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REVIEW

Alginate Matrices for Protein Delivery – a Short Review

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Summary

Growth factors are powerful molecules that regulate cellular growth, proliferation, healing, and cellular differentiation. A delivery matrix that incorporates growth factors with high loading efficiencies, controls their release, and maintains bioactivity would be a powerful tool for regenerative medicine. Alginate has several unique properties that make it an excellent platform for the delivery of proteins. Mild gelling conditions can minimize the risk of protein denaturation; moreover, alginate can serve as protection from degradation until protein release. Various modifications have been proposed to tune alginate binding and release proteins, simultaneously adjusting alginate degradability, mechanical stiffness, swelling, gelation properties and cell affinity. The primary objective of this article is to review the literature related to recent advances in the application of alginate matrices in protein delivery in regenerative medicine. A special emphasis is put on the relevance of delivery of growth factors and chemokine.

Key words

Alginate • Biomaterials • Protein release • Growth factor release

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Introduction

Alginate is an anionic and hydrophilic polysaccharide derived from the cell walls of brown seaweed and several bacteria strains. This linear copolymer consists of homopolymeric blocks of 1,4-linked β-D-mannuronate (M) and α-L-guluronate (G) residues. Blocks can be composed of consecutive G residues (G-blocks), consecutive M residues (M-blocks), and alternating M and G residues (MG-blocks) (Fig. 1). Depending on the source of alginate, the composition (i.e. M/G ratio) and sequence of blocks may differ (Szekalska *et al.* 2016, Lee *et al.* 2012). Alginic acid is insoluble in water; however, alginate monovalent salts and alginate esters are water-soluble and form stable viscous solutions (Venkatesan *et al.* 2015).

Alginates exhibit various advantageous properties desirable in biomedical applications. They are considered to be biocompatible, non-toxic and non-immunogenic. Moreover, alginates can be gelled with multivalent cations under gentle conditions, which offer a welcome opportunity for entrapment of sensitive materials such as proteins, cells or DNA. Alginates or their gels can also be chemically and physically modified to alter their properties, functions and applications (Pawar *et al.* 2012). In addition, alginates can be easily processed into various three-dimensional scaffold materials such as hydrogels, microspheres, microcapsules, sponges, foams and fibers (Venkatesan *et al.* 2015, Jerome *et al.* 2016, Sun *et al.* 2013, Ahmed *et al.* 2015). Due to the abovementioned properties and potential applications, alginates have drawn enormous attention from researchers in the field of regenerative medicine. They have been broadly investigated as scaffolds for tissue engineering, including wound healing, bone regeneration and cartilage repair (Sun *et al.* 2013, August *et al.* 2006, Kuo and Ma 2001, Venkatesan *et al.* 2015, Koehler *et al.*2018, Lee *et al.* 2012). They are intended to mimic the cell and organ-interactive functions of the extracellular

matrix, as well as to create chemically beneficial environments by presenting ligands that specifically bind to cell receptors. During the past two decades, alginate matrices have been also extensively investigated as carriers for delivery of biomolecules such as growth factors, which can further promote and regulate new tissue development (August *et al.* 2006). The primary objective of this article is to review the literature concerning recent advances in the application of alginate matrices in protein delivery in biomedical applications with a special emphasis put on the relevance of growth factors and chemokine delivery. A summary of recently published articles is presented in Table 1. Earlier studies were covered in the work of Gombotz and Wee (1998).

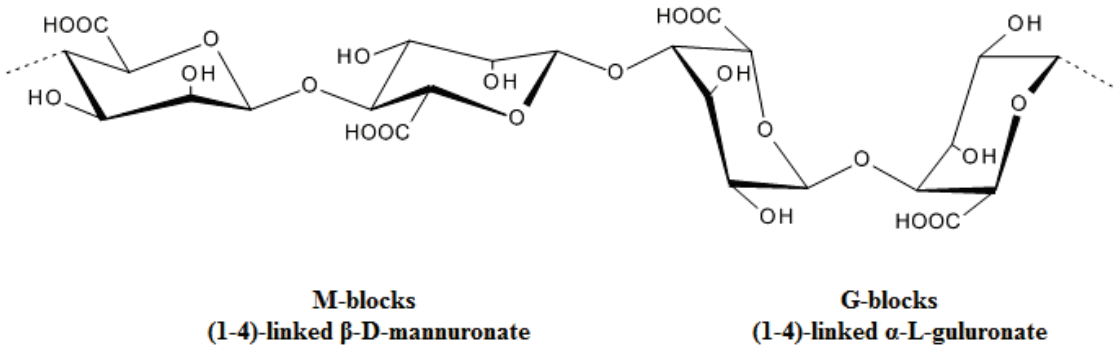

Fig. 1. The chemical structure of alginate.

Table 1. List of growth factors and other proteins encapsulated in alginate matrices studied in tissue engineering research. Recent studies.

Protein(s)	References	Preparation technique/ modifications/form	Releasing time/medium/ method of quantification
Vascular endothelial growth factor (VEGF)	Lee *et al.* 2003	Needle extrusion/external gelation, microbeads	DMEM, 12 days, gamma counter
	Gu *et al.* 2004	Needle extrusion/external gelation, microbeads	PBS and fetal calf serum, from 2 up to 19 days, ELISA
	Silva and Mooney 2007	Combination of two molecular weights of alginates (irradiated and not), oxidation, casting between glass plates	PBS, 32 days, gamma counter
	Freeman *et al.* 2008	Study of protein interactions with alginate sulfate (SPR analysis)	
	Freeman and Cohen 2009	Alginate-sulfate/alginate freeze-dried microporous scaffolds, post loading of growth factor	DMEM, 8 days, ELISA
	Jay and Saltzman 2009	Emulsification technique, microparticles	20 % FBS-M199, 10 days, ELISA
	Silva and Mooney 2010	Combination of two molecular weights of alginates (irradiated and not), oxidation, injectable hydrogel	PBS, 30 days, gamma counter

