular smooth muscle cell adhesion was found as well (Miller et al. 2007). Since chemistry was similar between all PLGA surfaces investigated, this study provided strong evidence of the influence of nanometer features on optimizing fibronectin interactions and subsequently vascular cell adhesion. Similar results were also found that nanostructured titanium implant surfaces promote bone cell responses leading to accelerated calcium deposition improving integration with surrounding bone compared to conventional titanium surfaces (Ergun et al. 2008; Webster et al. 1999; Yao et al. 2008).

Collagen, the major ECM component of most of these tissues, has been proved as a substrate or scaffold for cell attachment, proliferation, and differentiation (Elsdale and Bard 1972; Strom and Michalopoulos 1982). Wang et al formed a gene activated collagen substrate via assembly of complexes of plasmid encoding SDF-1alpha into a collagen substrate to create a microenvironment favoring stem cell homing. In vivo experiments showed that local release of SDF-1alpha from the transfected cells on the gene activated collagen substrate could effectively home CD117(+) stem cell to the surrounding tissues(Wang et al. 2010). Moreover, the nanosized collagen fibrillar structure (50–500 nm in diameter) has been demonstrated to enhance cell/matrix interactions (Grinnell and Bennett 1982; Kuntz and Saltzman 1997). For serving as a scaffold for regenerative cells, the nanosized fibers like collagen may be help to improve the cell/scaffold interactions and to be a better environment for cell growing. Several techniques, for example, self-assembly, phase separation, and electrospinning are developed to fabricate porous scaffolds composed with nanosized fibers (Wei and Ma 2008).

For example, electrospun poly(L-lactide-*co*-ε-caprolactone) (PLCL) fibrous scaffolds of varying fiber diameters (ranging from 300 nm to 7 mm) were used as scaffolds for culturing human umbilical vein endothelial cells (HUVECs). A higher cell adhesion and proliferation potential was found cultured with nanosized PLCL scaffolds (Kwon et al. 2005). Ma's group developed a thermally induced phase separation method to fabricate nanofibrous scaffold (Zhang and Ma 1999). With this method, nanofibrous poly(L-lactide) (PLLA) scaffolds with diameters ranging from

50 to 500 nm has been prepared using tetrahydrofuran (THF) as solvent (Zhang and Ma 1999, 2000). Nanofibrous scaffolds of PLLA prepared from this technique have demonstrated to adsorb/absorb cell adhesive proteins (fibronectin and vitronectin) 2–4 times higher and an almost twofold increased osteoblast attachment in comparison to solid walled PLLA scaffolds (Yang et al. 2004a).

So, an ideal 3D-scaffold for tissue regeneracy should have similarity to native ECM in terms of both chemical composition and physical nanostructure. Nanostructured biomaterials having physical features in the nanometer range, such as nanocrystals, nanofibers, nanosurfaces and nanocomposites, have gained much interest recently in regenerative medicine (Thomas et al. 2006a; Layrolle and Daculsi 2006).

Furthermore, except ECMs, intrinsic regulators (e.g., growth factors and signaling molecules) are another prime factors that have critical roles in regulating cell behaviors during the tissue reparation (Solanki et al. 2008; Kiritsy and Lynch 1993). For example, during the cutaneous wound repair process, the growth factors (platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), and fibroblast growth factor (FGF) et. al) play important roles in all of three repair phases (hemostasis and inflammation, granulation tissue formation, and matrix formation and remodeling) (Kiritsy and Lynch 1993). However, direct injection of growth factors into a regeneracy site is generally not effective because of their rapid diffusion and short lifetime. To enable growth factors to efficiently exert their biological effects during the tissue regeneracy process, a drug delivery system should be used (Tabata 2003).

6.3 Technological and Biological Opportunities for Therapeutic Devices

6.3.1 Functionalized Nanoparticles for Cell Tracking

Over the past decade, cell tracking is becoming more and more important for optimizing cell delivery or accurate biodistribution studies in regenerative medicine as mentioned above.

Nanoparticles, because of the size-dependent properties and dimensional similarities to biomacromolecules, are suitable as contrast agents (Chan et al. 2002; Bruchez-Jr et al. 1998; Chan and Nie 1998; Dubertret et al. 2002; Jaiswal et al. 2002; Ishii et al. 2003; Dahan et al. 2003; Lidke et al. 2004) or probes for biomedical imaging (Josephson et al. 1999; Bulte et al. 2001).

Magnetic nanoparticles in magnetic resonance imaging (MRI), QDs and other bioengineered nanoparticles are commonly used for cell labeling and tracking which provide several unique features and capabilities. Firstly, the size-dependent optical and electronic properties can be tuned continuously by changing the particles size (Alivisatos 1996). Secondly, nanoparticles have big specific surface which

can be useful for surface modification in order to target a specific organ or tissue in human body (Rhyner et al. 2006). These nanoparticles are functionalized by diverse techniques in order to meet the requirements for cell imaging and cell tracking.

6.3.1.1 Magnetic Nanoparticles

Magnetic nanoparticles, especially superparamagnetic iron oxide particles (SPIO), have a variety of applications on molecular and cellular imaging for enhancing magnetic resonance contrast. The popularity of SPIO particle is mainly because of these following several properties: (1) they provide the most change in signal (albeit hypointensity) per unit of metal which can obtain sharp images with different iron concentration; (2) they are composed of biodegradable iron, which is biocompatible and can thus be reused/recycled by cells using normal biochemical pathways for iron metabolism; (3) they can be magnetically manipulated and change their magnetic properties according to size (Bulte and Kraitchman 2004).

Normally, SPIO consist of two components, an iron oxide core and a hydrophilic coating. Typically, the core is magnetite (Fe_3O_4) and/or maghemite (γ Fe_2O_3) which plays important role in the MRI. In some case, SPIO nanoparticles can be functionalized simply by doping some other metal ions during the preparation process. Groman et al. (2007) fabricated one new mixed ferrite colloidal magnetic iron oxides by adding informational atoms (Lanthanide) during formation of the iron oxides core. The new functionalized nanoparticles, not only can be visualized by iron-based MRI, but also can be quantized by neutron activation (Eu, Sm, La, Tb add) and even visualized histologically using time resolved fluorescence (Eu, Tb added).

The SPIO nanoparticles must be functionalized by hydrophilic coating in order to stabilize iron oxide crystals in aqueous colloidal solutions or in vivo, reduce unspecific protein adsorption or cell interactions in vitro or in vivo. Most commonly, surface molecules are biocompatible hydrophilic polymers, for example polysaccharides-dextran (Weissleder and Papisov 1992). A rich dextran density can also enhance circulation time because of the flexible dextran layer forming a "molecular brush" (Papisov et al. 1993) because of the low protein adsorption in plasma. As a fact, long circulation time is critically necessary for better targeting and tracking cells. For this purpose, other biological macromolecules have been investigated for functionalizing iron nanoparticles, e.g. poly(sialic acid), heparin etc., but because of their high cost, efforts have been directed to the design of synthetic hydrophilic macromolecules.

Among these synthetic macromolecules, block-copolymers such as poloxamers and poloxamines has been widely used for enhancing circulation time in vivo because of the effect from poly(ethylene glycol) (PEG) molecules extended in the solution (Moghimi and Hunter 2000). To achieve coupling PEG on iron oxide particles, associated dextran on particles was oxidized and poly-L-lysine (PLL) were attached to the surface by electrostatic force. Finally, methoxy(polyethylene glycol)-O-succinyl succinate was immobilized on PLL covered particles (Weissleder et al.

1995). Then, Butterworth et al. (2001) developed a new method for the grafting of PEG onto magnetite particles by the use of trimethoxysilane-PEG which is more convenient for controlling the grafting process. The PEG immobilized iron oxide particles produced showed greatly enhanced colloidal stability with respect to uncoated particles.

Targeting specific tissue, organ, or cells precisely are necessary for cellular imaging and tracking. Passive targeting is easier to achieve in application which can make nanoparticles accumulate in a specific tissue or organ (liver, spleen, cancer et al.) by simply controlling the particles size (Thorek et al. 2006). But passive targeting is not a universal method for targeting because of its limitation. In order to achieve active targeting of SPIO against specific tissue, organ or cells, it is necessary to first conjugate targeting agents onto the SPIO surface directly or onto its hydrophilic coating. In this case, reactive moieties (i.e., amines, sulfhydryls, carboxyls, etc.) are needed in order to immobilize targeting moieties (i.e., antibodies, folic acid, galactose etc.). Take dextran coated SPIO particles as an example, these hydroxyl groups on dextran molecules were oxidized by sodium metaperiodate (Weissleder et al. 1995), then further modification can be applied. Josephson et al. (1999) crosslinked dextran with epichlohydrin, then amination was used to induce amino groups on SPIO particles. Finally, particles were functionalized by tat peptide and fluorochrome for imaging cells (Josephson et al. 1999; Groman et al. 2007; Koch et al. 2003).

In order to improve specific interactions with certain kind of cell, targeting moieties, for example peptides, antibodies, small molecule (folic acid, galactose etc.) are normally immobilized on SPIO particles. HIV tat peptide, which contains a membrane translocating signal, was immobilized on surface for efficiently transporting the iron oxides into cells (Josephson et al. 1999; Koch et al. 2003; Lewin et al. 2000). Monoclonal antibodies (mABs) are the proteins which can only interact with a specific substance, and achieve precisely targeting for one kind of cell. Bulte et al. coupled mouse anti-transferrin receptor mAB OX-26 with magnetic nanoparticles and then magnetically labeled oligodendrocyte progenitors (Bulte et al. 1999) and neural precursor cells (Bulte et al. 2003) by receptor-mediated endocytosis for monitoring cell migration. Schellenberger et al. (2002, 2004) found that Annexin V conjugated nanoparticles could detect apoptotic cells at nanoparticle concentrations as low as 0.1 μg Fe/ml in vitro. Targeting moieties are immobilized with PEG as space, for reducing unspecific interactions and increasing specific interactions. Iron oxide nanoparticle surface was modified by folic acid (FA) with PEG as spacer successfully (Kohler et al. 2004; Sun et al. 2006). Then the cell uptake properties were obviously increased after FA immobilized on particles. The specific interactions was found with FA receptor overexpressed cell line—HeLa, but not with non FA receptor overexpressed cells—MG-63 (Sun et al. 2006).

Moreover, Fe^{2+} released from iron oxide nanoparticles may have potential toxic effects on the cells. In order to prevent Fe^{2+} toxic effects of SPIONs, the gold-coated shell was combined on the surface, which is well know as a stable metal. More importantly, gold has well-defined surface chemistry with thiol or amine moieties. This offers an attractive and convenient route for further functionalization of the

SPIONs with biomolecules through thiol- or amine-coupling chemistry (Niemeyer and Ceyhan 2001).

Magnetic nanoparticles labeled cell not only make cell visible in the regenerative medicine, but also may be helpful for guiding cells into usable tissues for transplantation with the help of magnetic fields. Sasaki et al. (2008) fabricated novel magnetic nanoparticles coated with chitosan. When bound to fibroblasts and exposed to an external magnetic field, these magnetic nanoparticles improved cell seeding into the center of a 3D scaffold.

6.3.1.2 Quantum Dots

QDs are crystalline semiconductors typically less than 10 nm in diameter that have been studied for over 20 years. Recently, more and more applications are developed in biomedicine including regenerative medicine field (Bruchez-Jr et al. 1998; Chan and Nie 1998). In the past decades, several production methods are available, from photolithography to wet chemical synthesis. The QDs produced in colloidal solutions are the most useful for biomedical applications since high-quality nanocrystals can be prepared in large quantities at low costs (Rhyner et al. 2006).

QDs have unique optical and electronic properties comparing with organic dyes and fluorescent proteins because of higher molar extinction coefficients, emission wavelengths size tunable and long term photostability (Yu et al. 2003; Cui et al. 2007; Maysinger et al. 2007). These properties have made QDs a topic of intensive research in tracking cell migration, differentiation and metastasis (Rhyner et al. 2006).

The highest quality QDs are composed of II–VI, IV–VI or III–V semiconductors (Lemon and Crooks 2000; Rogach et al. 1999). The most common QD structure is a CdSe core functionalized with a thin shell of ZnS in order to reduce potential toxicity of core (Rhyner et al. 2006). No acute and obvious CdSe QD toxicity has been detected in studies of cell proliferation and viability in live cells (Jaiswal et al. 2002; Winter et al. 2001; Parak et al. 2002; Derfus et al. 2004) and animal models (Larson et al. 2003; Akerman et al. 2002). However, cytotoxicity was observed when Cd^{2+} was released by oxidization the CdSe in air or UV. This happened when the QD surface coating was not stable enough. But after larger molecules, such as proteins (e.g., streptavidin and bovine serum albumin) are used to functionalize on the surface, slower oxidation is found of the core (Alivisatos et al. 2005). Bioconjugation of QDs with biomolecules, such as arginine-glycine-aspartic acid, did not show any toxic effect on hMSCs as compared with unlabeled human umbilical vein endothelial cells (hMSCs) (Shah et al. 2007).

In general, surface modifications or functionalization must be taken place after the QDs are synthesized in order to transfer to an aqueous phase for medical applications. To accomplish this, the hydrophobic surface ligands can either be exchanged with bifunctional ligands or the entire QD can be coated with an amphiphilic polymer layer. In recent work, Gao and colleagues (2004) encapsulated luminescent QDs with a biocompatible copolymer and linked this amphiphilic polymer to

tumor-targeting ligands. Using either subcutaneous injection of QD-tagged cells or systemic injection of multifunctional QD probes, sensitive and multicolor fluorescence imaging of cells can be achieved under in vivo conditions which may be quite useful for regenerative medicine applications (Rhyner et al. 2006).

Although optical imaging with QDs is highly sensitive, a limitation in depth is a major disadvantage. Other imaging techniques, such as MRI, are more suited for tomography and 3D imaging. By functionalizing on QDs, dual imaging can be achieved. For example, gadolinium, which is visible by MRI, was used to link on the surface of QDs by polymer conjugated lipids (Mulder et al. 2006). These functionalized QDs can be easily detected both by fluorescence imaging and MRI in vitro (Mulder et al. 2006). Furthermore, these Gd-based dual-modality nanoparticle probes are promising for in vivo apoptosis after immobilized Annexin A5 on the surface through a PEG spacer (van-Tilborg et al. 2006). The dual imaging nanoparticle probes can be achieved by linking QDs with Fe_2O_3 or FePt as well (Gu et al. 2004).

Normally, fluorescent QDs require excitation from external illumination sources to fluoresce, which limits their application for imaging living opaque subjects because of the resultant strong autofluorescence background and a paucity of excitation light at non-superficial locations. So et al. (2006) reported self-illuminating quantum dot conjugates designed by mimicking a natural bioluminescence resonance energy transfer (BRET) system, with a mutant of R. reniformis luciferase as the energy donor and quantum dots as the acceptor, and have demonstrated that BRET emission can be imaged in cells and small animals. These self-illuminating QD conjugates can emit long-wavelength (from red to near-infrared) bioluminescent light in living cells and in living animals, even in deep tissues, and can be applied for multiplex in vivo imaging (So et al. 2006).

6.3.1.3 Other Nanoparticles

Except wildly used magnetic nanoparticles and QDs, diverse nanoparticles made by either organic or inorganic are applied as cell tracking or imaging probes.

For the specific application, bioactive inorganic particles, for example hydroxyapatite (HA) particles, were functionalized by fluorescence molecules. Zaheer et al. (2001) synthesized a near-infrared (NIR) fluorescent bisphosphonate derivative that exhibits rapid and specific binding to hydroxyapatite (HA). They demonstrate NIR light–based detection of osteoblastic activity in the living animal, and discuss how this technology can be used to study skeletal development. Fluorescence imaging of osteoblastic activity in living animals has also met with success using an active probe: a tetrasulfonated heptamethine indocyanine conjugated to the hydroxyapatite-binding ligand pamidronate (Rao et al. 2007).

A large group of organic nanoparticles such as liposomes, dendrimers and polymersomes have not only been developed for drug delivery, but can also be applied to in vivo optical imaging. Therien and colleagues (Ghoroghchian et al. 2005) reported the synthesis of NIR-emissive polymersomes (polymer vesicles with a

diameter of 50 nm to 50 mm) through the cooperative self-assembly of amphiphilic diblock copolymers and conjugated multi(porphyrin)-based NIR fluorophores. Dendrimers were previously used as carriers for magnetic resonance imaging (MRI) contrast reagents and, recently, McIntyre et al. (2004) designed a polyamidoamine dendrimer-based fluorogenic substrate to image tumorassociated matrix metalloproteinase-7 in vivo. A boronated dendrimer labeled with a vascular endothelial growth factor (VEGF) and an NIR dye Cy5 has been shown to selectively bind upregulated VEGF receptors in mouse breast carcinoma (Backer et al. 2005).

When multiple fluorescent dyes are attached to the same molecule, such as an antibody, the fluorescent intensity can decrease instead of increase owing to dye–dye quenching. However, when a viral capsid is used as the scaffold for labeling, more than 40 Cy5 dyes can be loaded onto a single virus particle via specific chemical coupling and no fluorescence quenching is observed due to the large intermolecular distances (Soto et al. 2006). This approach has resulted in the synthesis of highly fluorescent viral nanoparticles with a defined structure and a size of 30 nm in diameter. The local dye concentration was reported to be as high as 1.8 mM without significant quenching (Wu et al. 2005). Cowpea mosaic virus nanoparticles labeled with Alexa dyes have been used successfully to visualize the vasculature and blood flow and for imaging human fibrosarcoma-mediated tumor angiogenesis in living mouse and chick embryos (Lewis et al. 2006).

6.3.2 Functionalized Nanomaterials for Tissue Regeneracy

Besides multifunctionalized nanoparticles for better understanding tissue regenerative process through cell tracking and cell imaging, functionalized nanomaterials could become important for tissue regeneracy. They could be utilized directly for tissue regeneration or as cornerstone for artificial tissues. Li et al tried to guide magnetic nanoparticle polymer/DNA complexes after systemic administration to the heart with a magnetic field. They showed that an epicardial magnet could effectively attract the complexes in the left side of the thorax, resulting in strong reporter and therapeutic gene expression in the left lung and the heart (Li et al. 2008). Zhang et al conjugated magnetic nanoparticles with adenoviral vectors-encoded hVEGF gene. In a rat acute myocardial infarction (AMI) model, they injected magnetic nanoparticle/adenoviral vector (hVEGF) complexes intravenously. With magnetic targeting, the complexes significantly improved left ventricular function and exhibited higher capillary and arteriole density and lower collagen deposition in the infarcted area (Zhang et al. 2012).

The principles of the design of an ideal 3D scaffold for tissue engineering remain unclear. The scaffolds should mimic the structure, composition and biological functions of native extra cellular matrix (ECM) as much as possible. Moreover, most of the scaffold fabrication strategies have not given importance to mimic the nanoscale physical features of the natural ECM. It is well known that cells and proteins interact at the nanoscale (Thomas et al. 2006a; Cao 2008). For instance, researchers have

engineered a variety of scaffolds made from nanotubes, nanofibers, and nano composites that can be used to grow lifelike networks of cells from the liver, bladder, kidney, bones and cardiovascular system. These artificial tissues could be developed into new therapies for patients with diseased or damaged organs.

It is still early, but many laboratories are experimenting with a wide variety of nanomaterial scaffolds that can be infused with cells to form artificial tissues, such as bone and liver. It appears possible to repair damaged nerves by injecting them with nanomaterials that form bridge-like lattices. Other nanostructures show promise as foundations for growing three-dimensional networks of blood vessels.

Considerable efforts have been made to develop ideal scaffolds for tissue engineering so far. Various techniques such as solvent casting/particulate leaching (Mikos et al. 1994), gas foaming (Mooney et al. 1996; Nam et al. 2000), and phase separation/emulsification (Nam and Park 1999a, b) have been employed to fabricate conventional porous polymeric foams. Peptide self-assembling, phase separation, and electrospinning are normally techniques used for fabricating nanostructured scaffold materials.

6.3.2.1 Self-Assembly

Self-assembly is a process in which molecules and supramolecular aggregates organize and arrange themselves into an ordered structure through weak and noncovalent bonds (Murugan and Ramakrishna 2007; Whitesides and Grzybowski 2002). It is a common process in nature, for example, collagen has a triple helix secondary structure, which consists of three polypeptide chains in an extended left handed helix (Ramachandran 1988). Self-assembly could be used to produce natural or synthetic polymers into nanoscale structures including nanofibers (Zhang 2003; Hartgerink et al. 2001; Chiti et al. 2003), especially scaffolds based on peptides and proteins. The biological ECMs made by this technique are able to interact with cells at the molecular level to control the processes of tissue regeneracy effectively.

Several studies report promising results of this strategy. For example, a peptide amphiphile (chemical compound possessing both hydrophilic and hydrophobic properties) nanofiber network could be mineralized with hydroxyapatite to recreate the nanoscale structure of bone (Hartgerink et al. 2001). Certain peptide amphiphiles can be designed in order to get functionalized nanomaterials for specific applications. For example, these amphiphile nanofibers have been designed to mimic the collagen structure-building protein-like structural motifs that incorporate sequences of biological interest (Fields et al. 1998; Yu et al. 1998, 1999; Berndt et al. 1995). These nanofibers have been also applied to promote rapid and selective differentiation of neural progenitor cells into neurons (Silva et al. 2004). Self-assembly was also used successfully to encapsulate chondrocytes within a self-assembled peptide hydrogel scaffold for cartilage repair (Kisiday et al. 2002; Engel et al. 2008). Self-assembly of PAs can be promoted by various factors such as pH change, presence of Ca^{2+} ions, and drying on surface. Hong et al. (2003) developed another kind of

peptide containing 16 alternating hydrophobic and hydrophilic amino acids and studies the effect of amino acid sequence and pH on self-assembly into nanofibers (Thomas et al. 2006a).

6.3.2.2 Phase Separation

Phase separation techniques have been used to prepare porous polymer membranes for purification and separation purposes. In last two decades, it is becoming a frequently used and convenient method to prepare porous tissue regenerative scaffolds. A variety of biodegradable polymers have been fabricated into three-dimensional porous scaffolds using phase separation techniques (Zhang and Ma 1999; Ma et al. 2003; Gong et al. 2006). In order to meet the requirement of nanoscaled scaffold for tissue regenerative process, a novel phase separation technique has been developed to generate nanofibrous structures by manipulating the phase separation process (Zhang and Ma 2000; Chen and Ma 2006). The poly(L-lactic acid) (PLLA) fibrous scaffold contains nanofibers ranging from 50 to 500 nm in diameter (Chen and Ma 2006), which is similar to natural collagen fibers in size (Elsdale and Bard 1972; Hay 1991). Nanofibrous scaffolds of PLLA prepared from this technique have demonstrated to adsorb/absorb cell adhesive proteins (fibronectin and vitronectin) 2–4 times higher and an almost twofold increased osteoblast attachment in comparison to solid walled PLLA scaffolds (Zhang and Ma 2000). Due to the substantial surface area difference, degradation is much more rapid in such nanofibrous scaffolds, in which the overall mass loss is 51 % while mass loss in solid-walled nonfibrous foams is only 6 % after 15 months (Chen and Ma 2006).

One limitation of the early nanofibrous materials generated using the phase-separation technique is the lack of interconnected macropores, which are critical for cell seeding and recruiting, mass transfer, vascularization, and tissue organization. To overcome this problem, phase separation techniques are used in combination with other scaffold fabrication techniques such as porogen leaching. The combined technique provides broader control over porous architectures from macro-, micro- to nanoscales (Chen and Ma 2004; Wei and Ma 2006; Zhou et al. 2005; Gong et al. 2008; Ma et al. 2005a). Gong et al. (Zhou et al. 2005; Gong et al. 2008) fabricated well connected PLLA scaffolds via porogen leaching with phase separation technique in which gelatin particles was used as porogens. The biological performance of the scaffold was evaluated by in vitro chondrocyte culture and in vivo implantation. In comparison with the control scaffold fabricated with NaC1 particles as porogen under the same conditions, the experimental scaffold had better biological performance because the gelatin molecules were stably entrapped onto the pore surfaces (Gong et al. 2008). Surface modification was also taken place in order to improve the biocompatibility of these PLLA scaffolds. Ma et al. (2005a) immobilized collagen and introduced basic fibroblast growth factor (bFGF) on PLLA scaffold. Chondrocyte culturing on the collagen immobilized PLLA surfaces showed significantly improved cell spreading and growth. Incorporation of fibroblast growth factors in the collagen layer further enhanced the cell growth (Ma et al. 2005a).

6.3.2.3 Electrospinning

Electrospinning, as another method to produce nanoscale fibers, is a simple and cost-effective fabrication process that uses an electric field to control the deposition of polymer fibers onto a target substrate (Engel et al. 2008). The generated fibers can mimic the structural profile of the proteins found in the native ECM. The use of electrospinning process in biomaterials field was first reported by Martin and Cockshott (1977) as early as 1977. Since then, electrospinning process has been continuously investigated for the fabrication of nanofibrous matrices for divers applications (Reneker and Chun 1996; Li et al. 2002; J-s and Reneker 1999; Fong et al. 1999; Yoshimoto et al. 2003; Ma et al. 2005b; Chiu et al. 2005). Various synthetic polymer, PLA (Zong et al. 2002; Zeng et al. 2003), PLGA (Li et al. 2002), PCL (Silva et al. 2004; Yoshimoto et al. 2003), poly(dioxanone) (PDS) (Boland et al. 2005) and synthetic polypeptide (Huang et al. 2000), natural proteins such as collagen (Matthews et al. 2002; Huang et al. 2001; Matthews et al. 2003), silk protein (Li et al. 2005), elastin fibrinogen (Wnek et al. 2003) etc, are used for fabricating biodegradable scaffold. Electrospun nanofibers have been shown to support cell attachment and proliferation of a variety of cells as they have large surface area and well-interconnectivity of inter-fiber spaces, in addition to the nano sized diameters mimicking the physical nanoscaled dimensions of native ECM (Thomas et al. 2006a).

Biomolecules such as growth factors, drugs, and genes can be directly mixed into the polymer solution and electrospun to prepare functionalized polymer nanofibers. These functionalized bioactive nanofibers have potential applications in both tissue regeneracy and drug delivery systems. Co-spinning of growth factors for cells in future may enable to fabricate scaffolds with controlled release of cellular nutrients. Luu et al. (2003) and Liang et al. (Ye and Huang 2005) have encapsulated plasmid DNA in PLA-PEG and co-electrospun with PLGA in DMF and electrospun the mixture into nanofibers. Release of plasmid DNA from the scaffolds was studied for 20 days and found that the release of DNA sustained over 20 day period with a maximum release occurring at 2 h. Verreck et al. (2003) prepared polyurethane nanofibers containing model drugs itraconazole and ketanserin to study the pattern of drug release. Co-spinning of growth factors for cells in future may enable to fabricate scaffolds with controlled release of cellular nutrients (Thomas et al. 2006a).

Bioactive nanoscale fillers, e.g. hydroxyapatite (HA), tricalcium phosphate (TCP) et al., are incorporated into polymer solution to electrospun nanocomposite nanofibers for better interactions with cells. Thomas et al. (2006b) examined the physical property changes after nanoHA incorporated into PCL nanofibers. They demonstrated that it is possible to tailor subtle mechanical properties in a nanofibrous matrix by incorporating nanofillers of desired amount. Higher percentage loadings of nanoHA resulted in poor dispersion of the nanoHA powder as particle size of nanoHA used was ~100 nm. If the particle size of HA is small enough (~20–40 nm), PCL/nanoHA composite with more than 20-wt% produces fibers with well dispersed nanoHA (Thomas et al. 2006b). It has been reported that chondrocyte adhesion and proliferation on polymer/nanoHA composite materials are better than

the pure polymer (Hong et al. 2005). MSCs seeded onto nanocomposite scaffolds exhibited well cell spreading and growth on PCL/nanoHA nanocomposites, revealing favorable cell-matrix interactions (Thomas et al. 2006a).

