Hydrogels for protein delivery in tissue engineering

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\begin{abstract}
Tissue defects caused by diseases or trauma present enormous challenges in regenerative medicine. Recently, a better understanding of the biological processes underlying tissue repair led to the establishment of new approaches in tissue engineering which comprise the combination of biodegradable scaffolds and appropriate cells together with specific environmental cues, such as growth or adhesive factors. These factors (in fact proteins) have to be loaded and sustainably released from the scaffolds in time. This review provides an overview of the various hydrogel technologies that have been proposed to control the release of bioactive molecules of interest for tissue engineering applications.

In particular, after a brief introduction on bioactive protein drugs that have remarkable relevance for tissue engineering, this review will discuss their release mechanisms from hydrogels, their encapsulation and immobilization methods and will overview the main classes of hydrogel forming biomaterials used in vitro and in vivo to release them.

Finally, an outlook on future directions and a glimpse into the current clinical developments are provided.
\end{abstract}

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\section{1. Introduction}

Controlled drug delivery has seen rapid advances in the last few decades with the introduction of novel biomaterials and technologies that found application in all fields of pharmaceutical and biomedical sciences. Particularly, with the advent of protein therapeutics, the need for controlled delivery systems able to enhance protein’s pharmacokinetic and pharmacodynamic properties became more urgent. Nowadays, proteins are used in the treatment of many diseases but also in the area of tissue engineering, where supply of biomolecular cues that mimic the environment of natural tissues and promote the communication between cells proved crucial for achieving effective tissue repair or replacement. Therefore, modern tissue engineering aims at assisting the re-growth of functional tissues by combining cells and engineering materials with signaling biomolecules [1,2]. Biomolecular signals that are mainly growth factors or other cytokines, chemotactants, adhesion proteins and many others, must be locally delivered in their active form and with a sustained release profile. Among the existing technologies, hydrogels – water-swollen, cross-linked polymer networks – have emerged as particularly promising materials for tissue engineering, as they can act both as scaffolding materials and/or releasing matrices for biologically active and cell modulating substances. Their water content, soft nature and porous structure mimic biological tissues and make them suitable to accommodate cells and to encapsulate and release water-soluble compounds like proteins in a controlled fashion.

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manner and to direct the protein specifically used to release growth factors in a controlled and effective proteolytic degradation upon oral administration. Hydrogels are freely and possess short diffusion distances through the ECMs, owing to the large size and the hydrolytic and filtration of vascular endothelial growth factor (VEGF) (bFGF) has a half-life of only 3 min after intravenous administration; accordingly and differentiation, migration, adhesion and gene expression upon recruitment or implanted cells and promote the re-growth of tissues and organs not capable of self-healing otherwise [4–6].

Growth factors are large polypeptides that modulate cellular proliferation, differentiation, migration, adhesion and gene expression upon binding to specific receptors on the surface of target cells. Examples of growth factors and their applications in tissue engineering are listed in Table 1. Unlike other proteins (i.e. hormones), growth factors act locally and possess short diffusion distances through the ECMs, owing to their short half-lives. Typically, growth factors are enzymatically or chemically degraded or deactivated in physiological conditions in a very limited time frame. To mention, basic fibroblast growth factor (bFGF) has a half-life of only 3 min after intravenous administration; similarly, less than 30 min are needed to halve the plasma concentration of vascular endothelial growth factor (VEGF) [8] and platelet-derived growth factor (PDGF), isolated from platelets, has a half life of less than 2 min when injected intravenously [9].

Therefore, the exogenous administration of growth factors for tissue engineering faces important limitations associated to their rapid inactivation and/or elimination after intravenous delivery, their poor transdermal absorption due to the large size and the hydrolytic and proteolytic degradation upon oral administration. Hydrogels are frequently used to release growth factors in a controlled and effective manner and to direct the protein specifically to the wound site [10,11]. Spatiotemporal control over the delivery of these key signaling molecules not only enhances tissue regeneration, but also prevents unwanted and potentially harmful side-effects at other locations than the target. Various strategies have been investigated to achieve controlled protein delivery from hydrogels. They comprise ‘direct’ and ‘indirect’ delivery approaches. Direct release occurs through physical encapsulation, non-covalent binding, covalent immobilization to the delivery system via hydrolytically or enzymatically degradable linkers and the use of double carriers, where protein loaded micro/nanospheres are embedded in hydrogels and the release of the active is based on a combination of mechanisms (diffusion and/or degradation). ‘Indirect’ approaches rely on gene therapy and cell transplantation. Gene therapy is realized through the expression of genetic material encoding for the desired protein that is delivered in the target tissue, while in cell transplantation, specific proteins are secreted by cells that are encapsulated in a hydrogel [12,13].

In the following section the ‘direct’ approaches for protein delivery in tissue engineering will be discussed.

### 2. Proteins in the healing process: an introduction to their delivery approaches

Cell fate and behavior are highly influenced by a number of factors and interactions with the surrounding microenvironment. Signaling molecules create an effective communication between cells and extracellular matrix (ECM) and orchestrate the complex cascade of events that leads to successful regeneration of damaged tissues [3]. Of all molecules currently identified as key components of the wound healing process, growth factors play a pivotal role in the information transfer mechanism between cell and ECM. Although the natural dynamics of the cellular microenvironment are difficult to emulate in an artificial setting, it is currently widely accepted that the self-healing capacity of an organ or tissue can be augmented by the integration of growth factors. In fact, they accelerate the proliferation and differentiation of recruited or implanted cells and promote the re-growth of tissues and organs not capable of self-healing otherwise [4–6].

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### 3. Protein release strategies from hydrogels

Proteins can be loaded into hydrogels through a manifold of mechanisms and strategies. They can be physically entrapped in hydrogels or adsorbed to the matrix by specific interactions, non-covalent or covalent binding via degradable linkers and subsequently released via diffusion, swelling, erosion, degradation or a specific trigger such as pH, temperature, etc. Also, a combination of multiple delivery systems can be used to achieve modular release of multiple proteins. Depending on the release mechanism, different release profiles can be obtained. Table 2 overviews the technologies further described in the following sections.

