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Matrices and Scaffolds for DNA Delivery in Tissue Engineering

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Abstract

Regenerative medicine aims to create functional tissue replacements, typically through creating a controlled environment that promotes and directs the differentiation of stem or progenitor cells, either endogenous or transplanted. Scaffolds serve a central role in many strategies by providing the means to control the local environment. Gene delivery from the scaffold represents a versatile approach to manipulating the local environment for directing cell function. Research at the interface of biomaterials, gene therapy, and drug delivery has identified several design parameters for the vector and the biomaterial scaffold that must be satisfied. Progress has been made towards achieving gene delivery within a tissue engineering scaffold, though the design principles for the materials and vectors that produce efficient delivery require further development. Nevertheless, these advances in obtaining transgene expression with the scaffold have created opportunities to develop greater control of either delivery or expression and to identify the best practices for promoting tissue formation. Strategies to achieve controlled localized expression within the tissue engineering scaffold will have broad application to the regeneration of many tissues, with great promise for clinical therapies.

Keywords

gene therapy; regenerative medicine; biomaterials

2 Introduction

Tissue engineering and regenerative medicine aim to create functional tissue replacements, typically through creating a controlled environment that promotes and directs the differentiation of stem or progenitor cells, either endogenous or transplanted. Cells continually sense and respond to their environment, with signals initiated by diffusible factors, extracellular matrix (ECM) proteins, cell-cell interaction, or mechanical stress. Scaffolds serve a central role in many tissue engineering and regenerative medicine strategies by providing the means to control the local environment. The scaffold functions to create a space for tissue formation, and can mimic the natural environment by providing a substrate for cell attachment that presents chemical, biological, and/or mechanical cues. Additionally, scaffolds have also been employed as controlled release vehicles that can maintain therapeutic concentrations of diffusible tissue inductive factors [1–3]. The major challenge of tissue engineering thus lies in presenting the appropriate combination of signals, which may need to vary temporally and spatially, to the tissue progenitor cells such that it induces the formation of functional tissues.

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Gene delivery from the scaffold represents a versatile approach to manipulating the local environment for directing cell function [4]. Vectors for gene delivery consist of DNA or RNA that may be packaged with proteins, polymers or lipids to create particles that can effectively overcome the extracellular and intracellular barriers to gene transfer. Gene therapy approaches can be employed to increase the expression of tissue inductive factors or block the expression of factors that would inhibit tissue formation [4,5]. Additionally, gene delivery has the potential to provide protein expression for long periods of time at effective concentrations, and target any cellular process by altering expressing of a specific protein. From a pharmaceutical perspective, nucleic acids have their “information” encoded in the linear sequence of bases, and not their three-dimensional conformation, as is the case for proteins [6]. The physical properties of nucleic acids are determined in large part by the sugar backbone [6], and not by the nucleic acid sequence, which makes gene delivery a versatile approach that enables interchange of nucleic acid sequences or delivery of multiple sequences from a single system, without the need to adapt the delivery device.

In this review, we present the major challenges towards the application of gene delivery toward tissue engineering and regenerative medicine. The primary challenge is delivery, which is consistent with most applications of gene therapy. Research at the interface of biomaterials, gene therapy, and drug delivery has identified several design parameters for the vector and the biomaterial scaffold that must be satisfied. In addition to the established challenge of delivery, we present the evolving challenges associated with the relationship between the cellular microenvironment and gene transfer, and between transgene expression and the subsequent tissue formation. Strategies to overcome these challenges will have broad application to the regeneration of many tissues, with great promise for clinical therapies.

3 Vector design

Nucleic acids (e.g., DNA, RNA, siRNA) can be delivered alone, or packaged using viral or non-viral vectors to increase expression of a therapeutic gene or knockdown expression of a specific gene (i.e., RNAi). For delivery, vectors must evade the immune system and be transported to the cell microenvironment for internalization, typically into an endosome, from which the vector must escape prior to being degraded as the endosome transitions into a lysosome. To induce expression of an encoded gene, the nucleic acids must dissociate from any packaging component and traffic to the nucleus for expression. For delivery of siRNA or similar strategies, the nucleic acid only needs to be present within the cytoplasm for activity.

Viral vectors are composed of either DNA or RNA surrounded by a capsid, which provides greater efficiency than non-viral vectors yet provokes an immune response that can lead to clearance of the vector or infected cells [7,8]. A variety of viruses have been utilized as gene delivery vectors, with adenovirus and retrovirus among the most common, and lentivirus, and adeno-associated virus (AAV) among the more promising vectors for future therapies. A thorough description of these and other viral vectors is available in several excellent reviews [9–12]. Naked plasmid and non-viral vectors initiate inflammatory responses that are milder than viral vectors, yet lack their intrinsic efficiency. Plasmid alone is able to transfect cells *in vivo*, but generally has a low efficiency *in vitro*. Alternatively, the nucleic acids (e.g., plasmid, siRNA) are complexed with cationic polymers or lipids, with the design of these transfection reagents dependent upon the nucleic acid properties, such as size [13,14]. Complexation with polymers or lipids protects against degradation, creates a less negative particle relative to naked plasmid, and facilitates internalization and intracellular trafficking [15,16].

In selecting among the available vectors for delivery to promote tissue formation, multiple aspects of the vector must be considered such as the immune response to the vector, the target cell population for gene delivery, the required duration of expression, and the stability of the

vector. First, the immune response elicited from the vector limits transgene expression [17, 18], yet the local inflammatory response can potentially influence regeneration [19–22]. The extent of the immune response can determine whether the vector could be delivered more than once. Next, the different vectors have differential activity based on cell division. Some viruses can infect both dividing and non-dividing cells (e.g., neurons), whereas others are effective only in dividing cells. Many non-viral vectors are restricted to dividing cells. A third consideration is the required duration of expression, which is based on the progenitor cells and the requirements of the tissue. Some viruses integrate their DNA into the chromosome and thus provide permanent expression, whereas many applications in regenerative medicine require expression only during the healing or regenerative process. In addition, the vector must retain its bioactivity throughout the conditions used for scaffold fabrication. Some viral vectors inactivate rapidly at room temperature and may not be appropriate for incorporation into biomaterials. Non-viral vectors generally have good stability, though plasmid degradation and aggregation of DNA complexes are significant concerns. Finally, the level and duration of gene expression may need to be modulated to avoid side effects resulting from excessive protein activity at the target site, or inappropriate activity at a distant site. Expression can be modulated using inducible promoters that are either tissue specific or activated by small molecules [23].