As mentioned earlier, electrospinning of collagen into nanofibers have opened the door to make nanofibrous matrices mimicking nano structures of bone for bone tissue engineering. However an ideal scaffold for bone tissue engineering should mimic not only the nanofibrous physical structure but also the chemical composition. Electrospun nanofibrous nanobiocomposite scaffolds based on Type I collagen and nanoHA have been prepared as biologically inspired scaffolds mimicking the chemical and morphological features of natural ECM (Thomas et al. 2007).

Very recently, Badami et al. (2006) have electrospun PLA as well as PEG-PLA di-block copolymers of PEG-PLA into fibers with diameters ranging from 140 nm-2.1 mm and cultured MC3T3-E1 mouse calvaria-derived osteoprogenitor cells on the scaffolds up to 14 days. The results of study focussed on the effect of fiber diameter on spreading, proliferation and differentiation of osteoblastic cells on fibrous scaffolds with and without osteogenic factors The authors concluded that in the absence of osteogenic factors such as β-glycerophosphate and Lascorbat-2-phosphate, cell growth (cell density) was lower on polymer fibers than smooth polymer surfaces, while in the presence of osteogenic factors cell density on fibers was equal or greater than that on smooth surfaces (Thomas et al. 2006a).

Venugopal et al. (2005) have coated collagen over electrospun PCL by soaking the PCL matrix in collagen solution (10 mg/mL) and cultured human coronary artery smooth muscle cells. It was observed that SMCs migrated towards inside the nanofibrous matrices and formed smooth muscle tissue in 72 h. According to the authors, PCL scaffold supporting the cell growth needs collagen support for migration of cells inside the nanofibrous matrices. In another study He et al. (2005) showed that collagen coated PLLA-CL nanofibers exhibited enhanced cell attachment, spreading and viability of human coronary artery endothelial cells. It was found that coating of collagen on PCL scaffold definitely favored cell proliferation.

The co-use of these adhesion proteins and biodegradable synthetic polymers enables the construction of cell-adhesive scaffolds for vitally functioning engineered tissues (Kwon and Matsuda 2005; Kwon et al. 2001; Chen et al. 2000; Almany and Seliktar 2005; Zhang et al. 2005a). Co-electrospinning is a feasible approach to provide a compromise solution for overcoming the shortcomings of synthetic and natural polymers that is producing new porous nanofibrous biomaterials with good biocompatibility and improved mechanical, physical and chemical properties and biological performance.

Stitzel et al. (2006) have recently fabricated a vascular graft scaffold from electrospun polymer blends of Type I collagen (45 wt%), elastin (15 wt%) and PLGA (45 wt%). They found that by controlling the compositional ratio of collagen, elastin, and PLGA have resulted in improved electrospun fiber characteristics and physical strength of the vascular graft.

Core-shell types of multi component nanofibers by coaxial electrospinning are of another interesting mixed polymer system in tissue engineering for bioactive scaffolds. Functionalization of fibers without affect the core is desirable in tissue engineering and in controlled drug delivery for preserving an unstable biological agent from an aggressive environment and delivering a biomolecular drug in a sustained way. Co-axial electrospinning is a method for incorporation of water-soluble macromolecules as the core of nanofibers during electrospinning. The production of coreshell nanofibers from co axial electrospinning was first demonstrated by Sun et al. (2003). Zhang et al. (2004) fabricated bi-component nanofibers of PCL and gelatin in the form of a core-shell structure by coaxial electrospinning. A quantitative analysis of the effect of gelatin concentration on the diameters of core and shell of nanofibers was carried out that when the concentration of gelatin was below 12.5 w/v % the diameter of core and shell were, respectively, less than 200 nm and 400 nm. Zhang et al. (2005b) have fabricated collagen-PCL nanofibrous scaffold (collagen-r-PCL) by coaxial electrospinning and compared the surface biocompatibility with electrospun neat PCL, electrospun neat collagen scaffold and collagen coated PCL scaffolds by culturing human dermal fibroblasts. As compared to neat PCL scaffold and collagen coated PCL, scaffold human dermal fibroblasts cell density on collagen-r-PCL linearly increased over period. However, cell proliferations data of collagen-r-PCL are not significantly differ from those of electrospun neat collagen. In another study Jiang et al. (2005) fabricated biodegradable core-shell nanofibers with PCL as shell and protein containing PEG as core for controlled release of incorporated proteins such as lysozyme and BSA.

Recently, a group based in Singapore developed an alternative approach to wound healing, which they termed autologous layered dermal reconstitution (ALDR) (Chong et al. 2007). This technique relies upon novel TE scaffolds which consist of electrospun fibers made of PCL and gelatin, between 300 and 600 nm in diameter, with a total thickness of only 28 μm. The scaffolds were seeded with human dermal fibroblasts, which remained viable in the scaffold for all time points tested (up to 2 weeks) and doubled in population approximately every 3 days. Although no in vivo results are currently available, ALDR using electrospun scaffolds should offer a distinct advantage over traditional techniques. Namely, ALDR will allow for a rapid, layer-by-layer buildup of tissue in deep wounds, with dermal fibroblasts distributed throughout. This can occur because the electrospinning process takes place on top of a commercially available polyurethane wound dressing. As little as 48–72 h after implantation, the wound dressing can be removed, and another scaffold/wound dressing construct placed in the wound site. This is repeated until the wound area is fully repaired. Since each scaffold will be individually seeded with dermal fibroblasts prior to implantation, this layer by layer technique eliminates the long in vitro culture times otherwise needed for cellular infiltration and growth within larger, single-layer scaffolds. The end result is a continuous layer of tissue, wherein the use of a porous, nanostructured scaffolds allows for rapid cellular proliferation and integration between layers (Khang et al. 2010).

6.3.2.4 Nanocomposite Scaffold

Nanocomposite scaffold are made of regenerative scaffold with certain nanostructure system, for example, bioactive molecules and particles. Nanocomposites can be reinforced polymers or ceramics with low quantities of nanometric-sized particles (silicate, carbon nanotubes (CNT)) which give them improved properties. The properties of nano-composite materials depend not only on the properties of their individual parents but also on their morphology and interfacial characteristics. This rapidly expanding field is generating many exciting new materials with novel properties. Nanocomposites have attracted a great deal of attention in biomedical applications also. Many natural tissues such as bone possess a composite micro/nano structure. These complex composite structures play roles for the physical and biological properties of the tissues. To mimic the natural tissue structure, biomedical polymers, bioceramics and other organic/inorganic materials are to be combined for superior properties. Composite materials often show an excellent balance between strength and toughness and usually improved characteristics compared to their separate components. Recently, Kothapalli et al. have shown that by incorporation of 50 wt% nanoHA into PLA scaffold, the yield strength increased 150 % and compression modulus almost doubled in comparison to pure PLA. Addition of nanoHA can improve osteoconductivity to the polymer scaffolds (Kothapalli et al. 2005).

One of the most interested nanocomposite biomaterials for bone regeneracy is comprised of biodegradable polymers with nanoHA or other calcium phosphate bioceramic composition. Experiments prove that micro/nanometer features on biomaterial surfaces can be used to guide cell behavior along a desired biological response (Webster et al. 1999; Liu and Webster 2007). Xu et al. found that structural signals from microstructured substrates comprising arrays of square-shaped or round-shaped microwells could influence the migration, proliferation, and osteogenesis of the stem cells on the substrate. The results open a window to control stem cell functions by the combination of structured microwells with the manipulation of cellular signaling (Xu et al. 2014). In bone-regeneracy applications, promising results have been obtained with the nanophase materials ceramics and metals, with which increased osteoblast adhesion, proliferation and calcium deposition have been observed compared with conventional materials (i.e. with micrometer-scaled grains) (Webster and Ejiofor 2004).

Biologically inspired nanobiocomposites of collagen and nanoHA for bone substitute have a long history in biomedical field (Clarke et al. 1993; Rovira et al. 1993; TenHuisen et al. 1995; Itoh et al. 2001). There is possibility of enhancing the functionalities of collagen by incorporating other bone materials such as HA, bone morphogenic proteins (BMP) etc. A combination of collagen and nanoHA materials is bioactive, osteoconductive and osteoinductive and seems to be a natural choice for bone grafting. I.e., it mimics the bone components. The unique characteristics of this biocomposite is the spatial orientation between HA and collagen macromolecules, which seems to be the source of the mechanical strength of the composite. Conventionally, collagen/HA nanocomposites can be made by blending or mixing

the collagen and HA or by biomimetic methods (Du et al. 1999; Tampieri et al. 2003; Liao et al. 2004; Itoh et al. 2004; Yang et al. 2004b). However most of the collagen/HA composites are conventionally processed by anchoring microHA particles into the matrix of collagen, which makes it quite difficult to obtain a uniform to a homogeneous composite graft. Further, large size crystalline microHA, which is in contrast to natural bone apatite, may take a longer time to remodel into bone tissue up on implantation. In addition some of the composites exhibit very poor mechanical properties, probably due to the lack of strong interfacial bonding between constituents. There is a chance for improving osteointegration by reducing the grain size HA particles by activating the nucleation of ultra fine apatite growth into the matrix. This may lead to enhance mechanical properties and osteointegration with improved biological and biochemical affinity to the host bone (Thomas et al. 2006a).

Nanoparticle within regenerative medicine has been addressed mainly towards the development of entrapment and delivery systems for genetic material, biomolecules, such as growth and differentiation factors, and bone morphogenetic proteins and also as reinforcing- or bioactivity-enhancement phase for polymeric matrices in 3D scaffolds for tissue regeneracy (Engel et al. 2008).

Controlled delivery of biomolecules is crucial in the support and enhancement of tissue growth in tissue regeneracy applications. Nanotechnology approaches in delivery systems can enhance the success of specific therapeutic agents, such as growth factors and DNA among others, which are of paramount importance for tissue regeneracy (Reddy et al. 2006). Carriers in the nanoscale enable the intracellular delivery of molecules and the possibility of reaching targets that are inaccessible normally, such as the blood–brain barrier, tight junctions and capillaries, whereas the control over biomolecule dosage and delivery period are increased. The ultimate challenge is to develop artificial nanocarriers that can target cells with efficiency and specificity similar to that of viruses (Mastrobattista et al. 2006).

Examples of nanoparticles for delivery systems include currently microspheres, microcapsules, liposomes, micelles and also dendrimers. The different types of nanoparticles have been developed as solid, hollow or porous. The most common development methods are molecular self-assembly, nanomanipulation, bioaggregation and photochemical patterning (Allemann et al. 1993; Cade et al. 2004).

Biodegradable polymers are the most commonly used materials in drug delivery. Polylactic acid (PLA), polyglycolic acid (PGA), polyethylene glycol (PEG) and its copolymers have been used widely in combination with hydrogels to attain nanocarriers that exhibit different release properties. Particularly important for the development of nanoparticles for delivery purposes are ‘smart’ or ‘stimuli-responsive’ polymers that can undergo conformational changes, such as swelling or shrinkage, on variations in temperature, pH and magnetic field (Engel et al. 2008). Neffe et al. formed a 3D architectured hydrogel consisting of gelatin and lysine connected by urea junction units. The 3D architectured hydrogel could provide growing pores during the degradation, hence supporting cell adhesion and providing tailorable microscopic and macroscopic elastic properties of environment for cells. It could

effectively induce bone regeneration without requiring addition of cells or growth factors (Neffe et al. 2015).

In sophisticated tissue-engineering strategies, the biodegradable scaffold is preferred to serve as both a 3D substrate and a growth factor delivery vehicle to promote cellular activity and enhance tissue neogenesis (Jain et al. 2008). A novel approach has been described for fabrication of tissue-engineering scaffolds capable of controlled growth factor delivery whereby growth factor containing microspheres are incorporated into 3D scaffolds with good mechanical properties, well-interconnected macroporous and nanofibrous structures (Wei et al. 2006).

Incorporation of microspheres into scaffolds significantly reduced the initial burst release. Sustained release from several days to months was achieved through different microspheres in scaffolds. Released platelet derived growth factor (PDGF) was demonstrated to possess biological activity as evidenced by stimulation of human gingival fibroblast DNA synthesis in vitro. The successful generation of 3D nanofibrous scaffold incorporating controlled-release factors indicates significant potential for more complex tissue regeneracy (Jain et al. 2008). Growth factors are able to be incorporated on regenerative scaffold by other techniques, e.g. layer-by-layer self assembly. Collagen scaffolds functionalized with acid fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF) via assembly with heparin/PEI or chondroitin sulfate (Mao et al. 2005; Ma et al. 2007). The results prove that both aFGF and bFGF can be successfully deposited onto the scaffold. The FGFs in the multilayers obviously enhances fibroblast proliferation and viability (Mao et al. 2005; Ma et al. 2007).

We show here that the bioactive aFGF has been successfully deposited onto the TCPS sheet surface in the presence of heparin via a layer-by-layer manner. The aFGF built in the multilayers obviously enhances fibroblast proliferation and viability.

For the skin tissue engineering, Chung et al. (2006) explored the use of poly(ε-caprolactone) (PCL) grafted with nanostructured chitosan (CS) as a regenerative scaffold for the growth of human dermal fibroblasts. Resultant nano-CS/PCL surfaces exhibited significantly higher surface roughness values as compared to smooth CS/PCL surfaces: 106.0 nm compared to 3.6 nm, respectively. Furthermore, these nano-CS/PCL constructs exhibited significantly ($p<0.001$) higher rates of fibroblast proliferation and viability as compared to smooth CS/PCL surfaces or nano-rough PCL surfaces. As such, the technique of solvent spin-etching for polymers may represent an inexpensive means to prepare nanoscale TE scaffolds as improved artificial skin grafts (Khang et al. 2010).

6.4 Applications for Therapeutic Devices

After several decades development, dextran and other polymer-coated SPIONs are currently used in a number of biomedical applications; for example, Endorem® (Geurbet, France) is a commercially available contrast agent based on SPIONs

surface coated with dextran (Corot et al. 2006). It is a suitable contrast agent for labeling human MSCs (hMSCs) and human ESCs (hESCs) as it does not need a transfection agent (which may damage the stem cells) to facilitate its cellular uptake. Feridex ® and Sinerem® are other commercially available dextran-coated SPIONs that are combined with commercially available transfection agents, such as Fungene™, Superfect™ or Lipofectamine (Bulte and Kraitchman 2004; Corot et al. 2006). The use of transfection agents at higher concentrations may increase toxicity and, at lower concentrations, may not lead to sufficient cellular uptake (Bulte and Kraitchman 2004). Thus, the amount of transfection agent needed to enhance internalization is optimized carefully before combining it with SPIONs. The amount also depends on the stem cell type to be labeled.

With the further development and investigation, more and more products will be commercially available.

6.5 Barriers to Practice and Prospects

Although research on nanoparticles for non-invasive detecting is developing continually, there are still a lot of barriers which should be overcome.

Every technique and or application has it limitations, and the use of iron oxides or quantum dots for molecular and cellular imaging is no exception. (1) Resolution of MRI and fluorescence imaging is not good enough. For better understanding of cell behaviors, high resolution is required to investigate one single cell's migration, proliferation, and differentiation. (2) Better targeting and lower dose for imaging. Highly specific targeting is necessary for labeling interested cells only in order to reduce dose and get higher resolution. (3) for cellular imaging, as labeling is not permanent and self-replicable like reporter genes, with dilution of label upon cell division, iron oxide detection may rapidly become impossible, both in vitro (Bulte et al. 2001; Schaffer et al. 1993) and in vivo. (4) Finally, careful iron oxide titration and cellular differentiation studies need to be performed, as labeling may lead to inhibition of differentiation into certain cell types, without affecting cell viability or proliferation (Kostura et al. 2004).

For the scaffold for tissue regeneracy, they have not been used extensively but major contributions are expected in two areas. The first is growth of complex tissue, where microfluidic structures ensure a steady blood supply, thereby circumventing the well-known problem of providing larger tissue structures with a continuous flow of oxygen as well as nutrition and removal of waste products. The second, and probably more important function of microfluidics, combined with micro/nanotechnology, lies in the development of in vitro physiological systems for studying fundamental biological phenomena (Jain et al. 2008).

6.6 Conclusions and Future Challenges

Nanomaterials are considered as a new class of materials possessing superior properties over its microscale counterparts. Nanostructured biomaterials having physical nanofeatures such as nanocrystals, nanofibers nanosurfaces, nanocomposites, etc. have gained much interest in regenerative medicine.

The coregistration of in vivo fluorescence imaging with anatomical imaging modalities such as MRI helps traverse the shortcomings of fluorescence imaging, such as limited tissue penetration of photons and low three-dimensional spatial resolution, and provides complementary information. The development of multifunctional probes is attracting increasing attention and several studies have already appeared - from iron-oxide- and dendrimer-based dual MRI–fluorescence imaging contrast agents.

Effective and innovative imaging approaches are in great demand as new proteins and genes, particularly within the field of oncology, are being discovered at an ever-increasing pace. This provides a constantly multiplying library of molecules and pathways to be studied for prevention, diagnosis, and treatment of diseases. The full potential of new discoveries is however limited by the void between the advances in bioscience and the means to accurately, effectively and—critically—non-invasively image the molecular interactions in biological systems. Many challenges clearly remain in the pursuit of ideal SPIO probes for molecular imaging; increased target affinity, less complex conjugation schemes, reduction of cost, a means for MRS to avoid sequestration in lysosomes and more effective activatable probes. With persistent advances, this system continues to demonstrate its potential as a means to probe deeper into our biological universe.

Nanostructured scaffolds are interested in regenerative medicine, mainly because of their resemblance of nanomorphology and physical nanofeatures to natural extra cellular matrices. The nanoscaled features such as surface roughness and topography of nanocrystalline biocermics and nanofibrous scaffolds promote the cell behavior such as adhesion, proliferation and migration and differentiated functions. Polymeric nanofiber based nonwoven matrix is among the most promising nanostructured biomaterials for native ECM analogs. Electrospinning is a versatile technique to fabricate nanofibrous matrices of polymers for tissue engineering scaffold applications. One of the particular advantages of electrospinning in regenerative medicine is the ability to co-spin various components such as cell adhesive proteins and other cell-growth factors along with biodegradable synthetic or biopolymers. An ideal 3Dscaffold for tissue engineering should have similarity to native ECM in terms of both chemistry and physical nanostructure. Electrostatic co-spinning of nanocomposite fibers of polymers with nanoHA to fabricate hybrid scaffolds of improved mechanical properties and cellular behaviors has been established in our group. The unique characteristics of collagen/nanoHA composite system in native bone is the special orientation between HA and collagen molecules. Therefore, future efforts in nanofibrous collagen/nanoHA composite are required mimicking exactly the complex nano structured architecture of collagen matrix with the c-axis orientation of nanoHA particles (Thomas et al. 2006a).

Regenerative medicine aspects that focus on TE have evolved into two main strategies. The first strategy consists of an elegant approach in which stem cells harvested from the patient are expanded and seeded on 3D scaffolds within a bioreactor. The resulting hybrid construct is then implanted into the patient (together with growth factors) as a tissue matrix. However, the need to harvest and expand stem cells poses great efficacy and efficiency problems that define the success of the entire process. The second strategy relies on the development of intelligent materials that would be able to send signals to the stem cells already present in the diseased or damaged tissue niches that would then trigger the regeneracy process. Nanotechnology is a powerful tool for creating these 'smart' materials. This approach is challenging and is still far from being achieved. Among other advantages, it would raise the possibility to have such cell-free materials ready 'off the shelf' and to be able to use them as and when required (Engel et al. 2008).

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Chapter 7
Biointerface Technology

Joachim Rychly

Abstract The application of biomaterials to regenerate tissues requires research of the interface between the synthetic material and the living tissue. Because biomaterials represent a synthetic extracellular matrix that controls the cell biology by mechanism of cell adhesion, basic mechanisms of cell adhesion are addressed. The technology of designing instructive materials involves chemical modifications by grafting of chemical groups, adhesion ligands and growth factors. Physical characteristics of the materials are created by modifications of the surfaces structure and stiffness of the material. Because stem cells have emerged as promising cells to address the challenge of tissue regeneration the control of stem cells by the characteristics of materials is discussed. Insights into the mechanisms at the biointerface that are involved in the regulation of stem cells by materials will advance the development of innovative biomaterials in regenerative medicine. Another challenge in designing surfaces of medical implants is the prevention of infections due to a bacterial biofilm. Antimicrobial strategies involve both chemical and physical characteristics of the material surface.

Keywords Cell adhesion • Mechanotransduction • Surface grafting • Ligands • Surface coating

7.1 Introduction: An Historical Perspective

The biointerface is the interface between a nonviable material and the biological tissue or a cell. Mechanisms of the interaction between a material and the biological tissue control the reaction of the tissue and may also determine the fate of the material. The application of materials as medical implants or prostheses has a more than 2000 years history. To replace limbs, eyes, teeth, part of the skull or bone, beside wood or ivory the ancient cultures used mostly different metals. The first polymer

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as an implant was introduced by the British ophthalmologist Harold Ridley in 1949, when he used poly (methyl methacrylate) to replace a cataracted lens of a patient (Ridley 1952). He made the observation that the eyes of pilots who had shards of canopy plastic in their eyes due to enemy machine gun fire, tolerated this material, without ongoing reactions. In addition to implants, also ex vivo devices, like dialysis equipments or heart lung machines form a biointerface, in that case mostly with cells of the blood.

With the introduction of hip implants, vascular grafts or the kidney dialysis, first principles of application of medical materials were given by the late 1960s. The principal demand for a medical material was that the interaction of the material with the biological system should not provoke harmful reactions. The term "biocompatibility" originally refers to material characteristics of having no toxic effects or inducing mutagenesis and inflammation. The goal of the early biomaterials was to achieve a biological "inertness". The challenge of the new generation of materials is to create bioactive surfaces that are suitable to specifically control the biology of the tissue. In the field of regenerative medicine the control of stem cell plays a significant role. Therefore, the designing of implant materials is focussed on the question how characteristics of the materials are able to steer all the biological functions of a stem cell, which include self-renewal, differentiation to a specific cellular phenotype, secretion of bioactive factors, or migration. The development of such bioactive material surfaces requires the interdisciplinary collaboration between disciplines of engineering and the life sciences. The progress in this field depends on both the understanding of the biological mechanisms and the development of technological methods. The driving force for the design of bioactive material surfaces is the understanding of the complex mechanisms on the cellular level that determine the regenerative processes in the different tissues of the organism. Therefore, in this chapter first a review of cell biological mechanisms will be given with a focus on the adhesive interactions of cells with the extracellular matrix. These interactions play a key role at the cell-material interface and basically, the aim of material design is to control the cell biology by modifications of the chemical and physical properties of the material surfaces.

7.2 Background/Principles

7.2.1 Mechanisms of Cell Adhesion

Cells are regulated by different signals induced by soluble factors, cell-cell contacts and the interaction of cells with the extracellular matrix. Proteins of the extracellular matrix, like collagens, fibronectin, laminin, elastin are secreted by cells and differ in their composition depending on the type of tissue. For example, collagen I is a characteristic matrix component for bone, collagen II for cartilage or laminin for the basal membrane of the epithelium and endothelium. The composition and

structure of the extracellular matrix is dynamic and vary which determine its function. This is obvious during processes of the development and tissue differentiation. For example, during the development of branched organs like mammary gland, kidney, gut and lung the branched units are surrounded by a microenvironment that change in composition and spatial distribution over the time (Rozario and DeSimone 2010). The spatio-temporal expression and deposition of extracellular matrix provides instructive differentiation signals. In the mouse development, myogenic differentiation occurs as laminin, collagen IV and entactin expression increases, whereas fibronectin expression decreases (Godfrey and Gradall 1998). Although the control of stem cell differentiation by the extracellular matrix appears complex, defined matrix molecules induced specific differentiation of stem cells. Embryonic stem cells are normally not competent to differentiate to trophoblastic cells, however on collagen IV but not on laminin, fibronectin or collagen I the cells developed to a trophoblastic lineage (Schenke-Layland et al. 2007). Also directed differentiation of multipotent adult stem cells was dependent on the type of matrix protein. Neural stem cells developed to neurons, astrocytes and glia cells on laminin but not on fibronectin (Flanagan et al. 2006). Osteogenic differentiation of human mesenchymal stem cells was induced on laminin-5, collagen I and vitronectin (Klees et al. 2005; Kundu and Putnam 2006; Salasznyk et al. 2004). The studies also revealed that differentiation to the same phenotype might be differentially regulated by different matrix proteins (Kundu and Putnam 2006). As already mentioned, the extracellular matrix is a highly dynamic structure, which is constantly undergoing remodelling, i. e. assembly and degradation. Experiments using fluorescence time lapse-imaging demonstrated that in a cell culture individual fibrils of fibronectin were stretched and displaced (Sivakumar et al. 2006). Motile osteoblasts actively mediated fibronectin assembly by adding globules of matrix molecules to existing fibronectin fibrils and reorganized the extracellular matrix by shunting matrix material from one location to another or exchanged fibrillar material between fibrils. Remodelling of the extracellular matrix is the result of multiple processes, which requires at least two events: synthesis and proteolytic degradation of the components (Daley et al. 2008). Among the proteolytic enzymes, matrix metalloproteinases (MMPs) play a dominant role in the degradation of the extracellular matrix. Although matrix protein degradation remains a principal physiological function of MMPs, there is evidence that also other substrates, like peptide growth factors, tyrosine kinase receptors, chemokines are a target of MMPs, which indicates a more extensive involvement of MMPs in a variety of physiological processes (Page-McCaw et al. 2007; Stamenkovic 2003). The interaction of cells with the extracellular matrix is mediated by receptors of the integrin family which enable a bidirectional signal transduction (Hynes 2002; Takada et al. 2007). Integrins function as heterodimeric transmembrane receptors consisting of one ß and one α-subunit. In human, 18 α-subunits and 8 ß-subunits are described, which form at least 24 different receptors (van der Flier and Sonnenberg 2001; Wehrle-Haller and Imhof 2003). The combination of the ß with the α-subunit determines the binding specificity for the ECM ligand and a simplified classification into three classes yields a group of integrins, which binds to the RGD sequence (amino acids

Arg-Gly-Asp) of fibronectin or vitronectin, receptors which bind to laminin and integrins that bind to collagens (Wiesner et al. 2005). Activation of integrins which induces signal transduction involves conformational changes in the extracellular domain to expose the ligand-binding site (Luo et al. 2007). The conformational changes also enable an increased binding avidity which leads to a clustering of hundreds or thousands integrin interactions with matrix ligands into tightly bound adhesive units (Legate et al. 2009). To connect integrins with the actin cytoskeleton in integrin mediated signal transduction, the formation of adhesion complexes at the interface between cell and substrate plays a dominant role. In these focal adhesions 157 molecules have been identified that are assembled in a "integrin adhesome" and enables signal transduction (Zaidel-Bar et al. 2007). Failure to establish functional adhesions and thus the assembly of cytoplasmic scaffolding and signalling networks can have severe pathological effects (Winograd-Katz et al. 2014). Upon integrin binding to a ligand focal adhesions mature. First nascent adhesions are organized within the lamellipodium. During maturation the adhesions grow into dot-like structures, which then become elongated to form fibrillar adhesions (Geiger et al. 2001; Wehrle-Haller and Imhof 2002; Zaidel-Bar et al. 2003). This process is facilitated by the α- actinin-actin structures and requires myosin II (Choi et al. 2008). Super-resolution fluorescence microscopy enabled a nanoscale mapping of the organization of proteins in focal adhesions (Kanchanawong et al. 2010). The functions of some of the numerous proteins assembled in focal adhesions have been elucidated. For example, by its polarized orientation, talin has a role in the organization of proteins inside of focal adhesions (Kanchanawong et al. 2010). Talin facilitates the interaction of integrins with the cytoskeleton by direct binding to the integrin tail, or vinculin plays a role in the formation and growth of focal adhesions (Gallant et al. 2005; Humphries et al. 2007; Zhang et al. 2008). FAK appears to be responsible for turnover of focal adhesions and actin polymerization and is a major component in further downstream signalling events (Zhao and Guan 2009). Downstream, integrin signalling shares common pathways of growth factor receptors, like activation of MAP-kinases (Miyamoto et al. 1996; Moro et al. 1998). Beside the cross-talk between integrins and growth factor receptor pathways, also the physical proximity and lateral collaboration at the cell membrane between integrins and growth factor receptors are important to induce signalling and in consequence a biological function (Schneller et al. 1997).

