Polymeric scaffolds for gene delivery and regenerative medicine

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The purpose of this chapter is to introduce the fundamentals behind tissue engineering and gene therapy and the materials utilised in both fields. The application of gene therapy using these materials as scaffolds in regenerative medicine is then explored.

1.1 Tissue Engineering

Tissue engineering offers the possibility to create tissues in vitro and replace failing or malfunctioning organs in vivo [1, 2]. There are inherent difficulties in current organ and tissue transplantations strategies because acute donor shortages have stranded a significant number of patients on the waiting list. This list has increased from 19,095 patients in 1989, to 74,800 by February 2001 in the USA alone [3, 4]. Furthermore, those patients fortunate enough to receive transplantations may require immunosuppression therapies for the rest of their lives to defend against the associated risks of rejection. The lack of donor tissue availability and donor site morbidity further hampers transplantation of tissues. Replacement with mechanical devices is limited by an increased risk of inflammation and infection. Mechanical devices also lack the mechanism for self-repair and such devices will not grow concurrently with the patient [4-6].

The potential impact of tissue engineering from both a therapeutic and an economic standpoint is enormous. Organ failures and diseases are increasing with human life

expectation [7, 8]. The success of alternative donor sources from other species like the pig still remains in doubt, because of potential transferable diseases such as the pig endogenous retrovirus [3]. One form of tissue engineering involves the creation of tissues by transplanting cells removed from the patient or a close relative and seeded into an implant which serves as both a substrate and a physical support for the isolated cells. Using cells from the same genotype should avoid many of the problems associated with immune rejection of foreign tissue. As these cells are capable of proliferation, a small number of harvested cells can be expanded to a sufficient cell mass to replace the organ function. Therefore, it is not required to sacrifice the entire organ of the donor [9].

Tissue engineering can be classified into two main areas: *in vivo* and *in vitro*. Once a tissue can be generated on a large scale *in vitro*, it can then become a viable supply of new tissue for patients. *In vitro* tissue engineering thus requires a specifically designed environment for regeneration. This is in contrast to the *in vivo* approach, where the living body provides the microenvironment with the appropriate biochemical and biomechanical stimuli for tissue regeneration. To date, most effort has concentrated on creating tissue with singular cell types *in vitro* and thus the formation of only simple avascular structures. Therefore, *in vitro* tissue engineering, whilst theoretically desirable, has concentrated on tissues such as dermis, epidermis and articular cartilage [10, 11].

Tissue regeneration *in vivo* attempts to achieve natural regeneration of tissues and organs by harnessing the natural healing process of the body. For large defects, it is necessary to use a scaffold as support for the tissue to grow. The scaffold may be used either with or without cell seeding prior to implantation. Scaffolds without cells just serve to imitate the natural extra-cellular matrix (ECM) of the body. Tissue regeneration is, in this case, dependent on

the ingrowth from the surrounding tissue in a process, which is known as tissue induction. Infiltration of progenitor or stem cells from the site of implantation into the scaffold also plays an important role. Vascularisation of a scaffold is a typical example of tissue induction [12].

Tissues engineering using cell-seeded scaffolds have been applied to tissues such as liver [13, 14], blood vessels [15, 16], nerve [17], skin [11], cartilage [18] and bone [19]. Significant challenges to this approach include the design and fabrication of a suitable scaffold able to promote cell adhesion and to support cell growth, proliferation and differentiation and induced formation of natural tissue. In many cases, biocompatible, biodegradable polymers are used to either induce surrounding tissue and cell ingrowth or to serve as a temporary scaffold for transplanted cells to attach, grow, and maintain differentiated functions [20].

The hypothesis that cells on polymer scaffolds could give rise to organised tissue originates from the following biological observations:

1. Most tissues undergo constant remodelling [21].
2. Dissociated mature cells can reorganise themselves into their native histological structures when placed in ideal cell culture conditions [22].
3. While isolated cell populations are capable of histological reorganisation, this is limited when they are delivered as a cell suspension because they lack a template to guide restructuring [23].
4. The quantity of tissue for implantation is restricted by diffusion requirements for gas and nutrient exchange [22].

The strategy of tissue engineering generally involves the following steps (Fig. 1): Depending on the target organ, a suitable cell source is identified, isolated, and produced in sufficient numbers [3, 24]. A biocompatible material that can be used as a cell substrate or cell-encapsulation material is isolated or synthesised and processed into the required shape [9]. The material is seeded uniformly with cells, which can then be grown in a bioreactor [25] and finally the material-cell construct is placed into the target *in vivo* site, where depending on the site and the structure, vascularization may be necessary [23].