These considerations for using gene delivery for tissue regeneration will need to be continually reassessed with the emerging developments in the design of the both viral and non-viral vectors. Novel vectors are being identified using a range of strategies, such as rational design [24], directed evolution [25–27], and high throughput screening [28,29]. Evading the immune response has been attempted by modifying regions of the viral capsid [30,31] or the viral genome [32], or removing CpG (cytosine and guanine separated by a phosphate) dinucleotides from the plasmid [33]. For non-viral vectors in particular, intracellular trafficking of a plasmid is a limiting factor, which is illustrated by the observation that intracellular levels of plasmid were 2 to 3 orders of magnitude greater than the number of adenoviral particles [34]. Vectors are thus being designed with endosomal escape moieties [35,36], or degradable components to facilitate dissociation of the plasmid from the vector [37–40], or nuclear localization signals [41]. As these vectors are applied to tissue engineering and scaffold-based delivery, an additional design parameter that must be considered is the interaction between the vector and the material. Low-affinity interactions between vector and material will produce rapid release, while increasing the affinity can be employed to slow the release and retain the complexes within the scaffold [42]. Excessively strong binding affinities, however, will limit cellular internalization [42].

4 Biomaterial Design

Biomaterials serve a central role in regenerative medicine applications, and several basic requirements have been identified. Scaffolds have been fabricated from a range of natural and synthetic materials, with biodegradable materials being desirable to avoid a second surgical procedure. The degradation time for the scaffold should ideally match the time necessary for tissue regeneration. Degradation of the material can create a path for cell infiltration, or cell infiltration can be supported by highly porous scaffolds, which also allows for the initial transport of oxygen and nutrients and the removal of metabolic waste and degradation products. Cell migration can be directed by the matrix surface, with the composition mediating the binding of integrin receptors [43]. Porosity and pore size, both of which influence the rate of cell infiltration, can be manipulated using different amounts and sizes of porogen in case of synthetic polymer scaffolds, or the extent of cross-linking for hydrogels. The porosity is a determinant of the mechanical properties, which can regulate cellular processes such as differentiation, cell organization, and differentiated cell function [44–48], and must be sufficient to create and maintain a space for tissue formation. Finally, the architecture of the scaffold can function to organize cells within the environment, such as channels that align cells

or domains that maintain cell-cell connectivity [49]. The design aspects of the scaffold can influence tissue formation, while the addition of gene delivery from the scaffold can provide an additional mechanism to promote the desired responses leading to functional tissue formation.

Gene delivery from the scaffold enables localized expression, as the biomaterial can enhance gene transfer relative to traditional delivery systems (e.g., injection) [4]. Targeting a cell population or anatomical location by injection or systemic delivery is complex, but direct delivery of the vector from the scaffold can localize transgene expression primarily to the implant site. In addition to localized delivery, the scaffold can protect the vector against extracellular barriers that reduce their therapeutic efficacy by protecting them from attack by immune responses or and limiting degradation by serum nucleases or proteases [50]. Biomaterials have been able to increase the half-life of viral vectors and reduce the immune response that normally targets the virus [51]. Additionally, scaffold-based delivery has the potential to maintain effective levels of the vector for prolonged times, which extends the opportunity for cellular internalization and increases the likelihood of gene transfer. Sustained release formulations can compensate for vectors lost due to clearance or degradation. Alternatively, interactions between the biomaterial and vector can retain vectors locally and prevent clearance. Delivery from most biomaterial systems likely occurs through a combination of vector interactions with the matrix and subsequent release, with the vector and material designed to regulate these interactions. We categorize the scaffolds according to two basic mechanisms by which the DNA is incorporated: i) Scaffold Encapsulation and ii) Surface Immobilization. The following paragraphs describe the capabilities and challenges for these approaches.

4.1 Scaffold Encapsulation

Many materials used for controlled drug delivery are also used to fabricate tissue engineering scaffolds. Release from the scaffolds occurs by a combination of polymer degradation and vector diffusion (Figure 1). A critical aspect associated with the encapsulation of gene therapy vectors is that the scaffold fabrication method must be compatible with the vector integrity. Methods for scaffold fabrication can involve high temperatures, organic solvents, and the generation of free radicals or shears stresses that may damage the vector. Even if the vector is stably encapsulated, it can still be damaged by the degradation products [52]. The sections below describe the design and performance of scaffolds formed from hydrophobic polymers and hydrophilic polymers (i.e., hydrogels) with encapsulated gene therapy vectors.

4.1.1 Scaffolds fabricated from hydrophobic polymers—Vector release from hydrophobic polymer scaffolds occurs by a sequence of polymer degradation, dissolution of the vector and subsequent diffusion from the polymer [53]. At some point during the encapsulation of the vector in the polymer, the vector is typically dried and must be stabilized using protectants such as sucrose [6]. If the activity can be preserved during dehydration and rehydration, the solid form of the vector is more stable and can provide a longer shelf-life. Once implanted *in vivo*, body fluids wet the polymer resulting in an initial release of any vector that is weakly surface associated. For degradable polymers such as the copolymers of lactide and glycolide (PLG), the fluid can degrade the polymer and penetrate into pores to dissolve entrapped vector for subsequent release by diffusion. Because PLG is a bulk-degrading material, short-term degradation does not significantly affect the three-dimensional architecture, which is in contrast to surface eroding materials that would lose their shape during degradation. Alternatively, non-degradable materials (e.g., poly(ethylene-co-vinylacetate), EVAc) have been used for gene delivery and tissue regeneration, and water penetrates into a pre-existing network of pores through which the entrapped vector can be dissolved and released by diffusion [54].