#### 3.1. Physical entrapment of growth factors into hydrogels: general principles and release mechanisms

Physical encapsulation of growth factors in order to obtain a controlled release of the active is a frequently applied technique for local growth factor delivery in tissue engineering. Its relatively simplicity represents a clear advantage over more sophisticated encapsulation/release methods and the majority of hydrogel systems relies on this loading process. Loading is done by incubation of the preformed gel with the protein of interest or by adding the protein to the hydrogel in situ.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Abbreviation</th>
<th>Action</th>
<th>Tissue engineering applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular endothelial growth factor</td>
<td>VEGF</td>
<td>Migration, proliferation and survival of endothelial cells</td>
<td>Blood and lymphatic vessels</td>
</tr>
<tr>
<td>Insulin-like growth factor</td>
<td>IGF-1</td>
<td>Proliferation, apoptosis inhibition</td>
<td>Cartilage, skin, nerve, kidney, bone, muscle</td>
</tr>
<tr>
<td>Hepatocyte growth factor</td>
<td>HGF</td>
<td>Proliferation, migration, differentiation of mesenchymal stem cells</td>
<td>Liver, muscle, bone</td>
</tr>
<tr>
<td>Epithelial growth factor</td>
<td>EGF</td>
<td>Proliferation and differentiation of fibroblast, epithelial, mesenchymal and glial cells</td>
<td>Skin, nerve</td>
</tr>
<tr>
<td>Nerve growth factor</td>
<td>NGF</td>
<td>Proliferation and survival of neural cells</td>
<td>Nerve, brain, spine</td>
</tr>
<tr>
<td>Bone morphogenetic protein</td>
<td>BMP-2/3/7</td>
<td>Differentiation and migration of osteoblasts, renal development</td>
<td>Bone, cartilage, kidney</td>
</tr>
<tr>
<td>Platelet derived growth factor (2 polypeptide chains A and B forming homodimers AB and B and heterodimer AB)</td>
<td>PDGF-AA/BB/AB</td>
<td>Embryonic development, proliferation and migration of endothelial and smooth muscle cells</td>
<td>Bone, skin, muscle, blood vessels</td>
</tr>
<tr>
<td>Transforming growth factor</td>
<td>TGF-α/β</td>
<td>Proliferation and differentiation of basal, neural, bone-forming cells, keratinocytes, anti-proliferation epithelial cells</td>
<td>Brain, skin, cartilage, bone</td>
</tr>
<tr>
<td>Angiopoietin fibroblast growth factor</td>
<td>Ang-1/Ang-2/VEGF</td>
<td>Blood vessels maturation</td>
<td>Heart, muscle, blood vessels, bone, skin, nerve</td>
</tr>
</tbody>
</table>
was released down a concentration gradient demonstrated that bone regeneration was enhanced when rhBMP-2 engineering applications. For example, Brown et al. and Boerckel et al. bene-

root of time

Generally speaking, the majority of the gel matrices other hand, when the hydrogels pores are smaller than the protein di-

size and the water-content of the gel ('free-volume') [44]. On the other hand, when the hydrogels pores are smaller than the protein di-
ameter, swelling or erosion/degradation (bulk or surface) is needed for release. Generally speaking, the majority of the gel matrices reported to date exhibit diffusion controlled release, following Higu-

chik's kinetics, implying that the release is proportional to the square root of time [45]. This release profile was demonstrated particularly beneficial for the delivery of several growth factors for tissue engi-

neering applications. For example, Brown et al. and Boerckel et al. demonstrated that bone regeneration was enhanced when rhBMP-2 was released down a concentration gradient [46,47]. The diffusional spatiotemporal growth factor presentation proved effective not only for bone regeneration, but also for other engineered tissues like blood vessels [20,21].

The Ritger–Peppas equation is often used to fit release data and de-
terminate the underlying release mechanism: \( M_t/M_\infty = k t^n \) with \( M_t/M_\infty \) the fractional drug release at time, \( t \) and \( k \) a constant incorporating structural and geometric characteristics of the device. If \( n = 0.5 \), the re-
lease is governed by Fickian diffusion. If \( n = 1 \), molecules are released by surface erosion, while both mechanisms play a role if \( n \) has a value be-

between 0.5 and 1 [48,49].

Swelling-controlled systems depend on water uptake and changes in drug diffusivity within the matrix. Swelling increases polymer flexibility and makes pores bigger, resulting in higher drug mobility with some-
times \( n \) values that exceed 1. As a consequence, drug release depends on Fickian diffusion, polymer disentanglement and dissolution in water [50].

Erosion-controlled systems have increased in number since the development of synthetic biodegradable polymers. In these systems, the mobility of the drug in the homogeneous non-degraded polymer matrix is limited and drug release is then governed by the degrada-
tion rate of the polymer, porosity increase, and drug diffusion mainly in the surface of the polymer matrix.

The possibility to tailor the release kinetics of proteins from hydrogels can be achieved through the use of excipients or by changing the cross-links of the polymer network. In this particular instance, the use of synthetic polymers is extremely advantageous because they offer the opportunity to fine tune their chemical structure to achieve modular release. However, also natural polymer networks can be tailored to some extent by changing polymer concentration and crosslink density. Also exten-
tive work on the development of hybrid networks in which natural polymers are synthetically modified or combined with synthetic poly-

ermers, has been proposed to combine benefits of both kinds of polymers with respect to mechanical properties, tailorability in terms of biodegradability as well as biocompatibility and cytocompatibility.

The geometry of the hydrogel-based depot also affects the release rate and the duration. Generally, the bigger is the device, the longer the release consequently lasts. In recent years, patterning techniques utilized in tissue engineering (i.e. stereolithography, bioprinting) have been used to adjust surface area and consequently modulate release profiles of the biomolecules [51–53].

Deviation from the described release behaviors is observed when a specific stimulus – typically temperature, pH, presence of certain mole-
cules – leads to a physical or chemical change in the network structure,

Table 2
Overview of the main release mechanisms and strategies to load and modulate the kinetics of proteins released from hydrogels.