7.2.2 Cellular Mechanotransduction

Cells are able to sense mechanical forces, which control their physiological functions. Physical forces act or are generated at the interface between the cell and the extracellular matrix (Geiger et al. 2009; Mammoto and Ingber 2009; Puklin-Faucher and Sheetz 2009). Therefore, the cellular components that facilitate cell adhesion to the extracellular matrix have a primary role in the cellular sensory machinery and are able to integrate and transduce mechanical signals. Transduction of mechanical

forces is bidirectional. While cells are able to sense forces from outside they also generate forces to the extracellular matrix, which is facilitated by the cytoskeleton and regulated for example by actin polymerization (Galbraith et al. 2007; Giannone et al. 2007; Ingber 2006; Kumar et al. 2006). Myosin II is responsible for the contractile nature of the stress fibres to exert forces to the extracellular matrix (Katoh et al. 2001; Peterson et al. 2004). Integrins function as primary sensor and mechanotransducer and facilitate the mechanical coupling between inside and outside the cell (Schober et al. 2007; Wang et al. 1993). Transition of the β integrin subunit from an inactive state to an active conformation can be induced by mechanical forces (Cluzel et al. 2005; Kim et al. 2004; Puklin-Faucher et al. 2006). Mechanical loads directly applied to integrins induce an accumulation of focal adhesion molecules and a direct physical link to the cytoskeleton by immobilizing of signalling proteins, like FAK to the actin cytoskeleton (Cox et al. 2006; Michael et al. 2009; Riveline et al. 2001; Schmidt et al. 1998). As found in proteomic analyses, activation of integrins induces adhesive complexes, in which many cytoskeleton-binding proteins and proteins with a broad range of cellular functions are enriched (Byron et al. 2015). The recruitment and assembly of some proteins depend on mechanical tension generated by myosin II-mediated contractile forces (Schiller and Fassler 2013). To convert mechanical forces into biochemical signalling events, proteins at the adhesive interface are stretched and expose binding sites (Brown and Discher 2009; Vogel and Sheetz 2009). Vinculin binds to talin rod due to mechanically stretching of the talin molecule (del Rio et al. 2009). Recently, filamin A has been identified as a mechanotransductive substrate within the cytoskeleton. When strain is applied, β integrin binding to filamin A increased which enables its cytoskeletal anchorage, whereas the protein FilGAP dissociates from filamin A (Ehrlicher et al. 2011). Detailed studies revealed that fibrillar fibronectin can be extended by stretch more than eightfold and the mechanically induced unfolding of fibrillar fibronectin alters the displayed binding sites (Klotzsch et al. 2009; Vogel 2006). Fibronectin contains different recognition sites for binding of serum proteins, other matrix proteins, cell adhesion proteins distributed over more than 54 domains that can be switched on and off be mechanical forces (Vogel and Sheetz 2009). Interestingly, the mechanical properties of the fibronectin fibres are regulated, old fibres become more unfolded with age than newly deposited fibres. Further, due to differences in the mechanical strain, fibrillar fibronectin is more unfolded on rigid than on soft substrates (Antia et al. 2008). To identify mechanosensitive transcription pathways, a gene expression screen in epithelial cells revealed that the transcription factors YAP and TAZ were differentially expressed and localized to the nucleus when the cells were plated on substrates with increased stiffnesses (Dupont et al. 2011). In this case, YAP/TAZ only accumulated in the nucleus when the cells actively generated tension. This study also demonstrated that both factors are functionally required for the differentiation of mesenchymal stem cells to osteoblasts. In addition to a mechano-biochemical conversion near the adhesion site, there is evidence that cells are able to transduce mechanical signals directly to the nucleus due to a structural connectivity between extracellular matrix and cell nucleus (Maniotis et al. 1997; Wang et al. 2009). In this model, the cell is a “hard wired” tensegrity network which

refers to a stable interconnected cytoskeleton that resists mechanical stresses and maintain shape stability (Ingber 1997; Stamenovic et al. 1996). The connection between cytoskeletal filaments and the nuclear membrane is facilitated by a LINC complex (linker of nucleoskeleton and cytoskeleton) containing nesprins, sun and lamin proteins (Crisp et al. 2006; Haque et al. 2006). Through lamin A, which binds transcription factors, mechanical forces could directly alter gene expression in the nucleus (Dechat et al. 2008). In addition, mechanically induced expansion or contraction of nuclear pores may alter transport processes into the nucleus (Feldherr and Akin 1990). Such direct force transmission between cell membrane and nucleus may induce a fast induction of gene expression and may explain a rapid increase of calcium in the nucleus (Pommerenke et al. 2002).

7.2.3 Interaction with the Extracellular Matrix in the Stem Cell Niche

The stem cell niche is a specialized microenvironment in various organs which provides an anatomical compartment to maintain a pool of stem cells (Jones and Wagers 2008). The microenvironment, which involves soluble factors, the interaction with other cells and an extracellular matrix, regulate stemness, survival, differentiation, and migration out of the niche (Kolf et al. 2007). To mimic the mechanisms in a niche by bioactive material surfaces, the extracellular matrix is of primary interest. Evidence exists that the composition and mechanical properties of extracellular matrix determines the fate of stem cells in a niche, e. g. controls the balance between self-renewal and differentiation (Brizzi et al. 2012; Daley et al. 2008). The extracellular matrix of stem cell niches mainly consists of basement membrane components, like collagens, laminins, fibronectin, glycosaminoglycans (Votteler et al. 2010). Specific interactions of a matrix protein with a stem cell regulate the stem cell population within a niche. As an example, in skeletal muscle stem cells, binding of the receptor syndecan-4 in a complex with Wnt7a to fibronectin stimulates the expansion of these cells and newly activated stem cells remodel the niche by a transient increase in the expression of fibronectin (Bentzinger et al. 2013). Mesenchymal stem cells are localized in a perivascular niche and are exposed to signals from vascular cells (Crisan et al. 2008). Studies stressed the assumption that the type of extracellular matrix may determine the direction of stem cell differentiation, because on extracellular matrix derived from endothelial cells, mesenchymal stem cells developed markers of endothelial or smooth muscle cells (Lozito et al. 2009). Thus, dynamic remodelling of the extracellular matrix at a specific time and in a tissue-specific manner within a niche functions as an important switch to trigger stem cell differentiation or mobilization. Stem cell niches in various tissues obviously differ in their extracellular matrix components and their functions (Gattazzo et al. 2014). In the hematopoietic stem cell niche, collagen VI apparently plays a role, because in functional studies it provided a strong adhesive substrate for different hematopoietic cells (Klein et al. 1995) and the matrix glycoprotein osteopontin appears to

localize hematopoietic stem cells to the endosteal bone surface (Nilsson et al. 2005). In the neural stem cell niche, two laminin-like proteins, netrin-4 and reelin regulate the migration of neural progenitor cells (Kazanis and ffrench-Constant 2011). Beside regulation of the fate of stem cells mediated by specific adhesion to a matrix component, binding and presentation of growth factors by the extracellular matrix controls the cells inside the niche. The activity of these factors can be facilitated by receptor cross talk between integrins and growth factor receptors in the cell membrane (Brizzi et al. 2012). Because effects of the matrix are mediated by cell adhesion receptors like integrins, specific expression and activation of integrins play a role in controlling the stem cell population. Differential expression of integrin-β1 has been observed to regulate cell restriction and mobility of stem cells in the epidermal stem cell niche (Jensen et al. 1999). The fate of neural stem cells appeared to be dependent on the expression of β1-integrin (Yoshida et al. 2003). Neural stem cell differentiation was accompanied by a decrease in α5β1-Integrin. Nonetheless, because of the complex interaction of various factors in the niche, our understanding of the precise mechanisms how extracellular matrix determines the fate of stem cells is limited.

7.3 Technological and Biological Opportunities for Therapeutic Devices

7.3.1 Chemical Modification to Control the Biointerface

7.3.1.1 Modification of Chemical Groups

Chemical as well as physical characteristics of a material control the biological response of the tissue. For tissue regeneration, the key question is that, how the properties of a biomaterial specifically control the different biological functions of stem cells. Different steps of surface designing can generate a bioactive chemistry of a material. First, the chemistry is determined by the pure uncoated material. Next, the chemistry can be modified by grafting chemical groups on the surface, which alter the surface charge and the wettability. More specifically, molecules of the extracellular matrix or peptides which are characteristic of matrix domains and function as binding sites may be immobilized. Last, soluble factors, like growth factor may be incorporated into the material surface, which might be active as solid-phase ligand or which can be released by various mechanisms.

Dependent on the application regarding the tissue and function, materials for implants reach from metals to synthetic polymers and natural materials. All these materials differ in the chemistry of the surface. At the interface to a material surface the interaction of the cell is mediated by extracellular matrix proteins. However, prior to a matrix production of the cell, a first adhesive contact of the cell to the substrate can be mediated by a hyaluronan coat of the cell (Cohen et al. 2006; Evanko et al. 2007). The strength of this interaction differs in dependence on the

material to which the cell does adhere (Finke et al. 2007). For the subsequent integrin mediated adhesion, adsorption and organization of the extracellular matrix proteins to a material are required. The role of chemical variations of the surface to mediate adhesion dependent stimulation of biological functions of stem cells can be evaluated by generating polymers with different combinations of monomers. Combining 25 different monomers of acrylates to generate 576 polymers allowed a screening to identify materials with the ability to stimulate proliferation and differentiation of human embryonic stem cells (Anderson et al. 2004). Some of the polymers allowed for a high level of cytokeratin positive cells, indicating differentiation to epithelial cells. Interestingly, for some materials proliferation was observed only in the absence of retinoic acid as a soluble factor. This indicates an interaction of signals from soluble factors and the adhesive substrate. A relationship was also established between the ability of the polymers to adsorb fibronectin and cell adhesion (Keselowsky et al. 2003; Mei 2009). Polymers are not only capable to generate different amounts of adsorbed fibronectin, but also induce different activities of fibronectin (Mei 2009). Different techniques have been used to modify the chemistry of a material surface, which involved the use of self-assembled monolayers of alkanethiols, silanisation, plasma treatment, radiation grafting (Curran et al. 2005; Keselowsky et al. 2005; Ratner 1995). Grafting of functional groups using glow discharge plasma deposition was also successfully applied to modify titanium surfaces (Nebe et al. 2007). A major challenge of these modifications is the precise control of functional groups. The spectrum of functional groups comprises amino, methyl, hydroxyl, ether, carbonyl, carboxyl and carbonate. Specific alterations of the chemistry were found to guide differentiation and proliferation of mesenchymal stem cells (Curran et al. 2006; Phillips et al. 2010). -NH_2 and -SH modified surfaces stimulated osteogenic differentiation, whereas -OH and -COOH modified surfaces promoted chondrogenesis. Under specific culture conditions, –NH_2 surfaces enhanced the formation of adipogenic cells (Phillips et al. 2010). Generation of -CH_3 groups maintained the phenotype of mesenchymal stem cells (Curran et al. 2006). These biological responses of the cells depend on mechanisms related to changes in the cell-extracellular matrix interaction. Surface chemistry of a material can induce changes in the conformation of fibronectin, which modifies binding of integrins and induces short-term changes in focal adhesion formation (Keselowsky et al. 2004). Generation of –NH_2 groups on titanium surfaces using plasma polymerized allyl amine promoted the spreading of osteoblasts (Nebe et al. 2007). Titanium implants are widely used as bone substitutes, e. g. for artificial hip or knee joints. To stimulate bone regeneration at the interface to the bone tissue, titanium coating with calcium phosphate is a suitable approach because of the similarity with the mineral phase present in bone (de Groot et al. 1998; de Jonge et al. 2008). Similarly, calcium phosphate composites are applied as degradable scaffolds to heal bone defects (El-Ghannam 2005). The most successful technique to coat metallic implant with calcium phosphate has been the plasma-spray technique. Because coating must be at least 50 μm thick to completely cover the surface other methods including sol-gel deposition, electrospray deposition, electrolytic deposition have been applied and each has its advantages and disadvantages (de Jonge et al. 2008). Calcium phosphate

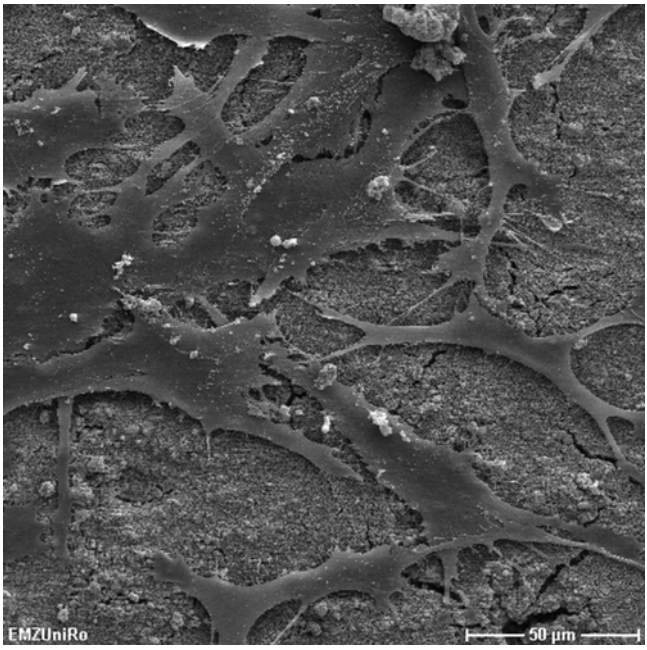

Fig. 7.1 Mesenchymal stem cells adhere, spread and form a flat morphology on hydroxyapatite coated surfaces

coatings are described to induce an increased bone-to-implant contact and therefore are regarded as osteoconductive (Barrere et al. 2003; Leeuwenburgh et al. 2006). To see, whether calcium phosphate surfaces may affect bone regeneration, a number of in vitro studies demonstrated that calcium phosphate promote the osteogenic differentiation of mesenchymal stem cells (Cordonnier et al. 2010; Moreau and Xu 2009; Muller et al. 2008; Sun et al. 2008). Although the mechanisms are not known, the observed strong adsorption of fibronectin and vitronectin, as well as a very flat morphology of stem cells on a calcium phosphate surface (Fig. 7.1) could support an osteogenic differentiation (Kilpadi et al. 2001; Walschus et al. 2009). Evidence exists that a substrate of graphene promotes cell adhesion and proliferation (Aryaei et al. 2014). To test the effect of such surfaces on cell reprogramming to pluripotent stem cells, embryonic mouse fibroblasts transfected with four transcription factors to induce reprogramming were cultured on graphene (Yoo et al. 2014). The results revealed an increased number of colonies on graphene that were undergoing reprogramming compared with cells on a glass surface.

7.3.1.2 Grafting of Cell Adhesion Ligands

To further specifically control cell adhesion, material surfaces can be grafted with complete molecules of the extracellular matrix or synthetic peptide sequences which represent binding sites of matrix proteins. The best known of these is the

RGD peptide containing the amino acids arginine, glycine, aspartic acid which is found in fibronectin, laminin, collagen type IV, tenascin and thrombospondin (Benoit and Anseth 2005; Comisar et al. 2007) and several other adhesion molecules. Structural modifications of the peptides from linear to cyclic RGD peptides are potent alternatives and can enhance affinity towards a receptor or stimulate cell adhesion (Durrieu et al. 2004; Maeda et al. 1994). In most cases RGD peptides are linked to polymers via stable covalent amide bonds. In this case an activated surface carboxylic acid group reacts with the nucleophilic N-terminus of the peptide (Lin et al. 1994). Alternatively, a coupling is possible in a two-step protocol. First, the surface carboxyl group is activated as an ester and followed by coupling the peptide in water (Jo et al. 2000). Beside synthetic polymers, other materials, including natural polymers, starch, dextran and inorganic materials have been coated with RGD peptides (Hersel et al. 2003). Among the inorganic materials, titanium and hydroxylapatite were successfully coated with RGD peptides (Fujisawa et al. 1997; Itoh et al. 2002; Rezania et al. 1999). On hydroxylapatite, RGD-peptides were immobilized via negatively charged anchoring groups, like glutamic acid, phosphonates or natural HA-binding amino acid sequences (Gilbert et al. 2000; Hersel et al. 2003; Itoh et al. 2002). To prevent unspecific protein adsorption, grafting of RGD peptides can be combined with passivation of the material surface using e. g. poly(ethylene glycol) (Banerjee et al. 2000; Drumheller and Hubbell 1995). Star-shaped poly (ethylene glycol) prepolymers were used to prevent unspecific protein adsorption and allowed the binding of RGD peptides for specific adhesion of mesenchymal stem cells (Groll et al. 2005). For a spatially and temporally controlled presentation of adhesive peptides, activation of the peptides using light or other triggers is discussed (Boekhoven et al. 2013; Petersen et al. 2008). Recently, a transdermal light triggering of cell-adhesive peptides on a hydrogel, subcutaneously implanted in mice was demonstrated (Lee et al. 2015). In these experiments cyclic RGD peptides were modified using a photolabile butyl ester as caging group. On exposure to UV-light the caging group is released and an active RGD peptide presented. Cell experiments on materials coated with matrix proteins or peptides revealed that integrin mediated interactions with the substrate are complex and require flexible and dynamic mechanisms. Therefore, the introduction of a spacer to bind RGD peptides or matrix proteins improved cell attachment (Craig et al. 1995; Kantlehner et al. 2000). When collagen was immobilized to a polyether ether ketone via glutardialdehyde, osteoblasts did adhere but spread only when polyethylene glycol as spacer was introduced (Fig. 7.2). To further enable a dynamic interaction of cells with the adhesive substrate and remodel the extracellular matrix, materials were cross-inked by enzyme-degradable peptide sequences. The combination of integrin binding and matrix degradation by cellular metalloproteinases allowed the cells to migrate through a gel, which mimics tissue remodelling (Lutolf et al. 2003a). Enzymatically mediated cell migration has been provided using materials from chemically crosslinked hyaluronic acid (Bulpitt and Aeschlimann 1999; Park et al. 2003). Further, elastase-sensitive sequences were generated by crosslinking elastin-like units which contained the adhesion motif REDV (Girotti et al. 2004). Cleavage of the polymer yielded a bioactive VGVAPG fragment which stimulated cell proliferation. This

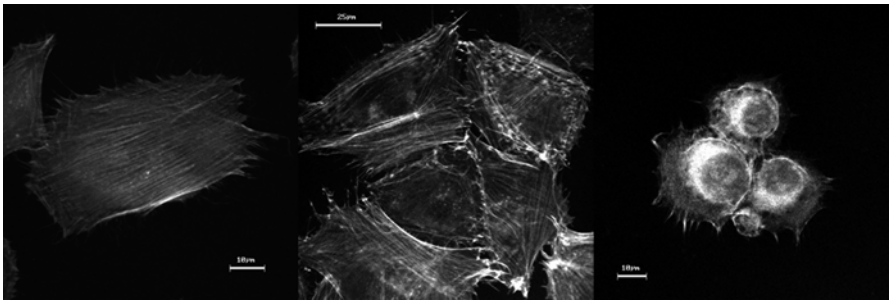

Fig. 7.2 The mode of collagen immobilization determines the spreading of osteoblasts. *Left*: On cover glass, which was coated by collagen I adsorption, cells spread and form actin fibres; *middle*: Cells spread and form actin fibres on a polyether ether ketone (PEEK) surface coated with collagen I, which was immobilized by glutardialdehyde (GDA) and polyethylenglycol was introduced as a spacer; *right*: Cells adhere but remain round without formation of actin fibres on PEEK, coated with collagen I, immobilized via GDA alone

functionality mimics dynamic processes of the extracellular matrix in vivo, whereby enzymatic activities can liberate cryptic binding sites. Although immobilization of matrix-derived peptides demonstrated support of cell adhesion, data of the biological specificity of such approaches are rare (Carson and Barker 2009). When titanium was passivated and grafted with the fibronectin fragment $FNIII_{7–10}$, this surface enhanced the osteogenic differentiation of mesenchymal stem cells relative to RGD immobilized surfaces (Petrie et al. 2008). This appeared to result from the specific targeting of the β1α5-integrin. The presentation of adhesion peptides in a structural organization that mimic fibrils of the extracellular matrix could further contribute to the biological outcome. RGD peptides in 3D-network of nanofibers promoted the osteogenic differentiation of mesenchymal stem cells (Hosseinkhani et al. 2006). In a three dimensional network of nanofibers the immobilization of the laminin epitope IKVAV induced the differentiation of neural progenitor cells into neurons (Silva et al. 2004).

7.3.1.3 Grafting of Antimicrobial Peptides and Organic Compounds

A challenge in designing regenerative implants is the combination with antimicrobial surfaces that prevent the formation of an infectious bacterial biofilm. While RGD peptides facilitate direct binding of cells via integrin receptors and are able to induce signal transduction, antimicrobial peptides are able to electrostatically interact with bacterial membranes and disrupt the structural integrity of the membrane (Alves and Olivia Pereira 2014). Antimicrobial peptides are components of the immune system of living organisms and protect them against microorganisms. They have certain common properties, like a highly cationic character and can adopt an amphipathic structure because of their high proportion of hydrophobic residues. An important feature is the ability to discriminate between host and microbial cells.

Two principal strategies have been explored to immobilize antimicrobial peptides to surfaces, i. e. layer-by-layer techniques and covalent immobilization. In the layer-by-layer approach anti-microbial peptides can be embedded in a multilayer of alternate adsorption of polyanions and polycations. The release and antimicrobial activity can be controlled by the number of layers deposited. When the antimicrobial peptide ponericin G1 was incorporated into a polyelectrolyte multilayer film with varying architectures, the peptide was released over 10 days and inhibited the attachment of *Staphylococcus aureus* (Shukla et al. 2010). The broad-spectrum antimicrobial peptide HHC-36 was impregnated into a three-layer coating of TiO_2, calcium phosphate and a phospholipid on titanium, which was highly effective against *S. aureus* and *Pseudomonas aeroginosa* (Kazemzadeh-Narbat et al. 2013). Covalent immobilization of antimicrobial peptides on a titanium surface was performed by first activation of the surface using O_2 plasma followed by silanization with 3-chloropropyl triethoxysilane (CPTES) which enabled binding of the peptide GL 13 K (Chen et al. 2014). This surface killed the bacteria by rupturing the cell membrane under flow conditions. To functionalize a polymer brush of Pluronic F-127 with an antimicrobial peptide, the polymer was terminally carboxylated to introduce an activated ester (Muszanska et al. 2014). The ester activation then allowed a coupling with the synthetic antimicrobial peptide. This anti-microbial polymer brush revealed both anti-adhesive and antibacterial properties against three bacterial strains. A critical factor for the immobilization of antimicrobial peptides to surfaces is the introduction of spacers. The antimicrobial activity of some peptides was completely lost when immobilized on solid surfaces in the absence of a spacer (Gabriel et al. 2006). A stretchable spacer probably allows that the peptide can permeabilize the membrane and enter the bacterium (Alves and Olivia Pereira 2014). The cationic polymer polyallylamine was tested as antimicrobial surface after covalently binding to a glass surface via methoxysilane (Iarikov et al. 2014). This surface effectively killed attached bacteria of *Staphylococcus aureus and S. epidermidis* up to 97 %. Other organic compounds derived from plants or algae attracted the attention for the generation of antimicrobial coatings (Vasilev et al. 2009). Furanones are compounds extracted from marine algae and have a strong antibacterial activity, when physically adsorbed to a biomaterial surface (Baveja et al. 2004). Another study demonstrated the immobilization of furanone loaded nanoparticles of poly(L-lactic acid) to a titanium surface by crosslinking the particles on a microarc-oxidized titanium surface (Cheng et al. 2015). This surface released furanone for 60 days with an antibacterial effect. Efforts are also made to combine anti-microbial activity and improvement of cell adhesion. Additional immobilization of RGD peptides on a polymer brush with antimicrobial peptides enhanced the adhesion of fibroblasts in a similar extension to RGD peptides alone on the surface (Muszanska et al. 2014). Coupling a polyelectrolyte multilayer of hyaluronic acid as antimicrobial surface with RGD peptides did also significantly improve the adhesion of osteoblasts (Chua et al. 2008).