A gas foaming/particulate leaching process has been employed to encapsulate plasmid into porous tissue engineering scaffolds [55,56]. The foaming method enabled the formation of highly porous scaffolds with interconnected pore structure and DNA incorporation without the use of organic solvents. Solvent evaporation has been used for fabrication of similar scaffolds, though the plasmid was rapidly released likely due to surface association [57]. In the gas foaming approach, lyophilized plasmid, PLG microspheres, and a porogen are mixed, compression molded, and then foamed. Released DNA was intact with a release profile that could be manipulated by changing the PLG molecular weight and lactide:glycolide ratio [56]. The foaming method provides a versatile method to encapsulate DNA in scaffolds with complex geometries, and scaffolds have promoted transgene expression *in vivo* after implantation subcutaneously, or into a cranial defect or the injured spinal cord [58–60]. An alternative method for controlling the release is to pre-encapsulate the DNA into the polymer microspheres prior to foaming [52], with scaffolds formed from the DNA loaded microspheres exhibiting a more sustained release of the plasmid. Implantation of plasmid loaded scaffolds subcutaneously has produced transgene expression lasting for over 3 months, with expression levels able to induce physiological responses [60]. Achieving high levels of transgene expression for long times was dependent upon the mechanical properties of the scaffold and the DNA loading [60]. Scaffolds without sufficient integrity would collapse, resulting in low levels of transgene expression, while low quantities of DNA were able to produce transgene expression but for shorter times.

The gas foaming process has also been employed to deliver polyplexes consisting of the plasmid complexed with the cationic polymer polyethylenimine (PEI). Encapsulating PEI/DNA polyplexes offers the potential to reduce the quantity of DNA that has to be delivered and also influenced the release profile [61,62]. Using sucrose as a porogen, DNA complexes were retained within the scaffold after an initial burst during the leaching step, while the release of the complexes was dependent on the porogen to polymer ratio [61]. Following subcutaneous implantation of the PEI/DNA-loaded scaffold, transfected cells were observed at the implant site up to 3 months, and the identity of the infiltrating cells was determined to be approximately 30% endothelial cells, 18% lymphocytes, and 52% other cell types that are suggested to be mainly fibroblasts based on morphology [62].

PLG scaffolds have been produced by alternative methods to create structures with different physical properties or handling strategies. Nano-fibrous PLG scaffolds have been formed by electrospinning a polymer solution mixed with plasmid, with the scaffold properties controlled by the composition of the polymer solution and processing parameters [63,64]. A thermally induced phase separation [65] was used to create porous DNA loaded scaffolds with a microcellular pore structure that was dependent upon the quenching temperature and duration, solvent/water ratio, and polymer concentration. Finally, an injectable scaffold was developed by dissolving PLG in the plasmid containing biocompatible water-miscible solvent glycofurol [66]. After injection, a solid polymeric implant was formed due to diffusion of the solvent from the site of injection. This technique allowed for 100% DNA incorporation efficiency and induced gene expression capable of promoting physiological responses.

4.1.2 Hydrogels—Hydrogels are formed by the crosslinking or self-assembly of a variety of natural or synthetic hydrophilic polymers to produce structures that contain over 90% water. Many natural materials, such as collagen or fibrin, have been widely used for tissue engineering applications for their innate cellular interactions and cell-mediated degradation [67]. Synthetic materials, such as poly(ethylene glycol) (PEG), lack the intrinsic cellular interactions yet allow for greater manipulation of the hydrogel properties relative to the natural materials. The hydrogels can be constructed *ex vivo* for subsequent implantation, or can be formed *in situ*. Cells have been encapsulated within hydrogels prior to gelation with minimal effects on viability, which is promising for the encapsulation of many gene therapy vectors. The extent

of crosslinking and the molecular weight between crosslinks are two parameters that influence the mesh size of the matrix and the swelling of the gel [68]. Depending on the hydrogel, degradation can be designed to occur through a variety of mechanisms, such as cell secreted enzymes (a cell responsive gel) or hydrolysis, and can be manipulated by the mesh size, ion exchange, and/or the strength of interactions. A larger mesh size results in more swelling, which enhances the degradation rate when the hydrolysable groups are located on the backbone, but reduces the degradation rate when the hydrolysable groups are located at the connection points of the matrix [69,70].

Vector release from a hydrogel is dependent upon the physical structure of the hydrogel, its degradation, and its interactions with the vector. In general, hydrogels produce high encapsulation efficiencies with release occurring through either diffusion alone (non-degradable hydrogels), or through a combination of hydrogel degradation and vector diffusion. Plasmid with 6000 base pairs, a typical size for use in gene delivery, has been reported as having a hydrodynamic radius of approximately 175 nm [71], which is substantially larger than the reported mesh sizes for some synthetic hydrogels. However, plasmid has significant conformational flexibility and may reptate through the hydrogel by undergoing random segmental motion to traverse between pores [72]. Additionally, the release rate can be increased by degradation of the hydrogel. The encapsulation of many non-viral and viral vectors can have limited release owing to interactions between the particle and the matrix [70,73].

The first tissue engineering scaffold that functioned as a gene delivery vehicle, was made of collagen [74]. Collagen is a natural biomaterial that can be used in several forms, including solutions, sponges, fiber, etc, gels at physiological temperatures, and is biodegradable. Collagen-based scaffolds have been employed with a range of non-viral and viral vectors, and are capable of inducing *in vivo* transgene expression and physiological improvements in applications such as bone regeneration [74,75], wound healing [76–80], muscle repair [81], and optic nerve repair [82]. The release kinetics and transfection efficiencies of DNA loaded collagen scaffolds are dependent on the vector, with polyplexes and lipoplexes having a slower release than plasmid alone [73]. Atelocollagen can be used as a less immunogenic alternative for collagen and has been loaded with plasmid DNA and siRNA. In contrast to collagen sponges [73], the release rate of plasmid from atelocollagen pellets is slow but can be increased by addition of glucose [83]. The pellets resulted in steady prolonged transgene expression *in vivo* up to 2 months [83]. siRNA delivery with atelocollagen accomplished gene inhibition *in vivo* up to 20 days [84,85].