Protein(s)	References	Preparation technique/ modifications/form	Releasing time/medium/ method of quantification
Basic fibroblast growth factor (bFGF)	Edelman *et al.* 1991	Heparin-Sepharose beads encapsulated in alginate microcapsules, needle extrusion/external gelation, post-loading of GF	PBS with 1 % gelatin, 16 days, gamma counter
	Lee *et al.* 2003	Needle extrusion/external gelation, microbeads	DMEM, 12 days, gamma counter
	Freeman *et al.* 2008	Alginate-sulfate/alginate microspheres, needle extrusion/external gelation	DMEM, 5 days, ELISA
Fibroblast growth factor 1 (FGF-1)	Moya *et al.* 2009	Needle extrusion/external gelation, microbeads	0.9 % NaCl and 0.25 % w/v $CaCl_2$, 21 days, gamma counter
	Khanna *et al.* 2010	Needle extrusion/external gelation, microbeads	9 % NaCl supplemented with 2 mmol/l of $CaCl_2$, 30 days, gamma counter
Platelet-derived growth factor-BB (PDGF-BB)	Freeman *et al.* 2008	Study of protein interactions with alginate sulfate (SPR analysis)	
	Freeman and Cohen 2009	Alginate-sulfate freeze-dried microporous scaffolds, post loading of GF	DMEM, 8 days, ELISA
Transforming growth factor (TGF-β1)	Mumper *et al.* 1993	Needle extrusion/external gelation, microbeads	PBS, PBS with 1 % BSA/, 0.1 N HCl, up to 28 h, ELISA and gamma counter
	Freeman and Cohen 2009	Alginate-sulfate freeze-dried microporous scaffolds, post loading of GF	DMEM, 8 days, ELISA
Recombinant glutathione-s-transferase (GST), Green fluorescent protein (GFP)	Momoh *et al.* 2015	Alginate with and without plasticizer (GLY), mixing, films	Simulated wound fluid containing 0.02 M $CaCl_2$, 0.4 M NaCl, 0.08 M Tris-buffer and 2 % (w/v) BSA, Multi scan EX Micro-plate photometer, Western blotting
Hepatocyte growth factor (HGF)	Freeman *et al.* 2008	Study of protein interactions with alginate sulfate (SPR analysis)	
	Ruvinov *et al.* 2010	Alginate-sulfate, needle extrusion/external gelation, microbeads	Medium M-199, 7 days, ELISA, Western blotting
Endothelial cell growth supplement (ECGS)	Tilakaratne *et al.* 2006	Hydrogel, external gelation	PBS, 142 h, Coomassie Plus Protein Micro Assay

Protein(s)	References	Preparation technique/ modifications/form	Releasing time/medium/ method of quantification
Epidermal growth factor (EGF), Insulin growth factor, FLT, Stem cell factor, Oncostatin, Thromboprotein, Interleukin 6	Freeman *et al.* 2008	Study of protein interactions with alginate sulfate (SPR analysis)	
Stromal cell-derived factor 1 (CXCL12)	Freeman *et al.* 2008	Study of protein interactions with alginate sulfate (SPR analysis)	
	Rabbany 2010	Ionotropic gelation method, freeze-dried hydrogels cross-linked with ethylenediamine, post-loading of chemokine	PBS containing 0.1 mg/ml BSA, 150 h *in vitro*, quantifying radioactivity, 14 days *in vivo*
	Wang and Irvine 2011	Emulsification technique, microparticles, post-loading of chemokine	Phenol red-free RPMI medium containing 10 % fetal calf serum, up to 24 h, ELISA
	Henderson 2011	Ionotropic gelation, freeze-dried hydrogels cross-linked with ED, post-loading of the chemokine	No release studies
	Duncanson and Sambanis 2013	Microcapsules prepared by electrostatic droplet generator	DMEM, 21 days, ELISA
	Chen *et al.* 2015	Microbeads with encapsulated murine or porcine islets	DMEM with 0.5 % BSA, 25 days, ELISA
Interferon gamma-induced protein 10 (CXCL10), CCL19 (Macrophage inflammatory protein-3 beta), CCL21 (C-C motif chemokine ligand 21)	Wang and Irvine 2011	Emulsification technique, microparticles, post-loading of chemokine	Phenol red-free RPMI medium containing 10 % fetal calf serum, up to 24 h, ELISA
Recombinant human bone morphogenetic protein-2 (rhBMP-2)	Jeon *et al.* 2011	Irradiated alginate, photocrosslinked heparin-alginate hydrogels modified with RGD sequences,	PBS, 21 days, ELISA
	Kolambkar *et al.* 2011	Irradiated RGD-modified alginate hydrogels casted into molds	PBS, 21 days, ELISA
	Priddy *et al.* 2014	Irradiated-oxidized RGD modified alginate hydrogels	PBS, 26 days, ELISA
	Quinlan *et al.* 2015	Spray-dried microparticles incorporated into collagen-hydroxyapatite scaffold	PBS, 28 days, ELISA
	McDermott *et al.* 2016	Irradiated and RGD functionalized alginate, injectable hydrogels incorporated into polymer constructs	PBS, 21 days, ELISA