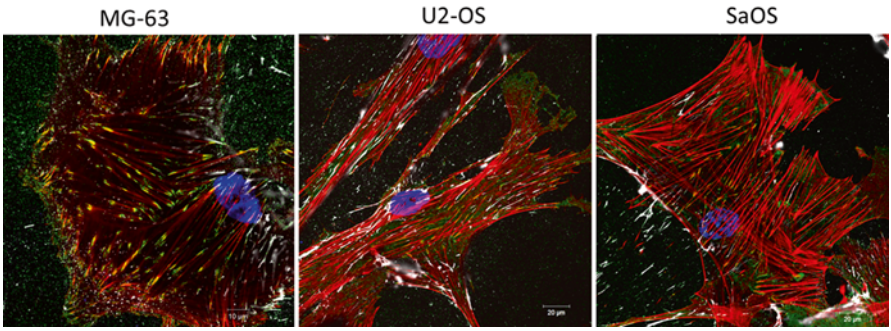

Fig. 7.3 Decellularized extracellular matrix from three different osteoblastic cell lines (MG63, U2-OS, SaOS) was deposited to a polished titanium surface. On these surfaces, a different appearance of focal adhesions was observed in cultured human mesenchymal stem cells. Whereas on matrix of MG-63 cells, cells developed well pronounced adhesions, on matrices of U2-OS and SaOS cells, focal adhesions were less expressed. (*green* – vinculin, *red* – actin, *blue* – nucleus)

7.3.1.4 Coating with Decellularized Extracellular Matrix

For tissue regeneration the natural extracellular matrix might supply an optimal support to control cellular processes. Several studies have documented that decellularization of whole organs, like heart, liver, lung, or kidney provides a scaffold of extracellular matrix that is able to regenerate tissue, when replenished with new cells (Arenas-Herrera et al. 2013; Ott et al. 2008). Different organs require different decellularization protocols including agents to generate an acellular organ that maintain its vascular structures. To enhance the biological functionality of implant materials, biomaterials can be coated by cell-generated natural extracellular matrix, which might preserve its composition and structure as found in vivo (Cheng et al. 2014; Fitzpatrick and McDevitt 2015). Implant materials, like titanium and polymers were used to coat with decellularized extracellular matrix (Fig. 7.3). For the generation of such hybrid materials several questions arise. Is the cell source important to obtain a matrix with a specific biological effect, which detergent can be used to remove the cells from the matrix, or is it possible to transfer the produced matrix to another material without loss of structural properties? A number of studies were aimed at stimulating osteogenic regeneration and used osteblastic cell lines, like SaOS-2 and MC-3T3 cells or mesenchymal stem cells as cell source to produce extracellular matrix (Datta et al. 2005; Pati et al. 2015; Thibault et al. 2013). Materials coated with decellularized matrix of these cells favoured the osteogenic differentiation of mesenchymal stem cells cultured on these substrates, also in the absence of osteogenic factors in the culture medium. When tested five different cell types to produce extracellular matrix, distinctive cellular responses of mesenchymal stem cells on these matrices have been observed (Rao Pattabhi et al. 2014). Matrix

derived from mesenchymal stem cells induced an increased proliferation of cultured mesenchymal stem cells on this decellularized matrix. Extracellular matrix, which was laid down by mesenchymal stem cells that were cultured in osteogenic differentiation medium for 3 days before, stimulated osteogenic differentiation of mesenchymal stem cells on this matrix. Extracellular matrix from two muscle cell lines induced a smooth muscle cell-like cell phenotype. Other experiments revealed that decellularized matrix from human fetal mesenchymal stem cells were more proliferative than adult mesenchymal stem cells (Ng et al. 2014) and matrix from bone marrow cells of young mice revealed a higher osteogenic capacity than matrix derived from cells of old mice (Sun et al. 2011). Indeed the composition of extracellular matrices from different cell types vary as shown in a comparison between bone marrow mesenchymal stem cells, articular chondrocytes and dermal fibroblasts (Lu et al. 2011). From experiments, in which mesenchymal stem cell derived matrix accelerated proliferation and multiple differentiation it was speculated that rather non-collagenous proteins are responsible for the differential effect of this matrix (Lin et al. 2012). In addition to a different composition of the matrix in dependence of the cell type, it became also obvious that mechanical properties of the matrices from different cell types vary (Prewitz et al. 2013). Mesenchymal stem cells produced a softer matrix than human neonatal dermal fibroblasts or human umbilical vein endothelial cells. The procedure to prepare cell-derived extracellular matrix appears to be important for the characteristics of the matrix. The goal of decellularization is to remove allogenic or xenogenic cellular antigens, but the selected procedure should preserve the composition, bioactivity and structural integrity of the matrix. Methods for decellularization include both chemical and physical treatments, but commonly used methods appear to be insufficient to achieve a complete removal of cellular components (Badylak et al. 2009). When comparing seven procedures, using a combination of freeze-thaw cycles or osmotic shock with different detergents, it was found that freeze-thaw in combination with NH_4OH and Triton X-100 combined with KCl were most effective (Lu et al. 2012). A more mild treatment was recently developed which appears to be superior in maintaining the structure of the decellularized matrix (Rao Pattabhi et al. 2014). The cell culture was treated with EDTA-PBS at 4 °C until the cells round up and detached from the substrate. To perform large-scale production of decellularized matrix for different applications, a transfer of coatings to a secondary surface would be required. To test this goal, extracellular matrix deposited by mesenchymal stem cells on tissue culture plastics was collected, mechanically homogenized and stored at room temperature up to 1 month (Decaris et al. 2012). After transfer to secondary tissue culture plates, the transferred matrix retained the ability to induce osteogenic differentiation in mesenchymal stem cells more sufficiently than on cell culture plastics. Because extracellular matrix also functions as reservoir and presenter of growth factors, efforts were made to immobilize growth factors into cell-derived extracellular matrix (Kim et al. 2015). Decellularized extracellular matrix derived from human lung fibroblasts was harvested, suspended and deposited on a polymer mesh scaffold. Heparin was bound via EDC chemistry, forming amide bounds with amine groups in the extracellular matrix. BMP-2 was then added to immobilize to heparin

and released at a controlled rate. This bioactive scaffold showed a significant increase of newly regenerated bone in a rat calvarial defect model.

7.3.1.5 Immobilization of Growth Factors

The extracellular matrix provides a reservoir for growth factors, which can be released and act as soluble ligands (Hynes 2009). Evidence exists that also matrix-bound growth factors stimulate cell functions via solid-phase signals (Wijelath et al. 2006). Specific binding sites have been detected in the extracellular matrix which can regulate the function of growth factors (Hynes 2009). Therefore, the immobilization of growth factors and other bioactive molecules plays a role in the strategies of designing the surface of implant materials for tissue regeneration (Cartmell 2009; Lee and Shin 2007; Silva et al. 2009). Growth factors bound to biomaterial surfaces may have enhanced activities compared with a soluble form of the factor, as it has been shown for TGF-β1 covalently linked to a polymer and stimulating matrix production (Mann et al. 2001). Different techniques have been applied to tether and control the release of bioactive factors (Place et al. 2009). The easiest way to add soluble factors is to load them into polymer matrix or to adsorb onto a composite (Soriano and Evora 2000; Ziegler et al. 2002). A variety of growth factors have been incorporated into hydrogels during the formation of the material in aqueous solution (Kanematsu et al. 2004). To tune the release of soluble proteins, the cross-linking density of the polymer can be modified (Hiemstra et al. 2007). bFGF could be released quantitatively from such hydrogels in 28 days. These techniques basically rely on the passive diffusion of growth factors from the matrix. Another strategy for protein release relies on a mechanical-responsive system (Augst et al. 2006; Lee and Mooney 2001). Many tissues, such as vasculature and musculature are mechanically dynamic. Mechanical compression could release factors from a material. Using a VEGF-containing alginate-hydrogel, it was shown that exposing mechanical strain to the hydrogel increased the release of VEGF (Lee et al. 2000). After implantation in mice, this mechanically induced release increased collateral vessel formation. Adding growth factors to ceramic materials is very convenient, because ceramics have a high affinity for proteins (Ziegler et al. 2002). Growth factors, such as TGF, FGF and VEGF were loaded to ceramics just by adsorption. The release patterns of most loaded ceramics seem to consist of an initial burst release of not bound protein followed by a second release dependent on the material/protein interaction (Habraken et al. 2007). Loading of calcium phosphate cements with growth factors was performed just by adding the protein to the liquid hardener, thereby distributing it equally through the cement. Bovine serum albumin can be used as carrier solution for growth factors to control the release of factors from the cement (Blom et al. 2002; Ruhe et al. 2006). Several in vivo studies proved the beneficial effects of growth factor loaded calcium phosphate scaffolds (Jansen et al. 2005; Kroese-Deutman et al. 2005; Ruhe et al. 2004; Seeherman and Wozney 2005).

More precise, growth factors can be immobilized to a material surface by covalent binding. This can be achieved by reacting of the side chains of polymers with

amino acids of a growth factor. Several growth factors have been covalently linked to polyethylene glycol, including TGF, EGF, bFGF (Bentz et al. 1998; DeLong et al. 2005; Kuhl and Griffith-Cima 1996). To control the release of covalently attached growth factors by the cells, synthetic hydrogels have been generated which contained protease sensitive binding sites (Lutolf et al. 2003a; Zisch et al. 2003b). In this case the hydrogels are prepared with functionalities of natural extracellular matrix, i. e. the ability to mediate adhesion and to respond to proteolytic degradation by enzymes, such as metalloproteinases which are secreted by cells. As structural building blocks, end-functionalized polyethylene vinylsulfone chains were used with thiol-bearing peptides. Cross-linking occurred by incorporation of bis-cysteine peptides, which can be cleaved by proteases. Growth factors, like VEGF and BMP were bound to these structures and could be delivered on cell demand (Lutolf et al. 2003a; Zisch et al. 2003a). Using this approach, an active liberation of VEGF was confirmed which resulted in a remodelled vascularized tissue, when the matrix was implanted subcutaneously in rats (Zisch et al. 2003a). Similarly, bone regeneration was demonstrated in a critical size defect by cell-mediated proteolytic release of BMP from a matrix (Lutolf et al. 2003b). A further more natural mechanism of the control of growth factor binding, modulation and release is the attachment of glycosaminoglycans to a material surface. These complex molecules have a tissue specific distribution and multiple physiological functions (Raman et al. 2005). Their sulphation patterns determine the specific interaction with proteins. One example is the binding of bFGF to heparin. Heparin has been widely incorporated into scaffolds to bind and release bFGF (Sakiyama-Elbert and Hubbell 2000; Zhang et al. 2006).

As demonstrated, the physiological effect of growth factors can be mimicked by designing of a modular peptide (Lee et al. 2010). This peptide contained a BMP-2 derived peptide sequence and hydroxyapatite-binding sequences inspired by the N-terminal alpha-helix of osteocalcin. The multifunctional fusion protein can bind to hydroxylapatite coated surfaces or bone structures and exert BMP activity. When this peptide was presented to mesenchymal stem cells, both immobililized or in solution, the construct was capable to promote the osteogenic differentiation of the cells (Lee et al. 2010).

Microspheres with encapsulated or surface bound growth factors present a system to persist and deliver growth factors at the target site (Arras et al. 1998; Cleland et al. 2001; Park et al. 2009b). For the fabrication of biodegradable polymer microspheres polyester like polylactide (PLA) and poly(lactic-co-glycolic acid) have been used. Applying a double emulsion technique, growth factors, such as bFGF, VEGF have been mixed into the particles (Perets et al. 2003). The loaded microspheres were incorporated into an alginate matrix or hydrogel. This approach enables the delivery of two or more growth factors with distinct kinetics. Microspheres containing PDGF were mixed with VEFG prior to processing into scaffolds, which resulted in a rapid release of VEGF and a slower, more even distribution of PDGF. When the scaffolds were implanted into rats, the distinct release kinetics of the growth factors stimulated the formation of a mature vasculature (Richardson et al. 2001).

7.3.1.6 Surface Loading and Release of Cations

Metals, like silver, zinc, copper and others are cytotoxic at higher ion concentrations. In a number of studies mainly silver was tested for antimicrobial coatings of implant materials. On a titanium surface, silver was successfully implanted by surface modification with a phosphonate monolayer. Adding of $AgNO_3$ to this layer enabled the formation of silver thiolate endgroups (Tilmaciu et al. 2015). On TiO_2 nanotubes, silver was deposited by electron beam evaporation (Lan et al. 2013). Other strategies are using silver nanoparticles, which will be incorporated or covalently linked in polymer assemblies (Vasilev et al. 2009). On glass, a self-assembled monolayer of silver nanoparticles was achieved through preliminary aminosilanization (Taglietti et al. 2014). All these silver coatings revealed antimicrobial activity in vitro and in some cases also in vivo. Silver was also combined with other antimicrobial agents. An amine-modified xerogel which was able to store and release nitric oxid was loaded with a $AgNO_3$ sol to promote a synergistic activity against bacteria (Storm et al. 2014). The challenge of silver coatings is to find a balance between antibacterial activity and cytotoxicity. Silver loading on a titanium surface in a micro-arc oxidation process at a concentration of 0.21–0.45 % in the coated layer revealed cytotoxicity to osteoblasts (Song et al. 2009). Similar to silver, copper ions are cytotoxic at higher concentrations. However, in contrast to silver, copper plays a role in physiological processes of the cell and defects in copper homeostasis are directly responsible for human diseases (Turski and Thiele 2009). Because copper is required for functional activities of several intracellular proteins, a possible role of copper in tissue regeneration was examined (Burghardt et al. 2015; Wu et al. 2013). These studies found that copper ions stimulate both proliferation and osteogenic differentiation of mesenchymal stem cells at a concentration of 0.1–0.3 mM (Burghardt et al. 2015). When copper was galvanically deposited on a titanium surface, in dependence on the concentration of copper ions released from this surface, copper induced both osteogenic differentiation of mesenchymal stem cells to mineralizing osteoblasts and the killing of adherent *Staphylococcus aureus* bacteria (Burghardt et al. 2015). Thus, by tuning the concentration of a copper release from a surface, copper enables the designing of implants with both antibacterial and regenerative properties (Fig. 7.4).

7.3.2 Physical Modifications to Control the Biointerface

7.3.2.1 Structural Organization of the Surface

The structure of a material surface can be categorized into topography and chemical patterning. The topography reflects the roughness of a surface which can be designed by ridges and grooves or by evenly or randomly distributed pits or protrusions. Chemical patterning is achieved by the spatial organization and immobilization of molecules in controllably size and position, mostly to control cell adhesion (Lim and Donahue 2007).

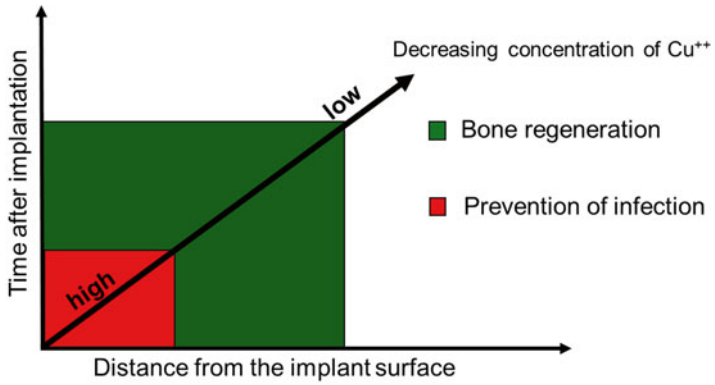

Fig. 7.4 Scheme of a strategy to design Cu^{2+} – containing titanium surfaces to both prevent bacterial infection and stimulate bone regeneration. By adjustment of an appropriate concentration of Cu^{2+} at the surface of a titanium implant, immediately after implantation and in the vicinity of the implant surface a higher concentration of Cu^{2+} will generate an anti-microbial effect. By decreasing concentration of Cu^{2+} in the time course after implantation and with greater distance from the implant surface, a stimulating effect on bone regeneration will be achieved (Reproduced from Burghardt et al. 2015, with permission from Elsevier Limited)

For clinical application of titanium implants different techniques have been used to roughen the surface, which include blasting, etching, and oxidation. A huge number of experimental data demonstrate that a rough implant surface has a beneficial effect on the bone response (Wennerberg and Albrektsson 2009). This concerns roughness in the micrometre level, whereas little is known about the effects of topographies in the nanometre level in vivo (Wennerberg and Albrektsson 2009). When testing the cell behaviour on topographies the scale plays an important role. It became obvious that cells are able to sense the micro- and nanoscale topography and react with bridging of grooves or conforming the surface structure (Millette et al. 1987; Teixeira et al. 2003; Walboomers et al. 1999). An attractive approach to generate defined structures on titanium or titanium alloys is the generation of nanotubes. By an electrochemical anodization process, self-organized oxide tube arrays with virtually perfectly organized hexagonality and a thickness of several hundreds of micrometers can be obtained (Roy et al. 2011). The diameter of the tube surfaces can be adjusted to any value between 10 and 250 nm and even with complex shaped surfaces (Bauer et al. 2006). The size of the nanotubes determines the amount of adsorbed fibronectin, which controls cell adhesion (Kulkarni et al. 2015). In general, the behaviour of the whole cell due to a topography correlates with an orientation of the cytoskeleton and the alignment of focal adhesions (Dalby et al. 2002, 2003). In addition to structural changes in the organization of cellular components, functional consequences have been observed. Osteoblastic cells expressed a higher RNA level of osteopontin and osteocalcin when cultured on a surface with grooves than on a flat surface (Matsuzaka et al. 2004). Generation of microgrooves on a PDMS membrane with the same height but different widths and spacings between

10 and 40 μm enhanced the reprogramming of mouse fibroblasts to pluripotent stem cells compared with a flat surface (Downing et al. 2013). An epigenetic mechanism was postulated that involves modification of histone by the physically induced shape change of the cell nucleus. Apparently, a defined size of pits or grooves is important on a structured surface. As shown, osteoblastic differentiation measured by the activity of alkaline phosphatase was stimulated more on 11 nm islands than on 85 nm islands (Lim et al. 2005). Although some conflicting results exist concerning the optimal size of nanotubes to promote an osteogenic differentiation (Oh et al. 2009; Park et al. 2009a), stimulation of cell spreading should be essential for a differentiation towards osteoblasts, which was observed at a smaller length scale of 15 nm (Park et al. 2007). Similarly, also cell proliferation depends on defined surface structures. Progenitor cells displayed a higher proliferation rate on 5–40 μm diameter posts compared with cells on a smooth surface (Mata et al. 2002). In addition to the size of posts created on a surface, the organization of a pattern controls the function of cells. When mesenchymal stem cells were cultured on disordered dots with nanosize the cells were induced to express osteocalcin and osteopontin in the absence of osteogenic supplements, demonstrating the stimulation of osteogenic differentiation (Dalby et al. 2007). In comparison, when the same nanofeatures were symmetrically organized, the cells did not express osteogenic proteins. From these data it is obvious that micro- and nanostructured surfaces stimulate various collective cell functions (Lim and Donahue 2007). In addition, efforts were made to see, whether surface topography affects adhesion of bacteria and may have a selective effect due to different characteristics of bacteria and eukaryotic cells. A nanometer sized titanium surface was found to reduce the adhesion of three different strains of bacteria (Puckett et al. 2010). Similarly, nanocolumnar structures on titanium, which were generated by a magnetron sputtering technique, strongly inhibited adhesion of bacteria but promoted adhesion and proliferation of osteoblastic cells (Izquierdo-Barba et al. 2015).

Chemical patterning which generates precisely defined micro- or nanometer areas for cell adhesion can be achieved by lithographic techniques (Nie and Kumacheva 2008). These techniques involve photolithography and printing techniques. Printing methods can be classified into techniques which involve the contact of a stamp with the substrate and methods which directly transfer “ink” to the substrate. Dip-pen nanolithography represents a relatively new direct writing technique, using the tip of an atomic force microscope to form a liquid meniscus between tip and substrate, and as a result of this procedure the ink molecules are transferred to the underlying substrate by chemical or physical adsorption (Piner et al. 1999). Micropatterning allows the spatial control of adhesion of the whole cell. By restriction of cell spreading the shape of cells can be controlled. Using mesenchymal stem cells, it was demonstrated that cell shape commits the direction of differentiation (McBeath et al. 2004). More rounded cells differentiated to adipocytes, whereas flat cells became osteocytes. The authors revealed that induction of mechanical tension of the cytoskeleton, which correlates with stress fibre formation and is mediated by the activities of RhoA and Rho kinase (ROCK) induces osteogenic differentiation. Blocking of RhoA and ROCK activities stimulated the adipogenic differentiation.

By generating fibronectin lines in the nanoscale which altered the cell morphology, the proliferation of embryonic stem cells was stimulated, which depended on an altered organization of the cytoskeleton (Gerecht et al. 2007). Generation of fibronectin lines with varying width of 10–80 μm and varying non-adhesive spacings between them allowed the control of the nuclear and cellular morphology in mesenchymal stem cells, as well as lateral contacts with neighbouring cells (Kasten et al. 2014). With decreasing width of the fibronectin lines, an increased migration of cells was observed.

In addition to control of the entire cell shape by adhesion patterns, the sensing of nanoscale adhesion sites by cells controls integrin mediated signal transduction and in consequence influences differentiation and proliferation. For example, the precise spacing between nanotopographic features of RGD-peptides for cell adhesion can modulate the clustering of integrins. A minimal distance of 58 nm between adhesive dots was required for integrin clustering, formation of stable focal adhesions and cell spreading (Arnold et al. 2004; Cavalcanti-Adam et al. 2007). The formation of a molecular gradient of the ligand spacing from 50 to 80 nm revealed that cells are able to sense the small differences in ligand spacing (Arnold et al. 2008). Differences which are little as 1 nm seem to affect cell polarization and migration.

7.3.2.2 Mechanical Characteristics of the Surface

Mechanical stimuli represent regulators of development and function in many tissues. It is generally accepted that the structure of the various tissues reflect the acting forces, which specifically control the physiological processes. In some cases, tissues are heterogeneously organized into mechanically distinct zones, for example the superficial, radial and tight zones of cartilage. Therefore, implant materials must provide some level of physical support to assist tissue function. Engineering strategies have been developed to steer the viscoelastic properties of implant materials, for example by cross-linking of polymers. Highly elastic gels of cross-linked hyaluronic acid with controllable viscoelasticity were generated for tissue engineering of vocal folds (Sahiner et al. 2008). For tendon repair, gels were combined with a type I collagen sponge to optimize the stiffness of the material, which was successfully applied in a patellar tendon model (Butler et al. 2008). Findings in several cell types provide evidence for the importance of the substrate stiffness as a physical signal for cells (Georges and Janmey 2005). Early experiments demonstrated that differentiation of mammary epithelial cells increased when grown on soft collagen gel substrate, as opposed to tissue culture plastic (Emerman et al. 1979). Neurons preferentially branched on soft tissues compared to stiff surfaces (Flanagan et al. 2002). Although in most of these studies, the influence of different mechanical properties is difficult to separate from the type and density of the chemical ligand, it is obvious that stiffness of the substrate plays a role in tissue development. The role of substrate stiffness in the context with regenerative processes was emphasised by the fundamental finding that stem cell lineage specification can be determined by

mechanical properties of the substrate (Engler et al. 2006). Mesenchymal stem cells were grown on polyacrylamide gels with varying compliance. These experiments convincingly demonstrated that the stiffness of the material defines the differentiation lineage (Discher et al. 2009; Zajac and Discher 2008). Soft substrates which mimic the mechanical properties of brain stimulated the neurogenic differentiation, intermediate stiffness leads to muscle cell differentiation and stiff substrates where found to be osteogenic. Similar experiments using adult neural stem cells have shown that softer substrates provoked neuronal differentiation, whereas stiffer materials induced the formation of glial cells (Saha et al. 2008). The mechanical properties of the substrates were also found to control the self-renewal of stem cells. Adult stem cells from skeletal muscle tissue revealed increased cell proliferation with rising stiffness of the matrix (Boonen et al. 2009). Mesenchymal stem cells were kept quiescent on a gel that mimicked the softness of bone marrow. In contrast stiffer substrates induced the entry of these cells into the cell cycle (Winer et al. 2009). The cells maintained the multilineage potential and could be differentiated both to adipocytes and osteocytes. These experiments provided evidence of mimicking the functional capacity of a bone marrow niche by tuning the mechanical properties of an artificial substrate. In addition to the control of proliferation and multipotential differentiation, sensing of substrate stiffnesses enables cells to migrate from soft to stiffer matrices, which appears of importance for stem cell translocation to sites of tissue regeneration (Gray et al. 2003; Kidoaki and Matsuda 2008). This phenomenon was termed “durotaxis” (Lo et al. 2000). To further explore mechanisms, how cells are able to sense rigidity of a substrate and response with a specific biological function, it was demonstrated that mesenchymal stem cells were able to assemble plasma fibronectin from the culture medium into fibrils of their extracellular matrix. The generated fibers were more stretched on a rigid substrate than on a soft material. More stretched fibronectin induced osteogenesis of the cells, which was dependent on the activity of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins (Li et al. 2013). However, some recent experiments revealed that mechanosensing of cells and in consequence the biological response is a result of many interdependent inputs from a complex and dynamic interaction between adhesion receptors, extracellular matrix and the synthetic material (Chaudhuri et al. 2014; Kumar 2014; Trappmann et al. 2012; Wen et al. 2014).

7.4 Applications for Therapeutic Devices

Progress in biomaterials design and engineering are converging to enable a new generation of instructive materials to emerge as candidates for regenerative medicine. The aim of the design of current biomaterials is to regulate tissue regeneration by modulating direct or indirect chemical and physical control over transplanted or host cells. The dilemma is that to influence cell behaviour, biomaterials must provide complex information (Place et al. 2009). Tissue engineered skin equivalents have been introduced into clinical practice in 1997. Since then tissue engineered

devices have been in clinical trials or already approved as therapies for tissues including cartilage, bone, blood vessel and pancreas. However, over-engineered devices make their translation to clinical use unlikely. The reconstruction of entire organs has largely given up and changed to smaller goals. For example, clinical advance in cardiac repair focus on coronar arteries, valves and regeneration of the myocardium. In principle, the aim is to develop synthetic materials that establish key interaction with cells that stimulate the innate organization and self-repair of the body.

7.5 Barriers to Practice and Prospects

A major hurdle for the progress in the application of biomaterials in the field of regenerative medicine lies not in the biomaterials but in stem-cell biology. The advancement of basic research in stem cell biology represents the driving factor for the development of biomaterials to regenerate a specific tissue. Current trends suggest that biomaterial development will continue to create more life-like multifunctional materials that are able to simultaneously provide complex biological signals (Chan and Mooney 2008; Howard et al. 2008). Much can be learned from the mechanisms that regulate cell fate in the stem cell niche. For example, the adhesion molecules that contribute to asymmetric stem cell division have begun to identified within the niche environment of hair follicle, intestinal epithelial, spermatogonial stem cells (Kanatsu-Shinohara et al. 2008; Ohyama et al. 2006; Tanentzapf et al. 2007). In addition to the general control of stem cell function, there is growing interest in the dynamic nature of stem cell niches which can change properties under certain conditions (Adams and Scadden 2008).

7.6 Conclusions and Future Challenges

Chemical and physical characteristics of biomaterials are able to control the biology of stem cells and significant advances have been gained in in vitro studies. By controlling the properties of biomaterials we may further improve the regulation of stem cell in a bioartificial system. Although stem cell function is regulated by a set of different signals from the environment, the control of the extracellular matrix has proven a valuable tool to guide the development and commitment of stem cells. The challenge is to engineer an artificial extracellular matrix, which is capable to directly control the behaviour of stem cells. In addition, the outcome of growth factors administration can be improved enormously with the use of slow-release constructs. A further step in the generation of bioactive materials will be the design of heterogeneous constructs and even complex organs, which will require both more insights the mechanisms of cell and developmental biology as well as innovation in biomaterial research.

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Chapter 8
Controlled Release Technologies for RNAi Strategies in Regenerative Medicine

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Abstract The ultimate goal in siRNA formulation for regenerative application centers around a gradual and sustained local release of intact siRNA which is adapted to the physiological requirements of the specific tissue. Although various issues in optimizing the siRNA delivery systems in terms of safety and efficiency still need to be addressed, progress towards the potential translation of RNAi-based therapies to clinical use has been made. An increasing number of promising siRNA-based gene therapies is currently in, or advancing towards clinical trials. Looking back to a period of only 17 years since the discovery of RNAi (Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC, Nature 391(6669):806–811. doi:10.1038/35888, 1998) and the introduction of siRNAs as the underlying principle of RNAi (Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T, Nature 411(6836):494–498. doi:10.1038/35078107, 2001), it is likely that regenerative applications will be explored in clinical studies soon as well.

Keywords siRNA • Chemical modification • Cell targeting

Abbreviations

ACI	autologous chondrocyte implantation
Ago	argonaute protein
BCL2	B-cell lymphoma 2
BMSCs	bone marrow stromal cells
cbfa1	core binding factor alpha1

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CD	cyclodextrin
Chi	chitosan
Ckip-1	casein kinase 2-interacting protein-1
CPP	cell penetrating peptide
DOPE	1,2-dioleoyl-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	1,2-di-O-octadecenyl-3-trimethylammonium propane
ECs	endothelial cells
EEP	ethyl ethylene phosphate
EPR	enhanced permeability and retention
GNAS1	guanine nucleotide-binding protein alpha-Stimulating activity polypeptide1
HA	hyaluronic acid
hASCs	human adipose stromal cells
HIF-1	hypoxia-inducible factor1
HIV	human immunodeficiency virus
hMSCs	human mesenchymal stem cells
LbL	layer by layer
LSD1	lysine (K)-specific demethylase 1A
miRNA	micro RNA
NP	nanoparticles
PCL	poly-ε-caprolactone
PEC	polyelectrolyte complex
PEI	polyethylenimine
PEG	polyethylene glycol
PEMs	polyelectrolyte multilayers
PHD2	prolyl hydroxylase domain protein 2
PLGA	poly(lactic-co-glycolic acid)
PLA	poly(L-lactic acid)
PLK1	tumor survival genes polo-like kinase 1
PSMA	progressive spinal muscular atrophy
RANK	receptor activator of NF-κB
RNA	ribonucleic acid
RNAi	RNA interference
RSV	respiratory syncytial virus
sFIt-1	soluble fms-like tyrosine kinase-1
shRNA	short hairpin RNA
siRNA	small interfering RNA
TCP	β-tricalcium phosphate
TRIB2	tribbles homolog 2
VEGF	Vascular Endothelial Growth Factor

8.1 Introduction

In 1998, double-stranded RNA (dsRNA) molecules were described to efficiently and specifically interfere with the expression of genes in catalytic amounts (Fire et al. 1998). This revolutionary discovery earned Andrew Fire and Craig Mello the Nobel Prize in 2006. Since then, therapeutic RNA interference (RNAi) strategies have been considered as a promising approach for targeting any disease-related gene including otherwise undruggable targets.