The release from hydrogels has been modulated through modifying the hydrogel or vector chemistry. Non-specific interactions between plasmid and a collagen matrix were promoted by modifying the collagen with poly-L-lysine (PLL) [86]. A high molecular weight PLL enhanced plasmid binding to the collagen and increased plasmid retention. Gelatin has been cationized with ethylenediamine to bind DNA within the gel. *In vivo* release demonstrated greater retention of DNA within the gel, a release rate determined in large part by the water content and extent of crosslinking, and persistence of DNA in the animal for 7–10 days [69, 87,88]. Alternatively, DNA complexes, which normally bind non-specifically to collagen, can be modified with a protecting copolymer that reduced interactions between the vector and material and enhanced release [73].

The innate ability of viruses to bind ECM proteins complicates the delivery of viral vectors from hydrogels formed from ECM components such as collagen [76]. However, collagen hydrogels have been employed for the delivery of viruses, and enabled persistent localized expression [89]. Adenoviruses, which have a high affinity for collagen, were encapsulated in a collagen hydrogel with greater virus retention at higher collagen densities [76,79], while canarypox viruses have been entrapped in gelatin sponges, with the porous sponge allowing

for a more rapid release. Implantation of the sponges supported transgene expression up to 96 days, with increasing levels for higher virus concentrations [90]. *In vivo* transgene expression induced by virus loaded gelatin matrices can be enhanced by increasing the gelatin concentration, but is limited up to 1.5% gelatin, as higher concentrations do not allow for uniform delivery by syringe [81]. Mixing collagen into the gelatin improved transgene expression but only for a total protein concentration less than or equal to 2%.

Hydrogels based on synthetic PEG have been extensively used for tissue engineering applications due to their flexibility and biocompatibility, and are being developed for localized gene delivery. PEG can be combined with additional subunits to alter its functionality and degradation rate, such as lactic acid, caprolactone, fumarate, and hyaluronic acid [69,70,91, 92]. Factors that increase the pore size or swelling ratio, such as PEG molecular weight and the extent of crosslinking, will normally increase the release rate [70], whereas factors that decrease the rate of degradation will slow the release [69]. More linear release kinetics can be obtained with slow degrading crosslinks, while a DNA burst can be induced using fast degrading crosslinks [91,92]. Incorporation of multiple degradable components with varied degradation rates could be employed to tune the release profile.

Viral vectors, non-viral vectors, plasmid, and siRNA have been incorporated into a variety of hydrogel materials, including collagen, gelatin, silk-elastin like polymers, chitosan, agarose, hyaluronic acid, fibrin, etc, that have been used as tissue engineering scaffolds (Table 1). Many of these gels can serve as an injectable, in situ, gel-forming depot, with different DNA release kinetics depending on properties such as the polymer density, DNA conformation, extent of crosslinking, swelling ratio, and the vector affinity for the material [93–95]. For viral vectors, scaffold encapsulation can stabilize the vector and minimize the immune response, both of which can contribute to the long-term expression [96]. For non-viral vectors, multiple studies have demonstrated the ability to control the release profile using various hydrogel parameters. However, a direct correlation between the hydrogel properties and the *in vivo* transgene expression remains to be determined and further studies are required.

4.2 Vector Immobilization

The immobilization of vectors to biomaterials, which has been termed substrate-mediated delivery, solid phase delivery, or reverse transfection, mimics the natural process of virus binding to extracellular matrix proteins [97,98]. Immobilization to the adhesive matrix co-localizes the vector and adhered cells [99,100], which can overcome mass transport limitations. Importantly, the vector can be immobilized to the scaffold surface following fabrication, thereby providing a method for gene delivery from scaffolds formed by processes that would normally inactivate the vector if they were encapsulated during fabrication.

The immobilization of DNA to the matrix may seem counterintuitive given the need for cellular internalization to achieve expression. Nevertheless, the relative affinity of the vector for the material must be modulated to maintain the vector locally, yet allow for cellular internalization. Molecular interactions between the vector and the polymer dictate whether the vector will be bound or released. Viral and non-viral vectors, which contain negatively charged DNA or RNA, potentially complexed with proteins, cationic polymers, or cationic lipids, interact with polymeric biomaterials through non-specific mechanisms, including hydrophobic, electrostatic, and van der Waals interactions that have been well-characterized for adsorption and release of proteins from polymeric systems (Figure 2) [101]. Non-specific binding depends upon the molecular composition of the vector (e.g., lipid, polymer, protein) and the relative quantity of each (e.g., ratio of amines on the polymer to phosphates in DNA (N/P)). Alternatively, specific interactions can be introduced through complementary functional groups on the vector and polymer, such as antigen-antibody or biotin-avidin, to control vector binding to the substrate. The effective affinity of the vector for the biomaterial is determined

by the strength of these molecular interactions, which may also be influenced by environmental conditions (e.g., ionic strength, pH), binding-induced conformational changes, or vector unpacking.

Hydrophilic materials provide more effective delivery relative to hydrophobic surfaces, and ionic surfaces did better than non-ionic surfaces. The surface composition was investigated using self-assembled monolayers (SAMs) on gold, which enable specific functional groups to be presented at the surface [102]. Hydrophilic, anionic surfaces provided for the most efficient binding and transfection relative to hydrophobic, or hydrophilic nonionic surfaces. Hydrophobic surfaces had similar adsorption as the hydrophilic, anionic surfaces, yet transfection was orders of magnitude less. More recently, the inclusion of PEG on the surface further increased expression relative to non-PEG surfaces, potentially by stabilizing the vector conformation [103]. Increasing exposure time of the surface to a vector solution leads to increasing quantities of immobilized vectors that are relatively stable on the surface [102]. This observation of hydrophilic surfaces can be translated to PLG scaffolds by protein-coating the scaffolds to increase their hydrophilicity and produce smaller complex sizes and more effective gene delivery [104]. Hydrophilic protein coated surfaces more effectively distributes the DNA among the cell population relative to the uncoated surfaces [42]. Alternatively, PLG can be modified to more effectively bind plasmid by incorporating cationic groups, such as PEI, cetyl trimethyl ammonium bromide (CTAB), and PLL, which can bind plasmid with an affinity that can be manipulated through the extent of modification [105–107].