1.2 Gene delivery

Gene therapy aims to treat diseases by delivering a foreign gene or DNA to the target cell or tissue for the expression of a desirable gene product, which is a protein [26]. It has been proposed for treating inherited single-order genetic disorders, cancer, cardiovascular diseases and infectious diseases, among many others [27]. For example, the demonstration of efficient DNA delivery with emulsion-coated stents on arterial walls in pig-stent angioplasty studies highlights the potential for gene therapy in cardiovascular diseases [28]. Other examples include delivering DNA that encodes for normal clotting factors which could cure haemophilia [29], delivering the p53 gene to colorectal cancer cells for enhanced responsiveness to antiangiogenic therapy [30], or in a genetic immunization approach transferring HIV DNA plasmids to AIDS patients in order to stimulate a strong T-cell mediated immune response [31].

Other than retroviral transfection that can integrate the foreign gene into the genome of the host, other modes of transfection can only produce transient foreign gene expression. As

such, many gene therapies are unsuitable for diseases that require prolonged expression of the gene product. Repeated doses of viral vectors cause immunologic and toxic side effects [32]. Sustained release of genetic vectors may prolonged the transgene expression. However, like the delivery of other delicate bioactive agents, controlled release of bioactive genetic vectors is a significant challenge. Before describing gene delivery from tissue engineering based scaffolds, it would be informative to discuss the mechanisms of gene delivery, introduce some of the materials commonly used in both gene delivery and tissue engineering and describe the ideal characteristics of a scaffold in tissue engineering applications.

1.2.1 Mechanism of transfection

The mechanism with which cells take up DNA as illustrated in figure 2 is complicated and has been the subject of numerous reviews [26, 33-35]. Typically negatively charged DNA is complexed with cationic vectors. With large particles, the route of uptake is predominantly phagocytic. If the particles are less than 150-200 nm in diameter, then the complexes can be taken up by endocytosis [36]. This involves the particles placed in pits that form endocytic vesicles by pinching away from the cell surface. These vesicles fuse with lysosomes followed by lysis. Receptor-mediated endocytosis can be promoted by the use of cell-specific ligands, such as transferrin, to target receptors on the surface of clathrin-coated pits that subsequently form the endosomes [37, 38]. The low pH and harsh enzymatic environment of the endosomes and lysosomes however would degrade the DNA if it is not shielded by a vector. Once escaped into the cytosol, the conventional wisdom is that the DNA should be dissociated from the vector prior to nuclear entry, through pores that are typically 10-50 nm in diameter. However, there is evidence that the DNA/vector complex

can enter the nucleus, probably through porous nuclear membrane as the cell undergoes mitosis. Whilst many of these steps have been identified as barriers to efficient transfection, the high efficiency with which naked DNA transfects muscle in comparison to vector-DNA conjugates/complexes highlights the immense complexities of gene delivery. We now describe some polymeric materials that have been investigated for scaffolds in tissue engineering and in gene delivery.

1.3 Materials for gene delivery and scaffolds in tissue engineering

The first step in the design of a gene delivery vehicle or a scaffold for cell transplantation is the choice of a suitable material. The material must be biocompatible and preferably biodegradable to avoid the risk of complications that may be associated with the long-term presence of a foreign material in the body. Over the last century, materials such as metals, ceramics and polymers have been extensively used for surgical implantations [39]. Metals and ceramics have contributed to major advances in the medical field, particularly in orthopaedic tissue replacement [40]. These materials, in comparison to polymers, however are difficult to process and lack biodegradability. In the case of gene delivery, materials should ideally protect the DNA during transport through the cell before decomplexing, or unpackaging, to release the DNA for nuclear entry. The use of polymers can be sub-divided into two categories: natural polymers and synthetic polymers.

1.3.1 Natural derived polymers

Proteins and sugars derived from natural extracellular matrices, such as collagen and glycosaminoglycan, have been used to repair nerve [41], skin [42], cartilage [18] and bone

[43]. Chemical crosslinking by glutaraldehyde has been proposed to control the stability and degradation rate of these matrices, whereas porosity has been controlled using both chemical and physical techniques [44]. However, collagenous scaffolds that have been crosslinked with glutaraldehyde can exhibit immunogenicity, calcification and fibrous scarring during long-term implantation [9]. Chemical crosslinking in gene delivery applications could also bind DNA so strongly that decomplexation does not occur or damage the bioactivity of the DNA [27]. Other collagen derivatives such as atelocollagen have however shown great promise as gene delivery devices [45].