RNAi is an endogenous process in the cells to control the gene expression through a reduced expression of undesired genes ('gene silencing'). This modulation is achieved either by cleavage of the mRNA and its subsequent degradation, or by inhibiting its translation. RNAi exerts its action through small interfering RNA (siRNA), microRNA (miRNA) or short hairpin RNA (shRNA), and can be also induced by their external delivery. Structurally, siRNA is a short double-stranded (ds) RNA of 20–25 bp in length and features a 2-nucleotide overhang at the 3′-end. Intracellularly, it is generated from longer dsRNA molecules through the action of the enzyme called Dicer. For the induction of RNAi, cells can be transfected either directly with the siRNA of choice, or with a DNA plasmid encoding for small hairpin RNA (shRNA). Upon transfection, the transcribed shRNA is then cleaved into siRNA by Dicer. Similar to siRNAs, miRNAs are small (21–25 bp), non-coding RNAs; however, in contrast to siRNA they are only partially complementary to their target mRNA molecules. Their mechanism of action comprises cleavage or degradation of mRNA. Independent of the nature of the small RNA molecule, i.e. siRNA or miRNA, their action relies on their incorporation into the endogenous RNA-induced silencing complex (RISC). The antisense strand of the siRNA duplex is incorporated as so-called guide strand into RISC and mediates, through Watson-Crick base pairing, the sequence-specific binding of RISC to its target mRNA. This brings the other RISC components such as the Argonaute (Ago) proteins into close proximity to the mRNA, leading to the subsequent mRNA cleavage by Ago. Due to its unprotected ends, the cleaved mRNA is then rapidly degraded by other nucleases. Beyond cleavage, RISC also plays an important cellular role by inhibiting endogenous mRNA translation via a related miRNA mechanism. The detailed mechanism of RNAi molecule is illustrated in Fig. 8.1 (Agrawal et al. 2003; Moore et al. 2010; Tebes and Kruk 2005).

Due to their sensitivity to nucleases, their highly negatively charged backbone and their considerable size (typically >13 kDa) (Agrawal et al. 2003), all of which contributing to poor bioavailability and cellular uptake, small RNA molecules are in principle unsuitable as therapeutic drugs. One strategy to overcome this barrier involves viral delivery vectors, which are highly efficient but may come with safety issues due to their risk of mutagenesis and immunogenicity (Pan et al. 2015; Levi et al. 2012). Alternatively, non-viral systems are explored for the formulation of DNA or small RNA molecules in nanoparticles. Nanoparticle formation may rely on, for example, ionic interaction with cationic polymers or cationic lipids, encapsulation into liposomes, or conjugation of the nucleic acid to a moiety capable of cellular entry. Since the discovery of RNAi, several clinical studies have explored

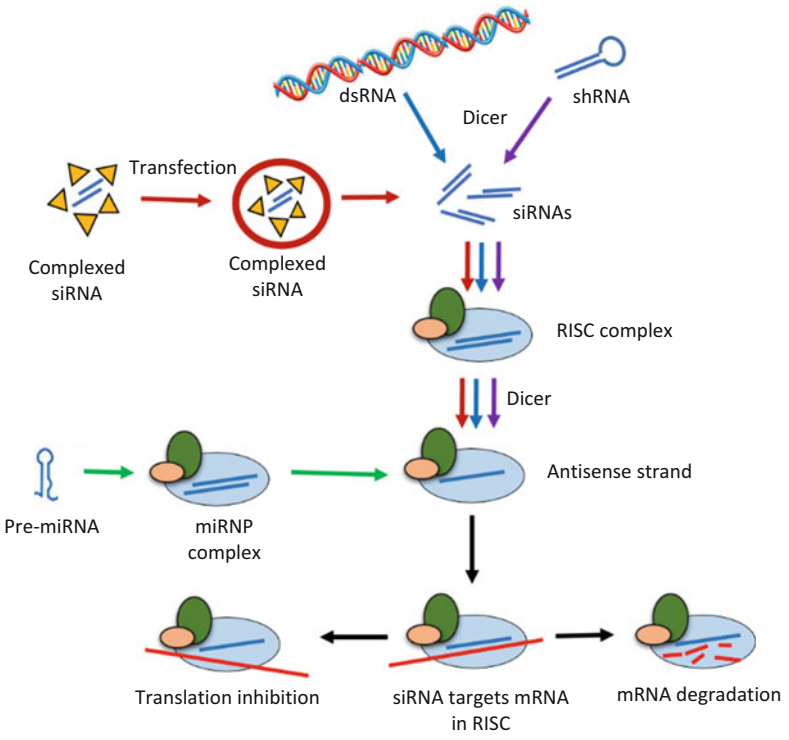

Fig. 8.1 RNAi mechanisms of shRNA, siRNA and miRNA in mammalian cells. *Purple and blue arrows*: Short hairpin RNA (shRNA) and double stranded RNA are processed by Dicer to form siRNAs in the cytoplasm. *Red arrows*: siRNA can also be delivered through transfection via endocytosis. The double stranded siRNA then complexes with argonate proteins to form the RISC complex. *Green arrows*: pre-miRNAs are processed into miRNAs which then complexes with argonaute proteins to form the miRNP complex. After elimination of sense strand from the double stranded RNAs, the antisense strand anneals to the target mRNA. *Black arrows* represent the last stages of the silencing process by siRNA, shRNA and miRNA; perfect or non-perfect match leads to mRNA degradation or inhibition of translation

its potential, with initially unsatisfactory results related to unwanted 'off-target effects' and/or issues regarding siRNA delivery (Kanasty et al. 2012). This indicates the requirement of further optimization of the delivery vectors, careful selection of the siRNAs and optimal application concepts. Nevertheless, several clinical studies are in progress (see below) (Kanasty et al. 2013).

The focus of this chapter will be on delivery systems and controlled release concepts for RNAi therapeutics and in particular on their use in regenerative medicine. Although RNAi-based therapies often target cancer due to the availability of attractive target molecules, there is an ever increasing number of approaches in other pathologies including regenerative medicine. In the latter case, these are often focussed on local delivery, that also allow for efficient delivery of siRNA to stem cells or other target cells for tissue engineering purposes. Thus, RNAi-based strategies

could for instance improve bone regeneration through targeting genes that have been recognized to negatively affect bone formation or stimulate bone resorption (Novina and Sharp 2004).

8.2 Background and Principles

Despite concerns regarding systemically administered siRNA due to adverse effects on other non-target tissues (López-Fraga et al. 2009), certain diseases, including viral infections, require administration via the blood stream. Systemic applications include intravenous and subcutaneous injection, delivery through the skin, lung (Lam et al. 2012) or nasal mucosa (Kanazawa et al. 2014). The reluctance to systemic siRNA delivery originates from the risk of systemic toxicity, inefficient targeting to the desired tissues as well as the above mentioned unfavourable physicochemical properties of siRNA (Durcan et al. 2008). Likewise, the highly negative charge of naked siRNA leads to rapid blood clearance caused by the reticuloendothelial system (RES), hampering efficient systemic application of siRNA due to short circulation times (Watts and Corey 2010). The introduction of chemical modifications is one possible approach to modulate the poor pharmacokinetic properties of siRNA, increase its stability and selectivity and avoid non-specific or off-target effects. For example, modifications at the 2′-position such as O-methyl (2′-O-Me) and fluoro (2′-F) substituents have been introduced (Corey 2007) (see below for further discussion).

Other formulation concepts to overcome the problems of low stability against nucleases and poor membrane permeability aim at neutralizing or overcompensating the negative charge by complexation with cationic polymers or lipids that improve the interaction with the negatively charged cell membranes and increase cellular uptake. Another way to address the uptake problem, is to hide the negative charge by encapsulation in neutral polymer nanoparticles, e.g. biodegradable polyesters. Apart from optimizing complex stability and transfection efficiency, the carrier nanoparticles can be chemically modified with targeting moieties, e.g. antibodies or cell penetrating peptides in order to improve biocompatibility and/or enable targeted delivery to the respective tissues or cells.

Standard systemic administration concepts achieve a comparably short protein knockdown. Many therapeutic applications, however, require more prolonged effects and thus a controlled delivery strategy. Prolonged delivery can be achieved by nanoparticle incorporation into implants that slowly release the nucleic acids over time and allow for fine-tuning of the release profiles. Biomaterials that are designed for the local delivery of RNAi therapeutics often feature a hierarchical structure. This means that the nucleic acids that are typically formulated in nanoparticles for stabilization and charge control are further embedded in a micro- and/or macroscopic matrix, such as microparticles, fibers, macroporous scaffolds or even combinations of these systems. The macroscopic devices thereby serve as controlled release systems for siRNA nanoparticles as well as a physical support, providing a large attachment area and direct contact area for invading cells ready to support tissue formation.

8.3 Formulation Strategies for siRNA and Nanoparticulate Systems

8.3.1 Naked siRNA

Direct injection of naked siRNA to the site of administration without any complexation has been shown inefficient in several cases, due to enzymatic degradation resulting in low siRNA half-life and efficiency. However, in some organs, such as eye, brain, skin and heart, the delivery of naked siRNA was reported to be effective in gene knock-down (Pan et al. 2015).

8.3.2 Chemically Conjugated siRNA

siRNA can be subject to chemical conjugation with (bio-)molecules to improve physicochemical properties and enhance cellular uptake. For example, a study published by Zhu et al. showed that siRNA conjugated with galactose-PEG at the sense strand was taken up more efficiently by hepatocytes via the asialoglycoprotein receptor. Cleavage of the disulfide bond between PEG and siRNA in the reducing intracellular environment by, e.g., glutathione caused the siRNA to be released (Zhu and Mahato 2010).

8.3.3 Viral Vectors

Viral vectors, such as lentiviruses, adenovirus-associated vectors and retroviruses have been developed for the delivery of siRNA-encoding DNA. Among them, adenovirus-associated vectors and lentivirus are the most commonly used viral vectors, due to their comparatively general safety, low immunogenicity and proper transduction efficiencies. Various applications of viral siRNA delivery have been investigated. For instance, by knocking down the BMP-2 inhibitor Noggin with lentiviral techniques, Levi et al. could increase the osteogenic capability of hASCs (Levi et al. 2012). However, the risk of mutagenic effects as well as immunogenic and inflammatory responses in body organs discourages to the use of viral vectors in clinical studies (Pan et al. 2015).

8.3.4 Liposomal Delivery

Liposome-based siRNA delivery vehicles are among the most common non-viral gene nanocarriers. In such liposome nanoparticles, the siRNA molecules are encapsulated in an aqueous core surrounded by a phospholipid bilayer. Liposomes for nucleic

acid delivery can contain various neutral and/or positively charged lipids. However, cationic lipids are preferable and commonly used in commercially available transfection agents such as Lipofectamine®. Cationic lipids facilitate loading of the negatively charged nucleic acids into the liposomes and expedite uptake through the lipid bilayer into the cells as well as endosomal escape. This approach was first described by Felgner et al. (1987). Lipoplex formation is achieved by simply mixing the cationic liposomes with the anionic nucleic acids, leading to self-assembly of the liposome/siRNA complexes as a result of electrostatic interaction (Buyens et al. 2012).

Commonly used cationic lipids include DOTMA and DOTAP. However, the use of cationic lipids *in vivo* is limited due to the surface charge and relatively large size of the lipoplexes. Thus, rapid clearance, immunogenicity and toxic side effects are induced, among others, by nonspecific interactions with serum proteins (Filion and Phillips 1997; Lappalainen et al. 1994). Stabilization of the liposomes is often achieved by the use of neutral helper lipids such as DOPE or cholesterol, which enhance cellular uptake by tailoring the lipoplex composition to the lipid composition of the cell membrane, facilitating the formation of endocytic vesicles (Marchini and Howie 2010).

8.3.5 Polymeric, siRNA Loaded Nanoparticles

Tightly packed polymeric nanoparticles (NPs) are formed by electrostatic interactions between polycationic polymers and negatively charged siRNA. Packing of siRNA as compared to longer nucleic acid molecules like for example plasmid DNA is considered as more challenging because siRNA molecules are comparably short and thus rather rigid, which is why they cannot be wrapped around the nanoparticle and condensed in the same way (Xu and Anchordoquy 2011). The net charge of the resulting NP depends on the ratio of positive charges (mostly from cationic amines or imines) of the polymers (nitrogen; N) and negative charges of the siRNA (phosphate; P) and is referred to as N/P ratio. In order to ensure high complexation efficacy and improve cellular uptake, the N/P ratio has to be sufficiently high, thus leading to positively charged NP. Since the adjustment of the N/P ratio is crucial as it has a notable impact on complex stability and cellular uptake efficacy, these interactions with the specific polymer should be well characterized in advance. A large number of studies exist on cationic polymers such as poly(ethylene imine) (PEI).

Among the cationic polymers, PEI is considered as a gold standard. This is based on its high transfection efficiency and favourable endosomal release characteristics for cytosolic delivery of siRNA after endocytosis, which is the prevalent uptake route of polycationic delivery vectors. The endosomolytic properties of PEI are caused by its high buffering capacity due to the high number of basic amino groups, acting as proton acceptors. This provokes more protons to be pumped into the endocytic vesicles to keep the acidic pH characteristic of endosomes. Eventually, this leads to osmotic swelling and rupture of the lipid bilayer. The phenomenon is commonly referred to as the proton sponge effect (Behr 1997). Branched PEI can be synthesized

by ring opening polymerization of aziridine, while the synthesis of linear PEI relies on the hydrolysis of poly(2-ethyl-2-oxazoline). On the negative side, the high charge density may lead to significant cytotoxicity, which depends on the molecular weight and structure of the PEI. Branched PEI is usually more toxic than linear PEI due to the higher density of amino groups, which in turn makes it more favourable for nucleic acid packing. Likewise, higher molecular weight PEIs like 25 kDa linear PEI show higher transfection efficiencies but at the same time exert higher toxicity. Thus, defined molecular weights and degrees of branching are required. Certain PEIs like the linear jetPEI or the branched PEI F25-LMW (Werth et al. 2006), meet these criteria and have been extensively used *in vitro* and *in vivo* (Höbel and Aigner 2013). Another way to circumvent this biocompatibility issue offer biodegradable PEIs, which are cleaved into smaller fragments with low toxicity and higher availability for rapid body clearance. Biodegradability is achieved using cleavable linkers based on, e.g., esters, carbamates, ketal linkages or disulfide bridges (Breunig et al. 2008). Concomitantly, transfection is supported by facilitated unpacking of nucleic acids after decomposition of the polymer (Islam et al. 2014).

An alternative approach to improve systemic biocompatibility is the grafting of polyethylene glycol (PEG) chains onto the PEI polymer, which shield the positive charge of the nanoparticle, thus reducing unspecific ionic interactions. The shielding is increased with the PEG chain length and the degree of grafting. The latter inversely correlates with transfection efficiency, whereas this unfavourable effect is less pronounced for siRNA delivery as compared to DNA (Malek et al. 2008). Enhanced biocompatibility was also observed by grafting of other natural oligomers/polymers like for example the non-ligand oligosaccharide oligomaltose (Gutsch et al. 2013; Höbel et al. 2010).

A formulation of siRNA with a PEI derivative, namely Staramine – a functionalized lipopolyamine – showed high cellular uptake with low systemic absorption and side effects after instillation. Intravenous (i.v.) administration of Staramine/siRNA nanocomplexes modified with methoxy-PEG enabled efficient siRNA delivery to the lung in mice, with reduced gene knockdown in liver, spleen, and kidney (Polach et al. 2012).

PEI-containing lipopolyplexes have been synthesized in order to combine the favourable properties of lipid delivery systems such as high transfection efficiency and comparably low cytotoxicity with the excellent siRNA complexation and endosomal release characteristics of PEI (Schäfer et al. 2010). In addition, lipopolyplexes showed higher storage stability as compared to polyplexes and retained biological activity over a prolonged time even in the presence of serum (Ewe et al. 2014).

8.3.6 Cyclodextrins

Modified cationic cyclodextrins are a possible alternative for cationic polymers in siRNA delivery, since they feature comparably high transfection efficiency paired with relatively low cytotoxicity (Islam et al. 2014). Grafts of β-CD and PEI were significantly less toxic than unmodified PEI (Forrest et al. 2005).

8.3.7 Natural Cationic Polymers

Minakuchi et al. introduced siRNA delivery using atelocollagen, which is positively charged and derived from natural collagen type I by pepsin protease-treatment (Minakuchi et al. 2004). Atelocollagen is less immunogenic as compared to collagen. Another protein-based nanoparticle system was developed by Zimmer et al., who used naturally occurring, arginine-rich protamine to tightly pack siRNA via electrostatic complex formation (Scheicher et al. 2015).

8.3.8 Micelles

Spontaneously forming micelles of fairly complex composition were introduced by Nelson et al. Micelles formed from a raft polymerized diblock-copolymer which consisted of a siRNA complexing hydrophilic block of polymerized dimethylaminoethylmethacrylate (DMAEMA) and a pH responsive, hydrophobic block of DMAEMA, butylmethacrylate and 2-propylacrylate that enables endosomal escape of siRNA from internalized micelles (Nelson et al. 2012). This concept of self-assembling polymers that can be tailored by their functional components holds great promise for siRNA applications (Jhaveri and Torchilin 2014).

8.3.9 Concepts Involving Biodegradable Polymers for Nanoparticle Formulation

Other concepts of NP formulation that involve biodegradable polymers for encapsulation of siRNA have been described for example for intradermal delivery. Jacobson et al. used supercritical carbon dioxide (SC-CO_2) to encapsulate siRNA in biodegradable PLA nanoparticles and showed a release siRNA *in vivo* over 80 days. Encapsulated siRNA showed minimal initial burst release from the surfaces of the nanoparticles (Jacobson et al. 2010). Methodically, siRNA and PLA were dissolved in a microemulsion with dichloromethane as lipophilic component and the microemulsion was sprayed into an extraction chamber with supercritical CO_2. The process allowed for precise adjustment of shape and particle size of the nanoparticles by varying extraction conditions, such as pressure of the supercritical extraction phase.

In another example, encapsulation of siRNA was achieved using PLGA by single- or double-emulsion solvent evaporation techniques. Addition of a small amount of cationic polymers such as PEI was shown to significantly improve siRNA complexation (Pantazis et al. 2012). PLGA/siRNA microspheres (with an average size of 5.45 ± 0.88 μm) have been successfully incorporated within calcium-based injectable bone cement (Wang et al. 2012) that is clinically used for fixation in osteoporosis patients.

A study by Jensen et al. demonstrated the delivery of siRNA by cationic lipid-modified PLGA nanoparticles as a dry powder formulation to the lungs after pulmo-

nary administration. The nanoparticles were prepared using a double emulsion solvent evaporation technique with DOTAP and PLGA dissolved in the organic phase. The use of DOTAP-modified PLGA facilitated gene silencing activity of the siRNA-loaded nanoparticles. In a next step, the authors demonstrated a spray drying step to successfully incorporate the nanoparticles in mannitol microparticles. The study introduces spray-drying as an optimal technique for engineering dry powder formulations of siRNA nanoparticles, enabling the local delivery of siRNA to lung tissue (Jensen et al. 2012).

8.3.10 Layer-by-Layer Techniques

A layer-by-layer (LbL) delivery method for siRNA uses electrostatic forces to fabricate multilayered films which could cover an implant material surface. The substrate is alternately dipped into polyanion and polycation solutions generating polyelectrolyte multilayers (PEM) in order to incorporate the nucleic acid and provide a controllable loading and release profile.

Song et al. used an LbL approach involving sodium hyaluronate and chitosan/siRNA nanoparticles to coat a titanium surface with a PEM film for gradual siRNA release over approximately 1 week (Song et al. 2015).

8.3.11 Cell-Penetrating Peptides

Cell-penetrating peptides (CPP), also known as protein transduction domains, are a class of peptides that are able to autonomously penetrate cellular membranes either directly or via endocytosis. They are usually short (up to 30 amino acids) and often positively charged. They can be either derived from naturally occurring protein transduction domains such as the HIV transactivator of transcription (Tat), low molecular weight protamine, or other rationally designed peptides. An example of the latter is the chimeric peptide Transportan that contains the amino terminus of the neuropeptide galanin and the wasp toxin mastoparan. They feature comparably low cytotoxicity and low risk of immunogenicity. Their positive charge enables them to form electrostatic nanoparticle complexes with nucleic acids, even though covalent approaches have been described as well (Hoyer and Neundorf 2012).

8.4 Biomaterials as Scaffolds for Sustained Release of RNAi Therapeutics

Tissue regeneration requires longer time periods, and siRNA-based approaches should thus be able to release siRNA over a relatively long period. RNAi technology is hence only applicable in regenerative medicine if the drug can exert its effect in a sustained manner. Local delivery of siRNA by simple injection of nanoparticles is

insufficient, due to the small size of nanoparticles which may cause their rapid dispersion from the target site, thus preventing continuous silencing at the target site over the desired longer time period (Krebs and Alsberg 2011). Biomaterial-based scaffolds have been widely applied to overcome such limitations. Biodegradable and biocompatible scaffolds, implanted in defect sites, are frequently used in tissue engineering approaches as cell carriers and / or to provide large attachment areas for invading cells and to promote cell proliferation and differentiation. By incorporating siRNA or siRNA-encapsulated nanoparticles into the scaffolds, a sustained release of siRNA over time is intended. The released siRNA can then locally exert its silencing effect, while the scaffold itself can aid in tissue regeneration by providing a conductive or inductive support for cellular growth and tissue formation inside the defect site. The siRNA release kinetics are governed by diffusion processes through the scaffold pores. Diffusion depends on interactions of the siRNA or the respective nanoparticles with the scaffold material by, e.g., electrostatic or hydrophilic interaction as well as on pore sizes, diffusion distances and the degradation rate of the biomaterial. Hence, the release over time can be adjusted to the physiological or therapeutic needs.

Considering the challenges in the delivery of siRNA to its specific site of action, scaffold-based siRNA delivery systems are generally designed to also meet various other criteria besides sustained and controlled release of the drug, such as high loading efficiency, structural integrity and suitable mechanical properties, favourable biophysical and morphological properties and high silencing efficiencies. To this end, the rational design of the biomaterial-based siRNA release systems is of high importance. The loading efficiency of siRNA into the carrier system naturally plays a critical role in determining silencing efficiencies. It is defined as the total amount of siRNA released over time plus the amount of remaining siRNA extracted afterwards divided by the theoretical siRNA loading. Integrity and cellular uptake of siRNA after *in situ* release also constitutes an important factor determining its activity (Krebs and Alsberg 2011).

As an example for a scaffold system, electrospun nanofiber scaffolds from a copolymer of caprolactone (CL) and 1 % ethyl ethylene phosphate (EEP), named PCLEEP, have been investigated by Rujitanaro et al. as controlled release system for siRNA. Morphologically, incorporating siRNA compexed with TKO (a commercially available siRNA transfection reagent with a cationic polymer/lipid formulation) into an electrospun PCLEEP scaffold led to bead-free fibers with narrowly distributed fiber diameters. When CL was copolymerized with EEP, siRNA release was significantly enhanced compared to release from PCL fibers. The transfection efficiency of released siRNA against GAPDH determined in NIH 3T3 cells seeded onto the scaffolds was partially retained for at least 30 days. Interestingly, siRNA entrapped alone in the scaffold was able to silence the desired gene by 21 % whereas release of the complexed siRNA showed slightly improved silencing (31 %) (Rujitanaroj et al. 2011).

In contrast to pure PCL which released only 3 % of naked siRNA (Cao et al. 2010), PCLEEP scaffolds showed about 90 % release of complexed siRNA and even more when naked siRNA was used (Rujitanaroj et al. 2011). However, the

authors determined the loading efficiency and found only about 60 % of the theoretical siRNA in the scaffolds. The release was normalized to these 60 %.

Another system involving polyesters was introduced by Nelson et al. who presented a new class of easily tunable biodegradable scaffolds for the delivery of siRNA loaded micellar NPs. This involved an *in situ* forming scaffold consisting of a biodegradable polyester urethane (PEUR) formed by an isocyanate functionalized three-armed cross-linker and biodegradable polyester macromers (PCL, PLA, PGA) (Nelson et al. 2012). In another study Nelson et al. showed that PEUR scaffolds are favourable in regeneration of excisional cutaneous wounds (Nelson et al. 2013) and allow for improved angiogenesis of regenerated tissue (Nelson et al. 2014). Unlike previous approaches, this study demonstrated the local delivery of siRNA for up to 3–5 weeks and the capability to fine tune the release kinetics from a scaffold *in vivo*. Therefore, siRNA release, scaffold degradation, and cell/tissue infiltration could be modified to optimize tissue regeneration (Nelson et al. 2014).

Chitosan scaffolds have been used by many groups for tissue engineering applications. Chitosan, as an example of a positively charged naturally derived polymer, can be easily processed to sponge-like implants via a freeze-drying procedure. These chitosan sponges provide very interesting properties for tissue engineering applications due to efficient swelling on the one hand and their porous structure, enabling cell migration into the implant, on the other hand. In a study by Jia et al., two fluorescently labelled siRNAs complexed with Lipofectamine® were loaded onto chitosan sponges in order to improve bone regeneration in a rat calvarial bone defect model. siRNA loading was performed by incubation of the chitosan sponge with the complexed siRNA and subsequent freeze drying. The *in vitro* release profile of siRNA was determined over 4 weeks, showing 20 % release within the first 8 days and a more rapid release in the following days along with the structural breakdown of the scaffold due to dissolution of the chitosan matrix. The release was determined relative to the theoretical loading and showed that the amount loaded to the scaffolds was equal to the released amount. The sponge-loaded fluorescently labelled siRNA demonstrated at least a fivefold increase in fluorescence intensity compared to the controls (sponges loaded with non-labelled, non-coding siRNA) (Jia et al. 2014).

A hydrogel delivery system composed of the biodegradable hydrogel Glycosil® (a thiol-modified analogue of heparin and thiol-modified hyaluronan cross-linked with a PEG diacrylate) and a chemically modified single-stranded RNA termed "agomirs" (miRNA enhancer) has been used to enhance endogenous miRNA-26a expression. miRNA-26a is known to modulate osteogenic differentiation of adipose tissue derived stem cells (ASC) (Luzi et al. 2008). The hydrogel served as scaffold for cell ingrowth and differentiation as well as a system for controlled and sustained release of the miRNA enhancer *in vivo*; however, data show a burst release of agomir which was released within a time span of 2 days. Still, significantly enhanced vascularization as well as bone formation in a controlled and sustained manner was observed (Li et al. 2013).