<table>
<thead>
<tr>
<th>Release mechanism from hydrogels</th>
<th>Characteristics</th>
<th>Some examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fickian diffusion</td>
<td></td>
<td>PEG–pi[HPMAmac] [15–17]</td>
</tr>
<tr>
<td></td>
<td>( M_t/M_\infty = k t^n )</td>
<td>PLA–PEG–PLA [18]</td>
</tr>
<tr>
<td></td>
<td>Concentration gradient</td>
<td>RADA 16 networks [19]</td>
</tr>
<tr>
<td></td>
<td>Generally limited to relatively short time spans</td>
<td>alginate [20]</td>
</tr>
<tr>
<td></td>
<td>Protein size = mesh size</td>
<td>dex–VS–PEG–4–SH [22]</td>
</tr>
<tr>
<td></td>
<td>Geometry determines release rate</td>
<td>dextran/(D,L)IA [23]</td>
</tr>
<tr>
<td></td>
<td>Water uptake allows protein to diffuse out</td>
<td>oppositely charged dextran microspheres [24]</td>
</tr>
<tr>
<td></td>
<td>Swelling can be mediated by degradation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controlled by physical or chemical erosion</td>
<td>PLA–PEG–PLGA [25,26]</td>
</tr>
<tr>
<td></td>
<td>Surface erosion mostly for physical gels</td>
<td>PEG/chol–PEG]/3–CD [27]</td>
</tr>
<tr>
<td></td>
<td>Constant release rate</td>
<td>PEG–P(D,L)IA [28]</td>
</tr>
</tbody>
</table>
| Hydrolytical or enzymatic degra-
|  | Protein size > mesh size | |
|                               | Stimuli lead to gel changes and drug release | nitrolintetraacetic acid/Cyrg [29] |
|                               | Stimuli: temperature, pH, biomolecules, drugs, magnetic field, etc | PEG–VS–MMI [30] |
|                               | | PEG acrylate–MMI [31] |
|                               | Adsorption of proteins through weak interaction (i.e. electrostatic, hydro-
|                               | phobic, H-bonding) | Fibrin-heparin [32,33] |
|                               | Affinity with binding sites: heparin, antibodies, chelating ions, peptides, |
|                               | molecular imprinting | PEG–I(3A,NU)Cu++ [34] |
|                               | | thiol acrylate PEG [35] |
|                               | Attachment of drugs to matrix via covalent bonds | polyacrylamide [36,37] |
|                               | Cleavable linker | PEG–RGD–MMP [38] |
|                               | Hydrolytical or enzymatic cleavage | |
|                               | Combination of multiple carriers: i.e. protein-loaded micro/nanoparticles |
|                               | embedded in hydrogels | PEG–GMA;gelatin [39] |
|                               | | Fibrin–PLGA [40] |
|                               | Release of multiple proteins through combination of mechanisms and rates | HAMC/PLGA [41] |
for instance, hydrogels swell or shrink in response to a certain trigger thereby modulating the release of encapsulated drugs/proteins.

3.1.1. Synthetic polymers for the physical encapsulation of growth factors

Among the various synthetic polymers studied for the delivery of growth factors in tissue repair, PEG-based networks play a prominent role.

Burdick et al. investigated the feasibility of a PEG-based hydrogel platform for the replacement and delivery of neurotrophins to the central nervous system. For this purpose, triblock copolymers based on PEG and acrylated poly(lactic acid) (PLA-b-PEG-b-PLA) were synthesized and hydrogels formed by photopolymerization. Three neurotrophins, ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) were encapsulated and released in vitro mainly by diffusion in 20 to 80 days depending on polymer concentration (10 to 30%) and neurotrophin size. In vitro, the released proteins stimulated the proliferation of a TF-1 cells transfected with the α-subunit of the CNTF receptor and stimulated outgrowth of a greater number of neuritis from retinal explants than in control culture conditions [18]. These types of hydrogels, initially described by Hubbell and coworkers [54], have been extensively investigated for growth factor delivery [55,56] because they offer several benefits, including the possibility to easily modify the network properties by changing the macromer chemistry and solution concentration. For instance, the degradation and swelling of the hydrogels are dictated by the number of lactic acid repeat units, the acylation efficiency, the molecular weight of the PEG core and the concentration of macromer in the prepolymer solution [57]. Also, the type of degradable unit (e.g., lactic vs caproic acid) alters network degradation rates [58].

Similar release and network concepts apply to other PEG/polyesters hydrogels, like those based on PEG-poly(lactic-co-glycolic acid) (PLGA)-PEG triblock copolymers [59]. These materials display lower critical solution temperature behavior and were processable avoiding the use of high temperatures to dissolve the polymer. It was shown that upon subcutaneous injection (rat model) the hydrogels were stable for one month [60]. TGF-β1 was loaded into these hydrogels and used as a slow releasing drug reservoir aimed for wound healing purposes. Significant levels of re-epithelialization, cell proliferation and collagen organization were observed [61]. Porcine growth hormone (pGH) and Zn-pGH were sustainably released from PLGA-PEG-PLGA hydrogels for 10–14 days in vitro with no initial burst and a good correlation with in vivo data, that confirmed a weight gain in hypophysectomized rats equivalent to conventional daily injections of 5 mg pGH for 14 consecutive days. Approximately 85% of the glycosylated granulocyte colony stimulating factor loaded in PLGA-PEG-PLGA hydrogels was released over a 12-day period and similarly to pGH, showed similar efficacy in rats as compared to daily i.v. injections [62]. Censi et al. studied the protein release from photopolymerized thermosensitive PEG-based networks formed upon gelation of methacrylated poly(hydroxypropyl methacrylamide lactate)-PEG- poly(hydroxypropyl methacrylamide lactate) (p(HPMAM-Lac)-PEG-p(HPMAM-Lac)) triblock copolymers [63]. Proteins are released from this network by Fickian diffusion and, similarly to previously described systems, network properties and release behavior can be tailored by tuning polymer chemistry and concentration [16,17,64]. The gels showed a good biocompatibility in rats [65] and their potential for cartilage tissue engineering application has been demonstrated [51].