Gelatin is a material that has shown potential in gene delivery applications. Our group has demonstrated the application of gelatin nanospheres for controlled gene delivery [46-48]. DNA release is controlled by the degree of crosslinking and stimulation of receptor-mediated endocytosis could be achieved by the covalent attachment of transferrin to the gelatin. This material has several advantages in gene delivery in that the gelatin nanosphere protects the DNA against degradation in serum and can be used for co-delivery of other biological agents such as chloroquine. Gelatin sponges seeded with adult mesenchymal stem cells and cultured in TGF-beta 3 supplemented media have produced cartilage-like extracellular matrix. *In vivo*, the scaffold was found to have good biocompatibility and immunological properties [49]. This suggests that gelatin has substantial potential as a gene delivery scaffold for tissue engineering.

Numerous other natural materials have found wide use in tissue engineering applications. Chitosan is a natural polysaccharide, whose structural characteristics are similar to glycosaminoglycans. Chitosan is highly soluble in an acidic environment and insoluble under neutral conditions. It has been used in a variety of biomedical applications, such as

haemodialysis membranes, drug and DNA delivery systems, artificial skin, orthopaedic and dental coating materials [50-54]. Chitosan has been demonstrated to be neither toxic nor haemolytic with strong protection against nuclease degradation [55, 56]. Administration of chitosan intravenously does not result in accumulation within the liver [57]. An example of its application has been with oral allergen-gene immunization with chitosan-DNA nanoparticles. The nanoparticles were effective in modulating murine anaphylactic responses indicating strong potential for prophylactic utility in treating food allergies [58]. The addition of cell targeting proteins such as transferrin to the chitosan-DNA complexes has also been shown to enhance reporter gene expression [50]. In specific cells, chitosan particles in the 50-100 nm size ranges have been shown to produce higher gene expression than PEI [59]. The degree of deacetylation can further be used to optimise transfection efficiency [60]. Chitosan is thus a strong candidate for effective gene delivery and tissue engineering.

Alginates are water-soluble polysaccharides that have the ability to form crosslinked gels in the presence of multivalent ions thus providing potential for a number of applications in gene delivery and tissue engineering [61]. Alginate is widely available (isolated from seaweed), readily forming a gel via calcium crosslinking, and exhibits reasonable biocompatibility. For example, alginate has been utilised in DNA vaccine based therapies [62]. Mucosal immunization using LacZ encoding DNA entrapped within alginate was shown to produce significant immune responses. However, a disadvantage in its use in tissue engineering applications is that the calcium ions on which gelation is dependent can be lost in ionic exchange either in culture or in vivo [23].

1.3.2 Synthetic polymers.

There are a wide variety of synthetic polymers that have been investigated for biomaterial and tissue engineering applications. Poly (vinyl alcohol) (PVA), poly (N-isopropyacrylamide) (PNIPAAm) and its derivatives have for example, been shown to have great potential as delivery vehicles for cartilage and the pancreas. The non-degradable crosslinks and the toxic crosslinking molecules used with polymers such as PNIPAAm however diminish their appeal [63-65].

The use of biodegradable materials has proven to be immensely important in medical applications over the last three decades. Polymers prepared from glycolic acid and lactic acid have found a multitude of uses in the medical industry, beginning with the biodegradable sutures first approved in the 1960s [9]. Since that time, diverse products based on lactic and glycolic acid and on other materials such as poly (ε-caprolactone) homopolymers and copolymers have been accepted for use as medical devices [66, 67]. PLGA has subsequently become established as a material with significant potential for controlled release of plasmid DNA [68, 69]. PLGA has also been used for controlled release of recombinant adenoviruses [70, 71]. Release can be achieved over periods longer than 10 days with significantly reduced immunogenicity *in vivo* [70, 72]. Sustained release of the adenovirus from the microspheres resulted in greater than 45 fold reductions in anti adenovirus titers in comparison to direct treatment of the adenovirus.

Other polymers that have been extensively investigated include polyphosphoesters [73], polyorthoesters [74], polyphosphazenes [75] and other biodegradable polymers [7]. The degradation mechanism varies with each of these polymer types. For example, polyorthoesters can be surface-eroding, while polyesters degrade in bulk. The degradation of polyphosphazenes can be controlled by changes in the structure of the side-chain rather

than the backbone as with the polyesters, whilst the properties of polyphosphoesters can be manipulated by adjusting either the backbone or the sidechain [73]. Polyanhydride derivatives such as copolymers of fumaric and sebacic acid (poly FA:SA 20:80) have found utility in both tissue engineering and gene delivery applications [76]. Other derivatives such as Poly [α-(4 aminobutyl) L glycolic acid] (PAGA); a biodegradable analogue of poly l lysine have good cytotoxicity characteristics and has been demonstrated to produce significant cytokine gene expression of mRNA and proteins *in vitro* and *in vivo* [77, 78]. Polyphosphoesters (PPE) and its derivatives have been utilised for biodegradable scaffolds in tissue engineering and controlled delivery of growth factors and DNA [73]. For example, PPE has been used to form nerve guide conduits and controlled release microspheres to provide prolonged site-specific nerve growth factor [79]. A derivative of PPE, poly(2-aminoethyl propylene phosphate) (PPE-EA) has achieved efficient gene expression and with tissue response better than either polyethylenimine or poly-L-lysine in mouse muscle [80].

