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Drug delivery in soft tissue engineering

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Introduction: Tissue defects, sustained through disease or trauma, present enormous challenges in regenerative medicine. Modern tissue engineering (TE) aims at replacing or repairing these defects through a combined approach of biodegradable scaffolds, suitable cell sources and appropriate environmental cues, such as biomolecules presented on scaffold surfaces or sustainably released from within.

Areas covered: This review provides a brief overview of the various drugs and bioactive molecules of interest to TE, as well as a selection of materials that have been proposed for TE scaffolds and matrices in the past. It then proceeds to discuss encapsulation, immobilization and controlled release strategies for bioactive proteins, before discussing recent advances in this area with a special focus on soft TE.

Expert opinion: Overall, minimal clinical success has been achieved so far in using growth factor, morphogen, or adhesion factor modified scaffolds and matrices; only one growth factor delivery system (Regranex® Gel), has been approved by the FDA for clinical use, with only a handful of other growth factors being approved for human use so far. However, many more growth factors are currently in clinical Phase I – II or preclinical trials and many delivery systems utilize materials already approved by the FDA for other purposes. With respect to drug delivery in soft TE, a combination of increased research efforts in hydrogel and support material development as well as growth factor development is needed before clinical success is realized.

Keywords: biomaterials, growth factors, hydrogels, soft tissue engineering

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1. Introduction

The concept of repairing or replacing damaged or lost tissue by integrating engineering and the chemical and physical sciences with the biological, life and clinical sciences was proposed almost two decades ago [1,2], and since then, research has made immense progress towards the ultimate goal of successful tissue replacement. Whilst still somewhat short of reaching this goal, however, we now understand that an effective tissue engineering (TE) approach should mimic the *in vivo* setting not only in terms of providing: i) a biocompatible scaffold of appropriate physicochemical properties, mechanical strength and biodegradation profile, ii) a cell source of appropriate progency or type, and iii) nutrients and other environmental conditions (pH, T, etc.), but also in terms of supplying appropriate biomolecular cues. In fact, inclusion of these cues has proven to be crucial in supporting cell migration, proliferation and differentiation [3-18]. Localized delivery of growth factors or other cytokines, chemotactants, adhesion proteins and many others is now understood to be effective and necessary in mimicking the natural microenvironment of cells within specific tissues. But, studies have also shown that mere dispersion of drugs or bioactive molecules in TE scaffolds and matrices presents many shortcomings (mainly in terms of release kinetics and extent, but also drug integrity) and drug

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Article highlights.

- An effective tissue engineering (TE) approach should mimic the *in vivo* setting not only in terms of providing: i) a biocompatible scaffold of appropriate physico-chemical properties, mechanical strength and biodegradation profile, ii) a cell source of appropriate progeny or type and iii) nutrients and other environmental conditions (pH, T, etc.), but also in terms of supplying appropriate biomolecular cues.
- Localized delivery of growth factors or other cytokines, chemotactants, adhesion proteins and many others is necessary to mimic the natural microenvironment of cells.
- Mere dispersion of drugs or bioactive molecules in TE scaffolds and matrices presents many shortcomings and drug delivery systems are necessary to ensure that bioactivity of drugs and proteins is protected and a sustained (or otherwise desirable) release profile is achieved.
- Growth factors are the main source of biomolecular cues in modern TE and delivery strategies need to be devised based on their unique properties, that is, hydrophilicity, considerable molecular weight, affinity to certain extracellular matrix molecules or motifs.
- Control over spatial and temporal release of growth factors from TE scaffolds and matrices is biologically highly relevant.
- To date, most clinical TE successes have been achieved with hard and connective tissue replacements (e.g., bone and cartilage), and 2D soft tissue regeneration (e.g., skin and cornea).
- Engineering of 3D soft tissues is a new and emerging field in TE, and requires the provision of the correct mechanical cues at the same time as biomolecular cues.
- Overall, minimal clinical success has been achieved so far in using growth factor, morphogen, or adhesion factor modified scaffolds and matrices clinically because satisfying results of *in vitro* experiments rarely translate into successful *in vivo* animal studies and even less frequently translate into successful human clinical trials.
- With respect to drug delivery in soft TE, a combination of increased research efforts in hydrogel and support materials development as well as growth factor development is needed before clinical success is realized.

This box summarizes key points contained in the article.

delivery systems are necessary to ensure drugs and bioactive molecules are protected and a sustained (or otherwise desirable) release profile is achieved.

The following sections provide a brief overview of drugs and bioactive molecules of interest to TE, biomaterials used for the fabrication of TE scaffolds and matrices, and common encapsulation, immobilization and controlled release strategies. A particular focus is placed on soft TE as a new and emerging field, which presents some additional challenges in terms of drug delivery, but also advantages that can be used for successful delivery of environmental cues in the form of biomolecules. This review closes with some concluding remarks on the likely direction future research in the field might take and the likelihood of clinical successes.

2. Drugs and bioactive molecules for tissue engineering

By and large, it is bioactive proteins that are of interest to TE rather than the small (hydrophobic) drugs that are of interest to many other medical applications. Specifically, growth factors, adhesion factors and (to a smaller extent) hormones are used in most TE applications. Some examples are bone morphogenic proteins (BMPs), transforming growth factors (TGFs), vascular endothelial growth factors (VEGFs), fibronectin, laminin and peptide mimics of these proteins.