Peptide based hydrogels were also used for protein delivery and release. A class of ionic self-complementary oligopeptides named RAD 16 and commercialized as PuraMatrix was introduced by Zhang et al. These peptides consist of alternating hydrophilic and hydrophobic amino acids that form β-sheets structures having one polar surface with complementary charged ionic side chains and a non-polar surface with alamines. These peptides are able to spontaneously self-assemble into stable macroscopic matrices or nanofibers. The monovalent cations that are needed for the peptide assembly are sequestered from the physiological environment [19]. The described family of peptides has been used to encapsulate and deliver several proteins for intramyocardial delivery. This peptide-based hydrogel has also been used to deliver platelet-derived growth factor BB (PDGF-BB) [66], stromal cell-derived factor-1 (SDF-1) [67] and insulin-like growth factor 1 (IGF-I) [68] to decrease myocardial infarct. The observed slow and controlled release of the active proteins has been ascribed to diffusion and to some extent to interaction between protein and polymer. The amphiphilic nature of the self-assembling polypeptide leads to interactions with the loaded protein, resulting in reduced diffusivity and slower release kinetics.

Hydrogels that showed degradation mediated release of model proteins and that potentially can be used to physically encapsulate and release growth factors with a zero order release kinetics are for example those formed by stereocomplexation between star PEG copolymerized with the stereoregulars D or L of PLA [28] or those based on PLGA-PEG-PLGA (ReGel) systems [25,26] and inclusion complexes of PEG/cholesterol-PEG-β-cyclodextrin [27].

Stimuli sensing hydrogels have also been investigated for growth factor release in tissue engineering. Recently, Weber et al. proposed a drug-sensing hydrogel based on glyrase sub-unit B (GyB), reversibly cross-linked by coumarmycin and able to release VEGF upon addition of an amidocarboxylic antibiotic, novobiocin. The polymer forming the hydrogel is based on polyacrylamide functionalized with nitrilotriacetic acid chelating a Ni2+ ion to which GyB can bind through a hexahistidine sequence. The addition of novobiocin causes the cross-links to be partly broken, resulting in opening of the network structure and release of VEGF [29]. Hubbell et al. synthesized hydrogels comprising multiamyl vinyl sulfone-terminated PEG, a monocysteine containing adhesion protein, and a bis(cysteine) metalloprotein substrate protein (MMP). These hydrogels were studied for tissue engineering purposes and both cells and vascular endothelial growth factor (VEGF165) were encapsulated in the hydrogel. The release of the encapsulated factor is triggered by matrix metalloproteinases (MMPs) [30]. MMPs are are zinc-dependent endopeptidases capable of degrading extracellular matrix proteins and they influence cell behavior such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense. MMPs sensing hydrogels were developed with the aim to mimic the natural processes involved in tissue repair. Other examples of semi-synthetic hydrogels were described by Phelps et al. whose design provides incorporated VEGF, enzyme-degradable sites and arginine-glycine-aspartic acid (RGD) cell adhesive ligands. Similarly to the system proposed by Hubbell, these matrices use PEG as the main building block because it has been shown to act as a protein-resistant and cell non-adherent background. The network is based on PEG diacrylate which is photocrosslinked in the presence of MMP-degradable polypeptide functionalized with two PEG-acrylate groups, a mono-PEG-acrylate RGD and a mono-PEG-acrylate VEGF. Recently, Phelps et al. have demonstrated the benefits of this approach in a mouse hind limb femoral artery ligation model. After 7 days, mice treated with implanted hydrogels showed a 50% increase in perfusion to the limbs and 100% increase in perfusion to the feet as compared to untreated mice. The matrix alone (not containing VEGF) performed as well as soluble VEGF injections, indicating that the engineered degradable and adhesive hydrogel has itself a beneficial healing or supportive effect. Engineered matrix containing VEGF performed better than injections of soluble VEGF, acting synergistically as a directive scaffold and a growth factor delivery vehicle [31].

3.1.2. Natural polymers for the physical encapsulation of growth factors

Hydrogels designed with natural polymers as building blocks display multiple advantages over synthetic polymer networks with respect to their biocompatibility, biodegradability and good cell adhesion properties. Therefore, extensive work is available on biopolymer-based hydrogels for cell and growth factor encapsulation in regenerative medicine [69–72].

The main classes of natural polymers studied in hydrogel formulations are polysaccharides and proteins/polypeptides.
3.1.2.1. Polysaccharides. Polysaccharides are in general hydrophilic polymers and are therefore very suitable for the design of hydrogels. The most commonly used polysaccharides in recent hydrogel research aimed for protein delivery are alginate, hyaluronic acid, chitosan and dextran (Fig. 1) [73,74]. Representatives of the hydrogel technologies based on these polysaccharides are briefly discussed below with respect to their use for growth factor delivery.

3.1.2.1.1. Alginate. Alginate is a linear polysaccharide composed of homopolymeric blocks of 1-4-\(\beta\)-D-mannuronic acid (M) and its C-5 epimer \(\alpha\)-L-guluronic acid (G), respectively, covalently linked in different sequences or blocks. This polymer block consists of consecutive G-residues, consecutive M-residues or alternating M and G residues. Depending on the block composition, alginate has different conformational preferences and behavior. G-rich blocks of the polymer are able to bind divalent cations of which Ca\(^{2+}\) is frequently used for crosslinking alginate to obtain hydrogels. Ca\(^{2+}\) ions bind to the G units of different polymers chains cooperatively in a so-called egg-box arrangement. The partial oxidation of alginate using sodium periodate makes the polysaccharide biodegradable [75,76], and these oxidized alginates are therefore particularly appealing for biomedical applications. An alginate hydrogel patch to deliver stromal cell-derived factor-1 (SDF-1), a naturally occurring chemokine that is rapidly overexpressed in response to tissue injury, was described by Rabbany et al. Alginate patches were loaded with either purified recombinant human SDF-1 protein or plasmid expressing SDF-1 and the kinetics of SDF-1 release were measured both in vitro and in vivo in mice. They demonstrated that SDF-1 plasmid- and protein-loaded patches were able to release therapeutic product over hours to days, with faster release for SDF-1 protein (in vivo \(K(d)\) 0.55 days) than SDF-1 plasmid (in vivo \(K(d)\) 3.67 days). Prolonged (induction of) SDF-1 release of SDF-1 resulted in accelerated healing (9 days) and reduced scarring of acute surgical wounds in Yorkshire pigs [77].