Protein(s)	References	Preparation technique/ modifications/form	Releasing time/medium/ method of quantification
Bone forming peptide-1 (BFP-1)	Luo *et al.* 2016	Freeze-dried porous scaffolds	PBS, 21 days, Fluorescence Spectrophotometry
Bovine serum albumin (BSA)	Leonard *et al.* 2004	Microparticles, external gelation	Water, Tris-buffer, 70 h, Fluorescence Spectrophotometry
	Castro *et al.* 2005	Beads, gelation	PBS/citrate buffer, 40 min, Coomassie Brilliant Blue dye binding assay
	Dai *et al.* 2005	Microcapsules, external gelation	HEPES buffer, 30 days, UV spectrophotometry
	Dai *et al.* 2006	Microcapsules, external gelation	HEPES buffer, 30 days, UV spectrophotometry
	Wells and Sheardown 2007	Needle extrusion/external gelation, microbeads, post-loading of protein	PBS, TRIS buffered saline, 0.15 % NaCl, 200 h, in some cases up to 250 days, Bradford assay
	Sivadsa *et al.* 2008	Spray-dried microparticles	PBS, 3 h, UV spectrophotometry of BSA-FITC
	Stoppel *et al.* 2011	Internal gelation, hydrogels, post loading of protein	Water, 2 h, BCA Protein Assay
	Mobus *et al.* 2012	Spray-dried microparticles	Water, PBS, modified simulated lung fluid, 48 h Coomassie assay
	Liu *et al.* 2013	Internal gelation combined with microfluidic emulsification, microcapsules	No data
	Kaygusuz and Erim 2013	Beads, gelation	Simulated gastric fluid, simulated intestinal fluid, 8 h, Bradford protein assay
	Hariyadi *et al.* 2014	BSA-loaded alginate gel microspheres by aerosolisation	PBS, simulated gastric fluid, simulated intestinal fluid, 24 h, BCA total protein assay
	Eldin *et al.* 2015	Microbeads, internal gelation	PBS, 5 h, UV spectroscopy
	Bajpai *et al.* 2016	Hydrogel discs, Diffusion Through Dialysis Tube (DTDT) technique	PBS, 5 h, UV spectroscopy
Lysozyme, Chymotrypsin	Wells and Sheardown 2007	Needle extrusion/external gelation, microbeads, post-loading of protein	PBS, TRIS buffered saline, 0.15 % NaCl, 200 h, Bradford assay
	Rahmani and Sheardown 2018	Hydrogels, external gelation	Sodium acetate and sodium phosphate buffer, 3 h, Bradford assay

Protein(s)	References	Preparation technique/ modifications/form	Releasing time/medium/ method of quantification
Myoglobin, Cytochrome C	Rahmani and Sheardown 2018	Hydrogels, external gelation	Sodium acetate buffer and sodium phosphate buffer, up to 3 h, Bradford assay
Kaposi fibroblast growth factor At-MTS	Maurice *et al.* 2004	Needle extrusion/external gelation, microbeads	Tris buffer, 2.5 h, mini-Bradford method

Model protein delivery

BSA is commonly used as a model protein for encapsulation of lipophilic drugs, chemicals, and nutrients in studies dealing with preparation of alginate-based carriers mostly in the form of microparticles by different processing methods. As examples, we mention several representative approaches.

Alginate microparticles prepared by spray-drying processes have been studied for their application in nasal and pulmonary drug delivery. Sivadas *et al.* (2008) prepared inhalable alginate particles (of an average diameter 3.23±0.25 μm) with a high encapsulation efficiency of 97 % with the preserved structure and thus bioactivity of BSA. The alginate particles released approximately 20 % of the loaded BSA over 24 h and then a slow release occurred, reaching a cumulative release of only 35 % after 180 h. Möbus *et al.* (2012) prepared Zn^{2+}-crosslinked alginate microparticles containing the model protein BSA *via* a simple one-step spray-drying process. The method led to particles with a diameter of 2-4 μm. The BSA release into the simulated lung fluid increased with an increasing content of protein in the alginate microparticles. For example, for microparticles with a 30 % initiating loading, almost 100 % of BSA was released within 24 h in comparison with 10 % loading microparticles with a cumulative release of 55 %.

Alginate hydrogels have also been studied for oral delivery of proteins. Hariyadi *et al.* (2014) prepared alginate microspheres that were 25 to 65 μm in size and with protein loading of 3.3 %. The BSA release from the hydrated microparticles reached less than 7 % in the simulated gastric fluid over 2 h, whereas 90 % of the protein load was gradually released in the simulated intestinal fluid over 10 h. The release from freeze-dried particles was significantly faster. Kaygusuz *et al.* (2013) prepared alginate particles containing montmorillonite (MMT) with a diameter of 2 mm. Incorporation of MMT significantly enhanced the BSA loading to 78 % in comparison to 40 % evaluated for conventional alginate beads. The presence of MMT also decreased the release both in simulated gastric as well as intestinal fluid.

BSA and hemoglobin were used as model proteins to evaluate the protein release from microbeads (1 mm) prepared from alginate with covalently bound long alkyl chains (Leonard *et al.* 2004). Under predefined processing conditions, the modified alginate enabled a high protein encapsulation (90-100 %). The microbeads prepared from such amphiphilic alginate derivatives exhibited almost no release of both BSA and hemoglobin, e.g. biomolecules that do not interact ionically with the alginate matrix. The authors highlighted a strong contribution of hydrophobic interactions between the tested proteins and hydrophobic alkyl chains in alginate hydrogels for the protein retention. However, addition of the enzyme lipase initiated protein release *via* dissociation of the physical hydrophobic network of the alginate microspheres.

BSA was also used to study the efficiency of carrier-microencapsulated liposome systems based on encapsulation of BSA-containing multivesicular liposomes (MVLs) into alginate microbeads (Dai *et al.* 2006). The proposed carrier preparation led to high encapsulation efficiency of BSA/liposomes and only to minor changes in the chemistry of the drug. The effect of various processing parameters on particle size and encapsulation efficiency of MVLs was evaluated. In addition to other parameters, the authors found that crosslinking with Ba^{2+} ions led to circular particles with a uniform size and with the highest BSA entrapment rate (up to 95 %) in comparison with crosslinking with Ca^{2+} and Al^{3+} ions. *In vitro* BSA release into HEPES buffer exhibited sustained BSA release over 2 weeks.

