20 Nanotechnology for Engineering Cellular Microenvironment and Gene Delivery

Janice H. Lai, Anusuya Ramasubramanian, Shaheen Jeeawoody, and Fan Yang

CONTENTS

20.1 INTRODUCTION

Tissue engineering aims at repairing and restoring lost tissue structure and functions caused by disease processes or traumatic events (Langer and Vacanti 1993). Driven by clinical demands, extensive research efforts have been dedicated toward regenerating a broad range of tissues, from the skin, cartilage, bone, and blood vessels to more complex organ-level systems such as the bladder. Most tissue engineering strategies involve using one or a combination of three key components: (1) cells; (2) scaffolds; and (3) biological signals. Cells are the building blocks of biological tissues and cell-based therapy involves delivering cells directly into the body to help regenerate

the lost tissue. For instance, autologous chondrocytes transplantation is a cell-based therapy for cartilage repair that involves harvesting healthy cartilage biopsy and then transplanting expanded cartilage cells back to a defected cartilage site in the same patient (Brittberg et al. 1994). Bone marrow transplantation is another example of cell-based therapy and has been widely used for a few decades to treat diseases such as leukemia. In addition to fully differentiated cell types, stem cells have gained tremendous attention as promising cell sources for tissue regeneration because of their ability to self-renew and differentiate into multiple types of cells in our body.

The success of tissue engineering is dependent on the ability to promote the desired cellular processes. Before stem cells can be broadly used for clinical applications, methods must be developed to control their lineage-specific differentiation with functional stability. Scaffolds and biological signals can be used collectively with cells to promote tissue repair and regeneration. Given the intricate processes involved in tissue development and regeneration, it is crucial to understand how microenvironmental cues regulate cell behavior. Such knowledge can then guide the rational design of tissue engineering strategies to promote the desired cellular processes and tissue formation.

20.1.1 The Multifactorial Cellular Microenvironment

In the natural extracellular matrix (ECM), cell behavior is regulated by a complex network of microenvironmental cues including biochemical and biophysical signals. Biochemical signals can arise from cell–cell interactions, soluble signaling, or insoluble ECM. Such biochemical signals play an important role in influencing cellular processes such as adhesion, migration, proliferation, and differentiation. Tissue development and remodeling are also tightly regulated by these signals. For example, in wound healing, the ECM orchestrates a cascade of biological events such as cell migration and proliferation, matrix synthesis, and angiogenesis. The ECM also serves as a reservoir for many growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factors, and transforming growth factor β (TGF-β), sequestering and protecting these growth factors from degradation (Schultz and Wysocki 2009). After tissue injury, the ECM modulates the release of these growth factors through proteolytic mechanisms and facilitates the wound healing process (Ferrara 2010).

Biophysical signals can regulate cell behavior via either extrinsic mechanical forces or intrinsic matrix stiffness. Fluid shear stresses in blood vessels can directly influence endothelial cell gene expression and biosynthetic activities, which, in turn, regulate blood vessel remodeling processes. Fluid shear and hydrostatic compression in bone tissue facilitate bone cell mechanical adaptation and tissue remodeling (Papachroni et al. 2009). In addition to extrinsic mechanical forces, intrinsic matrix stiffness has also been shown to play a critical role in regulating cell fate. Mesenchymal stem cells (MSC) grown on two-dimensional substrates with various elasticities demonstrated preferential differentiation toward tissue lineages with similar elasticities (Engler et al. 2006). Similar cellular behavior in response to matrix stiffness has been recently reported in three-dimensions as well (Banerjee et al. 2009; Huebsch et al. 2010; Pek et al. 2010).

Aside from macroscopic biophysical cues, microstructures and nanostructures of the ECM, such as surface topography and organization of different ECM components, also regulate cell behavior. Microscale and nanoscale topological features, such as grooves,

ridges, pores, and pits, are present in many tissues. For example, collagen fibers, an abundant component in connective tissue, possess hierarchical features on the microscale and nanoscale level (Buehler 2006). In the tendon, 20–280 nm diameter collagen fibrils are organized into fiber bundles of 1–300 μm diameter. These collagen fiber bundles are further aligned and organized into larger structural units, fassicles, to provide maximum tensile strength (Silver et al. 2003). Bone is a composite material that possesses hierarchical structures at the macroscale (cortical and cancellous bone), microscale (osteons and lamallae), and nanoscale (collagen fibrils, collagen molecules, and bone crystals) level (Rho et al. 1998). Not only do these multiscale hierarchical features confer mechanical and structural integrity to connective tissues, but they also interact with cells and bind to other ECM components (Kolácná et al. 2007).

Advances in nanotechnology facilitate better understanding of cell–matrix interactions at the nanoscale and inspire new strategies to control cell behavior. Numerous studies have demonstrated the effects of nanotopographical cues on cellular processes. When cultured on nanopatterned surfaces, both the cytoskeleton and nuclei of smooth muscle cells aligned to nanoscale gratings; proliferation was reduced and polarization pattern was altered in wound healing assays (Yim et al. 2005). Lamers and coworkers showed that the adhesion, morphology, and motility of osteoblasts are regulated by nanotopographical cues. Specifically, surface anisotropy regulated osteoblast morphology whereas spacing (ridge-to-groove ratio) of nanotopographical cues influenced osteoblast motility (Lamers et al. 2010). In addition to cell adhesion, morphology, motility, and proliferation, nanotopographical cues may also regulate the differentiation of stem cells. By controlling scaffold nanotopographical features, MSCs have been shown to undergo osteogenesis without osteogenic supplements (Dalby et al. 2007; Oh et al. 2009). Dalby et al. (2007) showed that a semidisorder array of nanopits of 120 nm diameter and 100 nm deep induced MSC osteogenesis, with enhanced osteoblastic gene expression and mineral deposition. In another study, human MSCs cultured on vertically oriented nanotubes of 70–100 nm diameter, but not less than 30 nm, differentiated into osteoblasts even in the absence of osteogenic supplement (Oh et al. 2009). All these findings highlighted the importance of nanostructures on influencing cell behavior. As such, designing scaffolds with optimized nanoscale features may provide a powerful tool to direct cell fate for tissue engineering applications.

20.1.2 Designing Scaffolds with Nanoscale Features

Scaffold design is an important tissue engineering strategy to control cellular microenvironment and promote tissue regeneration. Important criteria to consider for designing scaffolds include mechanical and physical properties, biocompatibility, and biodegradability. In contrast to delivering cells alone, scaffolds provide a three-dimensional environment in which the cells can proliferate, migrate, and differentiate. Scaffolds also allow the incorporation of biochemical or physical cues that may promote the desired cellular processes and tissue formation (Lutolf and Hubbell 2005; Shin et al. 2003). Cell adhesion peptides conjugated to scaffolds can enhance cell attachment in three-dimensions, and incorporating protease-sensitive peptide into hydrogel network allows cell-mediated matrix remodeling. Biological signals, such as growth factors, can also be conjugated to the scaffold and released through cell-mediated mechanisms.