Growth factors are ligands for cell-surface receptors. Their binding to membrane-bound receptors (mostly receptor tyrosine kinases, but also receptor seronine/threonine kinases and G-protein coupled receptors (GPCRs)) results in oligomerization and activation of the receptor through phosphorylation, which in turn activates intracellular signaling cascades ultimately leading to the transcription of target genes (**Figure 1**).

Growth factors of interest to TE are BMPs, TGFs, platelet-derived growth factors, fibroblast growth factors (FGFs), neutrophins, VEGFs and insulin-like growth factors (IGFs) [19]. Each group of growth factors represents a family of proteins that are partially homologous and sometimes show an overlap in their receptor specificity; however, the members of individual growth factor families may have vastly different functions. For example, while BMP-3 – the most abundant BMP in bone – inhibits osteogenesis, BMP-2 and BMP-7 are osteoinductive, and while VEGF-A induces the formation of new blood vessels from existing ones, VEGF-C induces the formation of new lymphatic vessels (**Table 1**) [19]. The reality that not only chemical identity or concentration of a growth factor but also duration and context of a particular growth factor signal can determine cell fate highlights the importance of highly localized and well-timed delivery of growth factors to developing tissues in order to achieve eventual clinical success.

Adhesion factors – the second-most studied group of bioactive proteins in TE – are normally bound irreversibly to the extracellular matrix (ECM) surrounding the cells of any tissue and act as a stress transmitter between a cell's cytoskeleton and the ECM. The extracellular domains of transmembrane proteins located on the cell membrane bind to the adhesion factors while the intracellular domains of the transmembrane proteins interact with the cytoskeleton. Since these adhesion factors can only exert their proper function when in an immobilized state and, therefore, act as antagonists when in a freely diffusible state, controlled delivery of these factors means presentation on scaffold/matrix surfaces in an immobilized state. Fibronectin and fibrinogen are currently the most important adhesion factors for TE [20,21], but vitronectin [22], laminin [23] and thrombospondin have also been investigated as potential candidates. Moreover, smaller subdomains within the proteins, which contain the receptor-binding domains, were identified and successfully expressed recombinantly and applied in TE over the past decade. In some cases, the very domain that binds to the receptor has been identified and

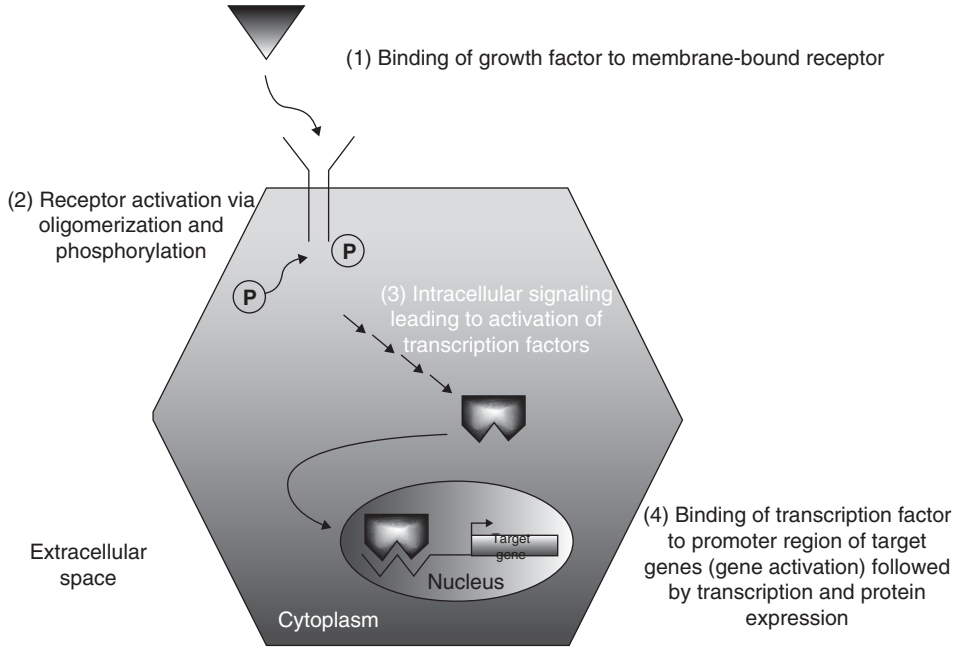

Figure 1. Growth factors bind to membrane-bound receptors, which results in oligomerization and activation of the receptor through phosphorylation and subsequent activation of intracellular signaling cascades that ultimately lead to the transcription of target genes.

successfully mimicked using synthetic peptides. A prime example is the tripeptide Arg-Gly-Asp (RGD) sequence in the receptor-binding domain of fibronectin and a number of other adhesion proteins, which bind to the integrin family of adhesion receptors [24-26].