The most common procedure of protein encapsulation in alginate matrix is an addition of protein to the alginate solution prior to crosslinking. However, the difference in protein concentration between the gel

and the vast volume of the crosslinking solution might cause diffusion, resulting in significant protein loss during gelation. Therefore, a quick encapsulation method performed post-synthesis would be more effective, minimizing protein loss and increasing loading efficiency. Wells *et al.* proposed a new encapsulation technique for high isoelectric point proteins, i.e. lysozyme (pI=11.0) and chymotrypsin (pI=9.1) and for a model protein albumin (pI=4.7) into alginate microcapsules; here, the microcapsules were incubated with a protein-containing NaCl solution and subsequently re-crosslinked with $CaCl_2$ (Wells and Sheardown 2007). The proposed method allowed for the incorporation of significantly higher amounts of isopositive proteins chymotrypsin and lysozyme that otherwise crosslinked alginate. Further, more than twice as much lysozyme was incorporated into the spheres when loaded from the solution containing degradative NaCl (0.15 %) (8 μmol/gram of alginate) contrary to a water solution alone (3.4 μmol/gram of alginate). On the other hand, similar BSA loadings were observed regardless the loading method used. The protein release depended on the supernatant type. The high pI proteins chymotrypsin and lysozyme showed relatively constant delivery into 0.15 % NaCl over more than 3 months. Whereas when released in a TRIS buffered saline, both proteins showed a sustained release lasting over 150 h. On the contrary, for the case of PBS, all three proteins were delivered in a burst release due to the high concentration of degradative phosphate ions within the medium. The group postulated that the sustained release of lysozyme and chymotrypsin indicates that electrostatic interactions of proteins with alginate play a dominant role in the release kinetics and affect diffusive properties of the protein in the alginate (a quick diffusion of negatively charged proteins and a slower diffusion of positively charged proteins). *In vitro* test demonstrated that the released lysozyme remained its active for at least 16 days.

Angiogenic factors delivery

Growth factors (GF) regulate a variety of cellular behaviors including growth, migration, differentiation, apoptosis, and cell survival. They act during development and play important roles in the maintenance of tissue homeostasis and wound healing processes. Secreted growth factors act on nearby cells through paracrine or contact juxtacrine signaling to mediate short-range cell-to-cell communications. Only when GF binds in a proper manner to a transmembrane enzyme-linked receptors in the membrane of the responding cell, the receptor will be activated and this process will promote a cascade of intracellular processes leading to changes in the behavior of the target cells through the direct regulation of transcription factors, or through the regulation of mRNA stability or protein translation (Lodish *et al.* 2000). Therefore regardless if GF is delivered in a soluble form *via* diffusion from the matrix or if it is bound to the substrate, the ligand site has to be available for their interaction with cell receptors.

Neovascularization and angiogenesis play an important role in wound healing as it involves the growth of new blood vessels to form granulation tissue. This dynamic process is highly regulated by signals from both serum and the surrounding extracellular matrix (ECM) environment. Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor beta (TGF), and platelet-derived growth factor-BB (PDGF-BB) are among the potent angiogenic growth factors in wound angiogenesis (Honnegowda *et al.* 2015, Bouis *et al.* 2006). Their loading into alginate hydrogels and subsequent protein release behavior can be controlled by regulating the conditions of microcapsule preparation.

A comprehensive study on VEGF encapsulation into alginate microbeads and VEGF release was performed by Gu *et al.* (2004). In this work, the influence of VEGF loading concentrations, alginate gelation parameters (alginate concentration, G-block content, types and concentrations of additives, as well as types and concentrations of cations in the gelling bath) and the releasing medium composition on VEGF loading and release was thoroughly examined. The authors reported that with increasing VEGF concentrations in the alginate mixtures (0.2, 2 and 20 μg/ml), the encapsulation yield decreased significantly (92.7 %, 69.4 %, and 48.3 %, respectively). After one day incubation in PBS or serum-containing media, the alginate gel with a loading of 20 μg/ml showed the highest initial protein burst (~50 % and ~70 %, respectively) followed by a continuous release into PBS at a rate of 4.4 % per day for 6 days. Other VEGF payloads showed a lower initial release (~10 % for 0.2 μg/ml and ~30-40 % for 2 μg/ml) followed by very low release levels. Neither VEGF encapsulation yield nor the release behavior was affected by the alginate concentration (1.5 %, 2 %, or 3 %). Also no significant difference in VEGF retention was observed for alginates with high and low G-block content. In

contrast, an increasing BSA loading concentration (added into the alginate matrix to mask electrostatic interactions) decreased the encapsulation yield.

Moreover, the concentration of $CaCl_2$ in the releasing medium greatly affected the profile of VEGF release; the amount of the eluted protein increased with increasing concentrations of Ca^{2+} cations. Specifically, approximately 22.3 % of VEGF was released into supernatant with 0.1 M $CaCl_2$ within 19 days. In contrast, almost 100 % of protein was released into the highest $CaCl_2$ concentration investigated (1 M). However, this system showed a very high initial burst where almost 85 % of VEGF was delivered within the first 72 h. The releasing medium with 0.5 M concentration of $CaCl_2$ led to a constant release rate of 6 ng/ml/day over 14 days. However, such high concentration of $CaCl_2$ cannot be used therapeutically.

A low concentration of Na^+ or Mg^{2+} cations (0.1 M) in gelling medium increased VEGF encapsulation yields (Na^+ 97.9 %, Mg^{2+} 98.9 %, control 92.7 %). As the concentrations of cations increased to 0.5 M or 1 M, the encapsulation yields were lower than the control. The authors attributed higher retention yields to an appropriate amount of the ions required to create a homogenous gel of smaller average pore size, which slowed the rate of the VEGF release. An addition of NaCl or $MgCl_2$ over 0.5 M into the releasing medium maintained the VEGF release in a similar manner to the $CaCl_2$ containing supernatant; however, those alginate hydrogels disintegrated in those supernatants after one week.