Recapitulating microenvironmental cues at the nanometer scale has gained increasing attention as a way to better mimic cell–matrix interactions. Designing scaffolds with more refined control of scaffold functionalities in both a spatial and temporal fashion would provide a powerful tool to guide the desired tissue development processes. Different types of nanomaterials have been reported for a wide range of tissue engineering-related applications (Figure 20.1). Natural biomaterials and synthetic polymers can form nanofibers through electrospinning and can be used as scaffolds for tissue engineering applications for bone, skin, and blood vessels (Kumbar et al. 2008; Ma et al. 2005; Matthews et al. 2002). For example, electrospun poly(ε-caprolactone) nanofiber scaffolds have been shown to support the osteogenesis of MSCs with increased mineralization and type I collagen production (Yoshimoto et al. 2003). Nanofibrous scaffolds based on collagen I with a coating of ECM molecules facilitated cell adhesion of human keratinocytes and accelerated early-stage wound healing in a rat model (Rho et al. 2006). Aside from forming nanofibers via electrospinning, amphiphilic peptides and polymeric dendrimers can self-assemble into nanofibers (Figure 20.2; Ma et al. 2005). These nanofibers have

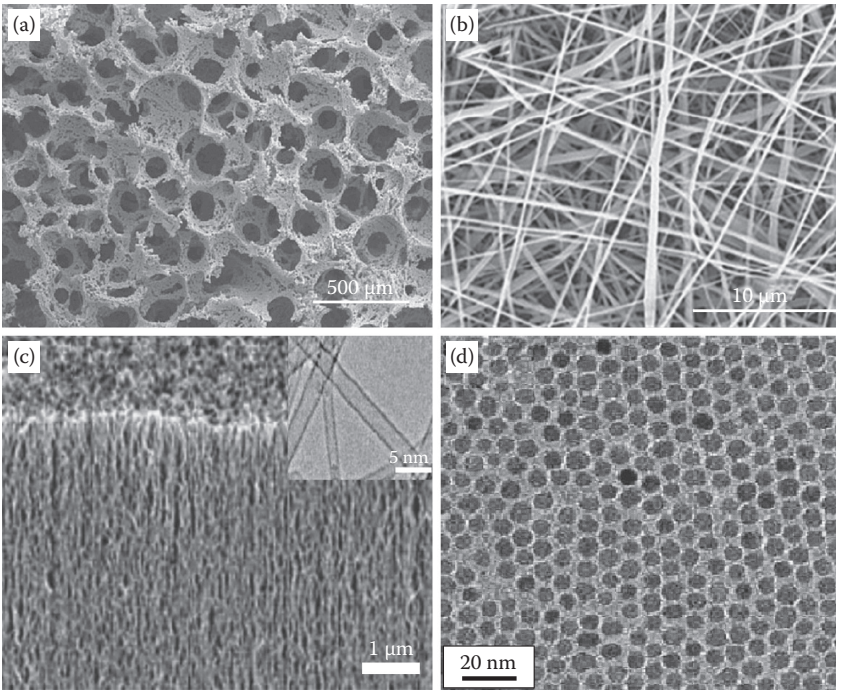


FIGURE 20.1 Examples of nanomaterials used in tissue engineering. (a) Poly(L-lactic acid) nanofibrous scaffold with interconnected spherical macropores. (b) Electrospun composite nanofibrous scaffold consists of synthetic biodegradable poly(ε-caprolactone), hydroxyapatite, and natural polymer gelatin (c) Vertically aligned, single-walled carbon nanotube forest. (d) Fe_3O_4 nanoparticles. (Reprinted from Zhang L. and Webster T. J. *Nano Today* 4(1), 2009. With permission.)

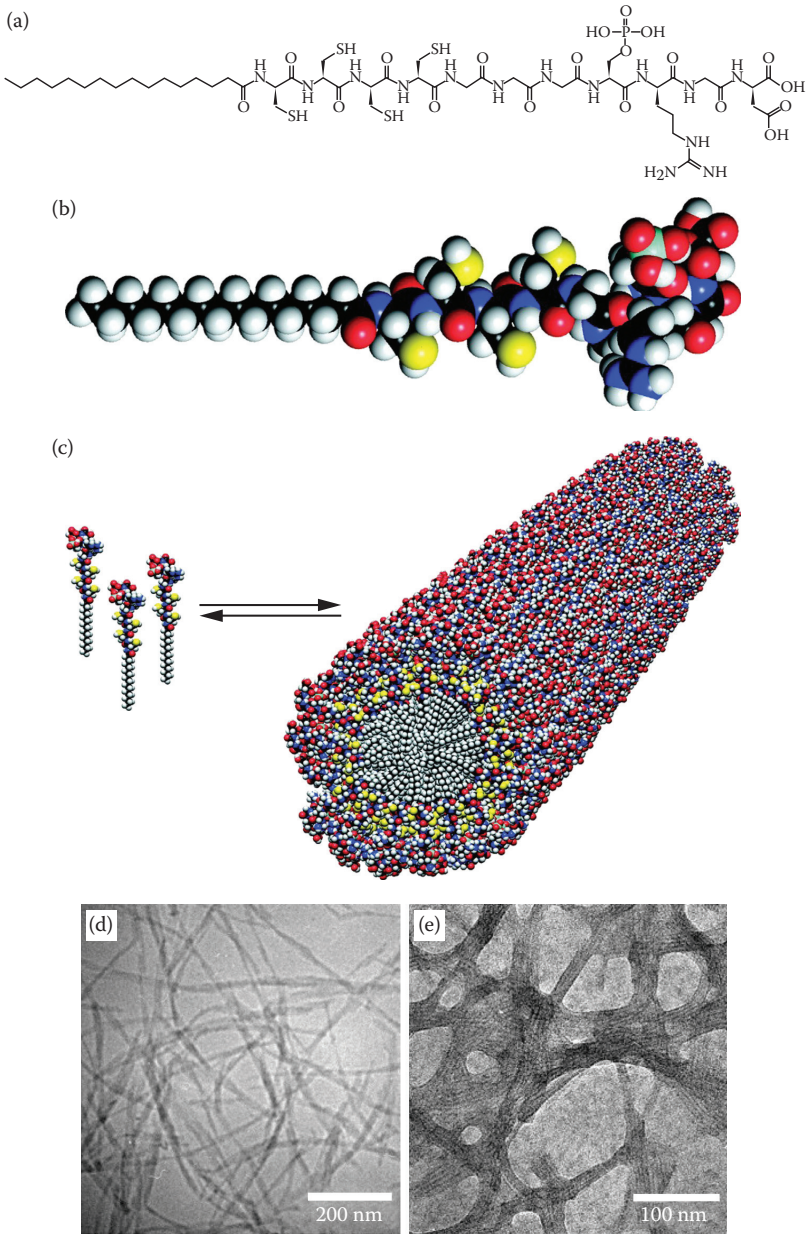
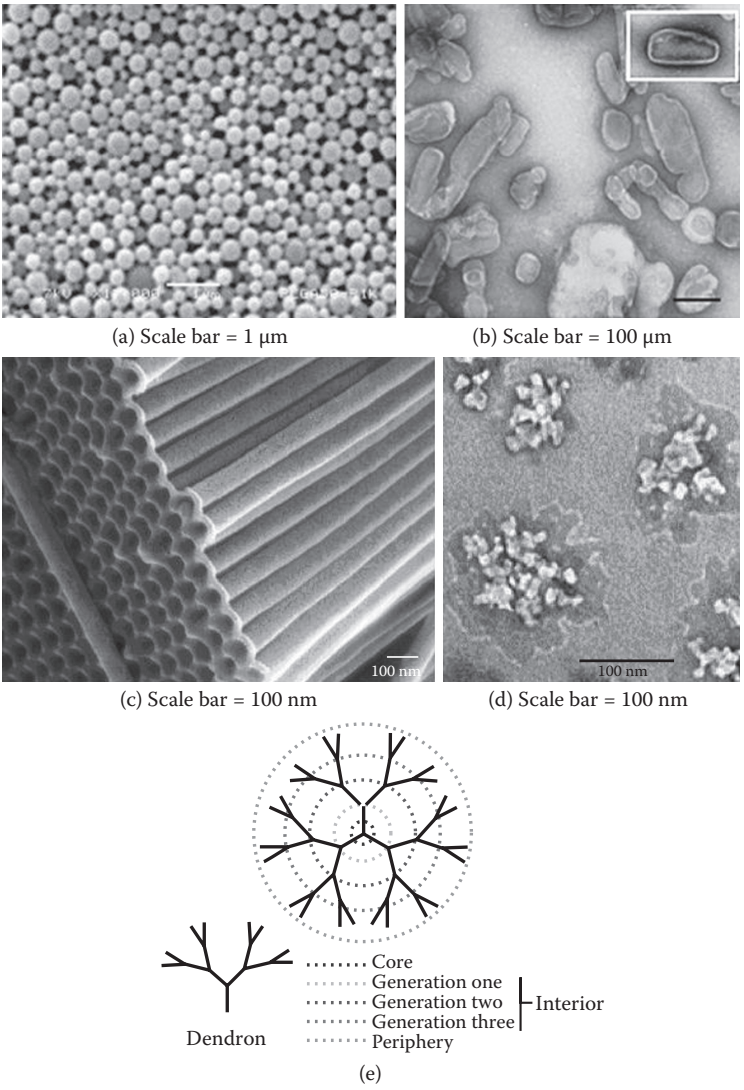