Although the biological properties of alginate are well recognized, some limitations in tailoring the mechanical properties, the pore size and the release kinetics of proteins from these gels still remain. Many researchers investigated several modified alginates to overcome these limitations [78–83]. For example, Ruvinov et al. examined the release of hepatocyte growth factor (HGF) from modified alginate hydrogels and

![Fig. 1. Most commonly used polysaccharides for hydrogel preparation for biomedical applications (M = mannuronic acid, G = guluronic acid).](image-url)

![Fig. 2. Biophysical properties and vascular endothelial growth factor (VEGF) release from alginate gels. Degradation of gels formed from high molecular weight non-oxidized alginate (triangles), binary molecular weight non-oxidized alginate (white squares) and binary molecular weight partially oxidized alginate (black squares) in phosphate-buffered saline (A). The viscosities of solutions of high (white bar), low (black bar) and binary (grey bar) molecular weight partially oxidized alginate were assessed (B). Release kinetics of VEGF165 from gels formed from binary molecular weight alginate, either partially oxidized (black squares) or non-oxidized (white squares) (C). Greater endothelial cell proliferation was observed in cells cultured with VEGF released from binary molecular weight partially oxidized alginate gels (grey), as compared to medium without VEGF (black) and control VEGF added to medium (white), as determined from the cell number counts from day 0 to day 4 (D). Reproduced from ref. [85].](image-url)
studied its potential to induce angiogenesis in vivo. They developed a cross-linkable affinity-binding alginate by introducing sulfoester groups into the uronic acids of alginate. It was shown that alginate-sulfate was capable to bind heparin-binding proteins, like growth factors, with equilibrium binding constants similar to that of heparin. The affinity binding to alginate-sulfate retarded the release of HGF. The bioconjugate HGF-alginate-sulfate was combined with alginate in an aqueous solution and a hydrogel was formed ex vivo by partial cross-linking in presence of CaCl₂. Upon injection into ischemic myocardial tissue the injectable hydrogel completed its cross-linking by sequestration of endogenous Ca²⁺. A cumulative protein release of 75% was observed after 6 h and in the following 5 days a cumulative release of approximately 85% was achieved [84]. This profile is typical of diffusion-based delivery systems that release the drug down a concentration gradient, as described for another alginate hydrogel reported in Fig. 2 [85]. A recent method employed to make alginate networks more stable and tailorable is through the preparation of interpenetrating polymer networks (IPN’s) [83]. An IPN is defined as a network comprising two or more polymers that are partially interlaced on a molecular scale but not chemically bounded to each other and that can be separated only after chemical bond breakage [86]. IPNs are designed to combine the advantageous properties of different polymers. This combination often translates in good modulation of the mechanical and biological characteristics of the final hydrogel, derived from the additive or synergistic effect of the polymers’ properties. Pescosolido et al. developed IPN hydrogels based on Ca²⁺-alginate and photocross-linked dextran-methacrylate derivatives, of which the mechanical properties were notably improved by the synergistic effect of the polymers composing the IPN system. These hydrogels proved effective in the controlled release of proteins [83]. In another study, it was demonstrated that complete delivery and a better control over VEGF release kinetics were achieved by cross-linking alginate microparticles with Zn²⁺ instead of Ca²⁺ [87,88].

Silva et al. described alginate hydrogels that were covalently modified with RGD sequences and loaded with growth factor VEGF, and that were lyophilized to form microporous scaffolds to induce neovascularization. The delivery system, modified with adhesive sequences and loaded with growth factor and cells, was used to induce the formation of a depot of vascular progenitor cells (outgrowth endothelial cells (OECs)) in vivo. It was observed that OECs were viable during the studied time frame and that the encapsulated VEGF induced the cells to migrate out of the scaffold. Local and controlled delivery of the morphogen was effective in stimulating the cells to repopulate the damaged tissue and participate in regeneration of a vascular network, while bolus injections was ineffective in preventing necrotic toe (Fig. 3) [89].

3.1.2.1.2. Dextran. Dextran consists of α-1,6-linked D-glucopyranose units with some degree of 1,3-branching. Various methods to cross-link dextran hydrogels have been developed and its high number of available hydroxy groups presents many options for derivatization and subsequent physical or chemical crosslinking [90]. For example, Hennink et al. developed a hydrogel system potentially suitable for the delivery of proteins relevant for tissue engineering, where a dextran backbone is derivatized with hydroxyethyl methacrylate (HEMA) moieties. This polymer in aqueous medium was chemically cross-linked by radical polymerization of the dextran-bound methacrylate groups to yield hydrogel based microspheres [91,92]. These microspheres released model proteins as well as therapeutic relevant proteins (IL-2 [93]; hGF [94]) in a controlled manner for days to weeks. The same group also developed a physically crosslinked hydrogel system by grafting the dextran backbone with either D- or L-oligo-lactate chains containing 5 to 15 lactic acid units. The hydrogel formation was driven by stereocomplexation and preclinical studies demonstrated the suitability of this hydrogel for the controlled delivery of IL-2. The density of oligo-lactates and the grafting density allowed tailoring of the release and degradation rate. The release of the encapsulated protein was dependent on diffusion [23]. Further efforts were addressed by Hennink et al. towards the development of dextran based delivery platforms for proteins, potentially suitable for growth factor and cytokine controlled release. Self-assembled macroscopic hydrogels based on oppositely charged dextran microspheres were prepared and their release behavior investigated. It was found that native proteins were released by diffusion/swelling [24].