Jay and colleagues investigated the VEGF release from alginate matrices gelled by various ionic crosslinkers (different salts and concentrations were studied) (Jay and Saltzman 2009). The mixing of alginate microparticles crosslinked by Zn^{2+} and Ca^{2+} allowed for a controlled and sustained VEGF release, the particles were non-cytotoxic, and the released VEGF retained its bioactivity. Lee *et al.* (2003) monitored the release of radiolabeled VEGF and bFGF from alginate beads into Dulbecco's Modified Eagle Medium (DMEM). The authors reported a sustained release profile of both growth factors with an almost zero-order release rate for the initial time periods and a small slowdown later in the experiment. Alginate hydrogels eluted approximately 55 % of the loaded bFGF within 12 days and approximately 70 % of VEGF within 10 days in DMEM.

The FGF-1 delivery from alginate microbeads in order to induce neovascularization *in vivo* was investigated by Moya *et al.* (2009). The release profile of radiolabeled FGF-1 revealed a high initial burst (~90 %) during the first 3 days, followed by a constant release of small amounts of FGF-1 over 3 weeks. The microbeads loaded with FGF-1 (total amount 150 ng) were implanted into a surgically created omental pouch in rats. Three weeks after implantation, the calculated vascular area for the FGF-1 loaded microbeads and for the bolus FGF-1/empty microbead control did not significantly differ, but both were higher than that observed for the empty microbead control. Six weeks after implantation, the vascular density evaluated for the FGF-1 loaded microbeads was significantly higher than the density observed for both control groups. Moreover, staining for smooth muscle actin revealed that 50 % of the vessels had associated mural cells. A trend of increased mural cell staining at 6 weeks for the FGF-1 loaded alginate beads was reported as well.

Diffusion is a predominant mechanism of growth factor release from non-degradable alginate hydrogels; however, protein release can be additionally tuned by matrix degradation. This scenario can be achieved by control over molecular weight, e.g. by gamma-irradiation of a polymer or by use of a binary molecular weight formulation to allow the polymers to more readily dissociate from the alginate matrix. The matrix degradation can be also enhanced by partial oxidation of urinate residues in order to make polymer chains susceptible to hydrolysis. Silva's group investigated the influence of alginate oxidation on the release of ^{125}I-labeled VEGF into PBS (Silva and Mooney 2007). The samples were prepared in the form of hydrogel blocks crosslinked with calcium slurry from mixtures of high (250,000 Da) and low molecular weight (50,000 Da) alginates subjected previously to oxidation with sodium periodate. The oxidized alginate matrix showed enhanced protein release into PBS especially at the beginning of the experiment. Approximately 60 % of the loaded VEGF was released within the first 7 days, followed by a slower release leading to approximately 80 % of overall released VEGF after 33 days. The non-oxidized alginate hydrogel delivered VEGF at a much slower rate, releasing only ~40 % of the entrapped VEGF after 30 days. The release profiles corresponded to degradation rates of the alginate matrices. The oxidized samples degraded more rapidly. After 6 days, approximately 45 % and 20 % of mass loss was detected for oxidized and non-oxidized alginate matrices, respectively. Within 40 days the oxidized hydrogels had

almost completely degraded (~80 % mass loss), while the non-oxidized alginates showed a mass loss of approximately 35 %. Importantly, *in vitro* tests revealed that VEGF released from the alginate matrix during the first 4 days was bioactive and even more effective than VEGF added directly to the culture medium. Moreover, the VEGF-alginate system promoted a 2.5 greater number of endothelial cell sprouts in comparison to the VEGF control. The *in vivo* experiment showed that the VEGF-alginate injectable system exhibited a controlled and localized delivery of growth factors in ischemic hindlimbs, releasing a higher level of VEGF in hypoxic regions than a bolus injection. The proposed injectable VEGF-alginate systems were further investigated *in vivo* elsewhere (Silva and Mooney 2010).

Most of the delivery systems are designed to deliver one growth factor in a single system; however, it has been shown that it is usually a certain combination of growth factors with different impacts on cell behavior which synergistically stimulate activity of cells in particular physiological *in vivo* processes (Wang *et al.* 2017). For example, in the first stage of angiogenesis, VEGF, FGF-2 and angiopoietin-2 stimulate disruption of preexisting blood vessels as well as migration and proliferation of endothelial cells into the place of the new formed immature vessels (Lee *et al.* 2011). Whereas in the second stage angiopoietin-1 and PDGF initiate pericyte recruitment into the place of vessel formation and thus promote stabilization of newly formed capillaries (Rouwkema *et al.* 2016). Therefore delivery of a cocktail of GFs to the place of implantation would be more effective in successful vascularization than delivery of individual growth factors.

Freeman and Cohen proposed a strategy for the sequential delivery of three angiogenic factors, i.e. VEGF, PDGF-BB and TGF-β1, from macroporous alginate-sulfate/alginate scaffolds (Freeman and Cohen 2009). The scientists took advantage of alginate-sulfate's ability to bind multiple heparin-binding proteins with equilibrium binding constants similar to those observed upon interaction with heparin (Freeman *et al.* 2008). Macroporous scaffolds prepared by a freeze-drying technique were incubated in single or triple protein solutions (100 ng of total protein loaded). The study revealed that for both alginate-sulfate/alginate and alginate scaffolds, the release patterns from the single and triple protein-loaded systems into DMEM coincided, indicating no mutual effect on the growth factor release rate and sufficient alginate-sulfate ability for multi-protein binding. The incorporation of alginate-sulfate into the alginate matrix significantly prolonged the protein release. The alginate-sulfate/alginate scaffold released VEGF with a small initial burst (20 %), followed by a slower releasing rate. The PDGF-BB and TGF-β1 were delivered continuously over an 8-day experiment. The VEGF and PDGF-BB release reached a plateau after 6 days, while TGF-β1 continued to be released into the medium. In contrast, pure alginate scaffolds delivered approximately 60 to 80 % of growth factors as an initial burst within the first day. Overall, the results demonstrated sequential delivery of growth factors, i.e. with an initial high level of VEGF followed by PDGF-BB, and a high level of TGF-β1 in later days. Moreover, the group showed that subcutaneously implanted triple factor-loaded scaffolds exhibited 3-fold greater blood vessel density and percentage of mature vessels than single growth factor-loaded or untreated scaffolds.