Zhang et al. introduced a new type of LbL film coatings based on polyelectrolyte multilayers containing poly(L-lysine) and negatively charged shRNA carrying cal-

cium phosphate nanoparticles. shRNAs against osteocalcin and osteopontin served as a proof of principle to show functional gene silencing in human osteoblasts. Using multilayered films they showed a more efficient silencing than for one layer only (Zhang et al. 2010).

A different concept for controlled nucleic acid release was introduced by Qureshi et al. (Qureshi et al. 2013). They used silver nanoparticles loaded with miRNA via a photocleavable linker. The nanoparticles provided stabilisation and immobilization of the miRNA as long as the nucleic acid remained immobilized. Upon UV-light exposure, the miRNA was released. Loaded silver-nanoparticles can be integrated into various biomaterials, such as PCL scaffolds and hydrogels and flashed upon implantation (Qureshi et al. 2015).

8.5 Modification of Delivery Systems for Cellular Targeting

Targeted delivery of therapeutics by linkage with targeting moieties as ligands for cell-type specific receptors offers the advantage of a higher local concentration of the drug in the desired tissue, thus requiring lower dosages and reducing off-target adverse effects. In this context, aptamers and antibodies as well as small organic molecules and peptides have been explored. These approaches have been mostly used for cancer targeting, e.g. (Zhou and Rossi 2014; Höbel et al. 2014), but there is also an application for bone regeneration (Zhang et al. 2012) that will be shortly described here.

An approach involving aptamers, i.e. oligonucleotides that bind to specific epitopes on the cell surface, has recently been developed which enable targeting of various diseases in different tissues in a cell-specific manner. This approach has been used, e.g., to target prostate-specific membrane antigen (PSMA)-expressing cells to treat prostate cancer in a mouse model after systemic delivery of siRNA directed against tumor survival genes like polo-like kinase 1 (PLK1) (Zhou and Rossi 2014).

Antibody-functionalized PEI nanoparticles for tumor cell-specific delivery of siRNA have been described as well. Cetuximab, an antibody specific for the epidermal growth factor receptor (EGFR), which is overexpressed in many human carcinomas, was covalently linked to the nanoparticles via a PEG spacer prior to siRNA complexation (Höbel et al. 2014).

Zhang et al. introduced a targeting peptide consisting of six repetitive sequences of the tripeptide aspartate-serin-serin grafted to DOTAP based liposomes for systemic intravenous siRNA application. The authors used this targeting system for delivery of siRNA against casein-kinase 2 interacting protein (Ckip) targeting to bone formation surfaces characterized by low crystalline hydroxyapatite. They compared *in vivo* targeting effectiveness of their peptide with a peptide of eight aspartates known to target highly crystalline hydroxyapatite typically found in bone resorption sites (Zhang et al. 2012).

8.6 Application

Biomaterials for the controlled release of RNAi therapeutics have been widely applied in tissue engineering. These systems address for example bone, cartilage or nerve regeneration as well as angiogenesis and skin repair for wound healing. In the following, applications in bone regeneration are given.

8.6.1 Bone and Cartilage

Two targets have attracted great attention for their application in bone regeneration: the receptor activator of NF-κB (RANK), which plays a major role in differentiation of bone-resorbing osteoclasts and antagonists of bone morphogenetic proteins (BMPs), especially noggin.

8.6.1.1 Targeting RANK

The receptor activator of NF-κB (RANK) and its ligand RANKL play a role as key molecules in differentiation and activation of osteoclasts. Binding of RANKL to its receptor triggers downstream signalling which leads to the transformation of precursor cells into mature osteoclasts. Therefore, inhibition of this pathway by silencing RANK is a promising approach to suppress osteoclast maturation and prevent bone resorption (Koide et al. 2010; Wang and Grainger 2010).

PLGA microspheres of about 5 μm in diameter were used to passively target osteoclast-precursor phagocytes and to deliver RANK siRNA (Wang et al. 2012). siRNA complexed with branched PEI (NP ratio = 20) was integrated into the microsphere by a double emulsion technique. In order to prevent passive release of siRNA from the microspheres (Wang et al. 1999) and to favour uptake by phagocyting osteoclastic cells, PLGA of a molecular weight of 57 kDa was used. siRNA released after phagocytosis in the osteoclastic cells exerted its function through inhibiting osteoclast precursors to differentiate along the osteoclastic line. PLGA microsperes were successfully integrated into a calcium phosphate cement for application (Wang et al. 2012).

Ma et al. used a thermosensitive chitosan hydrogel for delivery of siRNA directed against RANK (Ma et al. 2014). The fluorescence signal from labeled siRNA in the hydrogel showed a prolonged siRNA release profile *in vitro*, followed by a sustained gene silencing effect in a murine macrophage cell line for a period of up to 9 days. *In vivo* release investigations of Cy3 labeled siRNA in a subcutaneous local delivery setup in mice showed a burst release of about 75 % within the first day while the remaining siRNA was slowly released for 14 days.

8.6.1.2 Targeting BMP-2 Antagonists

BMP-2 as an osteoinductive factor was shown by several groups to play a major role in enhancing osteogenesis *in vitro* and *in vivo* (Cohen 2002; Wozney 1992). The effectiveness of this growth factor to improve bone formation lead to the registration of a BMP-2 releasing class 3 medical device, Infuse Bone Graft®. However, BMP-2 signalling is strongly regulated, e.g. by internal antagonists such as noggin, chordin and others (Yanagita 2005). In order to overcome this regulation, BMP-2 has to be delivered in high dosages to be effective, coming along with unwanted side effects. Therefore, siRNA application to increase the endogenous availability of BMP-2 by suppressing its active antagonists has been shown to synergistically improve BMP-2 effects (Schneider et al. 2014). Levi et al. investigated the effects of noggin silencing using lentiviral transfection. They found convincing effects of small hairpin RNA (shRNA) alone and in combination with BMP-2 *in vitro* on osteogenic differentiation of hASC and *in vivo* in a critical size defect that was treated with transfected hASC loaded onto a hydroxyapatite coated PLA scaffold. In the same study, the authors demonstrated that the system was also effective when a scaffold loaded with lentiviral/shRNA particles and BMP-2 was combined with pristine hASC. In another approach involving noggin silencing, Manaka et al. used a biodegradable hydrogel consisting of a PLA-*p*-dioxanone-PEG triblock co-polymer with a central PEG block (Saito et al. 2001) as delivery system for naked noggin siRNA (Manaka et al. 2011). They evaluated the biological efficiency of 1 nmol noggin siRNA in presence of extra BMP-2 (2.5 μg) *in vivo* in a mouse model. The results indicated that the siRNA releasing polymer melt implanted in a muscle pouch of mice caused ectopic bone formation with improved size and mineral content as compared to the control containing only BMP-2. Recently, another report on noggin silencing was published that involved a new transfection system based on cationic sterosomes (Cui et al. 2015). The authors investigated a transfection system consisting of cholesterol and stearylamin and found that it was more effective to silence noggin in mouse derived ASCs than Lipofectamine® 2000. They combined their transfection system with a hydrogel consisting of crosslinked methacrylated glycol chitosan and tested the siRNA loaded hydrogel in a critical sized calvaria defect. However, bone formation was not convincingly improved, possibly in part due to a lack of BMP-2 supplementation. Takayama et al, in contrast, injected siRNA against noggin in muscle tissue of mice, applied electroporation and eventually placed a BMP-2 loaded collagen sponge on top. The *in vivo* approach was unable to increase bone formation beyond the BMP-2 group, however, the density of newly formed bone was enhanced in the group with noggin siRNA and BMP-2 (Takayama et al. 2009). A study from the Alsberg group on PEG-based biodegradable and *in situ* forming hydrogels involving noggin silencing was recently published. They showed that release of PEI-complexed siRNA against noggin was controlled by the degradation rate of their hydrogel and allowed a sustained release over a 3–6 weeks period depending on the hydrogel composition. hMSC encapsulated in the siRNA containing hydrogel were successfully transfected and showed improved osteogenic differentiation (Nguyen et al. 2014). A study published by Kowalczewski and Saul

showed the adsorption of Lipofectamine® 2000 complexed siRNA against noggin on fibrin hydrogels. Complexed siRNA was not released in buffer but taken up by MC3T3-E1 mouse fibroblasts seeded onto the gels. Transfection was shown to correlate with the amount of complexed siRNA on the surface.

Kwong et al. investigated effects of siRNA against another BMP-antagonist, chordin, in hMSC (Kwong et al. 2008) and Schneider et al. performed similar experiments on hASC. Both studies found that silencing chordin improved osteogenic differentiation of human osteogenic cells in the absence of BMP-2. Moreover, in the presence of BMP-2, synergistic effects of the combination of siRNA against chordin and BMP-2 were found for hASC (Schneider et al. 2014). No release system, however, has been shown for this application yet.

8.6.1.3 Other Targets for Improved Bone and Cartilage Formation

Casein kinase 2 interacting protein 1 (Ckip-1) is another interesting target to improve osteogeneic differentiation that works via a bone morphogenetic protein-related signalling pathway. Song et al. used a multilayered film (LbL) of hyaluronic acid and chitosan for siRNA delivery from titanium surfaces (Song et al. 2015). siRNA was complexed with chitosan first and loaded to the LbL films as nanoparticle dispersion.

An approach using a dual siRNA release system was published recently by Jia et al. (2014). In addition to Ckip-1 they silenced Flt-1, a soluble VEGF receptor, to increase the amount of free VEGF in tissue and eventually improve bone healing. siRNA complexed with Lipofectamine® 2000 was loaded onto chitosan scaffolds. Rat-derived MSCs showed improved osteogenic differentiation and expression of von Willebrand factor *in vitro*, whereas proliferation in Lipofectamine containing sponges was found to be reduced compared to control sponges.

8.6.1.4 Cartilage

Regarding cartilage engineering, an interesting study was published by Perrier-Groult (Perrier-Groult et al. 2013). They addressed the problem that expansion of isolated chondrocytes in 2D culture is known to dedifferentiate the primary chondrocytes and eventually causes fibrocartilage formation. In order to keep the cells in a differentiated state they used siRNA against collagen type 1. After treatment of primary mouse chondrocytes with siRNA and electroporation, they could show that BMP-2 was more effective than TGF-β1 to induce typical chondrogenic marker when expanded cells were differentiated in an agarose hydrogel 3D culture.

8.6.1.5 miRNA for Bone Applications

Besides siRNA delivery, which has been the mostly investigated type of nucleic acid used in RNAi approaches, miRNA and shRNA-mediated gene silencing have also been subject to recent research projects. Li et al. investigated an RNAi approach using an identified miRNA, which positively modulates the coupling of angiogenesis and osteogenesis. They investigated the expression of different literature-known miRNAs in hMSC, mouse MSCs and mouse preosteoblast MC3T3-E1 cells during osteogenic differentiation. In contrast to other investigated miRNAs, miRNA-26a was found to be strongly upregulated in differentiated cells. A miRNA enhancer structure was used to investigate the effectiveness of miRNA-26a *in vivo* employing an hMSC loaded biodegradable hydrogel delivery system in a critical size calvaria defect model in mice. They found intriguing effects of miRNA-26a with 100 % bone regeneration compared to only 40 % for the cell loaded hydrogel system without miRNA-26a enhancer.

Another miRNA formulation was presented by Qureshi et al. in order to improve closure of critical size mouse calvarial defects. miRNA-148b was linked to silver nanoparticles via a photolabile linker. The conjugate PC-miR-148b-SNP, a derivative of miRNA-148b tethered to silver nanoparticles (SNPs), regulated the gene expression to enhance cortical defect closure in mice. They were added to hASCs and loaded to polycaprolactone (PCL) scaffolds showing enhanced levels of defect healing compared to controls (Qureshi et al. 2015).

Deng et al. investigated the effects of anti-miRNA-31 by lentiviral transfection of rat- and canine-derived ASC (Deng et al. 2013, 2014). miRNA-31 targets the special AT-rich sequence binding protein-2 (Satb2) that is involved in transcriptional regulation and chromatin remodeling and is relevant for osteogenic commitment of ASC downstream of Runx-2. Employing an anti-miRNA-31, they showed improved osteogenic differentiation *in vitro*. Additionally, Deng et al. loaded anti-miRNA-31 transfected ASC onto macroporous β-tricalcium phosphate (TCP) scaffold and showed improved bone healing compared to the non-transfected and miRNA-31 transfected cell-loaded scaffolds in critical size orbital defect models of different species (Deng et al. 2014).

8.6.2 Other Tissues

Local siRNA delivery enables a direct interaction with target genes in the intended tissue. In the following, a number of studies on various tissues that have been targeted with siRNA strategies are described.

A large body of evidence from preclinical studies confirms the successful use of pulmonary siRNA delivery in preclinical models, however none of them could yet proceed to clinical phase (Merkel et al. 2014). A study by Jensen et al. demonstrated a successful delivery of siRNA using cationic lipid-modified PLGA nanoparticles

as a dry powder formulation to treat severe lung diseases through pulmonary administration of siRNA (Jensen et al. 2012).

An anti-TGFβ1 hydrogel pressure-sensitive adhesives transdermal patch composed of polyvinylpyrrolidon, polyvinylalcohol and polyethylene glycol terephthalate could successfully reduce the expression of targeted gene and treat hypertrophic scars (Zhao et al. 2013). This study found that siRNA against TGFβ1 reduced TGFβ1-mediated collagen I synthesis and led to a more homogenous orientation of collagen I fibers.

Mittnacht et al. used hollow resorbable block-copolymer filaments as scaffolds to promote peripheral nerve regeneration. Scaffolds were loaded with chitosan/siRNA nanoparticles for local delivery of nanotherapeutics and the enhancement of nerve regeneration. The stable nanoparticles were instantly internalized by the cells without cytotoxic effects. The targeted gene was silenced by 65–75 % and neurite outgrowth was promoted even in an inhibitory environment (Mittnacht et al. 2010). Chitosan imidazole /siRNA nanoplexes in polyelectrolyte multilayers (PEM) were introduced by Hartmann et al. and an efficacious delivery, uptake and silencing in neuronal cell culture as well as in biologically functional neuronal implants was shown (Hartmann et al. 2013).

Hossfeld et al. successfully delivered chitosan/siRNA NPs from PEM to endothelial cells in porcine artery walls. RNAi strategies were also used in coronary arteries when inflammation occurred after stent placement. Chitosan/siRNA NP, incorporated into PEM that consisted of hyaluronic acid and chitosan were constantly delivered to the porcine artery wall *ex vivo*, suggesting that stents coated with biologically active siRNAs against cytokine receptors or adhesion molecules might be efficient in restenosis prevention (Hossfeld et al. 2013). Monaghan et al. reported on *in vitro* and *in vivo* effects of collagen based scaffold loaded with miRNA in wound healing processes. They found a specific impact on ECM remodelling after injury, which was shown to synergistically improve wound healing controlled by the dose of miR-29B present in the scaffold (Monaghan et al. 2014).

8.7 Barriers to Practice…

Several siRNA based therapies have entered clinical trial phases for FDA approval. None of these trials, however, is focussed on regenerative applications yet. When quantifying the extent of RNAi-based preclinical research during the past years, a steady increase of publications in the field is noticeable as a consequence of successful basic research and the promising general concept of RNAi. A Pubmed search using the keywords “siRNA” and “tissue engineering” or “regenerative medicine” shows that the annual number of publications has steadily increased over the last 10 years and still is increasing to date (Fig. 8.2).

First data on the efficiency of siRNAs in non-human primates were already published in 2006 (Zimmermann et al. 2006). In a proof-of-concept study on RNAi therapeutics in humans, lipid nanoparticle-formulated siRNAs directed against two

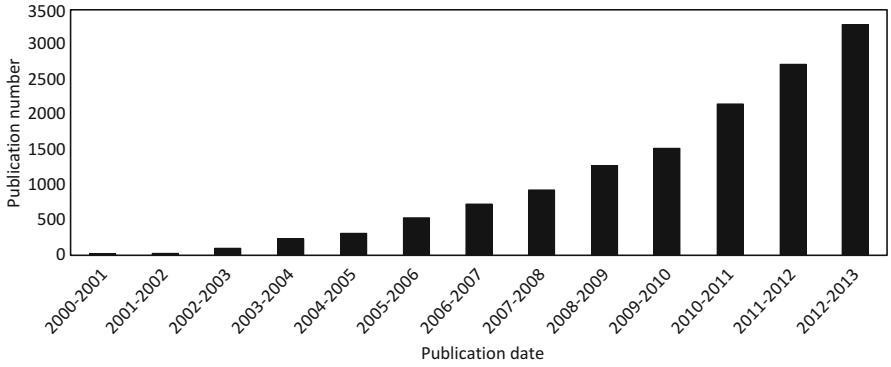

Fig. 8.2 Annual RNAi-related publications since year 2000

oncogenes in liver metastases were used (Tabernero et al. 2013). Even though siRNAs can theoretically silence any gene in the body, clinical studies still face major hurdles that have not been satisfactorily overcome. The problem of poor cellular delivery of naked siRNA due to its size, hydrophilic and anionic nature is addressed by rationally designed delivery systems. However, in many cases they still lack the efficiency of viral delivery systems and many of these vectors cannot be used in clinical studies due to safety concerns. Off-target effects of siRNA, rapid blood clearance by the RES system, immunogenicity and often cytotoxicity due to the unspecific interaction of the highly positively charged delivery systems with serum proteins remain issues that need to be tackled (Kanasty et al. 2012). Still, several drugs are currently in, or have already completed, early clinical phases (phase I or II; see e.g. (Kanasty et al. 2012; Draz et al. 2014) for review and Table 8.1 for some examples).

8.8 …and How to Overcome them

8.8.1 Approaches to Stabilize siRNA by Chemical Modifications

As described above, the problem of physiological stability due to rapid enzymatic degradation of naked siRNA leading to short half-lives in serum has been addressed by packing the nucleic acids into nanocarriers (Yamamoto et al. 2011; Dirin and Winkler 2013). On the other hand, chemical modifications of siRNAs also increase its inherent stability.

Natural oligonucleotides contain a phosphodiester (PO) backbone which is prone to degradation by nucleases *in vivo*. As defined by Dirin et al. (2013), the first generation of chemical modification to tackle poor physicochemical properties

Table 8.1 A choice of clinical trials on RNAi drugs, their targets and vehicles

Disease	Target	Vehicle	Drug name	Company	Status
Advanced solid tumours	Ephrin type-A receptor 2	Neutral liposomes	siRNA-EphA2	Anderson Cancer Center	Phase I recruiting
Metastatic solid tumors	E3 ubiquitin ligase Cbl-b	siRNA-transfected peripheral blood mononuclear cells *ex vivo* by electroporation	APN401	Comprehensive Cancer Center of Wake Forest University, Apeiron	Phase I recruiting
Choroid neovasculari-zation, age-related macular degeneration	VEGF 165 receptor	Solution of chemically modified siRNA for intravitreal injection	AGN 211745	Allergan	Phase II terminated
Advanced solid tumors	PKN3	Cationic liposomes	Atu027	Silence Therapeutics	Phase I completed
Adenocarcinoma of the pancreas	KRAS oncogene mutations	LODER (Local Drug EluteR) is a miniature biodegradable polymeric matrix	siG12D LODER (Local Drug EluteR)	Silenseed Ltd	Phase I completed
Optic atrophy	caspase 2 protein	Chemically modified siRNA	QPI-1007	Quark Pharmaceuticals	Phase I completed
Delayed graft function in patients receiving renal transplants.	pro-apoptotic p53	naked siRNA	I5NP	Quark Pharmaceuticals	Phase I completed
Treatment of relapsed or refractory cancers	M2 subunit of ribonucleotide reductase (RRM2)	Cyclodextrin nanoparticle decorated with human transferrin protein and polyethylene glycol for stability	CALAA-01	Calando Pharmaceuticals	Phase I terminated

https://clinicaltrials.gov/ and Burnett et al. (2011)

exchanged the PO group of oligodeoxynucleotide (ODN) molecules by phosphorothioate oligodeoxynucleotides (PS-ODNs). PS-ODNs show increased nuclease resistance, making a parenteral administration feasible. Furthermore, this modification improved pharmacokinetic properties and led to higher tissue distribution, decreased urinary excretion and longer residence time in various tissues as a result of higher plasma protein binding (Yu et al. 2001, 2009). However, the clinical application of PS-antisense ONs has been limited so far, due to poor pharmacodynamic properties, off-target effects and safety issues (Winkler et al. 2010; Stessl et al. 2012; Gekeler et al. 2006; Summerton 2007; Kling 2010; Pisano et al. 2008).

The second generation of chemical modifications focused on the ribose unit of the nucleic acids. The aims of these modifications include enhancement of nuclease resistance, improvement in efficacy, increased binding affinity and enhanced protein binding (Manoharan 1999). Gapmers which contain internal segments of PS-ODNs bound to 2′-OMe or 2′-O-methoxyethyl (2′-OMOE) were identified as improved structure over PS-ODNs (Prakash et al. 2008).

The third generation includes major structural changes represented by various ribose ring and phosphate modifications (Yamamoto et al. 2011). Such modifications include locked nucleic acids (LNA) and phosphorodiamidate morpholino oligomers (PMOs or morpholinos) (Summerton 1999). LNAs contain a methylene bridge between the 2′-O and 4′-C of the ribose unit, which leads to higher hybridization affinity and enzymatic stability (Fluiter et al. 2009). PMOs are constituted of an uncharged backbone, while the ribose moiety is substituted by a morpholino ring (Summerton 1999).

8.8.2 Opportunities of Local Delivery

In order to circumvent the mentioned obstacles for RNAi in regenerative applications, a combination of strategies for local delivery appears to be promising. This includes (1) the use of nanocarriers for the formulation of siRNA or other small RNA molecules, mediating protection against nucleases, efficient cellular uptake and intracellular delivery, (2) the incorporation of nanoparticle-formulated RNA into tissue-adopted implants, either macroporous scaffolds or hydrogels, that guide migration of regenerating cells into the material and (3) the targeted controlled release from the implants towards these cells in order to foster their regenerative function. In addition to chemical modifications and customization of the employed nanocarriers and implants, (4) chemical RNA modification appear to be beneficial.

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Chapter 9
Imaging Technology

Cajetan Lang and Sebastian Lehner

Abstract Regenerative medicine is a rapidly evolving area in the field of biomedical research. Thereby particularly cell-based advanced medicinal products raise new ethical questions concerning the development and testing of these products in humans. Moreover, on the level of non-clinical research new methods are necessary to study the specific properties of these advanced products.

Advanced medicinal products, such as stem cells based applications have the potential to survive in and interact long-term with the host organism. In contrast to postmortem tissue analyses, which are commonly used for conventional drug testing in animals, methods for serial and longitudinal monitoring of both the cells fate and the triggered biological mechanisms that leading to organ repair are needed for testing advanced cell-based products. Furthermore, the human origin of these products requires reliable testing of safety and mode of action in the clinical setting. Therefore new *in vivo* monitoring techniques with the potential for rapid clinical translation are urgently needed.

Keywords Cell tracking • Biodistribution • Cell labeling • Apoptosis • Angiogenesis • Inflammation

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9.1 Introduction: A Historical Perspective

Damage by disease or trauma and the consecutive inflammatory response leads to scar formation and functional loss instead of restoration of structure and function in many tissues and organs of the human body such as the heart, brain and pancreas. Current therapies either aim at the symptomatic treatment (congestive heart failure, Parkinson's disease, diabetes I) or the replacement of the whole organ by heterologous organ transplantation in case of the heart and pancreas. Lack of organ transplants and obvious limitations in symptomatic therapeutic approaches gave rise to the field of regenerative medicine, which aims to achieve functional recovery of damaged tissues by providing specific cell populations, alone or incorporated in biomaterial scaffolds, which enhance the body's intrinsic healing capacity (Daar and Greenwood 2007).

The role of "Imaging Technologies" in the field of regenerative medicine will be exemplified by the three most broadly investigated tissues in clinical trials – namely the ischemic heart (heart), substantia nigra (brain) and ceased pancreatic islets (pancreas). In the consecutive sections, the role of imaging will be discussed in detail on the basis of the heart.

First clinical applications to treat patients with Parkinson's disease, diabetes I and chronic ischemic cardiomyopathy with specific cell products to restore the function of substantia nigra, pancreatic islets or contractile myocardium have been performed starting around 1990 (Table 9.1).

Imaging technologies such as ultra sound, magnet resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET) and singe photon emission computed tomography (SPECT) are already indispensable tools for the assessment and stratification of structural and functional damage of the heart, brain and pancreas. Besides clinical hard endpoints, imaging also plays a major role for efficacy assessment of new therapies for the treatment of ischemic myocardium or Parkinson's disease. In contrast, proper function of transplanted pancreatic islets can be assessed indirectly by metabolic monitoring (Shapiro et al. 2000). In the above cited study of Steinhoff's group cardiac pump function was assessed by echocardiography and perfusion via SPECT thus assessing both function and perfusion deficit of the ischemically damaged myocardium. After coronary artery bypass graft (CABG) surgery combined with intramyocardial injection of autologous CD 133+ bone marrow stem cells, improvement of pump function and perfusion were measured with the same devices (Stamm et al. 2003).

Table 9.1 Ground-breaking reports in the field of regenerative medicine

	Year	Author	Cell-type	Disease
Heart	2003	Steinhoff et al. (2003)	CD 133+ BMSC	CIHD
Brain	1990	Lindvall et al. (1990)	Fetal dopamine neurons	PD
Pancreas	1990	Shapiro et al. (2000)	Cadaveric islets	DM 1

BMSC bone marrow stem cells, *CHID* chronic ischemic heart disease, *PD* Parkinson's disease, *DM 1* diabetes mellitus 1ype 1

Table 9.2 Early cell tracking studies in a clinical setting

	Year	Author	Cell-type	Label	Modality	Disease
Heart	2005	Hofmann et al. (2005)	BMMNC	FDG	PET	AMI
Brain	2006	Zhu et al. (2006)	Human neural stem cells	SPIO	MRI	Brain trauma
Pancreas	2008	Toso et al. (2008)	Islet Graft	SPIO	MRI	DM 1

BMMNC bone marrow mononuclear cells, *FDG-^{18}F* Fluorodeoxyglucose, *SPIO* super paramagnetic iron oxide, *AMI* acute myocardial infarction

Likewise molecular imaging – ^{18}F-Fluorodopa positron emission tomography (PET) – was used for measuring the dopamine synthesis in the putamen both before and after the transplantation of fetal dopamine neurons in a patient suffering from progressed Parkinson's disease in addition to clinical monitoring (Lindvall et al. 1990).

Therapeutic cell preparations are usually applied via injection of cell suspensions or aggregates into the organ of interest by using syringes or special catheters. In either case efficient disposition of the therapeutic cells, aiming at repairing the respective damages organ, is an absolute prerequisite for an effective therapy.

For this reason, few years later live imaging techniques were explored to assess the fate and biodistribution of transplanted cells (Table 9.2). Thereby direct labelling of the cells before application with a radioactive or superparamagnetic agents and consecutive MRI and PET scans served as a basis.

Data from clinical and preclinical studies assessing the retention of intramyocardially transplanted cells led to the perception that only a minor percentage of the applied cells stays at the site of injection (Hofmann et al. 2005; Lang et al. 2013). Similarly, massive cell loss from the injection site has been reported in the clinical trial, in which a patient with diabetes type 1 received FDG-labelled islets via infusion into the portal vein. A consecutive PET scan revealed that less than half of the injected activity had remained in the liver (Eich et al. 2007). Whereas comparable striking findings concerning acute cell loss following application in patients with Parkinson's disease has not been reported, a study aiming at treating subacute stroke patients with bone marrow stem cells found even lower engraftment rates in the brain (Rosado-de-Castro et al. 2013). In brief, bone marrow mononuclear cells were labelled *in vitro* with ^{99}mTc before intra-arterial injection into patients with subacute middle cerebral artery ischemic stroke. Whole-body planar scintigraphy revealed that less than 1 % of the detected activity was located in the brain, whereas 40 %, 6 % and 7 % could be located in the liver, spleen and the lungs respectively.