The prevalence amongst all these molecules is that they are: i) high in molecular weight (5 – 2000 kDa), ii) water-soluble and iii) very sensitive to their environment. It is a common misbelief that protein degradation solely occurs in harsh environments (extreme pH or T, solvents, mechanical shear), but simple exposure to hydrophobic surfaces can have the same effect, that is, loss of biological activity. This has far-reaching effects for TE as denaturation of host proteins on implant surfaces initiates the signaling cascade that ultimately gives rise to the foreign body response. Protein denaturation on surfaces also creates immunogeneic motifs, which can cause antibody formation. And whilst these antibodies may not always have a detrimental effect on a protein-loaded implant directly, they may impede on the interaction of the therapeutic protein and its receptor thus neutralizing its therapeutic effect and the usefulness of the implant. Thus, it is of utmost importance to ensure that proteins are released in their native form when incorporated into TE scaffold/matrices.

Although the majority of bioactive molecules delivered in TE are proteins, some small molecular weight drugs (e.g., antibiotics [27]) have been used in some TE approaches, and more recently nucleic-acid releasing systems were proposed [28-30]. Particularly, the use of nucleic acid-based drugs appears to be a highly sophisticated approach to some of TE's challenges, for example, the controlled and local delivery of growth factors and other bioactive molecules.

3. Tissue engineering scaffolds and matrices

Most TE approaches utilize biomaterial scaffolds and matrices to provide structural support to the developing tissue, whereby the terms 'scaffold' and 'matrix' commonly refer to a macro- or microporous material (e.g., a porous polymer sponge) and a nanoporous continuous material (e.g., a hydrogel), respectively [31]. However, this boundary is often blurred and the words are often used interchangeably. A selection of commonly used scaffold and matrix materials is listed in **Table 2**. For comprehensive reviews on the topic (including scaffold/matrix preparation/fabrication), refer to the excellent works by Hutmacher and others [32,33].

Most materials listed in **Table 2** can deliver drugs or bioactive molecules to developing tissues *in vivo*. However, depending on the biomaterial used and the characteristics of the drug to be delivered, this can either occur via encapsulation (physical entrapment) or immobilization (physicochemical, biochemical, covalent) of the drug within the scaffold/matrix material.

4. Encapsulation, immobilization and controlled release of bioactive molecules within TE scaffolds and matrices

Drugs and bioactive molecules may be encapsulated within biomaterials in a homogeneous or heterogeneous matter,

Table 1. Growth factors and other bioactive molecules of interest to tissue engineering [19,48].

Growth factor family	Example(s)	Function [48]	Tissues successfully treated to date [48]
Angiopoietin	Ang-1/Ang-2	Blood vessel stabilization and maturation	Heart, muscle, blood vessels
Fibroblast growth factor	bFGF (= FGF-2)	Migration and proliferation of endothelial cells	Bone, skin, muscle, nerve, blood vessels
Transforming growth factor	TGF-α/TGF-β	Proliferation and differentiation of basal or neural cells and bone-forming cells, anti-proliferative factor for epithelial cells	Brain, skin, cartilage, bone
Insulin-like growth factor	IGF-1	Cell proliferation, apoptosis inhibitor	Cartilage, skin, nerve, kidney, muscle, bone, cartilage
Platelet-derived growth factor	PDFG-AB/PDFG-BB	Embryonic development, proliferation and migration of endothelial cells	Bone, skin, muscle, blood vessels
Hepatocyte growth factor	HGF	Proliferation and differentiation and migration of mesenchymal stem cells	Bone, liver, muscle
Neutrophin	NT		Nerves
Nerve growth factor	NGF	Survival and proliferation of neural cells	Brain, spine, nerves
Keratinocyte growth factor	KGF		Skin, blood vessels
Bone morphogenic protein	BMP-2/BMP-3/BMP-7	Osteoinductive/prohibitive, differentiation and migration of osteoblasts, renal development	Bone, cartilage, kidney
Epithelial growth factor	EGF	Proliferation and differentiation of epithelial cells and regulation of their growth	Skin, nerve
Vascular endothelial growth factor	VEGF-A/VEGF-B	Migration and proliferation of endothelial cells	Blood and lymphatic vessels
Other bioactive molecules:			
Adhesion factors	Fibrinogen and fibronectin, laminin and vitronectin	Stress transmitter between cell cytoskeleton and ECM	Nerve, adipose, cartilage engineering
	RGD sequence	Receptor-binding domain of fibronectin and a number of other adhesion proteins, which bind to the integrin family of adhesion receptors	Various TE applications

BMP: Bone morphogenic protein; ECM: Extracellular matrix; FGF: Fibroblast growth factor; HGF: Hepatocyte growth factor; KGF: Keratinocyte growth factor; NGF: Nerve growth factor; PDGF: Platelet-derived growth factor; RGD: Arg-Gly-Asp; TE: Tissue engineering.

depending on their solubility in the matrix material. To be incorporated homogeneously, the drug must be soluble in the biomaterial or at least soluble in its precursors [31]. Given most bioactive molecules of interest for TE are growth factors or other water-soluble proteins, this is only the case when they are incorporated into a very hydrophilic biomaterial, which absorbs large amounts of water, and is then referred to as a hydrogel. In virtually all other cases of interest for TE, incorporation of the bioactive molecule occurs heterogeneously, for example, nano- or microdomains of the growth factor are dispersed throughout the biomaterial scaffold (**Figure 2**). If a protein drug were to be incorporated within a biodegradable but hydrophobic polyester, such as poly(lactic acid) or poly(ε-caprolactone) for example, it would have to be incorporated as a dry powder dispersed in the polymer matrix or as an aqueous solution dispersed in the polymer matrix via emulsion techniques. A play on the latter theme is the incorporation of bioactive molecules in micro- or nanoparticles [34], which are subsequently suspended in the polymer matrix (although release kinetics are then governed by other mechanisms).