FIGURE 20.2 Self-assembled nanofiber. (a) Chemical structure and (b) molecular model of peptide amphiphile highlighting the hydrophobic tail and peptide region functionalized with cell-adhesive ligand RGD. (c) Schematic of the self-assembly process of peptide amphiphile molecules into a nanofiber. (d) Transmission electron microscopic image of self-assembled nanofibers with 7.6 ± 1 nm diameter. (e) Self-assembled nanofibers after oxidative cross-linking. (Adapted from Hartgerink J. D. et al. *Science* 294(5547), 1684–1688, 2001. With permission.)

been used as scaffolds to support the proliferation and differentiation of many cell types such as neurons, chondrocytes, and MSCs (Holmes et al. 2000; Kisiday et al. 2002; Hosseinkhani et al. 2006). Biomimetic signals such as adhesive ligands and growth factor binding domains can be incorporated into the supramolecular and molecular design of these self-assembled polymers. For instance, amphiphilic



(a) Scale bar = 1 μm (b) Scale bar = 100 μm

(c) Scale bar = 100 nm (d) Scale bar = 100 nm

(e)

FIGURE 20.3 Examples of nanomaterials used for drug-delivery. (a) Nanoparticles. (b) Nanocapsules. (c) Nanotubes. (d) Nanogels. (e) Dendrimers. (Reprinted from Goldberg M. et al. *J. Biomater. Sci. Polym. Ed.* 18(3), 241–268, 2007. With permission.)

peptides with TGF-binding domains were shown to promote chondrogenic differentiation of human MSCs and enhance cartilage tissue regeneration in a full-thickness chondral defect rabbit model (Shah et al. 2010).

20.1.3 Biological Signals to Enhance Tissue Regeneration

Biological signals, such as growth factors and nucleic acids, are often used to promote specific cellular processes and tissue regeneration. Scaffolds can serve as a

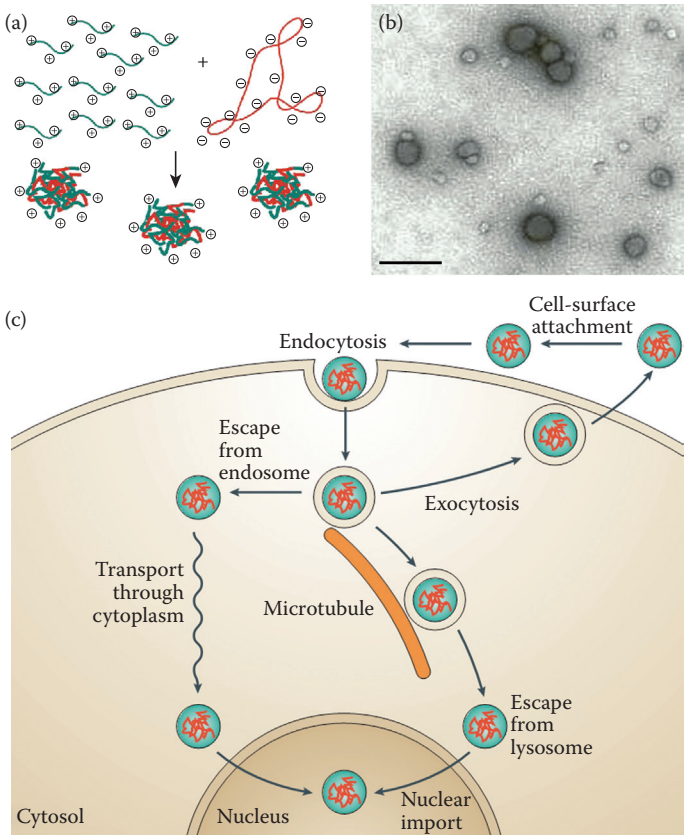


FIGURE 20.4 Gene delivery with cationic material. (a) Schematic of spontaneous polyplex formation by the electrostatic interaction between polycations (e.g., cationic lipids and polymers) and DNA. (b) Transmission electron microscopy (TEM) image of nanoparticles formed by plasmid DNA and PEI (scale bar = 200 nm). (c) Intracellular barriers to gene delivery. After attaching to the targeted cell surface, the polyplex must be internalized (e.g., by endocytosis), escape from the endosome, transport through the cytoplasm, move toward the nucleus and across the nuclear membrane, and unpackage. (Adapted from Pack D. W. et al. *Nat. Rev. Drug Discov.* 4(7), 581–593, 2005. With permission.)

delivery depot for bioactive molecules via various methods such as surface adsorption or covalent modification (Babensee et al. 2000; Tessmar and Göpferich 2007). Nanomaterials such as nanoparticles, nanocapsules, nanotubes, nanogels, and dendrimers have also been explored as delivery vehicles to enhance the controlled release and cellular uptake for various types of biomolecules (Figure 20.3; Goldberg et al. 2007). Advances in gene therapy provide a powerful tool to promote lineage-specific differentiation via directly regulating the intrinsic signals of stem cells. Today, technology is being developed with the potential to either "turn on" a target gene, through DNA delivery, or "turn off" a gene by small interfering RNA (siRNA) delivery. Current challenges in gene therapy include the need to identify optimal therapeutic gene targets as well as the lack of safe and efficient delivery methods. Cationic polymers can self-assemble with DNA to form nanoparticles via electrostatic interactions (Figure 20.4a and b), and different polymer designs have been used to overcome the hurdles involved in different steps during gene delivery. In this chapter, we will discuss recent advances in applying gene delivery as a tool to direct stem cell fate, with special focus on polymer-based gene delivery strategies using a nanomaterials approach.

20.2 GENE DELIVERY

20.2.1 Considerations for Effective Gene Delivery

Successful gene therapy requires a safe and effective delivery system to transport the DNA to the target cell nuclei. The basic considerations for developing carriers for gene delivery include safety, biocompatibility, stability, and the ability to transfer therapeutic genes to the target cells at high efficiency (Pack et al. 2005). To facilitate smooth translation into clinical practice, the ease of large-scale manufacture, administration, and cost-effectiveness must also be considered.