These findings suggest that new methods for reliable quantitative *in vivo* monitoring are an absolute prerequisite for further optimizing cell based therapies. In order the generate valid data on dose-response relationships, sophisticated methods for live imaging of biological mechanisms of these cell products are urgently needed.

Thereby molecular and cellular imaging in the field nuclear medicine offers a rapidly increasing line-up of methods to monitor and quantify processes such as angiogenesis and apoptosis in a clinical setting.

Besides further investigation on the mode of action of cell-based advanced products, new methods for appropriate safety testing is requirement for broad application in humans.

Conventional efficacy and safety testing strategies in the field of non-clinical development as established for small molecules or biopharmaceuticals are not sufficient and suitable for cell-based advanced therapies. Therefore specific testing requirements must be fulfilled for clinical trials or market approval by regulatory authorities such as the European Commission in the EU (Zscharnack et al. 2015).

Essential properties of cell-based products that have to be investigated are unintended biodistribution and migration of the applied cells, potential tumorigenicity and potential immunotoxicity or immunogenicity in the patient (Erben et al. 2014).

In contrast, pharmaceuticals in the field of regenerative medicine are mostly highly complex products containing of consisting of living cells. Therefore, interactions of the respective cell or product with the host organism rather than mere pharmacokinetics or -dynamics are central for the assessment of safety and dosage finding. Besides safety and efficacy testing, knowledge of the mode of action is paramount for targeted optimization of cell-based products. As living cells are more complex systems than chemical drugs, multidisciplinary approaches are needed to address this goal (Erben et al. 2014). Paying respect to the human origin of advanced cell-products preclinical models have to be chosen carefully regarding clinical translation. Thereby multimodal and molecular imaging offers superior options in this field for comprehensive *in vivo* monitoring of novel therapies.

The following points have to be addressed in the process of safety and efficacy testing of new cell products:

- **I Safety**: retention, biodistribution, migration, tumour formation
- **II Mode of action**: e.g. angiogenesis, apoptosis, inflammation in case of the heart

9.2 Background/Principles

In this chapter the role of "Imaging Technologies" in the field of regenerative medicine will be discussed. The focus will be on strategies based on stem cell applications, both alone and in biomaterial scaffolds for myocardial repair. Imaging of organ structure and surrogate end points for the effect is already established in clinical application in the field and cardiovascular imaging and has already been reviewed in detail elsewhere (Sogbein et al. 2014). Hence the focus of this chapter will be on *in vivo* monitoring of safety (I) and the mode of action (II) of cell based-advanced products for myocardial repair.

Table 9.3 Features of different imaging modalities

	Sensitivity	Resolution	Quantification	References
PET	+++	++	++++	Bonios et al. (2011), Terrovitis et al. (2009), Welling et al. (2011) and Fu et al. (2011)
SPECT	++	+	++	Bonios et al. (2011), Terrovitis et al. (2009), Welling et al. (2011) and Fu et al. (2011)
MRI	+	+++	–	Wu et al. (2010)
BLI	++++	–	++	Fu et al. (2011) and Terrovitis et al. (2010)

BLI bioluminescence imaging

9.2.1 Safety

9.2.1.1 Cell-Tracking: Biodistribution/Fate

The field of cellular *in-vivo*-imaging can be divided into "direct" and "indirect" cell tracking strategies.

For direct tracking, cells are labelled *in vitro* with radioactive tracers or paramagnetic iron particles for subsequent *in vivo* monitoring by positron-emission-tomographie (PET), Singe-Photon-Emission-Tomography (SPECT) or Magnetic Resonance Imaging (MRI).

For indirect labelling approaches cells are usually modified by the over expression of a certain reporter gene. Thus transplanted cells can be visualized *in vivo* after administration of the substrate for the respective gene product by the use of PET, SPECT or MRI.

For clinical translation optimal imaging methods for *in vivo* cell tracking should display the following characteristics:

(a) The used techniques should be highly sensitive, able to absolutely quantify the applied cell number and offer superior spatial resolution.
(b) Furthermore dynamic acquisitions should be possible for serial assessment of biological processes.
(c) The method has to be safe for clinical application in patients and must not have toxic effects on the therapeutic cells.

These aspects will be considered in the explanation of the different cell tracking techniques in the following section.

Direct Labelling

The most commonly used modalities are MRI and nuclear imaging with PET and SPECT (136). Each modality has specific advantages and disadvantages for tracking the fate of transplanted cells (Table 9.3).

MRI offers the highest spacial resolution and enables exact location of the applied cell product, yet sensitivity is relativly low (SPIO: 10^{-5} mol/l) (Rodriguez-Porcel 2010) and thus it's potential for absolute quantification. The contrast agent used for *in vivo* cell tracking experiments in most clinical studies is **superparamagnetic iron oxide (SPIO)**. *In vitro* studies could show that labelling cells with SPIO does not compromise viabilty or function and can thus be safely applied for MRI based cell tracking (Richards et al. 2012). Yet, several groups found that 1 day after transplantation of SPIO-labelled cells the majority of the tracer could be detected within macrophages adjacent to the site of injection (Terrovitis et al. 2008a) and therefore appears to be not sensitive enough for seriell monitoring of transplanted cell's fate. Further limitations of SPIO for clinical use are that production of iron oxide for clinical use has been stopped in Europe and the US (Psaltis et al. 2012) and the fact that many potential patients are equipped with implanted devices, such as impanted cardioverter defibrillators (ICDs), which can be a contraindication (Zhang and Wu 2007).

Currently, only SPECT and PET offer sufficient sensitivity for absolute quatification the retention of transplanted cells (Wu et al. 2010) and will therefore be discussed in detail. The strength of PET and SPECT is their high sensitivity (PET: 10^{-11}–10^{-12} mol/l and SPECT: 10^{-10}–10^{-11} mol/l) (Fu et al. 2011) and its potential for absolute quantification of the applied activity as the "percentage of the injected dose" (%ID). Moreover, rapid technological progress has overcome the limitations of low spatial resolution in nuclear imaging (Visser et al. 2009) and enables the detection of tiny anatomical structures at resolution of 1.6 mm in diameter by the use of microPET (Visser et al. 2009). If more detailed information on the anatomial structures is need, SPECT and PET can be easily combined with Computed Tomographie (CT) or MRI (Fu et al. 2011). Whereas PET offerst the best quantification, isotopes used for SPECT have longer radioactive half-live and thus enable longer time frames for cell tracking and biodistribution studies. Transplanted cells cannot be imaged with current technologies unless they are labelled with intracellular located tracers. The most commonly used ones are Indium-111 (^{111}In) for SPECT and [^{18}F]-Fluordesoxyglucose (FDG) for PET imaging. The main difference of these tracers is their radioactive half-live ($\mathbf{T}_{½}$) and thus their side effects and their scope of application (Table 9.4).

Whereas each of the above mentioned tracers has already been studied on the level of clinical trials (Hofmann et al. 2005; Zhu et al. 2006; Kang et al. 2006; Caveliers et al. 2007; Callera and de Melo 2007) only FDG (Hofmann et al. 2005;

Table 9.4 Characteristics of different tracers

	$T_{1/2}$	Imaging modality	Cell toxicity	Clinical application	Celltracking	References
FDG	110 min	PET	–	Yes	Studies	Hofmann et al. (2005)
^{111}In	2,8 d	SPECT	++	Yes	Yes	Schots et al. (2007)
SPIO	–	MRT	–	Studies	Studies	Fu et al. (2011)

$\mathbf{T}_{1/2}$: radioactive half-live

Kang et al. 2006) and ^{111}In (Schots et al. 2007; Caveliers et al. 2007) have been applied for cell tracking studies in the field of regenerative medicine. SPIO has been used in very experimental clinical trials for tracking of neural stem cells for the treatment of brain trauma (Zhu et al. 2006) and dendritic cells in melanoma patients (de Vries et al. 2005).

Radioactivity emitted from **FDG and ^{111}In** is present but not toxic for patients in the miniscule concentrations used for cell labelling (Hofmann et al. 2005; Caveliers et al. 2007). **^{111}In** is a commonly applied agent in clinical diagnostics (leucocyte scintigraphy) for the localisation of inflammatory foci in case of fever of unknown origin (Roca et al. 2010). The major advantage of indium is the relatively long half-live which enables longer tracking periods of the transplanted cells, yet a dramatic drawback – in contrast to FDG – is the cytoxicity that has been repeatedly described (Aicher et al. 2003; Brenner et al. 2004). Toxicity of the auger-electron emitter **^{111}In** is ascribed to the short range of the emitted low energy ß⁻particles which lead to deposition to its radiation dose inside the labelled cells (Hofmann et al. 2005). In addition, the long half-live of 67 h compared to 110 min for FDG leads to longer exposition to toxic radiation.

FDG is a well established tracer in clinical imaging and has been applied without any complications in three clinical trials for tracking stem cells delivered into the heart (Hofmann et al. 2005; Kang et al. 2006; Blocklet et al. 2006). Moreover, it has been shown that labeling stem cells with FDG does not compromise their viability and their differentiation and proliferation capacities (Wolfs et al. 2013), which is an indispensable prerequisite for clinical application. In summary, FDG appears to be an excellent tracer for tracking the acute fate and biodistribution of cells applied to regenerate damages organs. For quantitative fate tracking, the short half-life of 110 min and the efflux of un-phosphorylated FDG from transplanted cells has to considered (Botti et al. 1997).

Direct cell labeling is the method of choice for monitoring cell fate within the first few hours after transplantation. Within this period, advantages of this method outweigh the drawbacks, such as short half-live fo used tracers, tracer efflux from labelled cells and dilution of the tracer by cell division. An inherent problem of direct cell labelling it the impossibility of discriminating between viable and dead cells as well as extracellular from intracellular label (Wu et al. 2010). Due to short-half lives of the deployed cells and the effect of tracer efflux we suggest to use direct cell labeling in order to verify successful cell application and tracking early fate of the injected cell product (Fig. 9.1).

Low background radiation due to *in vitro* labeling leads to optimized quantification conditions, which is in contrast to indirect cell labelling with systemic administration of the radiopharmaceutical. The most sensitive technology to date for quantification of transplanted call in the early phase PET. As long as the time frame of interest is within one radioactive half-life and tracer efflux from the labelled cells is integrated the advantages of ^{18}F based tracers can be exploited. For cell tracking studies exceeding the initial hours after application, we recommend the use indirect cell labelling approaches which will be further explained in the following section.

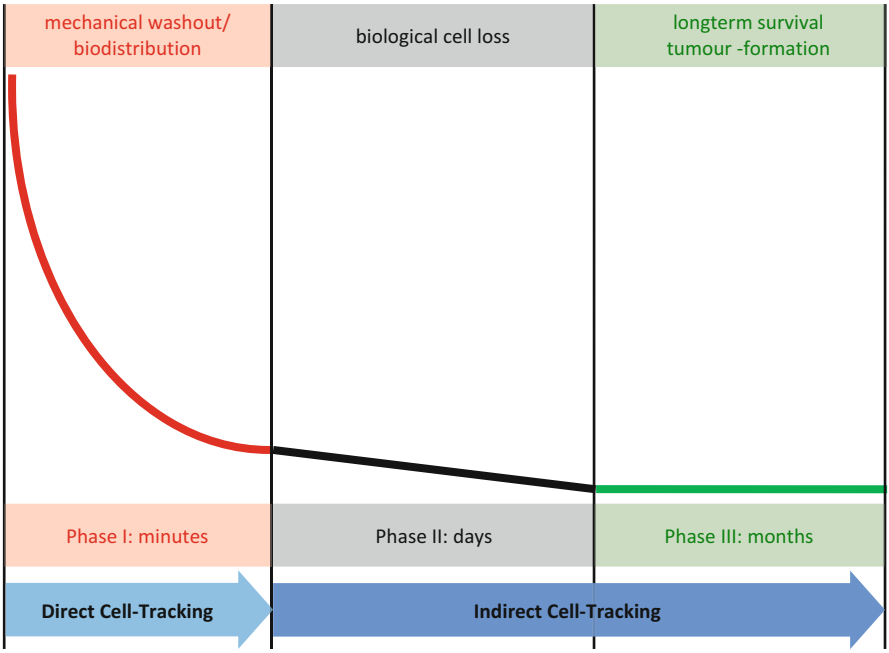

Fig. 9.1 When to use direct and indirect cell tracking

Indirect Cell Labeling

Direct cell labeling has been applied in several studies in the field of regenerative medicine (Psaltis et al. 2012) and is an established diagnostic tool in clinical application (leukocyte scintigraphy). In contrast, indirect cell labelling has been merely applied in a single clinical case report to date:

A 57-year-old patient diagnosed with glioblastoma multiforme received *ex vivo* expanded autologous $CD8^+$ cytolytic T cells, genetically engineered to express the interleukin-13 zetakine gene (therapeutic gene, encoding a receptor protein that targets the T cells to the tumor cells), and the Herpes Simplex virus 1 thymidine kinase (HSV1-tk) suicide gene/positron emission tomography (PET) imaging reporter gene (Yaghoubi et al. 2009). HSV1-tk phosphorylates ^{18}F-FHBG, trapping it within cells. Because mammalian thymidine kinases have low affinity for this reagent, it is possible to distinguish transplanted HSV1-tk expressing cells from surrounding tissues by PET (Naumova et al. 2014).

In contrast to this single clinical case report, many studies have explored various indirect cell-tracking techniques in preclinical studies. Yet, clinical translation has been hampered by concerns about the safety of genomic integration and about potential immune responses to some of the foreign reporter proteins.

Cells have to be genetically modified to over-express a “non-native” enzyme, protein or receptor. After administration of the respective substrate interaction with

the protein generates a signal that can be detected with dedicated devices such as PET or SPECT. Measurable signals are only generated by viable cells with intact metabolism and sufficient protein synthesis. Therefore, transplanted cells and their descendants can be detected with utmost specificity.

The most common technique used in small animal models is optical bioluminescence imaging (BLI) which uses the overexpression of the firefly protein luciferase (fluc). Administration of its substrate D-luciferin leads to enzymatic cleavage and emission of photons with a specific energy that can be detected and quantified with a ultrasensitive CCD camera (Cao et al. 2006). This method is very sensitive, cheap and non-toxic, but spatial resolution is low and the absorption of the emitted light precludes its use in most large animal and human studies (Fu et al. 2011; Terrovitis et al. 2010).

Another attractive reporter protein is the **human Sodium-Iodide-Symporter** (**hNIS**) which is predominantly located in thyroid tissue, the stomach and salivary glands (Dohan et al. 2003) in healthy individuals and can therefore be used as a reporter gene for cell tracking in limbs, brain and internal organ such as the heart [35]. hNIS mediates cellular accumulation of iodide and ^{99m}Tc-pertechnetate ($^{99m}TcO_4^-$) anions driven by the transmembrane sodium current and can be blocked by the competitive inhibitors thiocyanate (SCN^-) and perchlorate (ClO_4^-) (Dohan et al. 2003; Terrovitis et al. 2008b). Thus, cells expressing hNIS can be visualized following the intravenous administration of gamma-emitting $^{99m}TcO_4^-$ using SPECT and beta-emitting ^{124}I for PET imaging (Terrovitis et al. 2008b). In the clinical setting PET imaging offers superior sensitivity and acquisition of quantitative data compared to SPECT (Chen et al. 1997; Knesaurek and Machac 2006). Limitations regarding resolution can be overcome by the combined use with CT. Hence ^{124}I-PET/CT has become an attractive tool in clinical application for staging and pre-therapy dosimetry in patients with differentiated thyroid cancer (Freudenberg et al. 2011) and would be a ready-to-use technique for indirect cell tracking. hNIS based PET imaging has been used for tracking intramyocardially injected cells in small and large animal models respectively (Terrovitis et al. 2008b; Templin et al. 2012), which underlines its high potential for clinical translation. In contrast reporter proteins from viruses or fireflies its anticipated lack of immunogenicity in humans, hNIS might qualify for potential clinical application.

Another widely used reporter protein is the above mentioned **Herpes Simplex virus thymidine kinase** (HSV-tk). Transfected cells can be imaged via PET as phosphorylation of the administered radiolabel ^{18}F-FHBG or ^{124}I-Fialuridin leads to intracellular trapping of the tracer, thus enabling delineation of transplanted cells from the surrounding tissue. Yet, Miyagawa and colleagues could show that hNIS yields higher signal intensity and imaging contrast for PET than HSV-tk in a mouse model in which the respective adenoviral vectors were directly injected in to the myocardium (Miyagawa et al. 2005). Moreover, serial and longitudinal tracking of transplanted cells *in vivo* might be hampered by a strong immune response – caused by the viral protein- in the recipient organism (Miyagawa et al. 2005).

Indirect cell-labeling is the only method for long-term fate tracking, but bears inherent safety risks due to potential effects of genetic manipulation. Cell potency

and differentiation potential might be compromised whereas tumorigenicity and immunogenicity might be enhanced (Naumova et al. 2014). Moreover reporter-gene silencing could hamper quantitative monitoring of the transplanted cells.

9.2.1.2 Teratoma and Tumor Formation

Tumour and teratoma formation is the most feared side effect of stem cell based therapies (Lee et al. 2013). In order to prove safety of a therapeutic cellular product, the absence of spontaneous tumor formation in animal models is an indispensable prerequisite for clinical translation (Lukovic et al. 2014). Against the background that stem cell biology moves at a tremendous speed towards application in patients, Wu's group was the first to address the problem of undesired teratoma formation based on in-vivo imaging (Cao et al. 2006).

Whereas malignant germ cell tumors exhibit high uptake rates of both ^{18}F-FDG and ^{18}F-FLT, these tracers show unfavorable uptake properties in benign tumors (Cao et al. 2009). Therefore, following studies have used firefly luciferase (fluc) and Herpes Simplex Virus thymidine kinase (HSV-tk) as reporter probes to monitor teratoma formation from ESCs in the mouse model (Su et al. 2011; Pomper et al. 2009).

Absorption of the emitted light by penetrated tissue restricts fluc to preclinical use (Baril et al. 2010). In contrast HSV-tk has already been applied for *in vivo* cell tracking in patients and the respective reporter probe ^{18}F-FIAU is approved by the FDA (Yaghoubi et al. 2012). Yet, its potential immunogenicity in humans remains a major drawback for wide clinical application (Yaghoubi et al. 2012).

Cao et al. suggested a different approach based on the observation that teratoma formation is associated with high rates of neovasculogenesis and used ^{64}Cu-DOTA-RGD_4 which binds to $\alpha_v\beta_3$ integrin (Cao et al. 2009). This might, however, interfere with cellular therapies aiming at restoring the function of ischemically damaged tissues, as vasculogenesis is a wanted and expected therapeutic effect. Therefore, we recently introduced the human sodium iodide symporter (hNIS), a potential non-immunogenic protein (Yaghoubi et al. 2012), as a reporter gene for in-vivo monitoring of ESC-derived teratoma formation.

9.2.2 Mode of Action

9.2.2.1 Apoptosis

Apoptosis, as a consequence of myocardial infarction, plays a crucial role in both hypoxic and ischemic cardiac tissue as (Lehner et al. 2012; Fliss and Gattinger 1996). One interesting property of apoptosis is its potential reversibility, rendering apoptotic cells an interesting target for novel therapeutic approaches as well as serial non-invasive *in vivo* imaging to evaluate and monitor these therapies (Lehner

et al. 2014; Todica et al. 2014). Apoptosis is a highly regulated biological and energy dependent process which results in organized cellular and subcellular tissue degradation without recruitment of inflammatory cells. In healthy cells phosphatidylserine is expressed in the inner leaflet of the plasma membrane. Externalization of phosphatidylserine is one of the first events occurring during apoptosis and persists until the final degradation of the cell (Fadok et al. 1992).

Among different specific ligands particularly annexin-V has been studied (Sogbein et al. 2014). This naturally occurring 37-kD-protein protein binds calcium-depend with high affinity to negatively charged phosphatidylserine on apoptotic cells. For this reason, annexin A5 has been used for both *in vitro* and *in vivo* detection of apoptosis (Martin et al. 1995; van den Eijnde et al. 1999).

Data from ischemic rodent hearts suggested that ^{99}mTc-radiolabelled annexin-V based apoptosis imaging is feasible (Taki et al. 2004). Clinical studies show a pronounced accumulation of the ^{99}mTc based apoptosis tracer in the area at risk. Yet, these data obtained via SPECT cannot distinguish necrotic from apoptotic cells, due to lacking histological data from necropsies (Hofstra et al. 2000).

A promising PET based approach was recently developed on a preclinical level. Apoptosis following myocardial infarction in mice was monitored after the application of ^{68}Ga-Annexin A5 using a dedicated microPET. Highest accumulation rates were detected on day 2 after MI (Lehner et al. 2012). This opens up the opportunity to image and quantify the extent of phosphatidylserine expression as a surrogate marker for apoptosis, providing PET imaging as a useful tool for therapy assessment (Lehner et al. 2012).

Both ^{99}mTc-radiolabelled Annexin V and ^{68}Ga-Annexin A5 target at processes that overlap with necrosis or even viable cells. Thus specificity for apoptosis is unfortunately limited (Sogbein et al. 2014). Therefore novel PET radiotracers have been developed by Aposense Ltd. that are supposed to selectively accumulate within the apoptosis-related complex (Sogbein et al. 2014). Thereby, particularly ^{18}F-ML-10 which has recently been tested in a Phase IIa study might be of high interest for further clinical evaluation (Reshef et al. 2010). Yet ^{18}F-ML-10 has not been tested for the detection of apoptotic cardiomyocytes following ischemia reperfusion injury.

9.2.2.2 Angiogenesis

Angiogenesis plays a central role in the "repair process" of ischemically damaged myocardium. Therefore specific biomarkers for this biological process could contribute to monitor potential effects of cell-based medicinal products for cardiac repair.

Integrins are cell adhesion receptors which are involved in mediating migration of endothelial cells and regulating their growth, survival and differentiation (Rundhaug 2005; Hynes et al. 1999). Among these receptors, integrin $\alpha_v\beta_3$ is of particular interest, due to its high expression in activated endothelial cells during angiogenesis (Brooks et al. 1994) besides its essential role in the regulation of tumour growth, local invasiveness and metastatic potential (Ruoslahti 2002; Hood

and Cheresh 2002). Several studies reported successful molecular imaging of $\alpha_v\beta_3$ integrin expression after MI using radiolabeled antagonists containing the $\alpha_v\beta_3$- specific cyclic RGD peptide motif (Arg-Gly- Asp) (Sogbein et al. 2014).

Under normal conditions this glycoprotein remains inactive in the heart. One week after infarction a peak of tracer uptake can be measured, corresponding to the peak time of vessel formation (Meoli et al. 2004). Both ^{18}F and ^{68}Ga tracers targeting the $\alpha_v\beta_3$ integrin have been investigated and shown to be reliable tracers for *in vivo* monitoring of angiogenesis in different animal models of myocardial infarction, and a very preliminary study in humans (Rischpler et al. 2013). The time-consuming production of ^{18}F-galacto-RGD and the requirement for a nearby cyclotron led to the development of alternative tracers. Particularly ^{68}Ga tracers have the advantage of easy and fast production with a generator-produced radionuclide (Laitinen et al. 2013).

Laitinen et al. compared three tracers targeting the $\alpha_v\beta_3$ integrin, ^{18}F-galacto-RGD, ^{68}Ga-NODAGA RGD and ^{68}Ga-TRAP(RGD)$_3$ for *in vivo* monitoring of angiogenesis in a rat model of myocardial infarction. Angiogenesis could be successfully imaged and quantified using a dedicated microPET (Laitinen et al. 2013). The same group had previously shown that uptake of ^{18}F-galacto-RGD in the infarcted area 1 week after myocardial infarction predicts long-term LV remodeling (Sherif et al. 2012) and there demonstrated that early angiogenesis is an important prognostic factor for assessing left ventricular functional outcomes.

Recently, the SPECT tracer ^{99m}Tc-NC100692, which is a novel angiogenesis imaging agent manufactured by GE Healthcare has been introduced as a diagnostic agent for the detection of angiogenesis (Mozid et al. 2014). Meanwhile preliminary results on the use of this tracer for monitoring angiogenesis mediated by intracoronary transplanted bone marrow cells to treat ischemic myocardium has been published (Mozid et al. 2014).

Once established, imaging and quantification of angiogenesis could help to elucidate and monitor specific mechanisms underlying myocardial repair by advanced cell-products. This will help to optimize targeted cell therapies.

9.2.2.3 Inflammation

Inflammation is an inherent component of the post MI healing process. It has been suggested, that the quality of infarct healing shortly after injury determines the long-term outcome for the patient (Nahrendorf et al. 2010). During the biologically highly active healing period, the infarcted region undergoes extensive changes of tissue architecture. The exposure to cycling intraventricular pressure and myocardial contraction in this vulnerable phase can lead to deleterious changes in heart geometry and function. Within the first days after the infarction, expansion and left ventricular dilatation cause infarct rupture and death in the worst case whereas in the long term adverse remodeling leads to congestive heart failure with poor prognosis (Nahrendorf et al. 2010).

The prevention of this adverse process and the enhancement of endogenous wound healing could preserve left ventricular geometry and prevent heart failure.

Therefore, the inflammatory process as a target for cell-based advanced therapies has emerged recently (Ben-Mordechai et al. 2013). The common idea of most approaches is to shift this cell-mediated process from "pro-inflammation" to "anti-inflammatory" (Ben-Mordechai et al. 2013). Potential effects of new therapeutic approaches can be easily assessed in small animal models by means of immunohistochemistry and flow cytometry after harvesting the organs of interest. These methods are obviously not appropriate for clinical translation. ^{18}F-FDG Positron Emission Tomography (PET) has recently been proposed for longitudinal noninvasive assessment of myocardial inflammation in the mouse model (Lee et al. 2012). Thereby, ketamine/xylazine anesthesia was used to suppress glucose uptake of viable myocytes. Thus, FDG uptake in the area of myocardial infarction could be allocated specifically to infiltrating inflammatory immune cells. This finding could be confirmed by flow cytometry, suggesting that this FDG uptake can be attributed to activated monocytes/macrophages, which typically show high glucose utilization (Lee et al. 2012).

Other tracers that bind to inflammatory cells but do not show physiological myocardial uptake have been explored by Thackery et al. (2015). Yet, among the three tracers – ^{68}Ga-citrate, ^{68}Ga-DOTATATE and ^{18}F-FDG- tested in a mouse model, only ^{18}F-FDG combined with ketamine/xylazine suppression could reliably monitor post MI inflammation (Thackeray et al. 2015).

This preclinical protocol of combined FDG-PET and ketamine/xylazine suppression cannot be translated into clinical application. Yet, other strategies to decrease glucose and FDG uptake by myocytes have been successfully applied in patients including both fasting protocols and/or heparin application (Wykrzykowska et al. 2009; Cheng et al. 2010; Ishimaru et al. 2005; Minamimoto et al. 2011).

Although specific uptake by inflammatory cells remains to be validated in a modified clinical setting, we expect ^{18}F-FDG based inflammation imaging early after infarction to become a valuable tool in the clinical setting to determine the effect of therapies aiming at the modulation of inflammation during the wound healing phase.