Regardless of the type of the encapsulation (i.e., heterogeneous or homogeneous), this approach to drug delivery, that is, interdispersion of the drug within a matrix, presents several shortcomings (mainly in terms of release kinetics and extent). It has, however, been widely explored in the literature due to its simplicity. Most of these systems are hydrogel-based and utilize the hydrogel as both scaffold and controlled delivery platform [35,36]. Collagen-, fibrin- and chitosan-based hydrogels [35,37-38], as well as a multitude of poly(ethyleneglycol) (PEG) and other synthetic polymer-based hydrogels have been used for this purpose [39]. Natural materials possess the innate capacity to interact with cell-surface receptors and undergo a mainly cell-interaction dependent degradation, whilst easy manipulation of macro- and microscopic

Table 2. Biomaterials for TE scaffolds and matrices.

Class	Material	Applications [ref.]
Natural polymers	Alginate	Vascular TE [37,121-122]
	Chitosan	Bone, skin, neural, cartilage TE [123-128]
	Fibrin and fibrinogen	Cartilage and bone TE [37,122,129-130]
	Gelatin and pectin	Cartilage and soft TE [22,24,37,131-133]
	Hyaluronic acid	Eye, cartilage, skin, vascular TE [113,134-135]
	Collagen	Bone, cartilage, skin, peridontal and soft TE [33,35,124,136-140]
	Polyhydroxybutyrates	Bone [34]
Synthetic polymers	Poly(ε-caprolactone)	Skin, cartilage, bone, ligament, tendon, vasculature TE [141,142]
	Poly(lactic acid), poly(glycolic acid) and poly(lactic-co-glycolic acid)	Skin, bone, cartilage, ligament, [35,49,114,140,143-145]
	poly(ethyleneglycol) and its copolymers	Skin, vasculature, cartilage, soft TE [25,146-147]
	poly(ethyleneglycol) fumarates	Bone, cartilage TE [147-149]
	Poly(anhydrides)	Bone TE [65-67]
	Multiblock copolymers comprising poly(ethylene oxide) and poly(butylene terephthalate)	Skin, cartilage, muscle, bone [31]
Bioactive glasses and bioceramics	Hydroxyapatite, tricalciumphosphate and composites thereof	Bone TE [150-152]

TE: Tissue engineering.

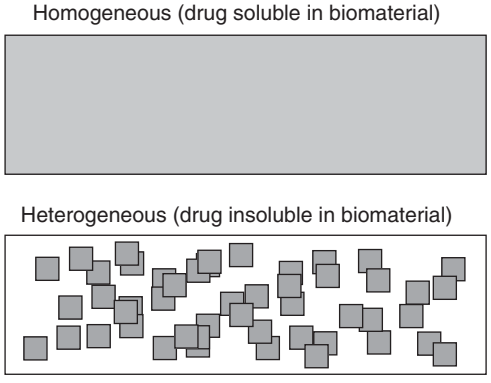

Figure 2. Protein drugs can be loaded within polymer scaffolds and matrices in a homogeneous or heterogeneous manner, depending on the solubility of the drug in the biomaterial. Whilst bioactive proteins, e.g., growth factors, can be loaded homogeneously into hydrophilic polymers such as hydrogels, they are heterogeneously distributed throughout the bulk of hydrophobic polymers in the form of protein particles or droplets of a highly concentrated protein solution in buffer.
Modified from [31].

properties of synthetic materials is traded against cellular recognition/interaction [36]. Apart from simple dispersion or physical entrapment in scaffolds and matrices, immobilization of growth and adhesion factors plays an important role in TE as the former two approaches often do not offer sufficient control over the temporal and spatial release patterns of bioactive molecules. Immobilization of bioactive drugs can be achieved via biochemical (i.e., affinity-based) or covalent links between the bioactive protein and the scaffold/matrix. However, when choosing a means of immobilization, considerations should include its affect on bioactivity of the protein or drug as discussed earlier. On the other hand, immobilization offers multiple ways to influence a proteins' release kinetics (if release is desired, that is) by fine-tuning the degradation profile of the scaffold material itself as well as the lability of the scaffold–protein bond.

In TE, the main goal of controlled release strategies is to provide therapeutically effective concentrations of drugs and bioactive molecules to the developing tissue for a specified time period. This means sufficient amounts of the drug need to be available, while at the same time the concentration must not be higher than the concentration at which the drug demonstrates toxic side effects. **Figure 3** illustrates how controlled release strategies maintain the level of a drug within this 'therapeutic window' for a much longer (sustained) time period than delivery without these strategies can achieve. However, employing controlled release strategies can also mean to enable delivery of drugs and bioactive molecules based on environmental cues. This is commonly referred to as 'programmed' or 'triggered' delivery.