The molecular properties of an increasing number of synthetic polymers such as molecular weight, molecular weight distribution, composition and molecular architecture can be manipulated to modify their physico-mechanical properties. Given the high processing ability for synthetic polymers, it is therefore possible to have porous materials with well-controlled microstructure and good mechanical properties.

1.4 Design criteria for polymer scaffolds in tissue engineering.

There are a number of biological, physical and chemical features desirable for the implementation of scaffolds for cell transplantation and tissue ingrowth. These are broadly split into five main categories:

1. Biocompatibility and/or cell-interactive properties
2. Porosity
3. Biodegradability
4. Mechanical compatibility
5. Controlled release function in the scaffold.

1.4.1 Biocompatibility and cell-polymer interactions

In vivo, or in culturing of cells in serum media, cells recognize synthetic materials through a complex protein layer, which forms immediately on the material upon contact with body fluids. This relation between material properties and cellular responses, mediated by the intervening protein layer, has complicated the development of biomaterials. Before the advent of biodegradable materials in the use of surgical procedures, polymer implants were intended to remain inert, thus unaffected by reactions with the surrounding tissues. As a result, cell-polymer interactions were first studied for the purpose of preventing or at least minimising the interactions [40]. Recent thinking favors the incorporation of biologically active structures into the material, to permit direct cellular interaction with the material [81, 82]. These advances are based on the molecular biology of cell adhesion; specifically, the identification of small domains in the adhesion proteins of the extracellular matrix has been critical. The active peptide sequence responsible for the interaction with the cell surface receptors can be synthesized and incorporated into the materials, either in the bulk or immobilized on the surface. These approaches toward biofunctionalization provide a means to control biological interactions directly through material design.

Hubbell and co-workers have characterized the conditions under which the minimal cell binding oligopeptide sequences such as arginyl-glycyl-aspartate (RGD), which are found in many cell adhesion proteins, is capable of supporting adhesion in cells such as the fibroblast [83]. It was observed that a surface density of 10 fmol/cm^2 of surface-coupled RGD ligand would be enough to promote normal cell spreading, clustering of the cell-surface receptors, and organization of a normal cytoskeleton, corresponding to about 140nm distance between peptide ligands [84].

Numerous schemes have been developed for the incorporation of such adhesion ligands into both biostable and biodegradable polymer surfaces. These methods are generally based on adsorption, physical immobilisation or covalent binding. For example, Langer and co-workers have developed copolymers of polylactide and lysine to provide sites for facile grafting of such peptide-based adhesion ligands [85-87]. Poly-L-lysine is highly effective at condensing and delivering DNA efficiently. Therefore the potential use of a scaffold with a PLL component for complexing DNA is also feasible. The cytotoxicity of PLL is however a serious drawback.

1.4.2 Pore size and morphology

Three-dimensional porous polymer matrices possess several advantages over conventional cell culture dish, including an increased area for cell anchorage and an increased volume for cell growth, migration and effective fluid-phase transport of nutrients . The morphology in addition to the size of the pores of the scaffold can critically determine the performance of an implanted scaffold, including the rate of tissue ingrowth. A high surface area favours cell attachment and growth, whereas a large pore volume is required to accommodate and

subsequently deliver a cell mass sufficient for tissue repair [88]. This has been demonstrated by transplantation of hepatocytes for the engineering of new liver tissue [89, 90]. When scaffolds are implanted *in vivo*, porous polymeric implants are often invaded by vascularized fibrous tissue. Predicting such behaviour is important because ingrowth in this manner can improve the survival of cells such as the hepatocytes, but has also shown to dramatically decrease the porosity of the implant [91, 92]. Implants show optimal vascular induction, where pores are large enough for cell and tissue infiltration but not large enough to allow fibrous deposit [93]. Highly porous biomaterials are also desirable for the easy diffusion of waste products from the implant [23, 91], which is a major requirement for regeneration of highly metabolic tissues.

The optimum porosity varies with tissue types such as cartilage with minimal porosity constraints, to liver which requires pores with a minimum diameter of 60μm [9, 90, 94]. A scaffold suitable for organ and tissue regeneration must also contribute towards the organisation and direction of cell growth and ECM production. Porous matrices with well-defined networks of interconnected pores take a significant role in this organization [88, 95]. The degree of porosity also has a significant impact on the rate of degradation. In the case of scaffolds composed of polyesters, the higher porosity can reduce the accumulation of the acidic degradation products, thus diminishing the impact of autocatalytic degradation [95]. In terms of gene delivery, it has been reported that cells seeded on PET matrices with a lower porosity (circa 87%) have higher gene expression levels than cells in matrices with a higher porosity (circa 90%). Thus porosity of a scaffold may also impact on transfection efficiencies [96].