Chemokine delivery

Chemokines are another group of proteins that play a key role in the wound healing process, particularly within the inflammatory and proliferative phases. They show an ability to induce directed chemotaxis in nearby responsive cells and specifically stimulate the recruitment of leukocytes to inflammatory sites (Rees *et al.* 2015, Graves *et al.* 1995).

A detailed study of the adsorption of chemokines stromal cell-derived factor 1 (CXCL12), interferon gamma-induced protein 10 (CXCL10), macrophage inflammatory protein-3 beta (CCL19) and C-C motif chemokine ligand 21 (CCL21) into alginate microspheres and subsequent protein release behavior was published by Wang and colleagues (Wang and Irvine 2011). First, the authors assessed the impact of medium ionic strength on CCL21 release. CCL21 release from the particles increased with an increasing ionic strength of PBS (30 mM, 150 mM and 750 mM of NaCl). This observation is consistent with electrostatic-mediated binding of CCL21 to the alginate matrix. Furthermore, it was reported that CCL21 loading efficiency increased with decreasing concentration of the protein mixed with microspheres (55 % for 50 μg chemokine/mg alginate and up 95 % for concentrations lower than 10 μg/mg). The chemokine post-loading into alginate microspheres increased in the order mCXCL10<hCCL19<hCXCL12 (i.e. ~50 %, ~70 % and ~90 %, respectively), inversely to

the evaluated release rates (ELISA method). CXCL12 loaded at the highest level; however, almost no protein release was detected. The loaded microspheres released chemokines at rates ranging from 7.2×10^{-6} to 3.2×10^{-3} ng/h/particle, covering the physiological range of chemokine release by individual cells. Moreover, in the cases of CCL21, CCL19, and CXCL10, release rates in the physiological range were maintained for at least 24 h. The authors stated that the logarithm of the effective diffusivity of each chemokine in alginate (obtained by fitting the release data to a Fick's law model) correlated well with the net surface charge of the proteins per residue, consistent with charge-mediated interactions between the proteins and the alginate matrix dominating the loading/release behavior. The authors outlined that the investigated systems released a bioactive protein generating functional attractant gradients, both in medium and collagen gels, that were able to attract cells more than hundreds of microns away to make contact with the individual microspheres.

The CXCL12 retention in alginate microcapsules dedicated to encapsulation of βTC-tet cells was investigated by Duncanson and Sambanis (2013). The microparticles were prepared directly from the co-solution of alginate and chemokine. The authors reported a large initial CXCL12 loss (87 %) during the capsule processing. The intracapsular chemokine concentration in the acellular microparticles decreased rapidly during the first 7 days after CXCL12 encapsulation, followed by significantly slower release rate for the next 14 days. When βTC-tet cells were encapsulated in the CXCL12/alginate matrix, a rapid decrease in intracapsular CXCL12 concentration was observed. One day after the cell encapsulation, the cellular microparticles contained a two-order lower concentration of CXCL12 (35 ng/ml alginate) than the microparticles without cells (1,100 ng/ml alginate). Furthermore, in contrast to acellular microcapsules, the intracapsular CXCL12 concentration significantly declined 2 days after the cell encapsulation, and at day 5, the chemokine was no longer detectable. The βTC-tet viability experiment revealed that the presence of CXCL12 in the encapsulation matrix completely abrogated cell apoptosis under hypoxic conditions.

Further work of Duncanson and colleagues presented the sustained release of CXCL12 from alginate microparticles designed for islet transplantation (Chen *et al.* 2015). The group also observed a significant loss of CXCL12 during the cross-linking process. The release rate of CXCL12 from 1.5 % alginate capsules was 1.75 ng/ml/h during the first 24 h and after 4 days stabilized at 0.18 ng/ml/h. The release profiles of CXCL12 for alginate concentrations of 1.5 % to 3.3 % showed no differences. Importantly, the encapsulation of pancreatic islets from C57Bl/6 mice within CXCL12/alginate microbeads resulted in long-term allo- and xenoislet survival and function, as well as in a selective increase in intragraft immunocompetent regulatory Tregs cells.

The delivery of ^{125}I-labeled CXCL12 from alginate matrices for wound healing applications was investigated by Rabbany *et al.* (2010). The authors applied surface immobilization of therapeutic agents. First, crosslinked porous alginate discs were covalently coated with spermine chloride thus generating a positive charge on the surface. Then, the discs were incubated overnight in a co-solution of radiolabeled CXCL12 (1 ng/µl) and heparin (1 U/µl) where polyanion heparin formed a polyelectrolyte complex with the coated spermine layer. The scaffolds delivered approximately 60 % of CXCL12 during the first 24 h *in vitro* (0.1 mg/ml BSA in PBS, 150 h, 37 °C). Thereafter, the protein delivery was minimal (~70 % of total CXCL12 released after 150 h). Also *in vivo* experiments revealed a fast CXCL12 release of ~ 60 % by 1 day. A beneficial effect of CXCL12 on wound healing in a large animal model was reported. The CXCL12-treated wounds significantly accelerated wound closure compared to non-treated controls. Moreover, 38 % of CXCL12-treated wounds were fully healed by day 9 with very little evidence of scarring as opposed to the controls. This proposed chemokine delivery vehicle was further investigated *in vivo* in later work (Henderson *et al.* 2011).