Protein drugs may be released from polymer scaffolds and matrices via simple diffusion of the drug (e.g., proteins incorporated in hydrogels with large mesh sizes) or via degradation of the polymer matrix (e.g., proteins incorporated in hydrolytically degradable hydrogels), whereby the degradation mechanism of the polymer plays an important role in determining the release kinetics of the bioactive molecule. In the case of non-degradable systems, release of the drug is diffusion controlled, that is, the diffusion coefficient of the drug is constant (because no change in its physical environment occurs) whilst its concentration within the matrix slowly decreases. Ergo, the rate of drug release constantly decreases with time

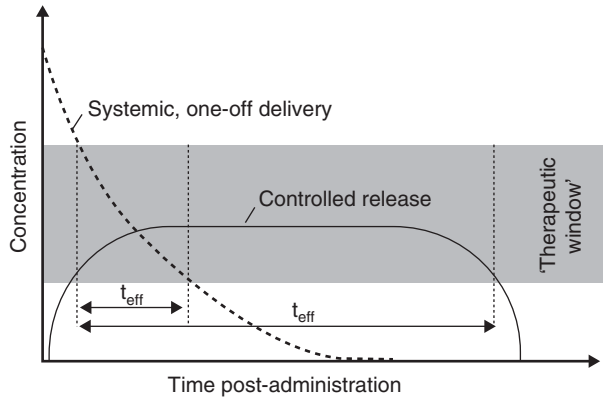

Figure 3. Controlled release strategies maintain the level of a drug within the 'therapeutic window' for a longer time period than one-off or systemic delivery. Without controlled release (dashed line) the drug is initially present at too high a concentration (at which it may possess toxic side effects), rapidly disperses in the tissue and passes through the therapeutic window for a very short time period (t_{eff}), before its concentration rapidly declines to too low a concentration to be effective. On the contrary, with controlled release (solid line) the drug is initially present at a very low concentration, which increases to a therapeutically effective level within a short time period. The concentration of the drug remains within the therapeutic window for an extended period of time (t_{eff}) before the drug is eventually depleted from the scaffold.

and a constant release rate cannot be achieved. This leads to the commonly observed 'burst-release' profile shown in **Figure 4A**, where initially large amounts of bioactive molecules are released with the rate decreasing to close to zero after a relatively short time. In this case, the biomaterials mainly serve as a vehicle to deliver bioactive molecules in a localized but non-sustained fashion. Hydrolytically degradable hydrogels with small mesh sizes may initially hinder an encapsulated protein in its diffusion from the material due to the physical entrapment, but as hydrolysis of the hydrogel proceeds crosslinks within the polymer network are cleaved and the mesh size increases. Eventually, the pores in the hydrogel will be large enough for the protein to diffuse out of the hydrogel. Degradation of the hydrogel occurs throughout the entire volume of the material as it is highly swollen with water. This leads to an increase in pore size that is equally fast throughout the entire hydrogel structure and, therefore, diffusivity of the protein through the matrix increases, ultimately leading to an increased release rate of the protein with time. However, as release occurs, the concentration of the drug within the hydrogel decreases, which slightly offsets the increase in release rate due to increased porosity. If properly balanced, these two effects can lead to a pseudo-zero-order release rate after an initial lag-phase as shown in **Figure 4B**. A similar effect can be observed for polymers degrading via bulk erosion/degradation. Hydrophobic, biodegradable polymers present a slightly different picture in that water will have much better access to the surface than the internal domains of the scaffold, leading to hydrolysis of the polymer on the surface rather than throughout the bulk of the material. Depending on the morphology of the implant and the degradation rate of the polymer, such surface degradation can release encapsulated drugs following an almost perfect zero-order mechanism, that is, the release rate of the drug is close to constant over time (**Figure 4C**). For a detailed review of drug release kinetics from and transport mechanisms within non-degradable and degradable polymer systems refer to Fu and Kao [32].

In synthetic and natural scaffolds/matrices that are highly permeable, it is sometimes difficult to retain protein drugs for prolonged periods and influence their release profile purely via the swelling or degradation rate of the biomaterial. Affinity interactions can help to prolong sustained release in these cases. In TE, the most common approach to improve the release kinetics of immobilized growth factors relies on the use of heparin-immobilized scaffolds [34,40-45] as most growth factors of interest to TE contain positively charged domains on their surfaces, which bind to (negatively charged) heparin or heparan-sulfate-containing proteoglycans present in the ECM. Early work utilized heparin-functionalized hydrogels to directly bind and subsequently release FGF-2 in a controlled manner [46,47]. Since then, more sophisticated heparin-based delivery systems were developed, for example, systems in which a synthetic linker peptide, capable of binding heparin, is covalently attached to a hydrogel [41,42,44]. The synthetic linker binds to heparin, which subsequently binds to heparin-binding growth factors. Conjugation capacity and subsequent release rate are dependent on the number of available binding sites, the affinity of factors towards their specific binding sites and the degradation rate of the scaffold [48]. Incorporating linker peptides that are sequestered by enzymatic activity and, therefore, triggered by cellular activity can exert further control over the release rate. For example, neutrophin-3 release from fibrin was extended to nine days post-administration and neural fiber density within spinal cord lesions was increased upon incorporation of a Factor XIIIa substrate into the linker peptide [41]. Other examples of heparin-based growth factor delivery systems include heparin crosslinked and modified collagens [40,43], synthetic scaffolds based on heparin-conjugated PLGA fabricated by a gas-foaming/salt-leaching method [45] and more recently heparin-conjugated PLGA scaffolds made from star-shaped PLGA, which displayed a threefold higher growth factor capacity compared to similarly heparin-conjugated scaffolds made from linear PLGA [49].