Fig. 3. Analysis of angiogenesis in ischemic hindlimbs after OEC transplantation. (A) Implantation of blank scaffolds, bolus injection of OECs and VEGF (same quantities as placed in scaffolds), transplantation of OECs on scaffolds lacking VEGF (alginate scaffold OEC), and transplantation of OECs on scaffolds presenting VEGF (alginate scaffold (VEGF) OEC). Photomicrographs of tissue sections from ischemic hindlimbs of SCID mice at postoperative day 15, immunostained for the mouse endothelial cell marker CD-31 (B), and human CD-31 (C). Reproduced from ref. [89].
More recently, dextran-peptide bioconjugates were synthesized by Shoichet et al. In their approach, the polysaccharide was modified with \( p \)-maleimidophenyl isocyanate (PMPI) thereby introducing maleimide functionalities in the backbone (Dex-PMPI). A peptide cross-linker, derived from collagen and susceptible to gelatinase A digestion, was synthesized with bifunctional cysteine termini and used to cross-link Dex-PMPI (Fig. 4). It was demonstrated that this type of hydrogel was cytocompatible and mimicked the degradation and remodeling of the ECM through the activity of cell-secreted enzymes [95].

Michael addition cross-linking reaction was used to induce network formation between dextran vinyl sulfone conjugates (dex-VS) and tetrafunctional mercapto poly(ethylene glycol) (PEG-4-SH). The release of several proteins including bFGF from hydrogels of different polymer concentrations was studied. Also these networks showed diffusional release and a certain extent of tailorable release of the bFGF [22].

In a recent study, Sun and coworkers demonstrated that the manipulation of the cross-link density of dextran based hydrogels loaded with multiple growth factors affects the neovascularization of ischemic and wounded tissues. Dextran-allyl isocyanate-ethylamine (Dex-AE) derivatives with different degrees of substitution were synthesized and mixed with PEG diacrylate at different ratios and subsequently, hydrogel formation was induced by photopolymerization. It was found that tissue ingrowth was favored by reducing the degree of substitution of cross-linking groups, which resulted in reduced rigidity, increased swelling, increased VEGF release rate, and rapid hydrogel disintegration. Furthermore, the release of multiple angiogenic GFs increased the size and number of newly formed functional vessels [96]. In another study, hybrid hydrogels based on combinations of glycidyl methacrylated dextran and gelatin, processed into different physical structures (microspheres, cylinders) have been synthesized and used to deliver growth factors, including BMP-2 and IGF-1 [97].

3.1.2.1.3. Hyaluronan. Hyaluronan or hyaluronic acid (HA) is a linear glycosaminoglycan composed of repeating disaccharide units...
of D-glucuronic acid and N-acetylglucosamine [98,99]. This naturally occurring polysaccharide is negatively charged under physiological conditions has molecular weights up to 10^6 Da. It is found mainly in the extracellular matrix (ECM) and in the synovial fluids of joints where it acts as a lubricant by which it reduces the friction of bones due to its unique viscoelastic properties [100].

Because of its biocompatible, attractive physical properties and possibilities for further chemical modifications, HA is an extensively studied polysaccharide for the development of delivery systems for tissue engineering applications [101–107]. However, unmodified hyaluronic acid in aqueous solution displays poor mechanical properties. Therefore, to be suitable for protein delivery, HA-gels need physical and/or chemical stabilization. Usually, the carboxylic groups of hyaluronic acid are derivatized to introduce functionalities that make the polymer suitable for cross-linking [108–113].

In situ photopolymerization was applied by Park et al. to crosslink thiolated-HA/alginate or thiolated-HA/glycidyl HA or photocross-linked glycidyl HA/acrylate 4-arms PEG [107] for engineering purposes. Many of the hydrogel systems based on gelatin and collagen are cross-linked using harsh conditions that exploit gluteraldehyde or water-soluble carbodiimides and that can be detrimental for the encapsulated proteins [108]. However, also milder preparation conditions were investigated. For instance, non-covalently crosslinked fibrillar collagen can be used to create hydrogels by entanglements of collagen fibers [127]. The mesh sizes of these entangled collagen fibers are quite large and therefore diffusion of only very large proteins can be controlled. Nevertheless, the protein release rate may still be lower than expected based on diffusion coefficients due to weak interactions between collagen and loaded proteins [127].

Gelatin has a long history in clinical use with examples of gelatin based systems with a variety of physical geometries. Gelatin derived from collagen can be positively or negatively charged depending whether collagen depolymerization is carried out at acidic or basic conditions. The charge of gelatin network can be utilized to complex oppositely charged proteins serving as additional feature to control release rate. Gelatin networks are gradually resorbed in vivo, a high initial burst occurred within the first 24 h. To overcome this issue, VEGF was later covalently bound to the matrix [128]. Gelatin based hydrogels have been used for the delivery of FGF-2 [129], HGF and VEGF [130]. Nowadays, gelatins can be produced in yeast cells recombinantly leading to slightly different polymer properties. The advantage is that properties such as molecular weight, amino acid sequences and iso-electric points can be tailored precisely. Degradation times, swelling properties and ultimately also drug release kinetics are affected by the design of recombinant gelatin. Only a few recombinant gelatin hydrogels have been investigated for their protein delivery properties [131,132]. Fibrin is a fibrous, non-globular protein derived from fibrinogen, a protein of the blood coagulation cascade. Fibrinogen, a water soluble protein, is converted into fibrin that polymerizes to yield a gel due to the action of thrombin. Fibrin networks are gradually resorbed in vivo by the secretion of fibrinolytic enzymes and as such has been widely used for formulating depot systems for proangiogenic growth factor delivery. Commercial fibrin glues are also used in the clinic for in situ application.