An extension of the immobilization approach is the precipitation of vectors onto the scaffold surface. Calcium phosphates (CaP) have been deposited onto polymer scaffolds for bone tissue engineering, and separately have been used as transfection reagents [108,109]. For immobilization, plasmid is co-precipitated with CaP as nanocrystalline CaP phases onto a surface [110]. The CaP vector is relatively non-toxic to cells, and the levels of transgene expression depend on the Ca/P ratio (maximal expression at ratio of 100–300) and the mixing conditions [109]. In the maximal expression conditions, 90% of the plasmid was bound to CaP, while 78% of the CaP bound plasmid actually resulted in complexed DNA in a size ranging between 25 and 50 nm. The removal of magnesium and addition of surplus calcium resulted in higher DNA precipitation and retention on the surface relative to the standard simulated body fluid [110]. The mineral solution lacking magnesium resulted in plate-like nanocomposite surfaces, consisting of many nanocrystals, in contrast with the larger spherical features (500 nm diameter) formed in the presence of Mg^{2+} . Even though all surfaces demonstrated cellular transfection, the mineral solution without Mg^{2+} resulted in the highest levels of gene transfer. A fast release was observed with precipitation in normal body fluid, and a greater surface retention was observed with the magnesium lacking and calcium enriched compositions. This suggests the occurrence of multiple mechanisms of gene delivery into the cells: DNA entering the cells after their release from the surface or smaller DNA particles directly ingested by the cells.

The specific binding of vectors to pre-fabricated scaffolds, which has been achieved using biotin-avidin and antibody-antigen binding, is an alternative to the above mentioned non-specific immobilization strategies. HA-based hydrogels with surface associated neutravidin was able to bind biotinylated PEI/DNA complexes [111]. Similarly, collagen and polyurethane films modified with anti-adenovirus antibodies were employed to binding adenoviruses, with [99] or without [112] the avidin/biotin chemistry. The specific binding was able to achieve dense surfaces of gene vectors that remained attached to the scaffold more effectively relative to the non-specific binding [99].

More recently, vectors have been immobilized in layers on surfaces using a technique termed polyelectrolyte layering [113–116]. Nanoscale films are created with alternating layers of

negatively charged plasmid and positively charged polymers that are deposited on materials such as stainless steel stents [113–116]. The duration of plasmid release can be manipulated through changing the degradability, conductivity, or electrochemistry of the polymer layers or substrate, while the released DNA is able to transfect cells without the aid of additional transfection agents [114,115]. Multilayered polyelectrolyte films have the ability to create highly tunable surfaces, and with the appropriate design of each layer, may potentially enable the sequential release of multiple gene sequences.

5 Cellular responses and transgene expression

A recurring theme throughout the gene therapy community has been that the *in vitro* results do not necessarily correlate to *in vivo* performance. Many *in vitro* studies are performed with cells cultured on tissue culture polystyrene, with the vector added to the culture media. Gene transfer occurs following vector transport to the cell surface, internalization, endosomal escape, vector unpacking, and nuclear transport. However, *in vivo*, these steps may present significantly different obstacles. Several recent reviews have described how viruses have evolved mechanisms and are being manipulated to overcome these hurdles [9], and how non-viral vectors are increasingly designed with a vector backbone and modular functional groups (e.g., endosomal escape, nuclear localization) that enable the vector to interface with cellular processes [15]. Though these strategies focus on manipulating the vector for enhanced delivery, a difference between *in vitro* and *in vivo* performance may lie with the cells that are the targets for transgene expression and their environment. The cell lines used with many *in vitro* studies may have adapted to *in vitro* culture and developed an enhanced ability to internalize and transport vectors relative to primary cells. In addition to differences in cells, the microenvironment *in vitro* and *in vivo* substantially differs. The microenvironment surrounding cells could potentially influence the processes involved in gene transfer. To design the environment, the vector properties will need to be considered, as various vectors may be limited at different steps in the trafficking process (e.g., lipoplexes at internalization, polyplexes at endosomal escape) [34]. The following paragraphs discuss the cellular response within the local environment of the scaffold, and how the design of the scaffold may influence cellular processes that are relevant to gene delivery.

5.1 Inflammation and vector activity

Biomaterial implantation induces a foreign body response, which together with the host response to the vector, may influence gene transcription. The foreign body response following biomaterial implantation has been described according to multiple stages: acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction, and fibrous capsule development [117,118]. Neutrophils are the first cells to arrive, followed by macrophages. Infiltrating macrophages could potentially have beneficial functions, such as secretion of angiogenic and other growth factors that promote cell proliferation, vascularization, and wound healing [119]. Fibroblasts arrive to contribute to either wound healing or fibrous capsule formation, and may be transfected by localized gene delivery [62]. The cell proliferation induced at the implant site may act to promote nuclear accumulation of the vector [120]. A long-term persistence of macrophages, however, can be problematic due to their production of oxygen radicals or formation of foreign body giant cells [119]. This inflammatory response to the material can be accompanied by an inflammatory response to the vector that can result in cell lysis or phagocytosis. Cells infected or transformed with viral vectors stimulate apoptosis by cell-autonomous mechanism or extracellular signals derived from other cells. Infection can also induce the expression of molecular markers that flag the affected cells for killing by for example natural killer cells. Unmethylated CpG motifs on plasmid can trigger antigen-specific immune defenses [7,33,121,122]. In summary, the inflammatory response induced by the biomaterial and the gene vectors has the potential to

degrade, clear, or inactivate the vector, inhibit promoter activity, attenuate gene transcription, eliminate transfected cells, or prevent repeated dosing.

The design of the vector and scaffold may be able to reduce the inflammatory response and thus enhance gene expression. For example, macrophage invasion is dependent on the type of cell adhesion molecules [119], and hydrogels may produce a more mild foreign body response compared with porous sponges. The material may be able to modulate some of the inflammation stemming from the vector, as the material could prevent some antibody responses. Alternatively, the vector design can modulate or reduce the inflammatory response and thus enhance transgene expression, such as by removing CpG motifs from plasmids [33, 121,123] or incorporating the pharmacological agent dexamethasone into a non-viral vector transfection reagent [124].