Initially, covalent immobilization of growth factors as a means of prolonging their availability to cells was considered impossible as it was thought to impede on their bioactivity. However, several groups have shown that tethering of growth factors and other bioactive proteins onto the surface of scaffolds and biomaterials can be valuable in retaining

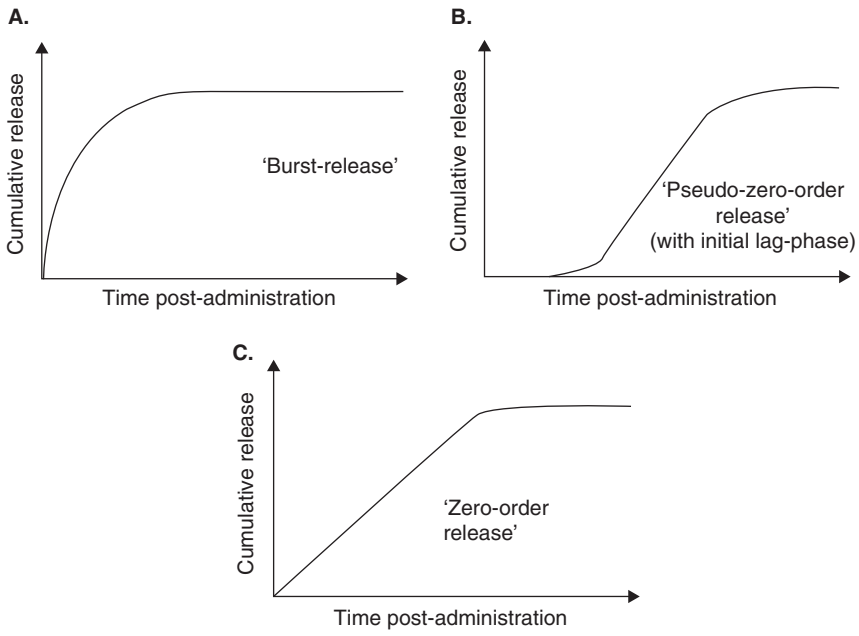

Figure 4. A. Purely diffusion controlled drug delivery often results in an undesirable 'burst-release' profile. **B**. Hydrolytically degradable hydrogels with small mesh sizes may initially hinder an encapsulated protein in its diffusion from the material due to the physical entrapment, but as hydrolysis of the hydrogel proceeds crosslinks within the polymer network are cleaved and the mesh size increases. Eventually, the pores in the hydrogel will be large enough for the protein to diffuse out of the hydrogel. Degradation of the hydrogel occurs throughout the entire volume of the material as it is highly swollen with water. This leads to an increase in pore size that is equally fast throughout the entire hydrogel structure and, therefore, diffusivity of the protein through the matrix increases, ultimately leading to an increased release rate of the protein with time. However, as release occurs, the concentration of the drug within the hydrogel decreases, which slightly offsets the increase in release rate due to increased porosity. If properly balanced, these two effects can lead to a pseudo-zero-order release rate after an initial lag phase. **C**. The rate of drug release from hydrophobic polymer matrices and scaffolds eroding via surface erosion is nearly constant, resulting in zero-order drug release kinetics.

them longer at therapeutically useful concentrations and in controlling their spatial and temporal distribution [50,51]. Tethered EGF, TGF-β1 and BMP-2 were able to elicit DNA synthesis and suitable cell responses of hepatocytes, increase ECM production and induce osteogenesis, respectively, whereas their unbound equivalents were not (or to a lesser extent) [50,51]. Similarly, covalent attachment of VEGF- and RGD-containing synthetic oligopeptides to PEG hydrogels resulted in complete vascularization of the construct via a cell-induced release of the angiogenic factor [52]. Increased temporal and spatial control over growth factor presentation by means of covalent immobilization was also demonstrated by Moore *et al.*, who were able to show that immobilized neutrophilic growth factors induce neurite outgrowth of primary neurons in macroporous scaffolds [53].

