

VEGF-loaded injectable hydrogel enhances plasticity in the injured spinal cord

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Abstract

We hypothesized that VEGF-containing hydrogels that gelify *in situ* following injection into a traumatized spinal cord, could stimulate spinal cord regeneration. Injectable hydrogels composed of 0.5% MVG alginate, supplemented or not with fibrinogen, were used. The addition of fibrinogen to alginate had no effect on cell proliferation *in vitro* but supported neurite growth *ex vivo*. When injected into a rat spinal cord in a hemisection model, alginate supplemented with fibrinogen was well tolerated. The release of VEGF that was incorporated into the hydrogel was influenced by the VEGF formulation (encapsulated in microspheres or in nanoparticles or in solution (free)). A combination of free VEGF and VEGF-loaded nanoparticles was mixed with alginate:fibrinogen and injected into the lesion of the spinal cord. Four weeks post-injection injection, angiogenesis and neurite growth were increased compared to hydrogel alone. The local delivery of VEGF by injectable alginate:fibrinogen-based hydrogel induced some plasticity in the injured spinal cord involving fiber growth into the lesion site.

Keywords: injectable hydrogel, VEGF delivery, nanoparticles, microparticles, alginate, spinal cord

Introduction

Injectable hydrogels are particularly interesting because they can adapt to lesions of any shape and can preserve intact tissues. Hydrogels have been used in regenerative medicine, serving as drug depots, bioactive agents delivery vehicles and materials in which to encapsulate and deliver cells [1]. Hydrogels also create a suitable environment for inducing tissue regeneration at the defect/lesion by providing an artificial extracellular matrix that can temporarily support cell attachment, proliferation and differentiation. Injectable hydrogels solidify *in situ*, maintaining cell viability and bioactivity while preventing damage to the surrounding tissue [2]. Injectable matrices require less invasive surgeries, shortening the surgical operation time and reducing the post-operative loss of function, pain and scar size [3].

Spinal cord injury (SCI) results primarily from severe mechanical trauma, leading to secondary injuries in addition to immediate axon and neuron destruction and loss of function. Currently, there is no clinical treatment or therapy that can restore lost function [4]. Several hydrogels have been investigated for use in spinal cord regeneration [1, 5, 6]. A recent review by Gilbert *et al.* lists articles focused on biomaterials used to treat spinal cord injuries [4]. Among them, alginate [7-10] was used but not as injectable matrices. Alginate has been widely used for drug delivery and cell encapsulation and as an injectable cell transplantation vehicle due to its biocompatibility, low toxicity and relatively low cost [11]. Its solid, non-injectable form has also been used for spinal cord regeneration [7-9, 12]. When co-injected with calcium in the lesion, alginate can gelify *in situ* and fill the spinal cord lesion (des Rieux, unpublished data). However, mammalian cells do not possess receptors for alginate, which limits their adhesion and proliferation. This limitation can be

overcome by supplementing alginate hydrogels with extracellular matrix (ECM) motifs/molecules that support cellular adhesion, such as laminin, fibronectin, collagen and RGD sequences [1], or fibrinogen [13]. Fibrinogen is a natural substrate for tissue remodeling that contains several cell signaling domains, including cell adhesion motifs [14]. Improved locomotor function as well as revascularization and axonal growth have been reported when fibrinogen or fibrin was implanted into injured spinal cords [15].

The exogenous administration of vascular endothelial growth factor (VEGF) to traumatically injured spinal cords has been recently investigated as a potential therapy for spinal cord regeneration. VEGF is a pro-angiogenic growth factor that has also been shown to have neuroprotective effects [16-18]. VEGF has been investigated in models of SCI and stroke, with mixed results [19]. Adverse effects could be due to a suboptimal delivery strategy [20]. Numerous nano- and micro-formulations have been developed for the sustained release of VEGF [21-24], including poly(lactide-co-glycolic acid) (PLGA) microspheres [25] and chitosan nanoparticles [26].