In an early clinical trial, VEGF was injected in an in situ forming fibrin matrix into the right popliteal region of a man suffering from claudication. Although substantial growth of newly formed vessels was observed, a high initial burst occurred within the first 24 h. To overcome this issue, VEGF was later covalently bound to the matrix and released by enzymatic cleavage [133,134]. Similarly, FGF-2 was electrostatically immobilized in fibrin hydrogels using heparin [135]. These strategies showed in animal model enhanced neovascularization and reduced fibrosis and inflammation.
Recently, Drinnan et al. reported on an elegant approach on the multimodal release of different proteins from the same hydrogel. They developed an injectable PEGylated fibrin gel designed for the release of PDGF-BB and TGF-β1 with distinct kinetics (Fig. 5). Growth factors were loaded into PEGylated fibrin gels via 3 mechanisms: entrapment, conjugation through a homobifunctional amine reactive PEG linker, and physical adsorption on the fibrin matrix. PDGF-BB was entrapped during thrombin-mediated crosslinking leading to its diffusion-controlled release over 2 days. TGF-β1 was both conjugated through the PEG linker and bound to the matrix via physical adsorption, delaying the release rate of TGF-β1 up to 10 days. Further, the release rate was highly correlated to gel degradation rate, indicating that TGF-β1 release was degradation-controlled [1136].

Fibrin hydrogels were also used to fabricate circuits for the controlled release of chondroitinase ABC (a bacterial enzyme capable of digesting the glycosaminoglycan (GAG) side chains from chondroitin sulphate proteoglycans) in spinal cord lesion treatment. Sustained delivery of the protein in vivo for about 3 weeks was demonstrated as compared to bolus injection. Furthermore, after 3 weeks, six times more bioactive chondroitinase ABC and 37% lower levels of inhibitory glycosaminoglycans (GAG) were found in the spinal cord when hydrogels were used vs intraspinally injected enzyme solution [1137].

3.1.2.2. Affinity-based drug release. Affinity based drug delivery systems utilize physical interactions between the therapeutic drug and the delivery system to manipulate drug loading and control release kinetics.

The easiest method to modulate release kinetics of loaded proteins is physical adsorption, involving the formation of ionic complexes by electrostatic interactions between charged polymers oppositely charged proteins [1138–140]. Many charged polymers like hyaluronan, chitosan, alginate, acidic gelatin can be potentially used to retard drug release [89].

Heparin sulfate is a naturally occurring, highly sulfated anionic glycosaminoglycan found in the ECM that is a natural matrix responsible for immobilizing and releasing various proteins that influence the natural processes of adhesion, migration, proliferation, differentiation. This glycosaminoglycan possesses a specific binding domain for many growth factors that interact with heparin via non-covalent bonding [32,141]. Besides enhancing matrix ability to retain the encapsulated protein, it has been shown that affinity binding with heparin also stabilizes the drug, preserving its structure and functionality during handling of the material [1142]. Heparin is generally incorporated within the delivery system through many strategies as addressed in recent state of art reviews of affinity-based drug delivery [11143,144]. Therefore, this paper deals only with some general aspects of this topic.

One approach to incorporate heparin in a hydrogel structure is to covalently link the heparin moiety to the polymer. Many examples of growth factor delivery from heparin modified polymer hydrogels have been reported. Hubbell used a bi-domain peptide that was covalently attached to fibrin on one site and to heparin on the other extremity. Growth factors of different affinity for heparin (bFGF>bNGF, BDNF, neutrophin-3) [32,33] were loaded and released with slower kinetics as compared to fibrin hydrogels. In a similar approach adopted by Kick et al. four arms thiolated PEGs were reacted with monomaleimide-functionalized low molecular weight heparin and cross-linked in aqueous medium after addition of heparin-binding peptide modified PEGs. This delivery system as well other similar hydrogels have been extensively used for the controlled delivery of many growth factors for different applications [145–148].

Heparinization was also applied to collagen [149,150], alginate [151], hyaluronan [152] and pluronic [153] using different chemistries and cross-link methods.

Polymers were complexed with growth factors binding heparin also by electrostatic interactions. Sulfonation of hyaluronan and uronic acid monomers of alginate allowed non-covalent interaction with heparin-binding proteins [80,84]. Although heparin bound hydrogels are definitely the most studied affinity systems to date, some challenges still remain such as unstable protein-heparin bonding in vivo, large amounts of heparin necessary to achieve sufficient drug loading and heparin-induced thrombocytopenia [154]. Affinity hydrogel systems based on the mechanism of metal-ion-chelation and binding of histidine tagged proteins have been proposed as an effective alternative to the heparin-containing systems. To mention, Metters et al. developed PEG-co-methacrylated iminodiacetic acid displaying affinity binding for histidine tagged proteins through divalent metal ions such as nickel and copper. The authors also elaborated a mathematical model to predict the release rate of proteins from the hydrogels [34].

Phage display is a technique that has been exploited to identify peptides with affinities for growth factors that could be coupled to the gel polymer [155]. As an example Anseth et al. developed an affinity peptide-functionalized PEG hydrogel with the ability to sequester monocoyte chemotactic protein 1 (MCP-1), a chemokine that induces the chemotaxis of monocytes, dendritic cells, and memory T-cells. Affinity peptides were immobilized in PEG hydrogels via a thiol-acrylate photopolymerization. The release of encapsulated recombinant MCP-1 from PEG hydrogels was tailored by altering the spacer distance between the affinity peptide and the crosslinking site [35]. Antibodies were also exploited for the specific binding of proteins onto scaffolds [156].

Molecular imprinting is a technique used to synthesize biomimetic polymer networks with template-shaped cavities that have affinity for specific molecules of interest (the templates), The nature of the interaction can vary from non-covalent bonds to metal coordination and even covalent bonds. This technique has been applied for drug and protein delivery and recognition. However, although some success has been achieved in the molecular imprinting of small molecule drugs, huge challenges still exist with imprinting of proteins [42,77,125,157].

3.2. Covalent binding

Proteins, mostly exploiting their reactive amine and thiol groups, can be covalently bound to polymer matrices via functional groups like hydroxy, amine, carboxyl groups that, if not naturally present in the structure of the polymer, have to be introduced by functionalization reactions, blending or co-polymerization. The release of the protein is mediated either via hydrolysis or reduction reactions or (cell-mediated) enzymatic cleavage. This type of mechanism leads to on-demand release of loaded proteins, mimicking the enzymatic activity naturally occurring in ‘healthy’ ECM. However, stability and maintenance of biological activity of the protein may represent an issue.