1.4.3 Biodegradability

When the ECM production is large enough to provide cells with a natural environment, the polymer scaffold degrades away at a controlled rate. Ideally, it should be completely resorbed, and natural physiological and metabolic pathways should eliminate the biodegradation products in order to avoid risks of unfavourable tissue reactions. The ideal lifetime of a biodegradable polymer scaffold depends on the application and particularly on the time required for the tissue or organ regeneration. Thus, the success of a biomaterial in tissue engineering depends largely on how its biodegradation rate can be controlled. Certain scaffolds are thought to exhibit accelerated degradation at times owing to autocatalysis, which may be a function of porosity. Poly(α-hydroxy acids) break down through a hydrolytic degradation pathway that leads to lactic acid/glycolic acid, which enter the tricarboxylic acid cycle and are eventually excreted. Thus, as these materials undergo hydrolytic breakdown, they release acidic by-products. An acidic environment can accelerate hydrolysis of these polymers [97, 98]. If the implants are structurally able to allow sufficient fluid flow through the interior, the by-products can be evacuated quickly. If however, diffusion is restricted by a non-porous environment, acidic by-products can accumulate within the implant resulting in adverse reactions from the surrounding tissue [99, 100]. One of the major advantages of degradation is that the scaffold can act as a controlled release device delivering growth factors or plasmids over a sustained period of time [101-108].

1.4.4 Scaffolds as controlled release devices

A last criterion, which can influence the choice of polymer as biomaterial, is the possibility of incorporating bioactive molecules, such as drugs, plasmids or growth and differentiation

factors [109]. Indeed, the controlled release of tissue-specific growth factors from the polymer scaffold may in some cases, considerably enhance the process of organ and tissue regeneration. Examples of growth factors include nerve growth factor (NGF) for nervous tissue regeneration [110], basic fibroblast growth factor (bFGF) for wound healing [24], bone morphogenetic proteins (BMPs) for cartilage and bone remodelling [111], and angiogenic growth factors for the control of vascularization [112]. In tissue-engineered devices, there are two potentially different delivery systems. Growth factors can be incorporated directly into the scaffold during or after fabrication [108, 113-116]. In a biodegradable system, the growth factor would be released as the scaffold degrades to induce tissue regeneration. Growth factor, directly incorporated into a biodegradable polymer scaffold, is released by a diffusion-controlled mechanism that is regulated by the median pore size [117]. The protein can also be released by an erosion mechanism or a combination with diffusion. Alternatively, the growth factor delivery device, in the form of microparticles, nanoparticles, or fibres can be incorporated into the scaffold [118]. This method of delivery is also desirable because growth factors have short biological half-lives. For example, platelet derived growth factor (PDGF) has a half-life of less than two minutes when injected intravenously [113].

Specific growth factors released from the delivery device to influence cell migration, proliferation and differentiation or improve engraftment of seeded cells can lead to more efficient tissue regeneration [113]. There should be no interference of the growth factor delivery device and the tissue engineered device. The two components should function synergistically. Growth factors released from a device may interact with matrix proteins in the scaffold or in the surrounding tissue to enhance their local bioavailability or provide increased stability [109, 119]. A novel approach to the use of growth factors is to

immobilise them onto the surface of scaffolds, which in conjunction with adhesive peptides, could mimic membrane-anchored growth factors such as heparin-binding epidermal growth factor [120].

1.5 Gene delivery from scaffolds

Many current tissue regeneration therapies as discussed above are based on the controlled release of proteins and growth factors from scaffolds that promote tissue formation. However a disadvantage of these systems is the decreased protein stability within the delivery system [121, 122]. The fabrication process to encapsulate growth factors can damage the bioactivity. The harmful factors include sonication, organic solvents, high temperatures and high concentration of surfactants [27]. Such conditions can promote therapeutic protein degradation, decreased potency and increased risk of immune toxicity. As a result, maintaining bioactivity of agents such as growth factors or recombinant cytokines has been difficult. A high dose is often required to maintain the protein in the therapeutic range. High doses, however, tend to cause systemic toxicity.

A critical direction in transplantation therapies has been the genetic modification of cells to prevent rejection of allogenic and xenogenic tissues [123]. The cells can produce therapeutic proteins in a localised manner at physiological levels for prolonged periods of time, thus avoiding local toxicity. The transplantation of transfected cells on porous polymer matrices has resulted in enhanced cell survival and vascularisation due to local expression of therapeutic proteins [124]. Transfected cells have also been utilised for the production of dopamine in the brain and BMP-2 growth factor in bone regeneration [125, 126]. Cell transplantation however, suffers from the need to harvest and grow the cells *in vitro*, which

is typically followed by inefficient seeding and cell survival during transplantation [122]. Such genetically altered cells often rapidly lose their transgene expression and their efficacy.