9.3 Technological and Biological Opportunities for Therapeutic Devices

This chapter aims at elucidating the relevance of "Imaging Technologies" in the context of regenerative medicine to the reader. Therefore, the field of cardiac repair has been chosen exemplarily to discuss the current state of the art in detail. In principal we want to answer these fundamental questions:

(1) **Why** to image?
(2) **How** to image?
(3) And **what** to image?

We have tried to answer the first two questions in the previous sections. The main reason for the necessity (**Why?**) of *in vivo* imaging technologies is the fact that

advanced cell-based therapies contain living units which requires new strategies for safety and feasibility testing in the clinical setting. The available techniques to achieve this goal (**How?**) have been elaborated in the previous section. Hence, the last question – **what to image** – remains.

Things that can be imaged are:

(a) Morphological and structural changes of the damaged heart
(b) Fate of the cell-based product (Fate)
(c) Mechanisms underlying myocardial repair (Mode of action)
(d) Surrogate Endpoints for efficacy assessment (LVEF, perfusion)

Imaging of both morphological and structural changes of the heart (a) and assessment of organ function based on surrogate endpoints such as left ventricular ejection fraction (LVEF) and regional wall kinetics are already established in clinical use. The most important techniques are magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), single photon emission tomography (SPECT) and ultrasound, as well as multimodality methods that rely on co-registration of images. All of them are established in clinical use (Naumova et al. 2014). Hence, the following section focuses on the fate of transplanted cells (b) and mechanisms underlying the corresponding myocardial repair (c).

EaCRCon the respective cell type used. The specific biological characteristics of this cell product dictate the type of safety concerns associated. Therefore the potential safety issues have to be anticipated and tested based on the existing knowledge about the product (cell) type (Erben et al. 2014). Based on this property, the Committee of Advanced Therapies of the EMA (the CAT) has launched a guideline on the so called "risk-based approach". This guideline proposes to identify the risks associated with the clinical use of an ATMP and risk factors inherent to the ATMP with respect to quality, safety and efficacy. Therefore, we tried to summarize available information on the fate of transplanted cells following injection and the potential mechanisms underlying myocardial repair.

9.3.1 Fate of Transplanted Cells: Engraftment and Biodistribution

The ultimate goal of cardiac stem cell therapies is to replace damaged heart. Irrespective of the underlying repair mechanism (Robey et al. 2008) efficient cell application is highly important. Various studies show that retention rates of injected cells are low, independently of the specific cell type, application route and in both clinical and preclinical trials (Hofmann et al. 2005; Lang et al. 2013; Erben et al. 2014; Lee et al. 2012; Lepperhof et al. 2014; Lang et al. 2014).

Thereby, engraftment of transplanted cells can be described – as suggested by Teng at al. (Teng et al. 2006)- as a function of time into three major phases, which are dominated by specific (patho-)mechanisms respectively (Fig. 9.2):

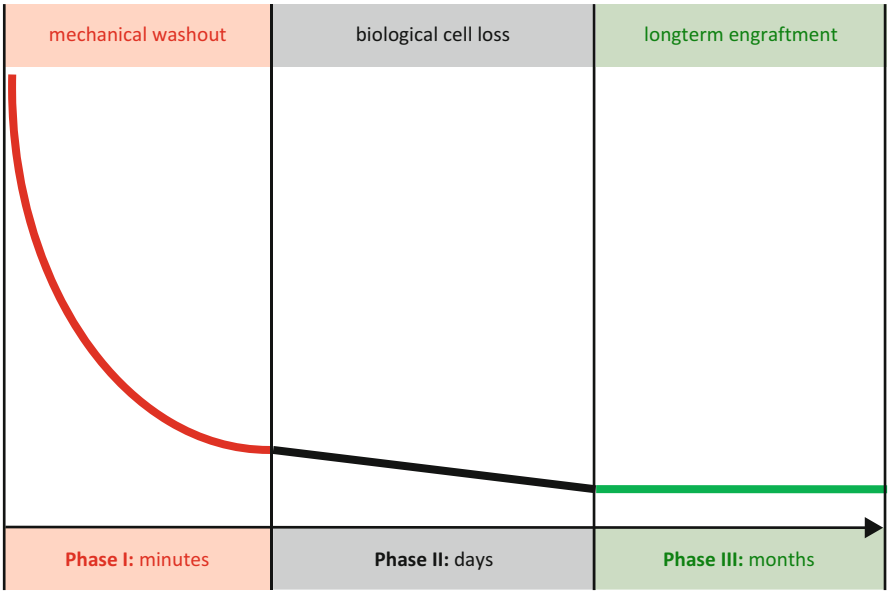

Fig. 9.2 Engraftment of intracardially injected cells

Phase I: Mechanical Cell Loss
Phase II: Biological Cell Loss
Phase III: Survival/Engraftment/Tumour formation

9.3.1.1 Phase I: Acute Mechnical Cell Loss

Acute cell loss within the first few minutes and hours after intramyocardial injection is most probably caused by mechanical contractions of the beating heart. As a consequence just applied cells are flushed via lymphatic and venous vessels into the sinus coronarius and further into the right atrium (Bonios et al. 2011; Terrovitis et al. 2009). From the right ventricle they either end up in the pulmonary capillary system or via the systemic circulation in organs such as liver, spleen and kidneys (Dow et al. 2005). The applied cells are trapped in the respective organ to a significant extent due to the relation of their diameter to the one of the organ's capillaries (Bonios et al. 2011). There is no consistent data concerning further cells loss by back leak of cells from the injections site caused by mechanical contraction or bLehnerLehnerding. Whereas Terrovitis et al. could significantly improve the fraction of retained cells by epicardial application of fibrin glue, we and others did not find this mechanism to have a measurable effect on engraftment (Teng et al. 2006).

9.3.1.2 Phase II: Chronic Cell Loss

The phase of chronic cell loss begins the first day post injection and persists until reaching an equilibrium of cell loss and survival. Murry's group could show that cell death peaks in the first week after transplantation. At day 32 % of the transplanted cells nuclei were apoptotic decreasing to a moderate amount of 1 % at day (Zhang et al. 2001). Three pathomechanisms are suggested to cause this massive cell death:

Cell-based products are mostly based on cell suspensions. The lack of a supporting matrix can cause "anoikis" which literally means "homelessness". Anoikis is a signal cascade which is caused by the loss of cell-matrix-contacts in cells that usually grow adherently and leads to programmed cell death, similar to apoptosis (Zvibel et al. 2002; Reddig and Juliano 2005). Preparation of therapeutic cells from cell cultures prior to injection often comprises enzymatic cleavage of surface proteins e.g. by the use of trypsin. As a consequence cells remain suspended in saline or culture medium until delivery. During this period and within the following hours, important survival signals are lacking due to no cell-matrix-contacts between transplanted cells and basal lamina.

Cell survival is further hampered by insufficient supply with nutrients caused by both the occluded coronary arteries in the treated myocardial area and clotting of cells within in the suspending agent. Hence, nutrient supply is restricted to diffusion (Robey et al. 2008). Electron microscope revealed that injected cardiomyocytes are irreversibly damaged by ischemia within the first days after transplantation (Zhang et al. 2001).

At last, cell death can be caused by the hostile environment in the inflammatory milieu of the infracted myocardium. The process of healing comprises infiltration with neutrophils and macrophages which produce reactive oxygen species and inflammatory cytokines (Nahrendorf et al. 2007).

9.3.1.3 Phase III: Long-Term Engraftment

The tiny fraction of cells that finally survive the burdens in Phase I and II, finally integrate more or less successful in to the target tissue. Due to the small amount of surviving cells and shortage of appropriate imaging modalities, few is known about the long-term fate. Currently no data from clinical studies are available about the long-term fate of transplanted cell products into the heart. Few large animal studies have reported detection of transplanted cells in the myocardium (Templin et al. 2012; Lee et al. 2015), yet reliable data on the fate of cells that left the site of injection is missing.

Data from the mouse model suggest that after 8 weeks less than 0.5 % of the applied cells survive at the site of application (Li et al. 2009). Cao et al. were the fist to assess teratoma formation by means of in-vivo imaging from intramyocardially transplanted pluripotent stem cells (Cao et al. 2006). The risk of teratoma or tumor formation from transplanted pluripotent and mulipotent cells is the most feared side effect concerning the safety of advanced cell products. Whereas few is known about

the long-term fate of non-proliferating cells, it has been shown that transplantation of pluripotent stem cells leads to teratoma formation at the site of cell injection in both immunocompetent and –compromised animals (Lee et al. 2013; Kolossov et al. 2006).

9.3.2 Mechanisms Underlying Myocardial Repair (Mode of Action)

To understand the potential mechanisms underlying myocardial repair attributed to cardiac stem cell therapies, dimensions of cell loss should be considered. Human heart tissue contains about 20 millions of cardiomyocytes per gram myocardium (Olivetti et al. 1990). Thus, a left ventricle weighing 200 g contains about four billion of cardiomyocytes. 25 % loss of this ventricle leads to congestive heart failure, whereas the acute loss of 40 % loss causes an acute cardiogenic shock in humans (Caulfield et al. 1976).

The magnitude of cells that would have to be replaced is in the magnitude of billions. The average cell numbers being transplanted in clinical trials range from 10^7 to 10^8 (Zimmet et al. 2012). Taking in to account the above mentioned tremendous cell loss in Phase I and II, which further minimizes the number of therapeutic cells in the target tissue, it seems to be clear that positive effects cannot be based on functional tissue replacement only.

In fact so called "paracrine factors" seem to play a crucial role in the process of myocardial repair (Hansson and Lendahl 2013). Adult stem cells secrete many cytokines and factors that have pro survival effects on adjacent cells (Xu et al. 2007). These factors have been shown to have many specific effects on the treated tissue, such as angiogenesis and neovasculogenesis (Kocher et al. 2001), reduced apoptosis (Xu et al. 2007) and immunomodulatory effects (Burchfield et al. 2008) (Fig. 9.3).

9.4 Applications for Therapeutic Devices

The previous section served as an introduction to currently or soon available techniques for in-vivo monitoring and quantification of effects in the field of regenerative medicine. As the heart is the most extensively studied organ in the field of regenerative medicine, the potential of the above mentioned "Imaging Technologies" will be explained on the basis of clinical studies in the field cardiac stem cell therapies.

Cardiovascular imaging has become a key component in the process of therapy planning, patient selection and the assessment of therapeutic effects. Therefore this chapter will focus on new techniques that predominantly play a role in the development and translation of new therapies in the field of regenerative medicine. Data

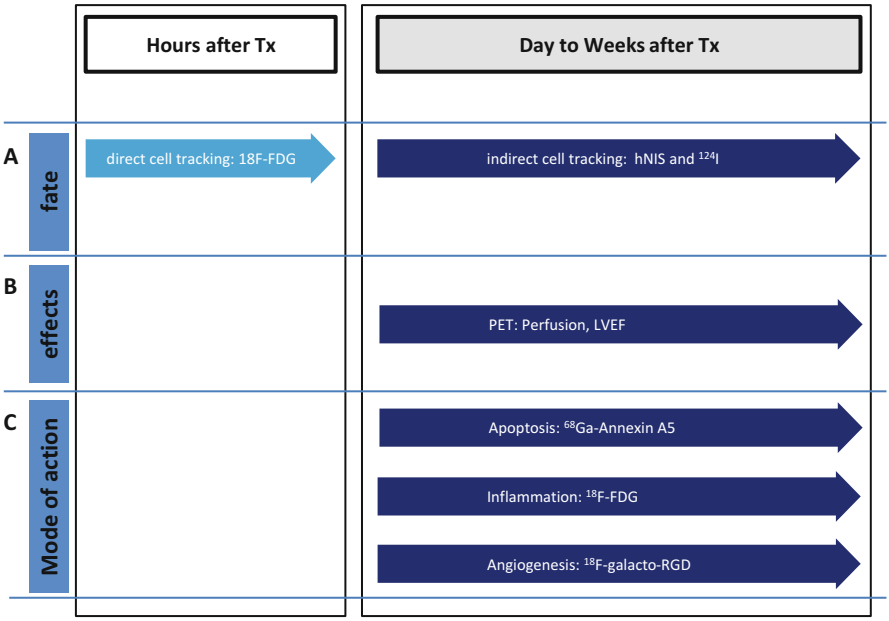

Fig. 9.3 Possible integrated PET-based imaging approach to assess cardiac stem cells therapies

from clinical studies will be summarized to illustrate the techniques that already reached the level of human application. Techniques with high translational potential for the field of regenerative medicine which are still in the phase of non-clinical development, but which we believe to have high potential for soon translation will be explained as well.

9.4.1 Cell Fate Tracking in Clinical Studies

The ultimate goal of cardiac stem cell therapies is to replace damaged heart. Thereby, efficient cell application is highly important, independent of the underlying repair mechanisms (Robey et al. 2008). Various studies show that retention rates of injected cells are low, independently of the specific cell type, application route and in both clinical and preclinical trials. In summary less than 10 % of the injected cells remain in the heart, whereas the majority can be detected in lungs, spleen and the liver as early as 1 h after application. In most published studies on the acute retention and biodistribution bone marrow stem cells were injected via catheter into a coronary artery. Cells were labeled *in vitro* with FDG, In^{111} or Tc-HMPAO respectively for consecutive monitoring by PET, SPECT or planar scintigraphy (Table 9.5).

Table 9.5 Biodistribution of i.c. and i.m. transplanted cells in clinical trails for the treatment of AMI

Author	Tracer	Device	Follow up p.i.	Heart %	Lungs	Spleen	Liver
Hofmann et al. (2005)	FDG	PET	75 min	2,1	>85		
Dedobbeleer et al. (2009)	FDG	PET	**60 min**	3,2	5,2	18	2,4
Musialek et al. (2011)	Tc-HMPAO	SPECT	**60 min**	4,9	16,5	13,5	24,0
Schachinger et al. (2008)	In^{111}	Planar Scinti	**60 min**	6,9	7,8	9	~30
Penicka et al. (2007)	Tc-HMPAO	SPECT/Scinti	**120**	2,9	>80		
			20 h	0,7			
Blocklet et al. (2006)	FDG	PET	**60 min**	5,5	–	29	45
			19 h	~0	–	29	71

In the first clinical study to monitor early fate of transplanted cells in-vivo, bone marrow stem cells were labelled with ^{18}F-FDG in-vitro and then injected intracoronary into patients with ST-segment–elevation myocardial infarction who had undergone percutaneous coronary intervention with stent implantation of the infarct-related coronary artery (Hofmann et al. 2005). In all patients 3D PET scans covering the upper abdomen and chest were obtained. The most striking result was the low retention rate of cells (~2 %) within the first hours after application. The remaining activity could be predominantly detected in the lungs, spleen and liver. Similar results have reproduced these findings, independently of the tracer, cell-type and imaging method used. All of the above mentioned studies have revealed important information about the initial fate of transplanted cells into the heart, but still have shortcomings regarding the study design. For future studies we highly recommend to use only imaging devices that enable absolute quantification of the measured activity and to integrate tracer efflux from the transplanted cells into the assessment strategy (Lang et al. 2013, 2014).

In contrast to more than ten clinical studies that have monitored the acute fate and biodistribution of stem cells transplanted into the heart, none is available concerning long-term fate and engraftment. Therefore we summarize relevant data from animal models that shed light on the long-term fate of intramyocardially transplanted cells.

Data from rodents are in line with clinical data concerning low acute retention rates and acute biodistribution. Furthermore the underlying pathomechanisms have been elucidated, as discussed above (Bonios et al. 2011; Lang et al. 2014). Various studies have shown the feasibility of monitoring intramyocardially transplanted cells by the use of different imaging modalities (Table 9.6). Cao et al. transplanted murine embryonic stem cells into the heart of immunodeficient nude rats following MI induction. The applied cells were transfected with a triple fusion protein expressing three reporter proteins – luciferase, monomeric red fluorescence protein, and

truncated thymidine kinase – and used both bioluminescensce and PET imaging for long term monitoring of cell fate. Measured signals from the transplanted cells increased until day 28, indicating tumor formation. Yet, this study demonstrated the value of indirect cell tracking for monitoring both engraftment and the risk of ectopic engraftment with consecutive teratoma formation (Cao et al. 2006).

Inspired by promising results from small animal studies, indirect cell tracking was tested in large animal models, using less sensitive PET and SPECT scanners which are in clinical use. Templin et al. transplanted human iPS cells overexpressing the reporter gene hNIS into pigs after induction of myocardial infarction by transient occlusion of the left anterior descending artery (Templin et al. 2012). Cells were injected -guided by 3-dimensional NOGA mapping- into the infarcted area. SPECT imaging following intracoronary injection of ^{123}I into each main coronary artery could detect the presence of transplanted cells up to 15 weeks after transplantation. The location of hNIS expressing cells could be verified by immunohistochemistry. Tumor formation could not be detected in any of the animals.

Another group showed the feasibility of cell tracking based on the transgene modification of pig MSCs with the reporter protein HSV-tk (Gyongyosi et al. 2008). Transgene MCSs were injected NOGA-guided intramyocardially 16 days after MI induction. PET scans following intravenous ^{18}F-FHBG injection could detect the transplanted cell survival up to 10 days after injection.

These preliminary results are encouraging but solely show the feasibility of qualitative monitoring of transplanted cells. Absolute quantification of transplanted cells based as percentage of the injected dose (%ID) is far from being reached.

Yet, mouse studies using bioluminescence have tried so quantify the signal from transplanted cells. Li et al. transplanted fluc-expressing murine cardiac stem cells into intramyocardially into mice, following surgical MI induction (Li et al. 2009). Transgenic cell could be detected via bioluminescence imaging until 8 weeks post injection. Signals measured *in vivo* in the myocardium were normalized to results from day 2. The percent bioluminescence signals were 1.7 ± 0.2 at day 21 and 0.4 ± 0.1 at day 56.

Recently, Lepperhof et al. published a study in which an elegant approach for stable transgene expression of the reporter protein FLuc was presented. Targeted integration of FLuc-expression cassette into the ROSA26 genomic locus using zinc finger nuclease (ZFN) technology strongly reduced transgene silencing in iPS-CM (Lepperhof et al. 2014). Therefore the signal measured bioluminescence *in vivo* imaging should be more appropriate for absolute quantification. Briefly, transgenic induced pluripotent stem cell (iPSC)-derived cardiomyocytes (CM) overexpression the aforementioned construct were injected in mice after MI induction by cryoinjury in the peri-infarct region. They claimed that up to 8 % of cells survived for 28 days at the site of injection. Yet, this 8 % refers to the signal detected on day 3 postinjection, due to difficulties with obtaining stable baseline signal intensity during the first day after cell injection (Lepperhof et al. 2014).

If the initial cell fate in the two studies above is similar to the fate of cells in both preclinical and clinical studies (showing massive initial cell loss of up to 98 % in with in the first hour), the retention rates of 0.5 % at 8 weeks (Li et al. 2009) and 8

Table 9.6 Preclinical cell tracking studies monitoring the fate of transplanted cell into the heart after MI-Induction

Author	Tracer	Device	Follow up p.i.	Heart %	Lungs	Spleen	Liver	Absolute quantification
Bonios et al. (2011)	FDG	PET	1 h	22.9 %±5.2 %	28.5 %±5.0 %	–	–	Yes
Terrovitis et al. (2009)	FDG	PET	1 h	17.8 +/– 7.3 %	–	–	–	Yes
Lang et al. (2013)	FDG	PET	25 min	5 %	–	–	–	Yes
Lang et al. (2014)	FDG	PET	25 min	5 %	35 %	–	–	Yes
Lepperhof et al. (2014)	D-Luc	PET	28d	8 % of signal at day 3	–	–	–	(No)
Li et al. (2009)	D-Luc	CCD	8 weeks	0.5 % of signal at day 2	–	–	–	(No)
Cao et al. (2006)	D-Luc	CCD	28d	+/teratoma	–	–	–	No
Templin et al. (2012)	^{99m}Tc	CCD	15 weeks	+	–	–	–	No
Gyongyosi et al. (2008)	18 F-FHBG	PET	10 days	+	–	–	–	No

% at 4 weeks (Lepperhof et al. 2014) might be even two orders of magnitudes lower – not much above 0 %.

9.4.2 In Vivo *Monitoring of Therapeutic Effects Underlying Regenerative Therapies to Damaged Heart*

Direct cell tracking has been used in the field of cardiology and cardiac surgery for more than 10 years in several studies. Due to safety issues, indirect cell tracking has been applied in a single case in a patient with a high grade glioblastoma (Yaghoubi et al. 2009). Likewise, new tracers for monitoring the mechanisms underlying the therapeutic effect of cell-based advanced therapies for myocardial repair have hardly been used in a clinical setting yet.

The most promising tracers are the ones aiming at both monitoring and quantifying the biological processes angiogenesis, apoptosis and inflammation *in vivo*.

9.4.2.1 Angiogenesis

Recently the first study testing a radiolabeled SPECT tracer for monitoring angiogenesis mediated by intracoronary transplanted bone marrow cells to treat ischemic myocardium was published (Mozid et al. 2014).

The applied tracer ^{99m}Tc-NC100692 is a novel angiogenesis imaging agent manufactured by GE Healthcare which is currently being investigated as a diagnostic agent for the detection of angiogenesis (Mozid et al. 2014). ^{99m}Tc-NC100692 contains the RDG motif with high affinity for the angiogenesis related integrin $\alpha_v\beta_3$. A phase 1 clinical study in which safety and biodistribution of ^{99m}Tc-NC100692 in healthy volunteers was tested, demonstrated only minimal amount of background activity in the thorax (Roed et al. 2009). Thus, it seemed to be a versatile imaging agent for angiogenesis detection in the heart. Consequently it was tested in ten patients after 3 and 8 weeks after myocardial infarction to measure the amount of angiogenesis in the healing process (Verjans et al. 2010). Based on this preliminary work the above mentioned study was conducted. Nine patients with chronic ischemic heart failure that were treated with either BMSCs or serum (control group) in the context of the REGENERATE-IHD trial (ClinicalTrial.gov Identifier: NCT00747708) underwent SPECT imaging following injection of ^{99}mTc-NC100692. Two patients with no history of cardiac disease showed no tracer uptake in comparison to study patients. The study could demonstrate that the application of this tracer in this context is feasible and safe. Unfortunately, there was no significant positive correlation between LVEF improvement and myocardial tracer uptake (Mozid et al. 2014). Nevertheless, the patient with the greatest improvement in LVEF received intracoronary BMSCs and had the greatest increase in tracer uptake (Mozid et al. 2014).

9.4.2.2 Apoptosis

^{99m}Tc-annexin-V was the first biomarker for apoptosis evaluated clinically, yet it labels both apoptotic and necrotic cells (Reshef et al. 2010). This lack of specificity stopped further clinical evaluation in the field of in vivo apoptosis imaging. Although PET probe for clinical imaging of apoptosis would be highly desirable this is yet an unachieved goal. Recently, ^{18}F-ML-10, a new apoptosis PET tracer has been described, which is capable of distinguishing between apoptotic and necrotic cells (Cohen et al. 2009). ^{18}F-ML-10 is the first tracer for apoptosis that has reached the stage of clinical development and has been successfully used in an Phase IIa study to image apoptotic neurovascular cells in patients with acute ischemic cerebral stroke (Reshef et al. 2010).

Apoptosis imaging in the context of monitoring effects of cell-based therapies to treat ischemic myocardium has not been applied to date. Despite its lack of specificity, the PET tracer ^{68}Ga-Annexin A5 was used in the first preclinical study to monitor a therapy in the field of regenerative medicine in a mouse model (Lehner et al. 2014).

In this pivotal study mice were treated with parathyroid hormone (PTH) after surgical MI induction. It has been shown that PTH treatment prevents adverse cardiac remodeling and improves postinfart cardiac function (Brunner et al. 2012; Zaruba et al. 2008). The proposed mechanisms of this positive effect are increased myocardial perfusion, neovascularization and enhanced cell survival and regeneration which result in a reduction of apoptosis (Zaruba et al. 2008). Two days after MI, ^{68}Ga-Annexin A5 PET scanning was performed. Immunohistochemistry revealed that apoptosis was significantly reduced in PTH treated animals. This finding corresponded to higher but not significant ^{68}Ga-Annexin A5 uptake in control animals within the infarction.

9.4.2.3 Inflammation

In vivo imaging methods, such as scintigraphy, SPECT and PET have been shown to be reliable for the identification of inflammatory foci in various inflammatory diseases (Basu et al. 2009). Recently, many groups started to investigate FDG-PET as a tool to identify vulnerable plaques in coronary and carotid arteries (Sogbein et al. 2014). The investigation of different specific immune cell populations is rapidly growing research area. Thereby macrophages have been a subpopulation of particular interest.

High levels of glucose metabolism make macrophages infiltrating the infarcted myocardium an attrative target for FDG-PET.

Lee et al. recently published the first preclinical study in which FDG-uptake could be directly attributed to macrophages infiltrating infarcted myocardium (Lee 2012). This finding was confirmed by a recent study demonstrating that FDG is superior to ^{68}Ga-citrate and ^{68}Ga-DOTATATE for *in vivo* monitoring of myocardial inflammation.

Yet, the need for glucose uptake suppression in the viable myocardium has been hampered by the lack of appropriate protocols. Whereas FDG-PET has been successfully used to monitor macrophages infiltrating the inflammatory myocardium post MI, both clinical and preclinical studies to use *in vivo* monitoring of myocardial inflammation as a maker to predict efficacy of advance therapies such as stem cells are still greatly lacking.

9.5 Barriers to Practice and Prospects

Imaging technologies play a central role in the uprising field of theranostics and individualized medicine. Particularly the field of tissue-engineering and the development of advanced cell-products requires new strategies for testing safety, in vivo fate, mode of action and efficacy of these products.

In contrast to conventional medicinal products and pharmaceutical, products based on living units such as cells, have the capacity to survive in the host organism after their application. At the one hand this offers unique possibilities such as functional tissue replacement, but bears the inherent risk of malignant behaviour of these living units in the host, such as tumour formation and unwanted migration or biodistribution.

The development of new tracers for reliable and specific monitoring of repair mechanisms such as apoptosis, angiogenesis and inflammation is cumbersome but tracer development is not a major safety risk for patients included in Phase I studies.

In contrast development of direct and indirect cell tracking methods is hampered by safety issues. Whereas cell labelling with FDG seems to be safe to date, several studies have reported compromised proliferation and differentiation potential of stem cells after labelling with ^{111}In (Aicher et al. 2003; Brenner et al. 2004). The use of indirect cell tracking is even more complicated and has therefore only been used in single patient with glioblastoma multiforme yet (Yaghoubi et al. 2009). Indirect cell tracking requires genetically modification transplanted cells. The overexpression of alien reporter proteins could potentially alter the quality of the cell based product and lead to an immune response in the host. The latter refers particularly to viral reporter proteins such HSV-tk. hNIS could be a more appropriate reporter protein which should potentially be not rejected due to its human origin. Yet, the impact of the reporter protein on the quality of the respective cell product has to be studies in detail before clinical translation.

9.6 Conclusions and Future Challenges

Imaging technologies in the field of regenerative medicine and tissue engineering are rapidly evolving. Imaging technologies such as ultra sound, MRI, CT, PET and SPECT are already indispensable tools for the assessment and stratification of

structural and functional damage of various organs. Recently, new methods for both *in vivo* cell tracking and monitoring the mode of action such as modification of angiogenesis, apoptosis and inflammation have been extensively studied. Whereas non-clinical development has already progressed a lot, clinical translation is still hampered by potential safety issues. Once these issues are solved, the next step is to combine the available techniques for an integrated theranostic approach. This will identify potential responders to new advanced therapies, monitor the safety, fate and biodistribution of the respective product and monitor both the effect and underlying mechanism.

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