We hypothesized that local VEGF delivery from injectable hydrogel would stimulate spinal cord regeneration, by enhancing neurite growth around the lesion and/or within the hydrogels. The objectives of the study were then to (i) study the impact of alginate-based hydrogel on cell proliferation, neurite outgrowth and spinal cord tissue and (ii) assess in a rat hemisection model the influence of local VEGF delivery from an *in situ* gelifying alginate hydrogel on angiogenesis, neurite growth and functional recovery.

Materials and methods

Preparation of hydrogels

A 0.5% (w/v) alginate (Pronova UPMVG (medium viscosity, 60% of the monomer units are guluronate), FMC BioPolymers, NovaMatrix, Philadelphia, PA) solution was prepared in MilliQ water, incubated with charcoal for 30 min and filter sterilized (Millex™, MA). The endotoxin level of NovaMatrix alginates is guaranteed to be below 100 EU/g.

A 50 mM calcium chloride (CaCl₂) (Sigma Aldrich, Saint Louis, MO) solution was prepared in MilliQ water and sterilized by filtration. Alginate hydrogels were formed by the co-injection of 0.5% alginate and 50 mM calcium chloride solutions.

Fibrinogen was added to alginate (5 mg/ml) based on the hydrogels' mechanical properties and compatibility with cell proliferation (data not shown). The fibrinogen used was a component of the Tisseel™ fibrin sealant kit, kindly provided by Baxter Innovations GmbH Inc., and was reconstituted with the supplied aprotinin solution at 100 mg/ml.

Rheological characterization of hydrogels

Dynamic viscoelastic measurements were performed on a Kinexus rotational rheometer (Malvern Instrument SA, Orsay, FR). Alginate solutions were loaded into the rheometer between the parallel plates (8 mm diameter) with a gap of 1 mm. Calcium solution was then added and record of the moduli started immediately (1 rad/s and a stress control of 1 Pa). A solvent trap containing water was used to prevent water evaporation. The moduli of the hydrogels without and with fibrinogen were

recorded overnight. The final moduli were obtained when a plateau was reached in the measurements.

Cell proliferation assay

NIH-3T3 cells (mouse fibroblast-like cells, CRL-1658) and SH-SY5Y cells (human neuronal-like cells, CRL-2266) (ATCC, Manassas, VA) were cultured at 37°C and 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% sodium pyruvate, 1% penicillin-streptomycin (PEST), 1.5 g/L NaHCO₃ and 10% fetal bovine serum (FBS). For SH-SY5Y cells, 0.6% nonessential amino acids (Invitrogen, Carlsbad, CA) were added to the above-mentioned medium.

Hydrogels with a volume of 300 µl were formed in 48-well plates and incubated for 60 min at 37°C (n=5). They were then washed with PBS, after removing calcium chloride solution used to gel the alginate and before to seed the cells to remove potential calcium chloride traces. A total of 30 000 NIH-3T3 cells/gel or 60 000 SH-SY5Y cells/gel were seeded on each gel (NIH-3T3 and SH-SY5Y cell doubling times are 20 and 48 hours, respectively). The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (MTS test) (Promega, Leiden, NL) was used following the supplier instructions. A 100 µl sample of the reagent was added to each well along with 200 µl of culture medium, and the cells were incubated for 1 hour at 37°C. The absorbance of the supernatants was read at 492 nm. The cells were washed in PBS, and fresh medium was added. The measurements were performed 6 hours after seeding (cell adhesion) and on days 3, 7 and 14 (cell proliferation).