20.2.1.1 Barriers to Gene Delivery

To develop highly efficient gene delivery systems, it is crucial to first understand the barriers that need to be overcome during the transport process (Figure 20.4c). In transit to the target cells, the genetic material must be protected from degradation and preserve its properties for effective transfection (Papisov 1998). It also needs to navigate through the extracellular space and arrive at the target cells. To achieve cell-specific targeting, receptor-mediated mechanisms can be incorporated into the delivery vehicle (Ferkol et al. 1996; Wu et al. 1991; Wu and Wu 1988). Cellular uptake usually occurs via endocytosis, which leads the carrier/gene complexes into an acidic intracellular vesicle called an endosome. The genetic material needs to escape from the endosome, continue to transport through the cytoplasm, and finally transport into the nucleus and get expressed. During this multistep transport process, the genetic material continues to encounter barriers such as the possibility of exocytosis, degradation in the endosome, or the presence of cytosolic nucleases (Pollard et al. 2001). Finally, in the cases in which a delivery system, such as a synthetic polymer, is used, the delivery vehicle must release the genetic material and permit transcription once the genetic material–carrier complex is delivered to the nucleus.

20.2.2 Methods for Gene Delivery

Gene delivery methods can be broadly divided into two categories, viral and nonviral approaches. The viral approach uses viruses as the delivery vehicle and takes advantage of the ability of viruses to efficiently transfer genetic information into cells. Despite its high gene transfer efficiency, clinical translation of the viral-based approach is limited by safety concerns such as pathogenesis and potential immunogenicity (Pack et al. 2005). Nonviral methods include the direct injection of naked genetic material, physical methods, and delivery with a gene transfer carrier such as synthetic polymers and lipids. Nonviral methods are potentially much safer, but often suffer from significantly lower transfection efficiency.

20.2.2.1 Viral Methods

In viral methods, a replication-deficient viral vector can be formed by replacing the coding region of the viral genome with a therapeutic gene, turning the virus into a gene delivery vehicle. Viral vectors can generally be classified as integrating or nonintegrating vectors. Retrovirus and adeno-associated viruses (AAV) can integrate their genome into the DNA of the host cell. They can achieve stable expressions in dividing cells, and are suitable for the treatment of chronic diseases, in which long-term gene expression is needed (Pack et al. 2005). Nonintegrating viruses, such as adenovirus and herpes simplex virus type I, transfer their genomes into the nucleus of the target cell as episomes without integration.

Retroviruses are enveloped RNA viruses that are present in all vertebrates. The three main classes of retroviruses are oncoretrovirus, lentivirus, and spumavirus. Oncoretrovirus was the first viral vector developed and has been widely used in clinical trials to treat diseases such as severe combined immunodeficiency (Heilbronn and Weger 2010; Thomas et al. 2003). A major limitation of using oncoretrovirus for gene delivery is that the virus can only be used to transduce dividing cells. Lentivirus, on the other hand, can enter the nucleus of both dividing and nondividing cells. Lentivirus has been shown to successfully deliver short hairpin RNAs into a wide range of dividing and nondividing cell types, including primary T cells, stem cells, and single-cell embryos, and achieve stable gene silencing (Rubinson et al. 2003). Lentiviral vectors have also been used to reprogram human somatic cells into pluripotent stem cells (Yu et al. 2007). The AAV is another commonly used vector for gene delivery due to their nonpathogenic nature and inability to self-replicate. The major limitation of AAV vector is that it can only accommodate small gene products of up to 5 kb (Kootstra and Verma 2003).

Unlike integrating viral vectors, the genome of nonintegrating vectors remains episomal, eliminating the possibility of insertional mutagenesis. The capacity to carry large genetic materials, the ability to transfect nondividing cells, and high gene transfer efficiency in a variety of tissues make adenovirus vectors and herpes simplex virus type I promising gene delivery vehicles (Kay et al. 2001; Thomas et al. 2003). However, high immunogenicity and transient transgene expression limit the clinical potential of these nonintegrating viral vectors as gene delivery vehicles.

Naturally evolved as effective delivery vehicles, viruses have been shown to deliver therapeutic genes at high efficiency in many *in vitro* and *in vivo* studies (Goldberg et al. 2008; Pack et al. 2005; Thomas et al. 2003). Although more than

70% of gene therapy-based clinical trials (as of July 2007) used virus-mediated gene delivery, there is still no FDA-approved viral vector-based gene therapy on the market (Check 2005; Green et al. 2008; Hollon 2000). Acute toxicity, oncogenesis, mutagenesis, and carcinogenicity are among the safety risks associated with viral gene therapy (Goldberg et al. 2007; Green et al. 2008; Pack et al. 2005; Thomas et al. 2003). High manufacturing costs, low cargo capacity, low quality control, and resistance to repeated infection are some of the additional characteristics of viral gene therapy that makes it an undesirable gene delivery method.

20.2.2.2 Nonviral Approaches

20.2.2.2.1 Physical Methods

The ability of DNA alone to transfect cells is very poor due to low cellular uptake and its susceptibility to nuclease degradation (Herweijer and Wolff 2003). Several physical methods have been developed to enhance DNA delivery efficiency including electroporation, pressurized intravascular delivery, sonoporation, laser irradiation, and magnetofection (Mehier-Humbert and Guy 2005). Electroporation increases the permeability of cell membranes to plasmid DNA by exposing the target cells to a series of electrical pulses. Pressurized intravascular delivery has been used to successfully transfect cells in a variety of tissue types including liver and skeletal muscle. Sonoporation, which usually involves the use of a low-dose ultrasound, is another physical method to enhance gene transfer efficiency. The application of ultrasound or laser irradiation leads to transient formation of small pores on the cell membrane and enhances permeability (Taniyama et al. 2002). Magnetofection involves the application of a magnetic field to enhance the uptake of plasmid DNA coupled with magnetic nanoparticles (Scherer et al. 2002). Most of these physical methods enhance the entry of DNA into cells by overcoming barriers posed by the cell membrane, but are often associated with significant cytotoxicity. Furthermore, the challenges associated with intracellular transport to the nucleus remain to be addressed (Geng et al. 2011; Guo and Huang 2011; Rychahou and Evers 2010).

20.2.2.2.2 Cationic Biomaterials for Nonviral Gene Delivery

Biomaterials, such as cationic polymers and lipids, are capable of condensing DNA into nanoparticles for enhanced delivery into target cells (Nguyen et al. 2009). Because of their lipophilic nature, cationic lipids can penetrate cell membranes and deliver DNA into cells, and have been widely used for nonviral-based gene delivery *in vitro*. However, clinical applications of cationic lipids for gene therapy are impeded by their unfavorable biodistribution, as they tend to accumulate in the lung and liver when delivered *in vivo*. Furthermore, they may cause high levels of cytotoxicity by disrupting the cell membrane.

Cationic polymers are promising gene delivery carriers due to their relative ease of manufacture and enhanced safety compared with viruses (Goldberg et al. 2008). However, polymer-mediated gene delivery is typically much less efficient than viral vectors due to the multistep barriers they need to overcome to achieve efficient expression (Green et al. 2008; Nguyen et al. 2009). The polymer must initially bind to, condense, or encapsulate the DNA in the form of a nanoparticle to protect it from