Both hydrolysis and enzymatic degradation of polymers play an important role in precisely timing the delivery of a drug or bioactive molecule within or in the proximity of a matrix/scaffold. By fine-tuning the composition of the polymer, the device geometry and the method of drug encapsulation/immobilization, virtually any biodegradable polymer can be engineered to achieve on-demand and pulsed protein delivery regardless of its degradation/erosion mechanism (i.e., surface vs bulk erosion) as demonstrated for bulk-degrading PLGA [35,54-59]. crosslinked hydrogels [60-64] and surface eroding polyanhydride [65-69]. To give two specific examples, Langer and co-workers developed a resorbable PLA/PLGA-based microchip which enables the patterned delivery of multiple agents [70], and Liu *et al.* developed a laminated device based on polyanhydride which enables multi-pulse release profiles for parathyroid hormone and bovine serum albumin, both of which are released in their bioactive forms [65]. Biomolecule- or pH-responsive hydrogels are so-called 'closed-loop' controlled release devices [36], in which the drug release rates are regulated by the system itself in response to *in vivo* stimuli, for example, the change in pH as a drug carrier passes through the stomach after oral administration [71,72], or the presence of high levels of glucose or other biomolecules [73,74]. More recently, research in this area has focused on creating biomolecule-responsive hydrogels via molecular imprinting techniques [75]. Externally-regulated drug delivery systems (or 'open-loop' controlled release devices [36]) can be activated

by a variety of external stimuli [76-78], including temperature [76], electric fields [78,79], magnetism [80] and light [81]. The incorporation of these approaches into TE scaffolds enables a highly desirable temporal and spatial control over drug release by means of a typically very small, but precisely controlled external stimuli.

5. Focus: soft tissue engineering

To date, most clinical TE successes have been achieved with hard and connective tissue replacements (e.g., bone and cartilage) and 2D soft tissue regeneration (e.g., skin and cornea), while the reconstruction of more complicated 3D soft tissues (e.g., cardiovascular and adipose tissues) presents itself as a more far challenging problem. The repair of soft tissue defects, such as those experienced after trauma or oncological surgery (e.g., mastectomy) by exploring TE approaches, has interested reconstructive and plastic surgeons for decades, but has so far eluded successful translation into the clinic. While current reconstructive procedures can relocate soft tissue from one area of the body to other areas, new treatments that are less invasive and more precise, and which offer faster recovery are needed [82]. Various methods of fat injection or grafts have been explored [83], and many new materials have been developed to treat soft tissue defects. However, by and large these treatments have severe limitations, such as donor-site morbidity and deformity, unsatisfactory and unpredictable results, complications from the toxicity of (permanent) implants and the foreign body response [83-86]. Adipose tissue is a key component necessary for soft tissue reconstruction [82], and adipose TE has emerged as an alternative to current treatments [85,87]. Adipose TE aims to generate fat tissue equivalent to the native tissue in terms of cellular and extracellular structure [88]. On a cellular level, adipose tissue largely consists of mature (i.e., terminally differentiated and non-proliferative) adipocytes, with pre-adipocytes, smooth muscle cells, endothelial cells, fibroblasts and mesenchymal stem cells being present in much smaller numbers. Upon appropriate microenvironmental stimulation, both mesenchymal stem cells and pre-adipocytes undergo further proliferation and differentiation. A multitude of growth factors and hormones as well as various tissue-inherent conditions (e.g., cell–cell or cell–ECM interactions) contribute to the conversion of progenitor cells to mature adipocytes and are, therefore, potential candidates for integrated TE approaches where scaffolds and bioactive molecules are used in unison to achieve tissue repair/reconstruction. For example, subcutaneous application of Matrigel combined with bFGF (or FGF-2) in mice resulted in the development of well-vascularized coherent adipose tissue consisting of mature adipocytes [89-91]. Thereby, addition of bFGF proved more effective when the growth factor was encapsulated in porous microspheres compared to application of the free protein. Just the application of microspheres loaded with both adipogenic and angiogenic factors (bFGF, insulin and IGF-1) without simultaneous application of matrigel has also been shown to induce adipogenesis *in vivo* [92-94]. These studies demonstrate how normal physiological mechanisms of adipose tissue formation can be exploited for *de novo* tissue formation, but neither approach allows control over the size or the shape of the newly developing tissue. Implantation of hollow support structures protects the developing fat from potentially deforming mechanical forces (as repeatedly demonstrated by Morrison and co-workers [18,95-102]) and has in combination with the use of matrigel as a filler and a vascularized fat flap recently been shown to produce a persistent adipose tissue, signifying an enormous step towards clinical application [102]. However, matrigel is derived from mouse tumor and, therefore, unacceptable for clinical applications; hence matrigel alternatives are needed. Due to their softness, hydrogels are the preferred material of choice and much research effort has been spent on the development of suitable synthetic [37,71,103-105] and natural (or native adipose tissue-derived) hydrogels [100,106-107]. As discussed earlier, drug incorporation into hydrogels can be achieved via encapsulation (if the mesh size is smaller than the biomolecule) or via affinity-based or covalent immobilization. For example, immobilization of RGD peptides on biodegradable PEG-based hydrogels improved adhesion of 3T3 fibroblasts, an adipo-progenitor cell line *in vitro* [108], and addition of angiogenic bFGF, VEGF and/or PDGF-BB resulted in improved vascularization as well as adipose tissue formation in the above mentioned TE chambers (e.g., filled with matrigel or collagen matrix) [18,96-98].