Dorsal root ganglion (DRG) culture

Hydrogels were formed on Organotypic Millicell culture inserts (30 mm diameter) (Merck Millipore, Overijse, BE). DRG from newborn rats (day 0 to 2) (Wistar) were

extracted and placed on hydrogels (5 DRG/insert) (n=10). Matrigel (BD Biosciences, Erembodegen, BE) was used as a positive control. Culture medium (DMEM supplemented with 10% FBS, 1% L-glutamine, 1% PEST and 1% nonessential amino acids + 50 ng/ml NGF (BD Biosciences)) was added to the basolateral compartment only. The cultures were incubated at 37°C for 48 hours before fixation. Light microscopic images of the DRGs cultured on hydrogels were recorded (ApoTome microscope, Zeiss). The number of neurites per explant (n=10) and the lengths of the longest neurites (n=6 if number of neurites >100) from each explant were measured using Axiovision software (Zeiss). The data are reported as means +/- SD.

VEGF formulation

VEGF₁₆₄ was produced and purified as previously described [26]. VEGF was encapsulated either in nanoparticles or in microspheres. VEGF was encapsulated in chitosan-dextran sulfate (CS/DS) nanoparticles as previously described [24, 26]. The encapsulation efficiency was 76%, the drug loading was approximately 5 µg VEGF/mg of nanoparticles, and the mean nanoparticle diameter was 300 nm. VEGF was encapsulated in PLGA microspheres using the TROMS technique [25] with an 80% encapsulation efficiency and a loading of 3 µg/mg microspheres. The mean microsphere diameter was 7 µm.

In vitro VEGF release profiles

The influence of the VEGF formulation on its release from alginate:fibrinogen hydrogels was studied *in vitro*. A 1 µg sample of VEGF₁₆₄ in its free form, encapsulated in microspheres [25] or encapsulated in nanoparticles [26] was incorporated into 500 µl of alginate, and hydrogels were formed by the addition of

calcium chloride (n=3). The hydrogels were incubated in PBS + 0.01% sodium azide and 0.5% BSA at 37°C for 1 month. The amount of VEGF released was measured using a sandwich ELISA (R&D Systems, Oxon, UK) and expressed as a percentage of the released VEGF.

Hydrogel injection into a rat hemisection model of spinal cord

The animal experiments were approved by the ethical committee for animal care of the health science sector of Université catholique de Louvain. Female Long Evans rats (UCL Animal facility, 180-200 g) were anesthetized using a rodent anesthesia System (Equipement Veterinaire Minerve, Esternay, FR) with vaporized isoflurane (Isoba, Schering-Plough Animal Health, Merck Animal Health, Boxmeer, NL) to perform a laminectomy at T9-10 and expose the spinal cord. A lateral hemisection resulting in a gap of 4 mm long up to the midline was created (n=12) [27, 28]. Hydrogels (10 µl) were injected using a double syringe system (Duploject™, Baxter Innovation) equipped with a 30 G needle and containing a 0.5 % alginate solution on one side and a 50 mM calcium chloride solution on the other side, into the cavity formed by the hemisection, and gelation occurred within 5 min. No gel was injected into the untreated operated animals (negative control). Then, the muscles were sutured together, and the skin was stapled. Post-operative care included the subcutaneous administration of Baytril (enrofloxacin, 2.5 mg/kg s.c., once per day for 2 weeks), buprenorphine (0.01 mg/kg s.c., twice per day for 3 days) and lactated Ringer's solution (5 mL/100 g, once per day for 5 days). In addition, the bladder was expressed twice per day until bladder function recovered. To study the influence of ECM component on the damaged spinal tissues, alginate hydrogels supplemented with fibrinogen were injected. To evaluate the influence of VEGF on spinal cord recovery,

alginate:fibrinogen hydrogels were loaded with 2 μg of VEGF (1 μg free and 1 μg encapsulated in nanoparticles) and injected into the rat spinal cord hemisection model.

Alginate:fibrinogen hydrogel influence on spinal cord tissues

At 4 weeks post-injection, the rats were transcardially perfused with 4% phosphate-buffered formaldehyde to fix the tissues.