Therefore, delivery of genes from scaffolds designed for tissue engineering is an attractive approach. Instead of cell transplantation, scaffolds providing controlled release of plasmids could transfect infiltrating cells during tissue induction, which could then promote healing, or tissue formation (Fig. 3). With regards to handling characteristics, such as fabrication and storage, plasmid DNA can be more robust in comparison to the protein it will encode the cell to produce [27]. Delivering this DNA in a controlled release fashion ensures that the cells can provide a localised sustained expression of the bioactive protein. Furthermore, scaffold delivery of multiple genes can be controlled to match the changing expression of genes that are required for optimal tissue induction or formation [122]. Additionally, plasmids that diffuse from the local site of delivery would not cause the toxicity that is associated with high doses of growth factors or proteins [127]. Finally, Xie and coworkers have shown that 3-D transfection may promote a higher gene expression level and longer expression time in comparison to 2-D transfection [96].

Plasmid delivery from scaffolds for tissue regeneration has been demonstrated by a number of groups. Bonadio and co-workers have pioneered this approach by entrapping plasmids encoding the gene for human parathyroid hormones in collagen matrices. This porous sponge was coined " the gene activated matrix' (GAM) [127]. When the GAM was placed in tibia or femur defects, dose dependent bone tissue growth was observed over 6 weeks. In contrast, sham controls resulted in no bone growth. A direct correlation was found between osteoclast numbers and bone healing with DNA dose. Even the lowest quantities of plasmid

resulted in some mRNA and protein expression. Mooney and co-workers used PLGA based porous scaffolds to provide controlled release of DNA to the localised target [122]. In aqueous conditions, DNA could be released for up to 1 month with intact biological activity. *In vivo* studies using platelet-derived growth factor encoding plasmids in the porous scaffolds implanted subcutaneously resulted in efficient localised healing. Berry and co-workers have shown that collagen scaffolds with entrapped plasmids placed within the proximal and distal ends of a severed rat optic nerve resulted in DNA delivery to the nerve cell body in the retina [128]. An additional approach to gene delivery has been the tethering of plasmid-polylysine vectors to the surface of scaffolds. Shea and coworkers showed that transfection by surfaces presenting DNA with both HEK293 and 3T3 cells resulted in expression levels up to 100 fold greater than bulk delivery of the complexes [129]. Positively transfected cells were observed only where DNA complexes were tethered, indicating the potential for spatial control over gene transfection. This is consistent with the report by Luo and Saltzman that physical concentration of DNA increases transfection efficiency [130]. Using a similar biotin-avidin based interaction, viral vectors have also been successfully tethered to a surface for efficient gene transfection [131].

Viral vectors have also been delivered from scaffolds. Viral vectors such as adenoviruses have been widely investigated because of the high efficiency of their transfection [132, 133]. Transgene expression mediated by adenoviruses is transient. The use of polymeric scaffolds to provide a sustained release of viral vectors is attractive. For example, Siemens and co-workers tested a number of polymer matrices for the delivery of the canarypox virus to prostate cancer cells. In their study, gelatin sponges were found to be most effective for viral gene delivery both *in vivo* and *in vitro* [134]. Kalyanasundaram and co-workers have shown that a combination of alginate and gelatin microspheres stabilised by calcium ions

achieved a sustained release of adenovirus with a nominal loss of bioactivity [135]. This maybe a more viable combination in a scaffold delivering viral vectors.

In summary, gene delivery scaffolds for tissue engineering should meet several design criteria:

1. The surface should permit cell adhesion and growth [82].
2. Neither the polymer nor its degradation products should provoke inflammation or toxicity when implanted *in vivo* [136].
3. The material should be reproducibly processable into three-dimensional structures.
4. There should be controlled porosity in order to provide a high surface area for cell-polymer interactions, sufficient space for extracellular matrix regeneration, and minimal diffusional constraints during *in vitro* culture [137].
5. The scaffold should resorb once it has served its purpose of providing a template for the regenerating tissue, since foreign materials carry a permanent risk of inflammation [138].
6. The scaffold degradation rate should be adjustable to match the rate of tissue regeneration by the cell type of interest [139].
7. Plasmids should be encapsulated and delivered without loss of bioactivity. [27, 122]

Such scaffolds should allow attachment of isolated cells to a polymeric support structure that has suitable surface properties for guiding the reorganisation and growth of cells. These should be designed so that the cells could survive by diffusion once the

cell-polymer construct was implanted. Ideally, the cell-polymer construct would become vascularized in conjunction with expansion of the cell mass following implantation, and both these processes could be influenced if desired by release of plasmids that promote vascularisation by encoding cells to produce therapeutic proteins [27, 122]. The ability of scaffolds to conform to all these conditions is also highly dependent on the manufacturing process.