5.2 Microenvironment and gene transfer

Relative to the standard *in vitro* culture, the *in vivo* cellular microenvironment can have different cell morphology or proliferation, matrix mechanics, or fluid transport that can influence multiple aspects of gene transfer. Tissue engineering scaffolds represent a bridge between the *in vitro* two-dimensional (2D) culture on polystyrene and the *in vivo* native three-dimensional (3D) environment. Signaling pathway activity, and ultimately the cellular response, can differ depending on whether the cells are present in a 3D or 2D environment. For example, autophosphorylation levels of focal adhesion kinase (FAK) are down-regulated in 3D cultures compared to 2D controls, even though phosphorylation levels of other adhesion-activated proteins remain similar, suggesting their independence from the FAK-regulated pathway [125]. Relative to culture on 2D substrates, cells cultured in 3D matrices exhibit a higher rate of cell adhesion, have a morphology that is consistent with *in vivo*, and adhere to the matrix through different sets of integrins [126]. Importantly, the matrix plays a fundamental instructive role in cell polarity and maintaining cell-cell interactions, which enable the formation of tissue structures [127–129]. Scaffolds can therefore provide a means to investigate and manipulate the contribution of cellular processes to gene transfer. The comparison of 3D matrices with 2D surfaces demonstrated an increased level and duration of transgene expression on the 3D matrix [130], which may result from the changes in the cellular microenvironment rather than simply differences in gene availability.

The mechanical properties differ between many 2D and 3D systems and are increasingly recognized as significantly regulating cellular processes [45,48,131]. Matrix elasticity influences a range of cellular processes, such as contractility, migration, differentiation, and potentially organogenesis [45,131], and gene delivery has been investigated as a function of this elasticity [132]. Cellular internalization of polyplexes and increased polyplex dissociation was observed on hydrogels with greater rigidity, as increasing the matrix elastic modulus (E) of alginate from 20 to 110 kPa increased transgene expression for 400%. More generally, a range of materials with varying mechanics was investigated with a similar trend observed. The role of the matrix mechanics was attributed to an increased rate of cell proliferation on the more rigid matrices, as cell proliferation can enable nuclear accumulation of the delivered plasmid [120]. Though gene transfer may be enhanced on more rigid materials, delivery on softer materials must also be developed since softer materials may be necessary to promote the desired cellular responses [131].

In addition to the proliferation, the microenvironment can influence endocytosis through the adhesion surface. Endocytosis occurs through multiple pathways, such as macropinocytosis, clathrin-mediated endocytosis, and caveolar endocytosis, with the particle size being the determining factor for the internalization pathway [133]. Particles with diameters less than 200 nm were internalized via clathrin-mediated endocytosis, while particles of 500 nm were mainly internalized through caveolae-mediated endocytosis. Both pathways are involved in the

internalization of immobilized complexes, however, inhibitors to the caveolae-mediated endocytosis more dramatically reduced the intracellular levels of plasmid [134]. Additionally, cells cultured on fibronectin had the highest levels of transgene expression, which may coincide with the fibronectin internalization preferentially through caveolae [134].

Finally, fluid transport at the material site may contribute to vector internalization. Fluid flow can impart a shear stress that will augment mass transport [135–137]. Cellular internalization of lipoplexes was increased more than nine-fold at 2.3 dyn/cm² compared to the static condition, but decreased again for higher shear stresses due to disruption of lipoplex–cell binding [138].

5.3 Transgene expression and tissue formation

The fundamental relationship between gene delivery, transgene expression and tissue formation remains a significant challenge in the design of tissue engineering scaffolds. Gene delivery can stimulate local protein production capable of activating autocrine and paracrine loops that may play important roles in tissue development and physiology [139]. Genes can be delivered *in vivo* that i) encode for the desired protein(s), or ii) encode for transcription factors that induce expression of the desired protein(s). Most approaches have focused on the delivery of genes encoding for growth factors (e.g., vascular endothelial growth factor (VEGF), bone morphogenetic protein (BMP)), in which case transfected cells act as bioreactors for localized secretion thereby conditioning the environment [60,140]. Delivery of DNA encoding for transcription factors, or active variants thereof, may provide a different functionality. These factors function intracellularly, and may provide a potent approach to direct differentiation of stem or progenitor cells towards a specific lineage. They can be employed to induce the expression of tissue inductive factors, similar to delivering a gene encoding for these factors. Zinc-finger protein (ZFP) transcription factors have been engineered that regulate the endogenous gene encoding VEGF-A [141], and led to induced expression of VEGF-A resulting in an enhancement of both angiogenesis and wound healing. Delivery of a gene encoding for a stabilized form of hypoxia-inducible factor-1alpha (HIF-1alpha), which lacks the oxygen sensitive degradation domain, induced expression of VEGF-A and promoted the formation of a mature vascular network that was not observed by direct delivery of the protein [142]. Transcription factors activating an endogenous genes can induce expression of all splice variants [141]. Alternatively, a single transcription factor has the potential to regulate multiple separate genes, which may prove advantageous for tissue regeneration.

6 Challenges and opportunities

The next major challenge for DNA releasing scaffolds is connecting localized transgene expression with the requirements for tissue formation. Natural tissues develop as a result of complex temporal-spatial patterns in the expression of various cytokines, growth factors, and matrix molecules in the cellular microenvironment [143]. Both ligands and receptors exhibit distinct expression profiles that correlate with a diverse range of developmental functions. Engineering mature and functional tissues will depend on the ability to direct cells into spatially complex arrangements on length scales ranging from micrometers to centimeters and guide dynamic organization, maturation, and remodeling of cells [144]. The application of gene delivery to biomaterials and regenerative medicine may provide the technology necessary to recreate these patterns that may be necessary for complex tissues, such as blood vessels and nerves. The following sections address the current capabilities and identify opportunities and challenges for the application of localized gene delivery to regenerative medicine.