6. Summary and conclusion

The last two decades of TE research resulted in a great deal of insight into the requirements for successful TE approaches. Whilst still somewhat short of reaching the ultimate goal of complete tissue replacement, we now understand that an effective TE approach should mimic the *in vivo* setting not only in terms of providing: i) a biocompatible scaffold of appropriate physicochemical properties, mechanical strength and biodegradation profile, ii) a cell source of appropriate progency or type, and iii) nutrients and other environmental conditions (pH, T etc.), but also in terms of supplying appropriate biomolecular and biomechanical cues [109]. Localized delivery of growth factors or other cytokines, chemotactants, adhesion proteins and many others is now understood to be effective and necessary in mimicking the natural microenvironment of cells within specific tissues. This review highlighted the importance of growth factors as the main source of biomolecular cues in any TE approach and discussed delivery strategies based on the properties these biomolecules possess, that is, hydrophilicity, considerable molecular weight, affinity to certain ECM molecules or motifs. It further highlighted the biological importance of being able to control the spatial and temporal release of the growth factors from TE scaffolds and matrices, and which strategies are currently used to achieve this level of control. A particular focus is placed on soft TE – and here in particular adipose TE – as a new and

emerging field in TE. The importance of providing the correct mechanical cues at the same time as biomolecular cues was highlighted and hydrogels were identified as well suited to this application due to their softness. However, most hydrogels are not capable of protecting the *de novo* forming adipose tissue from the mechanical forces commonly encountered *in vivo*, hence necessitating the addition of support structures, which then also need to be biodegradable, biocompatible and so on. Due to their large water content though, hydrogels are capable of incorporating hydrophilic growth factors and morphogens and early results suggest that this can be beneficial for both adipo- and angiogenesis during tissue formation *in vivo*.

7. Expert opinion

As researchers gain more and more insight into the importance of individual growth and adhesion factors and their synergistic interplay during healthy tissue development, more effort will be spent on facilitating the delivery of multiple growth factors with appropriate control over temporal and spatial distribution of these biomolecular cues. Synthetic polymer-based delivery systems have already been used to investigate the effect of combinations of growth factors on tissue development, and many growth factors showed synergistic effects [110-112]. For example, the sequential delivery of VEGF and PDGF from PLGA microsphere-containing scaffolds resulted in a significant increase in local blood vessel density and maturation of the newly formed vasculature [110]. Similarly, the immobilization of multiple neurotrophic growth factors in poly(2-hydroxyethylmethacrylate) and poly(L-lysine) scaffolds stimulated and guided neurite outgrowth in neurons [53]. However, whilst many studies indicate that immobilized growth factors and bioactive molecules are better suited to provide an appropriate temporal and spatial profile of cellular signals than signaling by growth factors interdispersed within or physisorbed on a scaffold or matrix, many (pre-) clinical applications of TE constructs are still based on one of the latter approaches. However, this may change now that researchers have understood that immobilization of a growth factor on a substrate surface does not necessarily have to result in a loss of activity of the bioactive molecule. Having said this, the immobilization strategy nonetheless has to be fine-tuned for the particular growth factor(s) used, the specific TE application and the scaffold material.

As more recombinant versions of growth and adhesion factors become available, more research efforts will be spent on incorporating these into TE scaffolds as this will enhance reproducibility of experiments and hopefully translate into better clinical outcomes. However, it is also feasible to directly incorporate plasmids encoding for the various growth factors into TE scaffolds and matrices, and stimulate growth factor synthesis *in situ* [113-116]. However, most applications taking this approach will require pre-encapsulation of the plasmid into carrier vesicles (e.g., liposomes) or nanoparticles [117-119], or condensation into polyplexes by addition of polyethylenimine or similar [120], prior to encapsulation in the scaffold/matrix in order to achieve satisfying gene transfection, as naked nucleic acids are unable to cross the cell membrane due to their opposing negative charge.

Release-on-demand/stimuli-responsive release will continue to be an area of intense research, although it would be beneficial if more collaboration among materials scientists, chemists and the medical profession was to occur as a concerted research effort in this area, which could significantly decrease the time it takes from scaffold/matrix discovery to clinical evaluation. Particularly, release-on-demand systems that enable pulsed delivery of multiple growth factors based on light- or ultrasound-triggers and systems that release their cargo based on cell-demand would be of interest from a clinical perspective. Some success has been reported already [52], but many systems still have to come a long way until they are ready to enter the clinic.

Overall, minimal clinical success has been achieved so far in using growth factor, morphogen or adhesion factor modified scaffolds and matrices because satisfying results of *in vitro* experiments rarely translate into successful *in vivo* animal studies and even less frequently translate into successful human clinical trials. So far, only one growth factor delivery system (i.e., Regranex® Gel, a PDGF carrier) has been approved by the FDA for clinical use, with only a handful of other growth factors being approved for human use so far. However, many more growth factors are currently in clinical Phase I – II or preclinical trials and many delivery systems utilize materials already approved by the FDA for other purposes; therefore, we are set to see some clinical success in the near future.

With respect to drug delivery in soft TE, we will need to see a combination of increased research efforts in hydrogel and support materials development as well as growth factor development before clinical success will be realized. In order to provide the appropriate mechanical and biomolecular cues, emphasis will need to be placed on material stiffness and degradation profiles.

Declaration of interest

The author states no conflict of interest and has received no payment in preparation of this manuscript.

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