The injured site was embedded in paraffin and sliced transversally in 12 μm thick sections. Every section was collected and processed for immunohistochemical analysis. Primary antibodies against neurofilaments (mouse anti-Pan neurofilament, 1/1000, Covance, Emeryville, CA), astrocytes (rabbit anti-GFAP, 1/1000, Abcam, Cambridge, UK), infiltrating macrophages (mouse anti-CD68 [ED1], 1/300, Abcam) and T lymphocytes (mouse anti-CD3, 1/300, Thermo Scientific, Fremont, CA) were used in combination with a secondary immunoperoxidase stain (biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, 1:200) or anti-rabbit IgG (VECTASTAIN Elite ABC Kit (Rabbit IgG), Vector Laboratories). Negative controls were generated by omitting the primary antibodies. Hematoxylin was used as a counterstain. Negative controls were performed by omitting the primary antibodies. Image acquisition was performed using a MIRAX (Zeiss, Zaventem, BE) or a Nanozoomer (Hamamatsu, Louvain-la-Neuve, BE) slide scanner, allowing the acquisition of entire sections. The level of staining in the injury zone (determined morphologically) (4-6 sections/rat, n=4) was quantified using Frida software (The Johns Hopkins University).

VEGF local delivery influence on angiogenesis and spinal cord plasticity

At 4 weeks post-injection, the rats were transcardially perfused with 4% phosphate-

buffered formaldehyde to fix the tissues.

Immunofluorescence was performed on 12 μm sections to quantify the endothelial cells (mouse anti-RECA, 1/75, AbD Serotech, Oxford, UK). An AlexaFluor 488 anti-mouse IgG2a antibody (Invitrogen) was used for the detection of RECA-1. Images were acquired with an AxioImager fluorescence microscope equipped with an ApoTome module (Zeiss). Quantification was performed on 0,63 mm^2 pictures taken in the injured zone (35-40 pictures/condition, 3 rats/condition) using an AxioVision script. Adjacent sections were stained for neuronal cells (β III tubulin, 1/1000, Covance) and neurite growth (mouse anti-GAP43, 1/10000, Millipore, Temecula, CA). An AlexaFluor 488 anti-mouse IgG2a secondary antibody (Invitrogen) was used for the detection of GAP43 and an AlexaFluor 568 anti-mouse IgG2a antibody (Invitrogen) was used for the detection of β III tubulin. Negative controls were generated by omitting the primary antibodies. Images were acquired with an AxioImager fluorescence microscope equipped with an ApoTome module (Zeiss). Quantification of the injured zone plus 100-130 μm of surrounding tissue (4-6 sections/condition, n=3) was performed using an AxioVision script.

Assessment of the functional recovery of the rats using Catwalk™

The functional recovery of the rats was evaluated using the Catwalk™ test (Catwalk 7, Noldus, Wageningen, The Netherlands) (n=7). Paw print recording allowed analysis of various aspects of walking steps such as the basis of support, the weight support, the length of contact and the sequence regularity of steps. Up to 5 runs were performed per animal and analysis was performed by blinded experimenters on the fastest uninterrupted run (<2 s) [29]. The rats were trained 2 weeks before surgery, and runs were recorded just before the surgery and once per week for 4 weeks

following the surgery. Many parameters were analyzed by the Noldus software and showed the same tendency. Only the weight support intensity of the left hind paw (the side affected by the surgery) was considered due to its relevance to our study. Runs were recorded as early as 1 week post-surgery even though some rats were not able to support their weight because the recovery of weight support was also a relevant parameter in our study. Weight support was defined as the ability of the rat to move with its body lifted from the floor with no belly dragging. Eleven rats/group were trained and recorded.

Statistical analysis

Statistical analyses were performed using PRISM (GraphPad Software, CA, USA). Two-way ANOVA with post hoc Bonferroni's multiple comparison tests were performed with a P-value between 0.05 and 0.001 (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Error bars represent the standard error of the mean in all figures.