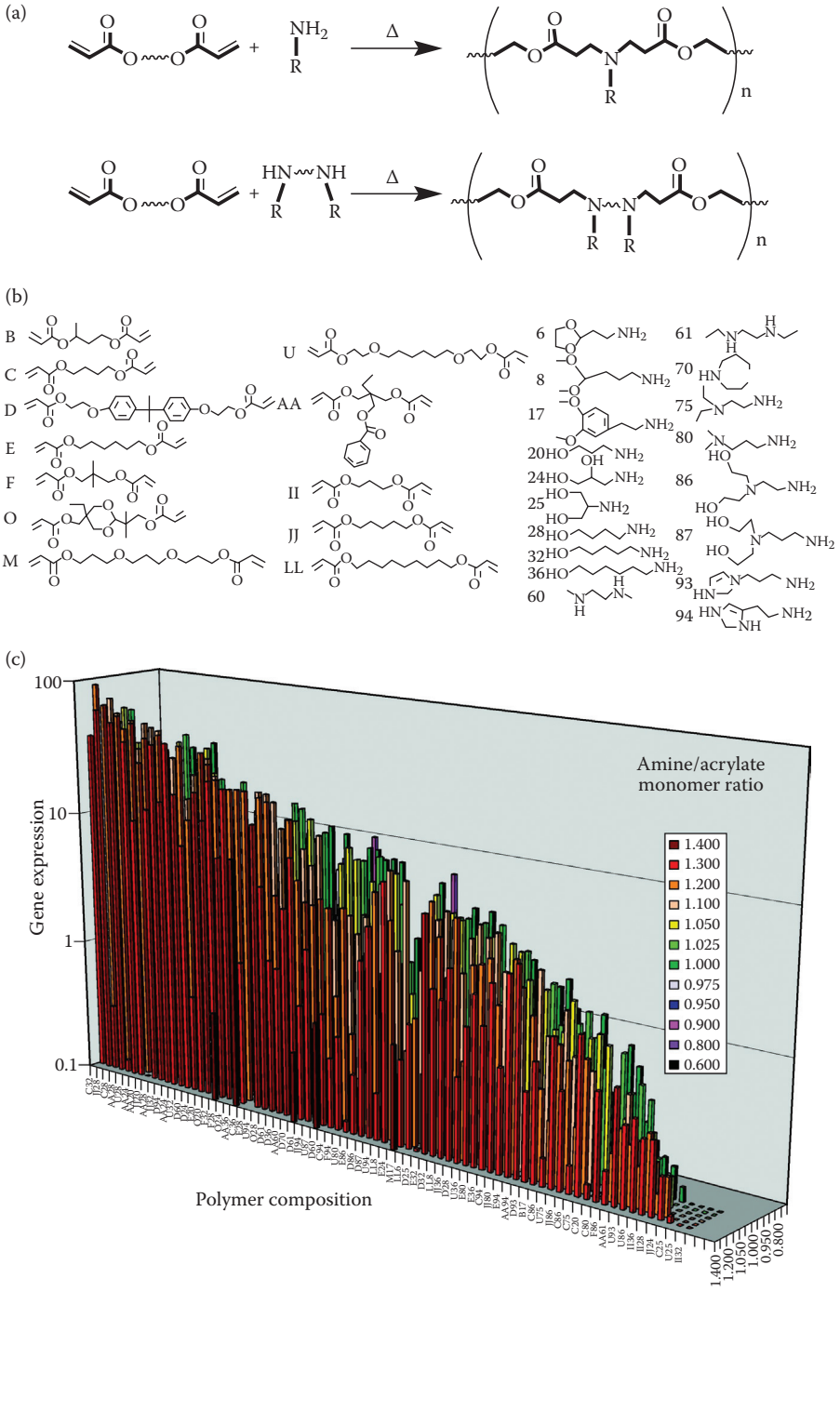
degradation. Once the nanoparticles get trafficked into the cell via endocytosis, they must be capable of escaping the endosome into the cytoplasm before endosomal degradation. Finally, the nanoparticles need to travel through the nuclear membrane and release the DNA cargo for gene expression. Two cationic polymers that have been widely used for gene delivery are polyethylenimine (PEI) and poly(L-lysine) (PLL; Goldberg et al. 2008; Green et al. 2008). However, PEI is nondegradable and has high cytotoxicity effects and a low transfection efficiency in comparison to viral vectors and high cytotoxicity effects (Green et al. 2008; Moghimi et al. 2005). PLL is biodegradable and can be conjugated with bioactive ligands to enhance target-specific delivery (Luo and Saltzman 2000). However, like PEI, PLL suffers from low transfection efficiency and high cytotoxicity (Brazeau et al. 1998). Because of the complex multistep barriers of the gene delivery process, it is very difficult to develop a polymer with high efficiency using the conventional design-driven, iterative synthesis approach.

20.3 HIGH-THROUGHPUT APPROACH TO DISCOVERING NOVEL MATERIALS FOR GENE DELIVERY

To accelerate the discovery of novel biomaterials for effective gene delivery, high-throughput screening has emerged as a novel approach to help better understand the structure–function relationships that govern polymer-mediated gene delivery. In a high-throughput screening approach, a large library of polymers with diverse chemical structures can be synthesized in a combinatorial approach, and the polymer/DNA nanoparticles can then be screened for their transfection efficiency and cytotoxicity using high-throughput screening assays. The versatility of polymer chemistry, combined with computational tools for predicting structure–function relationships, can be used in combinatorial library synthesis to generate a broad range of cationic polymer vectors with specific properties (Goldberg et al. 2008). Compared with the conventional, iterative approach, such a high-throughput approach greatly accelerated the development of novel polymers with enhanced gene delivery efficiency (Akinc et al. 2003a,b; Anderson et al. 2003; Green et al. 2008; Thomas et al. 2007). The results of such screening studies yield valuable information on structure–function relationships, which can guide the design of the next generation of polymer libraries synthesis for further performance improvement. Poly(β-amino esters) (PBAEs) are an excellent example of biodegradable polymers for gene delivery that have arisen from such combinatorial synthesis and high-throughput screening approaches (Green et al. 2008).

20.3.1 The Development of PBAEs for DNA Delivery

PBAEs constitute a particularly diverse class of cationic polymers for gene delivery. PBAE has several advantages including its biodegradability via hydrolytically degradable ester groups, reduced toxicity, capacity for structural diversity, and ability to trigger endosomal escape (Lynn and Langer 2000). Most importantly, PBAEs can be synthesized via a one-step reaction with conjugate addition between diacrylate and amine monomers, which makes it feasible for facile combinatorial synthesis (Figure 20.5a and b; Green et al. 2008). The first high-throughput effort to probe the


(a)
(b)
(c)
Amine/acrylate monomer ratio
1.400
1.300
1.200
1.100
1.050
1.025
1.000
0.975
0.950
0.900
0.800
0.600
Gene expression
100
10
1
0.1
Polymer composition

structure–function relationship of PBAEs synthesized a combinatorial library of 140 PBAEs composed of 20 amine monomers and 7 diacrylate monomers (Akinc et al. 2003a). Results from this screening showed that polymer structures can significantly influence the size and charge of the polymer/DNA nanoparticles, which has a significant impact on transfection efficiency. Lead polymers formed complexes smaller than 250 nm and had a positive ζ-potential, and demonstrated four to eight times higher transfection efficiency than PEI (Akinc et al. 2003b). Two leading PBAEs were further optimized by varying other parameters, including chemical structures of the polymer end groups, molecular weight, and polymer to DNA ratios (Akinc et al. 2003b). Using a semiautomated, parallel synthesis and screening process, Anderson et al. created a library of 2350 structurally unique, degradable PBAEs (Anderson et al. 2003). This combinatorial library was synthesized by diluting monomers in dimethyl sulfoxide, a low-viscosity medium, and using a fluid-handling robot and 12-channel micropipette to automate monomer mixing and thereby simultaneously setting up all 2350 reactions (Anderson et al. 2003). The library was later screened for its ability to bind DNA and transfect COS-7 cells, an easy to transfect cell line, under serum-free conditions. The screening identified a subset of 46 polymers with performance superior to the PEI control (Anderson et al. 2003; Green et al. 2008). These studies found that lead polymers can condense DNA into nanoparticles with smaller sizes and more positive surface charge, and such lead polymers also showed structural similarities (Figure 20.5c). Among the top nine polymers, all were formed from a conjugate addition of an amino alcohol and a hydrophobic diacrylate molecule (Anderson et al. 2005; Green et al. 2008). Moreover, the three top-performing polymers (i.e., C28, C32, and JJ28) all had converging structures (Anderson et al. 2005). These high-throughput studies highlight the importance of combinatorial synthesis and screening platforms in uncovering structure–function relationships and thereby optimizing polymeric gene delivery systems.