Bone morphogenetic proteins delivery

Bone morphogenetic proteins (BMP) are a group of cytokines that affect bone as well as cartilage growth and repair, enable skeletal tissue formation during embryogenesis and throughout adulthood. Therefore, they are the most important growth factors in bone formation and healing processes studied in bone tissue engineering. Regarding the use of alginates, several strategies of delivering BPMs within a biomaterial scaffold to the site of injury in order to stimulate the endogenous bone repair process has been proposed (Quinlan *et al.* 2015, Priddy *et al.* 2014, Bessa *et al.* 2008).

Quinlan *et al.* (2015) focused on the

development of collagen-hydroxyapatite scaffolds containing alginate microparticles for controlled delivery of rhBMP-2. The rhBMP-2 was encapsulated in spray-dried alginate microparticles (1 μg/mg alginate) with a yield of 45 %. The release profile from pure alginate microparticles into PBS revealed an initial burst (~40 %) during the first 7 days followed by a slower release rate for the next 7 days. At the end of the study (28 days), the microcapsules released 46 % of the initially loaded rhBMP-2. The rhBMP-2/microcapsules were then incorporated into three-dimensional porous collagen-hydroxyapatite scaffolds (CHA) with loading of 1.5 μg rhBMP-2 per scaffold. The alginate microbeads-CHA constructs exhibited a high initial burst of rhBMP-2, followed by a sustained delivery for the next 7 days. Nevertheless, after 28 days, the alginate microbead–CHA constructs delivered only 15 % of the originally encapsulated rhBMP-2. The authors suggested that a burst release was caused by the aggregation of the encapsulated protein at the surface of the spray-dried alginate microparticles, and thus rhBMP-2 might have diffused more quickly. However, the strong interaction of rhBMP-2 with hydroxyapatite could have hindered the release of a fraction of the protein, resulting in a significantly lower amount of rhBMP-2 released compared to the microparticles alone. An *in vitro* study of MC3T3-E1 murine pre-osteoblast cells revealed that rhBMP-2 released from alginate-CHA scaffolds maintained its pro-osteogenic effect by enhancing ALP activity of the cells.

Another work investigated the release kinetics of bone forming peptide-1 (BFP-1) labelled with 6-carboxy tetramethyl rhodamine from three-dimensional porous alginate scaffolds (PAS) using fluorescence spectrophotometry (Luo *et al.* 2016). Scaffolds of two protein concentrations (10 and 100 μg of BFP-1 noted as p-PAS-10 and p-PAS-100, respectively) were prepared by a freeze-drying process and protein release from disc specimens into PBS was monitored for 21 days. The results showed that both BFP-1/alginate scaffolds had an excellent sustained-release performance but with a different velocity of peptide release depending on the concentration of the loaded peptide. The p-PAS-100 released 70 % of BFP-1 in the first 9 days, while the p-PAS-10 delivered approximately 30 % of BFP-1 in a relatively sustained rate over 21 days. After 21 days in PBS, the p-PAS-100 scaffold was much larger than the p-PAS-10 one, suggesting faster degradation of p-PAS-100. The accelerated p-PAS-100 degradation might be a reason for quicker protein release from this system. *In vitro* studies revealed higher cell adhesion, aggregation, proliferation and alkaline phosphatase activity of MG-63 cells for the BFP-1/alginate scaffolds in comparison to the pristine alginate scaffolds, when outstanding bioactivities were observed, especially for the p-PAS-10 scaffold.

Alginate matrices do not sufficiently support the adhesion and ingrowth of bone cells; therefore, they are often enriched by a specific adhesive peptide sequence Arg-Gly-Asp (RGD) that mediates cell attachment (Ruoslahti 1996). The abovementioned strategy was used in the following studies. Priddy *et al.* (2014) investigated the effect of alginate oxidation on rhBMP-2 release. Irradiated and oxidized-irradiated sodium alginates were first modified with RGD peptide sequences (two sequences/polymer chain). Then, 150 μl of the alginate hydrogel containing 500 ng of rhBMP-2 was injected into the PCL nanofiber mesh. Only 20 % of the total loaded protein was released into PBS within 26 days. The oxidation of the irradiated alginates accelerated the BMP-2 release but the difference between the oxidized and non-oxidized samples was noticeable only till day 5. More than 95 % of the released BMP-2 was eluted by day 3 and day 8 for the oxidized-irradiated and irradiated alginates, respectively. Further, alginate matrices retained 35 % of the loaded rhBMP-2 after 26 days and the protein was still bioactive as observed by the induction of alkaline phosphatase activity and positive Alizarin Red S staining of MC3T3-E1 cells. Moreover, robust bone regeneration was observed in both groups through 12 weeks in *in vivo* studies. The histological analyses of bone defects showed enhanced degradation of oxidized-irradiated alginate and suggested the presence of more mature bone after 12 weeks of healing.

The rhBMP-2 was also incorporated into irradiated alginate covalently coupled with G_4RGDASSP peptide sequences (Kolambkar *et al.* 2011). The RGD-alginate solution mixed with rhBMP-2 was gelled with the calcium sulfate slurry and injected into cylindrical molds, giving plugs containing 500 ng of rhBMP-2. The release into PBS containing calcium and magnesium ions was monitored over 21 days. The sample released 71.2±3.8 ng of rhBMP-2 over 21 days; however, 98.6 % of the total released protein was detected within the first 7 days. The samples at day 0 and 21 were assayed for the amount of entrapped rhBMP-2. The rhBMP-2 amount in the initial alginate matrix (day 0) represented only 55.1 % of the total protein added (275.5 ng/500 ng), while

27.2±3.3 ng of rhBMP-2 was retained in the hydrogel at the end of the experiment (day 21). Taking into account the abovementioned results, only 25.8 % of the initial amount of rhBMP-2 (71 ng/275.5 ng) was released into the medium during the experiment and 9.9 % of the initial protein concentration (27.2 ng/275.5 ng) was still present in the alginate matrix at day 21, despite the fact that only a minute amount of rhBMP-2 was released. The authors hypothesized that the rhBMP-2 molecules can bind to the alginate matrix, masking the antibody binding site, or degrade over the incubation period. Thus, the actual amount of growth factor present in the hydrogels might have been higher.