1.6 Fabrication methods

The conventional processing techniques to produce porous structures used in the polymer industry are unsuitable for producing tissue engineering scaffolds. Indeed, additives are commonly used, such as surfactants, plasticizers, stabilizers and lubricants, which can be toxic to cells. The properties of the polymer, the components involved and the shape of the scaffold required dictate the choice of processing technique for the manufacture of polymer scaffolds [140].

The traditional methods for scaffold fabrication include fiber bonding, solvent casting, membrane lamination and melt molding [141, 142]. Mooney *et al* demonstrated that an effective method of stabilizing PGA scaffolds was to spray solutions of PLLA/PLGA in chloroform (1-15% w/v) over a PGA mesh using a nitrogen stream to atomize the polymer solution. Since PGA is poorly soluble in chloroform, the PGA fibres are effectively unchanged by the process [143]. Porosity in scaffolds is commonly formed by particulate leaching. This is achieved by evaporating chloroform from solutions of PLLA containing sodium chloride particles. These polymer films with entrapped salt particles are then

leached in water to remove the particles resulting in highly porous films [90]. These same films can then also be formed into hollow tubes [100]. A similar system has been used by Shoichet and co-workers where glucose crystals are dispersed within a PLGA solution in dimethyl sulfoxide (DMSO) [97]. Wan and co-workers have shown that coatings of various porosities can be obtained by immersing mandrels coated with a solution of PPE in chloroform into non-solvent immersion baths, followed by freeze or vacuum-drying. The porosity of the coatings decreased with an increase in polymer molecular weight, drying time before precipitation and concentration of polymer solution. These methods appear to be however, most effective for only thin films or structures with thin walls [144].

Novel methods of manufacturing scaffolds include a prototyping technology where a binder is expelled through a print head nozzle onto a powder bed. The scaffold is built by layers of powder and binder in a method that can create complex three-dimensional shapes. The highly toxic solvents however prevent incorporation of biological agents such as cells, plasmids or growth factors during fabrication [19]. Wintermantel *et al* have developed a novel method for scaffold formation using minimally invasive approaches. They introduced a thread like material and delivered it through an injection canal (e.g. cannula). A fluid stream acted as a carrier for the "lycra monfil™" based material, which was unreeled from a spool creating a porous scaffold in the form of a tangle [145]. Another minimally invasive technique has been demonstrated by Salem *et al* in which biotinylated polymeric microparticle and cell slurries were self-assembled into porous scaffolds upon co-injection with the crosslinking protein, avidin [146]. Langer and co-workers have produced macroporous polymer foams by a hydrocarbon templating method in which a viscous polymer solution of PLA in chloroform and a particulate hydrocarbon porogen such as paraffin was compacted in a Teflon mold. The polymer/solvent/porogen phase was then

extracted in a hydrocarbon solvent, such as hexane which is a non-solvent for the polymer but miscible with the polymer solvent. This resulted in the porogen becoming extracted and rapid precipitation of a porous polymer phase [147]. Vacanti and coworkers have shown that electrospinning forms scaffolds that are suitable for bone regeneration. In this process, polymer fibers with nanometer dimensions are formed by subjecting a fluid jet to a high electric field [148]. This same process has also been used to produce PLGA and PLA-PEG based scaffolds for DNA delivery [149].

Defined three-dimensional biodegradable foams have also been shaped by lamination of highly porous PLLA and PLGA membranes previously prepared by solvent casting and salt leaching. The membranes with the appropriate shape are solvent impregnated, then stacked up in a three-dimensional assembly with continuous pore structure. Computer-assisted modelling can then help to design templates with the desired implant shape. The *in vivo* pre-vascularization of these laminated foams has been demonstrated by the injection of a sufficient mass of hepatocytes for liver regeneration [91]. Finally Mooney *et al* have demonstrated that the use of supercritical carbon dioxide (CO_2) is one of the promising approaches to creating polymer scaffolds [150]. This is achieved by exposing poly(α-hydroxy acid)s to CO_2 gas (5.5 MPa, 72 hr). When the CO_2 gas pressure is decreased, the thermodynamic instability promotes the dissolved CO_2 to nucleate and form pores within the polymer matrix. As the method avoids the use of organic solvents [151], it is possible to then incorporate plasmids or DNA into the scaffold without loss of biological activity [122].