20.3.2 Combinatorial Approaches to siRNA Delivery Systems

Recent advances in RNA interference (RNAi) have provided another powerful tool for regulating cell behavior via gene silencing. RNAi is a gene-silencing mechanism that involves double-stranded RNA-mediated sequence-specific mRNA degradation and is a powerful mechanism for controlling cell behavior. However, the success of RNAi-based therapeutics requires an efficient delivery system to transport the 21–25 nucleotide double-stranded siRNAs into the target cells. Chemically synthesized siRNA often degrades rapidly *in vivo* and direct injection of naked siRNA often

FIGURE 20.5 (a) High-throughput synthesis and screening of PBAEs for gene delivery. PBAE synthesis by the conjugate addition of amines to diacrylate groups. (b) Acrylate and amino monomers used for the synthesis of the PBAE library. (c) Transfection proficiency of the PBAE polymer library. COS-7 cells were transfected with PBAE/DNA nanoparticles. PBAE polymers with different amine/acrylate monomer ratios were screened. (Adapted from Anderson D. G. et al. *Mol. Ther.* 11(3), 426–434, 2005 and Green J. J. et al. *Acc. Chem. Res.* 41(6), 749–759, 2008. With permission.)

leads to poor gene silencing (Urban-Klein et al. 2005). The low success rate of early studies highlighted the importance of developing delivery systems that would protect siRNA from degradation and facilitate its cellular uptake. Earlier trials showed that PEI can complex synthetic siRNAs into nanoparticles and help preserve the bioactivity of these molecules (Urban-Klein et al. 2005); however, PEI/siRNA complexes have significant toxicity effects (Akinc et al. 2008). In an effort to develop novel biomaterials for safe and efficient siRNA delivery, Akinc et al. synthesized a combinatorial library of lipid-like materials (lipidoids; Akinc et al. 2008). From a large library of more than 1200 structurally diverse lipidoids, Akinc and colleagues identified lipidoids that facilitate high levels of specific siRNA-mediated silencing of endogenous gene transcripts. Top-performing lipidoids also share common structures including amide linkages, more than two alkyl tails of eight to twelve carbons and a secondary amide (Akinc et al. 2008). Such information provides valuable guidelines for further optimizing siRNA delivery vectors.

20.4 A COMBINATORIAL APPROACH TO IDENTIFY SYNERGISTIC GENETIC SIGNALS

Gene therapy offers a promising approach for promoting desired cellular processes and tissue development by upregulating inductive genes via DNA delivery or downregulating inhibitory genes via RNAi delivery. Codelivery of multiple genetic signals may act synergistically to accelerate the desired cellular processes. Although extensive work has been performed on delivering single genetic signals for gene therapy, efforts using synergistic genetic signals are only beginning to emerge. The cellular microenvironment is highly dynamic and multifactorial. Many signaling pathways are interconnected, affecting cell fate in a synergistic or antagonistic manner. For example, bone morphogenic protein-6 (BMP-6) or insulin-like growth factor-1 (IGF-1) alone could not induce chondrogenesis, whereas on codelivery, such factors with TGF-β3 can synergistically promote chondrogenic differentiation (Indrawattana et al. 2004). Likewise, most tissue morphogenesis processes are tightly regulated by interactive signals that either turn on an activator gene, or turn off an inhibitor gene. Identifying synergistic genetic signals that promote the desired cellular processes would provide a powerful tool for directing tissue regeneration.

High-throughput screening has been explored to facilitate the discovery of potent gene targets in regulating stem cell differentiation. For example, Zhao et al. screened a synthetic siRNA library targeting 5000 human genes, which yielded 12 candidate suppressors for osteogenic specification in human MSCs (Zhao and Ding 2007). A recent study examined the effects of codelivering two genes, *BMP2* and core-binding factor α-1 (*Cbfa1*), on osteogenic differentiation of adipose-derived stem cells (ADSCs; Lee et al. 2010). *BMP2*- and *Cbfa1*-transduced ADSCs showed an upregulation of osteogenic markers and increased mineralization. Codelivery of *BMP2* and dexamethasone, a small molecule activator of osteogenesis, also led to increased expression of early-stage osteogenic markers in mouse embryonic stem cells (Blum et al. 2004). BMP-transduced, mouse muscle-derived stem cells also showed increased angiogenesis and mineralization when cotransduced with VEGF (Peng et al. 2002). These studies demonstrated that codelivering multiple genetic

signals may synergistically promote lineage-specific differentiation. A recent study has also explored the potential benefits of codelivering multiple inductive and suppressive genes on osteogenic differentiation (Ramasubramanian et al. 2011). The gene expression of three target genes, *BMP2*, an osteogenic inducer, as well as *GNAS* and *Noggin*, osteogenic suppressors, were modulated in a combinatorial manner using biomaterials-mediated gene delivery. Compared with *BMP2* DNA delivery alone, codelivery of *BMP2* DNA and either siGNAS or siNoggin significantly accelerated osteogenic differentiation in human ADSCs, with enhanced osteogenic gene expression and mineralization. These results suggest that inductive or suppressive genetic switches interact in a complex, nonlinear manner. Given the complex interactions among various genetic signals, it is highly desirable to develop high-throughput screening assays to facilitate the identification of synergistic genetic signals for gene therapy (Nguyen et al. 2009; Ramasubramanian et al. 2011).

20.5 CONCLUSION

Advances in nanotechnology have provided powerful tools to control cell fate and tissue regeneration. These strategies can be broadly classified into two categories, the "outside–in" or "inside–out" approaches. The outside–in approach focuses on engineering the cellular microenvironment via recapitulating the sophisticated structure and biological functionalities found in the native ECM. Nanotechnology facilitates the incorporation of nanoscale features into scaffold design to better mimic native tissues, which helps elucidate the complex interactions between cells and their surrounding microenvironment. The inside–out approach influences cell behavior via directly regulating genetic signals using biomaterials-mediated gene delivery. Toward this end, the use of cationic lipids or polymers in gene delivery systems offers great versatility in chemical modification to circumvent barriers in gene delivery. High-throughput synthesis and screening approaches have demonstrated great promise in accelerating the rapid discovery of novel biomaterials that can condense nucleic acids into nanoparticles for efficient gene delivery. Identifying potential synergistic signals using high-throughput screening would also provide potentially more powerful gene targets for promoting desired tissue formation. Finally, validating the efficacy of these strategies in appropriate animal models will be crucial for the successful translation of their final applications in the clinical setting.

Although significant advances have been made in applying nanotechnology for advancing tissue regeneration, challenges remain before such therapies can be successfully translated from bench-to-bedside. For example, extensive efforts have been dedicated to understanding individual types of microenvironmental cues (e.g., nanotopography), but how complex interactive cues regulate cell fate and tissue development remains largely unknown. Moreover, many of the molecular mechanisms underlying these effects have yet to be discovered. As for the application of nanomaterials for gene delivery, there is an impending need to develop technology that facilitates efficient targeting to specific cell types or tissues *in situ*. Biomaterial-based vectors allow the possibility of conjugating biofunctional moieties such as antibodies that target specific cells or tissues. In sum, nanotechnology will continue to provide a powerful tool for both fundamental and applied research in tissue regeneration.

The contributions of nanotechnology to the tissue engineering field will also become even more prominent as the field continues to evolve and better integrate with multiple disciplines, such as molecular and cell biology, materials science, computational biology, and medicine.

REFERENCES

Akinc, A., D. G. Anderson, D. M. Lynn, and R. Langer. 2003a. Synthesis of poly(beta-amino ester)s optimized for highly effective gene delivery. *Bioconjugate Chemistry* 14(5), 979–988.