On the other hand, the sustained delivery of rhBMP-2 from irradiated RGD-functionalized alginate was reported by McDermott and colleagues (McDermott *et al.* 2016). In this work, structural porous scaffolds produced from a copolymer of poly(L-lactide) and poly(DL-lactide) (PLDL) as well as electrospun poly(ε-caprolactone) (PCL) nanofiber mesh tubes served as platforms for alginate matrices. The pores and inner core of cylindrical PLDL scaffolds were infused with the alginate hydrogel, while for the PCL tube the alginate hydrogel was injected in situ. The resulting scaffolds, loaded with 500 ng of rhBMP-2, were incubated in PBS for 21 days. The PLDL/ALG such as PCL/ALG constructs delivered rhBMP-2 in a similar manner, i.e. there were no significant differences in release kinetics, total release and protein retention between groups. The scaffolds released approximately 40 ng within 21 days. The amount of rhBMP-2 retained in PCL/ALG scaffolds was 41.4±21.9 ng, while 71.1±20 ng of rhBMP-2 was detected in PLDL/ALG scaffolds. Considering the above information, the sum of the released and retained protein did not coincide with the initially loaded amount. The authors suggest that it may have been caused by partial protein degradation, losing protein prior to detection or inability of detection by the selected ELISA assay, and thus the release kinetics might have been underestimated.

The natural affinity between heparin and heparin-binding growth factors (e.g. VEGF, FGF-2, BMP) *via* electrostatic interactions between heparin's negatively charged sulfate groups and the proteins' positively charged amino acid groups can be exploited to control the release of growth factors from alginate matrices. Jeon *et al.* (2011) proposed an affinity-based growth factor delivery system using photocrosslinked heparin-alginate hydrogels to allow controlled, prolong release of therapeutic proteins. In this study, the BMP-2 release into PBS from four different photocrosslinked alginate systems, i.e. methacrylated alginate hydrogel (ALG), arginylglycylaspartic acid modified methacrylated alginate hydrogel (RGD-ALG), methacrylated heparin-alginate hydrogel (HP-ALG) and arginylglycylaspartic acid modified methacrylated heparin-alginate hydrogel (HP-RGD-ALG), was investigated over 3 weeks. It was reported that systems without photocrosslinked heparin released BMP-2 more rapidly than systems containing native heparin. Almost all loaded protein was released from ALG and RGD-ALG hydrogels within the first 7 days. In contrast, the BMP-2 release from the photocrosslinked HP-ALG and HP-RGD-ALG hydrogels was slower and sustained over 3 weeks without an initial burst release (~60 % released after 1 week, ~70 % after 3 weeks). As there were only minor differences in the release profiles of RGD-containing hydrogels and unmodified ones, alginate hydrogels can be modified with the RDG peptide sequence to allow cell adhesion without influencing the function and benefit of the photocrosslinked heparin. The *in vitro* studies confirmed that BMP-2 released from photocrosslinked HP-ALG hydrogels retained its biological activity and affected the behavior of the MC3T3-E1 preosteoblasts. Moreover, *in vivo* experiments showed that BMP-2-loaded HP-ALG hydrogels induced significantly more osteogenesis than BMP-2-loaded unmodified ALG hydrogels, with 1.9-fold greater peripheral bone formation and 1.3-fold greater calcium content 8 weeks after implantation. The photocrosslinked heparin-alginate hydrogels have been reported to exhibit sustained and controllable release of other growth factors, e.g. VEGF, TGF-β1, and FGF-2.

Conclusions

During the last 25 years, alginate properties, characterization and preparation have been extensively studied demonstrating their applicability as biomaterials. Due to outstanding physicochemical properties and especially mild crosslinking conditions, alginates are a valuable platform for protein encapsulation. Protein loading and release are strongly affected by the type and characteristics of the alginate matrix, by protein characteristics, by the mutual interactions between proteins and alginate matrix as well as by the processing method. Crosslinking type and crosslinking density significantly affect release through hindering the protein in a more or less crosslinked gel. Alginates with high G-blocks content yield stiffer, brittle and mechanically more stable hydrogels, while high M-blocks content leads to soft and

more elastic gels with higher water adsorption and easier ion exchange. Neutral biomolecules and proteins with a low pI diffuse through the alginate hydrogel network with diffusion coefficients depending on the ratio of their molecular weight to the network mesh size; they are released quickly, usually with a burst release effect. On the contrary, proteins with a high pI can interact ionically with a negatively charged alginate matrix what can strongly slow down if not block protein release. Several modifications have been proposed to tune alginate binding and release of proteins, adjusting at the same time alginate degradability, mechanical stiffness, swelling, gelation properties, protein-matrix interaction and/or cell affinity. However, the often observed high burst effect and short release times will still represent challenges for researchers. To this end, it will be necessary to put scientific effort on comprehensive understanding of interactions between particular proteins and a negatively charged alginate matrix. Such information could serve as engineering design criteria to develop new modifications of the alginate matrix to achieve well control over the protein release kinetics as well as preserving protein activity for prolonged release times. Additionally, further research activities should also reflect current deep knowledge about regeneration processes on cellular levels (e.g. during wound healing or vascularization) leading to requirements for multiply growth factor delivery. Following that, further important issue to be solved is estimation of optimal delivered GFs doses based on demands for regeneration of particular tissues; this could be achieved when combining proposed *in vitro* evaluation experiments with appropriate *in vivo* studies. Finally, alginate-based systems for delivery of proteins and especially of growth factors are of great interests in constructing of bioactive biomaterials. The proper selection of the alginate type, crosslinking method as well as processing method with respect to characteristics of delivered proteins could provide delivery systems with tailor-made characteristics for particular tissue engineering applications.

Conflict of Interest

There is no conflict of interest.

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