6.1 Concentration and duration

The concentration and duration of activity for tissue inductive factors at the regenerating tissue site are critical parameters involved in promoting cellular processes that lead to the formation of mature tissues [145], as excessive concentrations or inappropriate expression of growth factors may lead to abnormal tissue formation. Non-viral vectors delivered from the scaffold can induce localized transgene expression with a duration that may be significantly longer than the duration of vector release [4,66,139]; however, the precise relationship between delivery and duration of expression must be developed. Low dosages of DNA released from a scaffold had shorter durations of expression relative to larger dosages, with modest differences in the expression level [60]. Modifications to either the vector or scaffold properties will likely affect the duration of expression through either controlling the duration of delivery or altering the gene silencing mechanisms.

The number of cells expressing the transgene and the extent of transgene expression by the cells can impact tissue formation. High levels of VEGF secretion by retrovirally transduced myoblasts induced the growth of abnormal blood vessels, which would likely regress over time [146]. Decreasing the number of cells transplanted, which decreased the total dose of VEGF, served to reduce the region in which abnormal blood vessel formation was observed, while transplantation of cells that were selected for low VEGF expression resulted in the formation of normal, mature vascular structures. These results illustrate that a discrete threshold in micro-environmental concentration determines either normal or aberrant tissue formation, and indicates that gene delivery strategies must provide an appropriate concentration of tissue inductive factors.

6.2 Spatially patterned gene delivery

The spatially patterned delivery of DNA encoding for tissue inductive factors may be able to spatially direct cellular processes in order to recreate complex tissue architectures. For example, an injury at the bone/cartilage interface requires that both bone and cartilage be restored. Transplanted or endogenous progenitor cells have been shown capable of forming either bone [75,147,148] or cartilage [149,150] when presented with the appropriate factors. A scaffold that delivers bone specific and cartilage-specific factors within the defined regions of the scaffold may facilitate regeneration across this interface. The ability to release multiple factors from the scaffold, with spatial control, may be particularly important for the ultimate application of stem cell technology to regenerative medicine. These cells have the potential to differentiate down multiple pathways, and the environment must coordinate differentiation with the complexity of the tissue architecture.

Several methods have been developed *in vitro* to spatially control gene delivery and obtain patterns of gene expression (Figure 3). In the first method, vectors are deposited homogeneously across the surface, and the surface is manipulated to support cell adhesion in defined regions. This approach was employed to transfect and align cells [111]. As an alternative to manipulating cell adhesion, the deposition of the vector can be controlled. The spatially controlled deposition of gene therapy vectors can be achieved by several methods, such as spotting, printing, microfluidics, or pinning (i.e., the aqueous vector solution wets the hydrophilic but not hydrophobic region) [151–156]. The deposition procedure must retain vector activity, while the vector-material interactions must maintain the vector locally. Patterned transfection will be achieved only if effective concentrations of vector are present within the pattern and not outside the pattern.

Spatially controlled binding of gene vectors and transgene expression was achieved, resulting in directed neurite outgrowth [155,156]. Patterned stripes of vectors in widths as small as 100 μm were achieved using microfluidic deposition [156], resulting in transfection of cells only

within the pattern, with different pattern widths requiring varying amounts of DNA to achieve maximal transfection. A neuronal co-culture model was performed consisting of neurons and accessory cells, with the accessory cells transfected to induce the secretion of nerve growth factor (NGF). Neuron survival was observed primarily within the pattern, and the extending neurites remained within the pattern. Adenovirus has been similarly been patterned onto surfaces, with patterned expression of brevican inducing neurite extension between, and not within, the brevican-rich regions [155].

These reports illustrate the potential for spatially controlled gene delivery, yet a significant challenge remains in extending these techniques to 3D scaffolds. Microfabrication techniques, such as three-dimensional printing, laser ablation, and similar procedures are being developed to create scaffolds with controllable feature size, patterned topography, and the ability to reproduce the complexity of several tissues [157–160]. Inkjet printing, for example, has been applied to pattern and control cell attachment [161]. Merging these technologies with the gene therapy vectors may enable the extension of these 2D patterning and microfabrication strategies to 3D scaffolds.

6.3 Concentration gradients

Patterning of vertebrate tissues may be controlled by discrete cellular responses to different concentrations of diffusible inductive factors. Spatial gradients in the expression of various cytokines, growth factors, and matrix molecules are a characteristic feature of many developing tissues [143,162,163]. Gradients in the concentration of growth factors can similarly direct cell migration, create patterns of cellular differentiation among a progenitor cell population [164–167], and direct tissue organization into complex structures, such as branching networks of vascular or nervous systems. Both ligands and receptors exhibit distinct profiles of spatial expression that correlate with a diverse range of developmental functions. For neuronal development, spatial gradients have been implicated in the patterning of cell types [167]. In the neural tube, it is likely that Sonic hedgehog diffuses from the floor plate cells and establishes a concentration gradient in the ventral neural tube. Relatively small changes in the concentration of Sonic hedgehog can elicit the generation of distinct neural cell types [164]. Alternatively, concentration gradients can also function to direct axonal extension. NGF, brain-derived neurotrophic factor (BDNF), and netrins have all been implicated as chemoattractants that direct neurite extension in different neuronal networks for the central and peripheral nervous systems [168–171]. Manipulating the location of transfected cells secreting diffusible factors and their level of transgene expression, or creating gradients of transfected cells [155] can be employed to create and control concentration gradients [155,156]. The ability to effectively create, control, and maintain concentration gradients *in vitro* and *in vivo* is still under development, and could provide a valuable tool to guide progenitor cell differentiation and organize tissue formation.

6.4 Temporal control

Tissue development can often be characterized as occurring in sequential phases. Bone formation has been described with three phases: proliferation, matrix deposition, and mineralization, with differential gene expression between the different phases [172]. Strategies that could induce the sequential expression of genes at the appropriate time could potentially enhance tissue formation. Obtaining temporal control over gene expression with multiple factors is challenging, as transgene expression does not directly correlate with the release profile [60]. Sequential delivery, however, could potentially be developed through modifications to the biomaterial design. Encapsulation strategies may entrap one vector in a rapidly degrading polymer and a second vector in a slow degrading polymer [52]. The polyelectrolyte layering strategy could have one vector close to the material that would be released after different vectors located at the exterior [116]. Strategies under development for

drug delivery may also be adapted for the differential delivery of gene therapy vectors, such as stimuli-triggered release (e.g., chemical or electrical stimulus) [173–175]. An alternative approach to manipulating gene expression temporally is to manipulate the vector. Inducible or tissue specific promoters could be employed with the established delivery systems mentioned herein to provide expression at the appropriate time and in a desired cell population in response to promoter activity or external stimuli [176–179].