Akinc, A., D. M. Lynn, D. G. Anderson, and R. Langer. 2003b. Parallel synthesis and biophysical characterization of a degradable polymer library for gene delivery. *Journal of the American Chemical Society* 125(18), 5316–5323.

Akinc, A., A. Zumbuehl, M. Goldberg, E. S. Leshchiner, V. Busini, N. Hossain et al. 2008. A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nature Biotechnology* 26(5), 561–569.

Anderson, D. G., A. Akinc, N. Hossain, and R. Langer. 2005. Structure/property studies of polymeric gene delivery using a library of poly(beta-amino esters). *Molecular Therapy* 11(3), 426–434.

Anderson, D. G., D. M. Lynn, and R. Langer. 2003. Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery. *Angewandte Chemie (International ed. in English)* 42(27), 3153–3158.

Babensee, J. E., L. V. McIntire, and A. G. Mikos. 2000. Growth factor delivery for tissue engineering. *Pharmaceutical Research* 17(5), 497–504.

Banerjee, A., M. Arha, S. Choudhary, R. S. Ashton, S. R. Bhatia, D. V. Schaffer et al. 2009. The influence of hydrogel modulus on the proliferation and differentiation of encapsulated neural stem cells. *Biomaterials* 30(27), 4695–4699.

Blum, J. S., M. B. Parrott, A. G. Mikos, and M. A. Barry. 2004. Early osteoblastic differentiation induced by dexamethasone enhances adenoviral gene delivery to marrow stromal cells. *Journal of Orthopaedic Research* 22(2), 411–416.

Brazeau, G. A., S. Attia, S. Poxon, and J. A. Hughes. 1998. *In vitro* myotoxicity of selected cationic macromolecules used in non-viral gene delivery. *Pharmaceutical Research* 15(5), 680–684.

Brittberg, M., A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson, and L. Peterson. 1994. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *New England Journal of Medicine* 331(14), 889–895.

Buehler, M. J. 2006. Nature designs tough collagen: Explaining the nanostructure of collagen fibrils. *Proceedings of the National Academy of Sciences of the United States of America* 103(33), 12285–12290.

Check, E. 2005. Gene therapy put on hold as third child develops cancer. *Nature* 433, 561.

Dalby, M. J., N. Gadegaard, R. Tare, A. Andar, M. O. Riehle, P. Herzyk et al. 2007. The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. *Nature Materials* 6(12), 997–1003.

Engler, A. J., S. Sen, H. L. Sweeney, and D. E. Discher. 2006. Matrix elasticity directs stem cell lineage specification. *Cell* 126(4), 677–689.

Ferkol, T., J. C. Perales, F. Mularo, and R. W. Hanson. 1996. Receptor-mediated gene transfer into macrophages. *Proceedings of the National Academy of Sciences of the United States of America* 93(1), 101–105.

Ferrara, N. 2010. Binding to the extracellular matrix and proteolytic processing: Two key mechanisms regulating vascular endothelial growth factor action. *Molecular Biology of the Cell* 21(5), 687–690.

Geng, T., Y. Zhan, J. Wang, and C. Lu. 2011. Transfection of cells using flow-through electroporation based on constant voltage. *Nature Protocols* 6(8), 1192–1208.

Goldberg, M., R. Langer, and X. Jia. 2007. Nanostructured materials for applications in drug delivery and tissue engineering. *Journal of Biomaterials Science. Polymer Edition* 18(3), 241–268.

Goldberg, M., K. Mahon, and D. Anderson. 2008. Combinatorial and rational approaches to polymer synthesis for medicine. *Advanced Drug Delivery Reviews* 60(9), 971–978.

Green, J. J., R. Langer, and D. G. Anderson. 2008. A combinatorial polymer library approach yields insight into nonviral gene delivery. *Accounts of Chemical Research* 41(6), 749–759.

Guo, X., and Huang, L. 2011. Recent advances in nonviral vectors for gene delivery. *Accounts of Chemical Research*. DOI: 10.1021/ar200151m.

Hartgerink, J. D., E. Beniash, and S. I. Stupp. 2001. Self-assembly and mineralization of peptide–amphiphile nanofibers. *Science* 294(5547), 1684–1688.

Heilbronn, R., and S. Weger. 2010. Viral vectors for gene transfer: Current status of gene therapeutics. *Handbook of Experimental Pharmacology* 197, 143–170.

Herweijer, H., and J. A. Wolff. 2003. Progress and prospects: Naked DNA gene transfer and therapy. *Gene Therapy* 10(6), 453–458.

Hollon, T. 2000. Researchers and regulators reflect on first gene therapy death. *Nature Medicine* 6, 6.

Holmes, T. C., S. de Lacalle, X. Su, G. Liu, A. Rich, and S. Zhang. 2000. Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds. *Proceedings of the National Academy of Sciences of the United States of America* 97(12), 6728–6733.

Hosseinkhani, H., M. Hosseinkhani, F. Tian, H. Kobayashi, and Y. Tabata. 2006. Osteogenic differentiation of mesenchymal stem cells in self-assembled peptide–amphiphile nanofibers. *Biomaterials* 27(22), 4079–4086.

Huebsch, N., P. R. Arany, A. S. Mao, D. Shvartsman, O. A. Ali, S. A. Bencherif et al. 2010. Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate. *Nature Materials* 9(6), 518–526.

Indrawattana, N., G. Chen, M. Tadokoro, L. H. Shann, H. Ohgushi, T. Tateishi et al. 2004. Growth factor combination for chondrogenic induction from human mesenchymal stem cell. *Biochemical and Biophysical Research Communications* 320(3), 914–919.

Kay, M. A., J. C. Glorioso, and L. Naldini. 2001. Viral vectors for gene therapy: The art of turning infectious agents into vehicles of therapeutics. *Nature Medicine* 7(1), 33–40.

Kisiday, J., M. Jin, B. Kurz, H. Hung, C. Semino, S. Zhang et al. 2002. Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: Implications for cartilage tissue repair. *Proceedings of the National Academy of Sciences of the United States of America* 99(15), 9996–10001.

Kolácná, L., J. Bakesová, F. Varga, E. Kostáková, L. Plánka, A. Necas et al. 2007. Biochemical and biophysical aspects of collagen nanostructure in the extracellular matrix. *Physiological Research* 56, Suppl 1, S51–60.

Kootstra, N. A., and I. M. Verma. 2003. Gene therapy with viral vectors. *Annual Review of Pharmacology and Toxicology* 43, 413–439.

Kumbar, S. G., R. James, S. P. Nukavarapu, and C. T. Laurencin. 2008. Electrospun nanofiber scaffolds: Engineering soft tissues. *Biomedical Materials* 3(3), 034002.

Lamers, E., R. van Horssen, J. te Riet, F. C. van Delft, R. Luttge, X. F. Walboomers et al. 2010. The influence of nanoscale topographical cues on initial osteoblast morphology and migration. *European Cells* and *Materials* 20, 329–343.

Langer, R., and J. P. Vacanti. 1993. Tissue engineering. *Science* 260(5110), 920–926.

Lee, S. J., S. W. Kang, H. J. Do, I. Han, D. A. Shin, J. H. Kim et al. 2010. Enhancement of bone regeneration by gene delivery of BMP2/Runx2 bicistronic vector into adipose-derived stromal cells. *Biomaterials* 31(21), 5652–5659.

Luo, D., and W. M. Saltzman. 2000. Synthetic DNA delivery systems. *Nature Biotechnology* 18(1), 33–37.

Lutolf, M. P., and J. A. Hubbell. 2005. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nature Biotechnology* 23(1), 47–55.