Verheyen et al. recently reported on an efficient strategy to introduce methacrylamide groups on the lysine residues of a model protein (lysozyme) for immobilization and triggered release from a polyacrylamide or dextran hydrogel network. They designed a novel spacer
hydrogel blends composed of hyaluronan (HA) and methyl cellulose in media, and water sequestration within the scaffold onset strongly depended on the complex interplay between protein interpenetrating networks. The protein release kinetics and delivery et al. used PLGA microspheres in collagen gels and collagen-HA semi-multiple neutrophins with individual release rate PLGA microspheres into PLA-PEG-PLA hydrogels for the delivery of multiple nanoparticles for the release of VEGF for revascularization purposes and osteoblastic differentiation of cells in a synergistic manner or IGF-1(gelatin. This hybrid system allowed the independent release of BMP-2 drogel matrix, composed of glycidyl methacrylated dextran (Dex-GMA)/individual delivery of two different growth factors and embedded in a hy- nanoparticles for the release of VEGF for revascularization purposes 3.3. Dual/multiple delivery systems Biomedical hydrogels that can deliver multiple growth factors in a multimodal and provide desirable pore structure and porosity to potentially encapsulate cells, have considerable potential as future therapeutic tools in tissue engineering. The use of growth factors loaded microspheres embedded in the hydrogel structure is a common approach to multimodal protein delivery.

Microparticles of acidic and basic gelatin were used as carriers for the individual delivery of two different growth factors and embedded in a hydrogel matrix, composed of glycidyl methacrylated dextran (Dex-GMA)/gelatin. This hybrid system allowed the independent release of BMP-2 or IGF-1(Fig. 6) that facilitated cell attachment, proliferation, metabolism, and osteoblastic differentiation of cells in a synergistic manner [39,161]. Fibron hydrogels were complexed with heparin-functionalized PLGA nanoparticles for the release of VEGF for revascularization purposes [40].

A similar approach was adopted by Burdick et al., who formulated PLGA microspheres into PLA-PEG-PLA hydrogels for the delivery of multiple neutrophins with individual release rate [18]. Similarly, Biondi et al. used PLGA microspheres in collagen gels and collagen-HA semi-interpenetrating networks. The protein release kinetics and delivery onset strongly depended on the complex interplay between protein transport through the PLGA matrix and in the collagen-based release media, and water sequestration within the scaffold [162,163]. Physical hydrogel blends composed of hyaluronan (HA) and methyl cellulose (MC) were designed by Shoichet et al. for independent delivery of one or more therapeutic proteins, from 1 to 28 days, for the ultimate application of spinal cord injury repair. To achieve a diversity of release profiles, they exploited the combination of fast diffusion-controlled release of dissolved solutes from the HAMC itself and slow drug release from PLGA particles dispersed within the gel [41]. Methacrylated hyaluronan networks encapsulating alginate microspheres enhanced mesenchymal stem cell chondrogenesis following delivery of TGF-β3 in vitro and in vivo [164].

4. Conclusions and outlook

The importance of growth and adhesive factors and their synergistic interplay during healthy tissue development is nowadays a well-established concept. However, their administration in the form of bolus injection to promote tissue regeneration and repair has shown to be often ineffective and potentially harmful due to the short duration of action of the encapsulated protein drug. Such a delivery approach of growth factors has been applied in a number of clinical trials of unsatisfactory outcome. While some phase I clinical trials (angiogenic gene therapy and human growth factor FGF-1 infusion for coronary artery disease) [165,166] have reported promising results, phase II clinical trials, recruiting a larger number of patients, have not shown the expected benefits to patients. To mention, VIVA (Vascular endothelial growth facto r in Ischemia for Vascular Angiogenesis) trial conducted on 178 pa tients by intravenous and intracoronary infusion of VEGF [167] and FIRST trial comprising intracoronary infusions of FGF-2 on 337 patients [168], did not result in effective commercially available formulations for the treatment of cardiovascular diseases. On the other hand, positive outcomes resulted from clinical trials on growth factors administered through the use of delivery systems that controlled to some extent the protein release rate. In this way the problems associated with the applications of bolus injections are overcome. For example, after successful clinical trials, two growth factors BMP-2 (clinical trial BESTT) [169,170] and BMP-7 (clinical trial OP-1 Putty) [171,172] immobilized in collagen sponge and matrix, respectively, are currently commercially available for the treatment of bone fractures and defects. Also in the field of cardio vascular diseases, encapsulation of FGF-2 into alginate microcapsules led to some positive results in clinical trial phases (clinical trial Polymer) [173,174]. Finally, Regranex® Gel is the only growth factor delivery syst em of PDGF that obtained full FDA approval for clinical use.

Generally speaking, formulation of proteins in controlled delivery systems allow to sustain the release and lower the doses of potent growth factors, that, if administered by infusion, need supraphysiologi cal concentrations, leading to severe side-effects and do not assure therapeutic efficacy over a sufficient time-span, because of rapid degradation/elimination. Moreover, the design of smart delivery systems may offer the possibility to deliver multiple growth factors with inde pendent release rates and to load or attract cells for the regeneration of damaged tissues. This review summarizes the efforts that have been addressed on facilitating the delivery of single or multiple growth factors with appropriate control over temporal and spatial presentation of these biomolecular cues. Current available technologies that have demonstrated potential in the modulation of release profiles, according to the specific therapeutic needs, including traditional diffusion/swelling/degradation-mediated, on-demand, affinity and covalent binding based delivery are presented.

However, some challenges still exist. For example, highly inconvenient encapsulation methods often involving post-loading techniques and low encapsulation efficiency, the stability of the protein, the safety and biocompatibility of the delivery devices and the difficulties to translate in vitro results to in vivo situation. Further efforts in the design or optimization of materials with minimal tissue response, sufficient stiffness, appropriate degradation and release profiles are needed in order to make the translational step between academia and clinics successful.
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