1.7 Conclusions

Many of the barriers in tissue engineering and the gene delivery fields can be overcome through the merging of the two disciplines. The pioneering work by Bonadio, Levy,

Mooney and many other co-workers has highlighted the potential of gene delivery in tissue engineering devices. Plasmids delivered by porous biodegradable scaffolds can fill diseased tissue or defects to endow infiltrating repair cells with therapeutic properties that can enhance the healing process. Controlled release of plasmids can provide sustained expression of the growth factors and proteins in the regenerating site. Present and future work must now focus on combining the other factors that are critical in scaffold designs for tissue engineering such as porosity and biocompatibility with more sophisticated gene vector technology.

1.8 References

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Figure 1 Schematic representation of a typical tissue engineering approach. Specific cell populations are harvested from the appropriate tissue and seeded onto a biodegradable polymer scaffold.

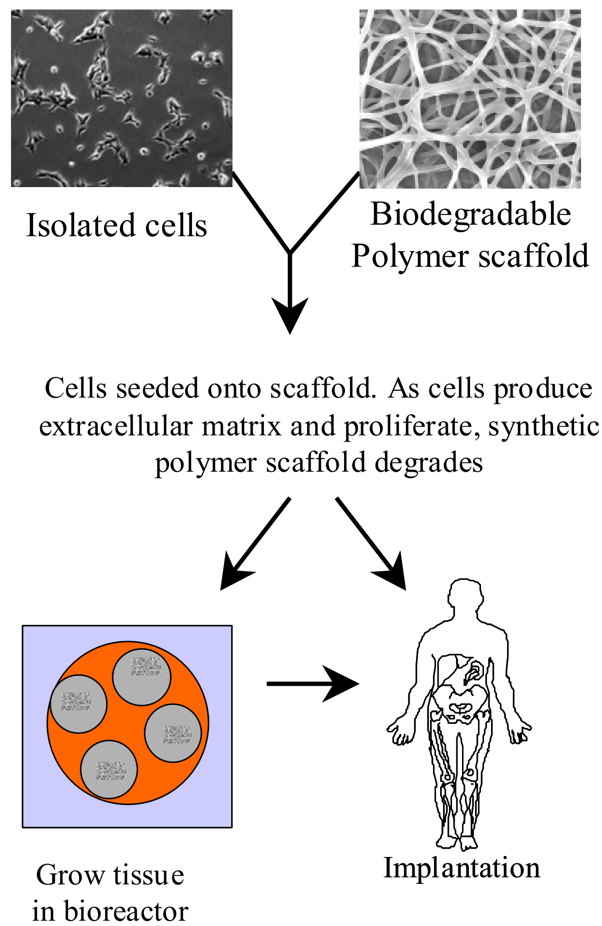

Figure 2: Schematic of mechanisms of transfection

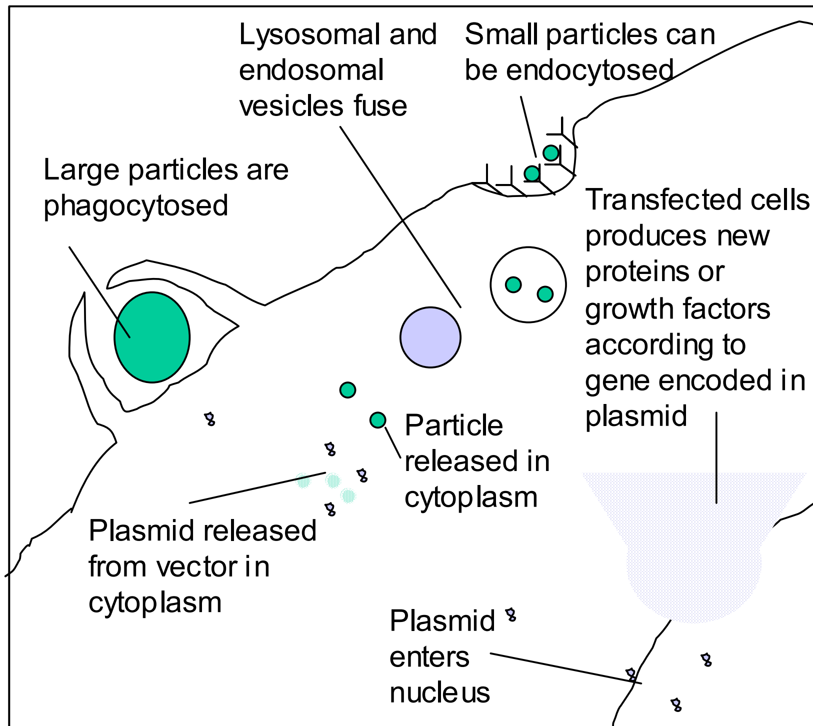

Figure 3. Schematic showing gene delivery from scaffolds approach to tissue engineering.