Lynn, D. M., and R. Langer. 2000. Degradable poly(beta-amino esters): Synthesis, characterization, and self-assembly with plasmid DNA. *Journal of the American Chemical Society* 122(44), 10761–10768.

Ma, Z., M. Kotaki, R. Inai, and S. Ramakrishna. 2005. Potential of nanofiber matrix as tissue-engineering scaffolds. *Tissue Engineering* 11(1–2), 101–109.

Matthews, J. A., G. E. Wnek, D. G. Simpson, and G. L. Bowlin. 2002. Electrospinning of collagen nanofibers. *Biomacromolecules* 3(2), 232–238.

Mehier-Humbert, S., and R. H. Guy. 2005. Physical methods for gene transfer: Improving the kinetics of gene delivery into cells. *Advanced Drug Delivery Reviews* 57(5), 733–753.

Moghimi, S. M., P. Symonds, J. C. Murray, A.C. Hunter, G. Debska, and A. Szewczyk. 2005. A two-stage poly(ethylenimine)-mediated cytotoxicity: Implications for gene transfer/therapy. *Molecular Therapy* 11, 990–995.

Nguyen, D. N., J. J. Green, J. M. Chan, R. Langer, and D. G. Anderson. 2009. Polymeric materials for gene delivery and DNA vacinnation. *Advanced Materials* 21(8), 847–867.

Oh, S., K. S. Brammer, Y. S. Li, D. Teng, A. J. Engler, S. Chien et al. 2009. Stem cell fate dictated solely by altered nanotube dimension. *Proceedings of the National Academy of Sciences of the United States of America* 106(7), 2130–2135.

Pack, D. W., A. S. Hoffman, S. Pun, and P. S. Stayton. 2005. Design and development of polymers for gene delivery. *Nature Reviews Drug Discovery* 4(7), 581–593.

Papachroni, K. K., D. N. Karatzas, K. A. Papavassiliou, E. K. Basdra, and A. G. Papavassiliou. 2009. Mechanotransduction in osteoblast regulation and bone disease. *Trends in Molecular Medicine* 15(5), 208–216.

Papisov, M. I. 1998. Theoretical considerations of RES-avoiding liposomes: Molecular mechanics and chemistry of liposome interactions. *Advanced Drug Delivery Reviews* 32(1–2), 119–138.

Pek, Y. S., A. C., Wan, and J. Y. Ying. 2010. The effect of matrix stiffness on mesenchymal stem cell differentiation in a 3D thixotropic gel. *Biomaterials* 31(3), 385–391.

Peng, H., V. Wright, A. Usas, B. Gearhart, H. C. Shen, J. Cummins et al. 2002. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. *Journal of Clinical Investigation* 110(6), 751–759.

Pollard, H., G. Toumaniantz, J. L. Amos, H. Avet-Loiseau, G. Guihard, J. P. Behr et al. 2001. Ca^{2+}-sensitive cytosolic nucleases prevent efficient delivery to the nucleus of injected plasmids. *Journal of Gene Medicine* 3(2), 153–164.

Ramasubramanian, A., S. Shiigi, G. K. Lee, and F. Yang. 2011. Non-viral delivery of inductive and suppressive genes to adipose-derived stem cells for osteogenic differentiation. *Pharmaceutical Research* 28(6), 1328–1337.

Rho, J. Y., L. Kuhn-Spearing, and P. Zioupos. 1998. Mechanical properties and the hierarchical structure of bone. *Medical Engineering and Physics* 20(2), 92–102.

Rho, K. S., L. Jeong, G. Lee, B. M. Seo, Y. J. Park, S. D. Hong et al. 2006. Electrospinning of collagen nanofibers: Effects on the behavior of normal human keratinocytes and early-stage wound healing. *Biomaterials* 27(8), 1452–1461.

Rubinson, D. A., C. P. Dillon, A. V. Kwiatkowski, C. Sievers, L. Yang, J. Kopinja et al. 2003. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nature Genetics* 33(3), 401–406.

Rychahou, P. G., and B. M. Evers. 2010. Hydrodynamic delivery protocols. *Methods in Molecular Biology* 623, 189–195.

Scherer, F., M. Anton, U. Schillinger, J. Henke, C. Bergemann, A. Krüger et al. 2002. Magnetofection: Enhancing and targeting gene delivery by magnetic force *in vitro* and *in vivo*. *Gene Therapy* 9(2), 102–109.

Schultz, G. S., and A. Wysocki. 2009. Interactions between extracellular matrix and growth factors in wound healing. *Wound Repair and Regeneration* 17(2), 153–162.

Shah, R. N., N. A. Shah, M. M. Del Rosario Lim, C. Hsieh, G. Nuber, and S. I. Stupp. 2010. Supramolecular design of self-assembling nanofibers for cartilage regeneration. *Proceedings of the National Academy of Sciences of the United States of America* 107(8), 3293–3298.

Shin, H., S. Jo, and A. G. Mikos. 2003. Biomimetic materials for tissue engineering. *Biomaterials* 24(24), 4353–4364.

Silver, F. H., J. W. Freeman, and G. P. Seehra. 2003. Collagen self-assembly and the development of tendon mechanical properties. *Journal of Biomechanics* 36(10), 1529–1553.

Taniyama, Y., K. Tachibana, K. Hiraoka, M. Aoki, S. Yamamoto, K. Matsumoto et al. 2002. Development of safe and efficient novel nonviral gene transfer using ultrasound: Enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. *Gene Therapy* 9(6), 372–380.

Tessmar, J. K., and A. M. Göpferich. 2007. Matrices and scaffolds for protein delivery in tissue engineering. *Advanced Drug Delivery Reviews* 59(4–5), 274–291.

Thomas, C. E., A. Ehrhardt, and M. A. Kay. 2003. Progress and problems with the use of viral vectors for gene therapy. *Nature Reviews Genetics* 4(5), 346–358.

Thomas, M., J. J. Lu, C. Zhang, J. Chen, and A. M. Klibanov. 2007. Identification of novel superior polycationic vectors for gene delivery by high-throughput synthesis and screening of a combinatorial library. *Pharmaceutical Research* 24(8), 1564–1571.

Urban-Klein, B., S. Werth, S. Abuharbeid, F. Czubayko, and A. Aigner. 2005. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA *in vivo*. *Gene Therapy* 12(5), 461–466.

Wu, G. Y., and C. H. Wu. 1988. Receptor-mediated gene delivery and expression *in vivo*. *Journal of Biological Chemistry* 263(29), 14621–14624.

Wu, G. Y., J. M. Wilson, F. Shalaby, M. Grossman, D. A. Shafritz, and C. H. Wu. 1991. Receptor-mediated gene delivery *in vivo*. Partial correction of genetic analbuminemia in Nagase rats. *Journal of Biological Chemistry* 266(22), 14338–14342.

Yim, E. K., R. M. Reano, S. W. Pang, A. F. Yee, C. S. Chen, and K. W. Leong. 2005. Nanopattern-induced changes in morphology and motility of smooth muscle cells. *Biomaterials* 26(26), 5405–5413.

Yoshimoto, H., Y. M. Shin, H. Terai, and J. P. Vacanti. 2003. A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering. *Biomaterials* 24(12), 2077–2082.

Yu, J. Y., M. A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian et al. 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318(5858), 1917–1920.

Zhang, L., and T. J. Webster. 2009. Nanotechnology and nanomaterials: Promises for improved tissue regeneration. *Nano Today* 4(1).

Zhao, Y., and S. Ding. 2007. A high-throughput siRNA library screen identifies osteogenic suppressors in human mesenchymal stem cells. *Proceedings of the National Academy of Sciences of the United States of America* 104(23), 9673–9678.