6.5 Multiple factor delivery

Tissue morphogenesis and regeneration are typically driven by the concomitant action of multiple factors [180]. The synergistic effect of growth factors has been reported for many developmental processes, such as angiogenesis [181], where mature blood vessels form by the combined action of VEGF and platelet-derived growth factor (PDGF) to form stable vessels. Although VEGF is able to initiate angiogenesis, PDGF promotes vessel maturation via recruitment of smooth muscle cells to the developing endothelium [182]. PLG scaffolds releasing both VEGF and PDGF formed a mature vascular network within and around the scaffolds [183]. As another example, spinal cord consists of multiple nerve tracts (e.g., corticospinal, rubrospinal), which respond to distinct neurotrophic factors [184]. Regeneration strategies may need to deliver multiple factors to target the different tracts, and these factors may need to be localized within specific regions of the spinal cord [185]. In addition to the lack of stimulatory factors that promote regeneration, an excess of inhibitory factors hinders nerve growth [186,187]. Regeneration strategies may need to provide both the stimulatory factors and block or degrade the inhibitors. The complexity of the biological systems combined with presence of multiple barriers to tissue formation may require multiple factors to be delivered, with a delivery technique that is spatially and temporally regulated. Layer by layer films have recently demonstrated to achieve sustained, multiagent delivery by controlling interlayer diffusion [188], and could potentially be used to deliver multiple gene vectors.

7 Conclusion

The combination of tissue engineering and gene delivery provides a promising core technology for regenerative medicine and functional tissue repair and replacement. However, many challenges remain in developing delivery systems with the necessary efficiency and control. The tissue engineering scaffolds function as a substrate for cell infiltration, organization, and differentiation, while gene therapy can provide the essential tissue inductive factors. Progress has been made towards the major challenge of achieving gene delivery within a tissue engineering scaffold, though the design principles for the materials and vectors that produce efficient delivery require further development. Nevertheless, the advances in obtaining transgene expression with the scaffold have identified opportunities for controlling expression temporally and spatially and to determine the best practices for promoting tissue formation with transgene expression. Finally, we note that the gene expression is occurring within the scaffold microenvironment, and that stimulus to promote tissue formation by the transgene expression must be considered within the context of the microenvironment, which contains architectural, mechanical, chemical, and biological cues. All aspects of the microenvironment created by the scaffold must be considered for its role in promoting tissue formation, and continued development of vector releasing scaffolds holds great promise for numerous applications in regenerative medicine.

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A) Hydrogels

B) Hydrophobic polymer scaffolds

Figure 1.

The rate of DNA release from hydrogels (A) and scaffolds (B) results from a combination of diffusion and material degradation. An initial burst may occur for vectors that are surface associated. Degradation of the biomaterial opens paths through the scaffold to allow for vector diffusion through the pores.

Figure 2.

A) Viral and non-viral vectors can interact with polymeric biomaterials through non-specific mechanisms, including hydrophobic, electrostatic, and van der Waals interactions, and more specific interactions, regulated by avidin/biotin or antibody/antigen interactions. B) An immobilized vector can be released by desorption for non-specifically immobilized complexes, or covalently coupled vectors may require degradation of either the tether or the biomaterial for release.

Figure 3.

Spatially patterned transgene expression:

A) Pattern created by controlled cell adhesion in a micropatterned hyaluronic acid-collagen hydrogel, scale bar: 20 μm [111], Reprinted from *Biomaterials*, 26, T. Segura, P. H. Chung, L. D. Shea, DNA delivery from hyaluronic acid-collagen hydrogels via a substrate-mediated approach, 1575–1584, Copyright (2005), with permission from Elsevier.

B) Pattern created by pinning aqueous solutions to hydrophilic regions on SAMs. Image demonstrates transfection within two circles. scale bar: 500 μm [153], Reprinted from *Acta Biomaterialia*, 1, A. K. Pannier, B. C. Anderson, L. D. Shea, Substrate-mediated delivery from self-assembled monolayers: effect of surface ionization, hydrophilicity, and patterning, 511–522, Copyright (2005), with permission from Elsevier.

C) Pattern created by lipoplex deposition on a polystyrene surface using soft lithography microfluidics, the microchannels are respectively 500, 250, and 100 μm wide, scale bar: 500 μm [156]. Reprinted from *Molecular Therapy*, in press, T. L. Houchin, K. J. Whittlesey, L. D. Shea, Spatially patterned gene delivery for localized neuron survival and neurite extension, Copyright (2007), with permission from Nature.

μm

Table 1

Hydrogels designed for DNA delivery, made of different biomaterials and assessed for different applications

Gene vector	Material	Application	Ref.
adenovirus	Collagen	wound healing myocardial tissue	[76,77,79,99]
adenovirus	collagen/gelatin	wound healing	[81]
canarypox virus	Gelatin	tumor inhibition	[90]
adenovirus	Fibrin	<i>in vitro</i>	[96]
adenovirus	Polyurethane	heart valve cups intra-aortic implants	[112]
plasmid	Collagen	bone regeneration	[74,75]
plasmid	Collagen	wound healing optic nerve regeneration	[73,75,80,82,86]
DNA complexes	Collagen	subcutaneous perivascular	[73,86]
plasmid	atelocollagen (glucose)	Intramuscular	[83]
PEI/DNA	PEG-HA	<i>in vitro</i>	[111]
plasmid	PEG-HA	<i>in vitro</i>	[70]
plasmid	pluronic (HA)	<i>in vitro</i>	[93]
plasmid	PEG-PLA-PCL	<i>in vitro</i>	[91,92]
plasmid	PEG/fumarate	<i>in vitro</i>	[69]
plasmid adenovirus	SELPs	Intratumoral	[94,95]
plasmid	Gelatin	Intramuscular	[87,88]