Results

Selection of alginate concentration based on the hydrogel moduli and injection in SCI

A high-molecular-weight alginate (MVG, >200 kDa) was selected to provide a long residence time. Three alginate concentrations were selected: 1, 0.5 and 0.25% (w/v). Among ECM molecules, Tisseel™ fibrinogen was chosen not only because it has been successfully employed in spinal cord regeneration studies [15, 30] but also because it is part of a FDA-approved product widely used in human surgeries.

To evaluate the suitability of the hydrogel moduli relative to the spinal cord modulus, the influence of alginate concentration and of fibrinogen supplementation on the

hydrogels' mechanical properties was studied. The storage G' and the loss G'' moduli at the end of the gelation process were compared (Table 1)(Supplementary data 1).

Hydrogels formed from 1% alginate solution shown the highest moduli ($G'=6040$ Pa). Reducing the alginate concentration by a factor two caused the hydrogel moduli to be divided by more than 40-times ($G'=134$ Pa). 0.25% alginate based hydrogel was ultra-soft-matter gel (30 Pa). All hydrogels had a predominant elastic behavior with a storage G' modulus stronger than the loss G'' modulus. The addition of fibrinogen did not affect the moduli of the 0.5 and 0.25 % hydrogels but the 1% alginate hydrogel moduli were 3-times lower ($G'=2560$ Pa).

In addition, a preliminary study was performed, co-injecting 1, 0.5 or 0.25% alginate solutions and a 50 mM calcium chloride solution with a double syringe system in a rat spinal cord hemisection to obtain injectable gels that gelify *in situ*. The 1% alginate gelified immediately, often clogging the injection system, making the injection difficult. 4 weeks post-injection, the rats were euthanized and the spinal cords stained for neurofilaments. The 1% alginate hydrogel was too dense to allow any cell infiltration and formed a compact mass at the injection site (Supplementary data 2). 0.5 and 0.25% alginate hydrogel-injected lesions were filled with cells. Residues of the 0.5% alginate hydrogel were detected in the lesion while none were seen for the 0.25% alginate hydrogel (Supplementary data 2).

A 0.5% (w/v) solution was then preferred to 1% and 0.25% alginate solutions since the 1% alginate solution was too viscous to be injected easily and did not allow cell infiltration and the 0.25% hydrogel was too soft and had almost disappeared 1 month post injection.

Hydrogel cytocompatibility

The influence of the hydrogel composition on cellular adhesion (6 h after seeding) and cell proliferation (between 3 and 14 days) was studied using two different cell types, mouse fibroblast-like cells (NIH-3T3 cells) and human neuronal-like cells (SH-SY5Y cells).

The levels of cell adhesion after 6 hours and proliferation after 3 days on alginate were the same regardless of the cell type and hydrogel composition (Figure 1). One and 2 weeks following seeding, NIH-3T3 cells grown on alginate proliferated (between a 2.5 and 3.4-fold increase in the OD). No proliferation on alginate was observed for SH-SY5Y cells. Fibrinogen supplementation did not influence cell proliferation. Thus, the *in vitro* release of fibrinogen from alginate hydrogel was evaluated. Most of the fibrinogen (73%) was released by diffusion after 3 days of incubation (Supplementary data 3).

Ex vivo influence of hydrogels on neurite growth from dorsal root ganglia

To evaluate the ability of hydrogels to support neurite growth from neuronal tissues, *ex vivo* dorsal root ganglia (DRGs) cultures were performed on alginate, with or without fibrinogen. Matrigel was used [31] to assess DRG viability for each set of DRG cultures (data not shown).

The hydrogel composition influenced the extent of neurite growth from DRG. Few neurites were observed when DRGs were seeded onto alginate (Figure 2a). The addition of fibrinogen stimulated neurite growth (Figures 2b). Significantly more neurites were observed when fibrinogen was added to alginate (Table 2). Based on these results, alginate hydrogel was supplemented with fibrinogen for the following *in vivo* study.

Influence of alginate:fibrinogen hydrogel on spinal cord tissues after spinal cord injury

The influence of hydrogel composition on the evolution of spinal cord injury was evaluated in a rat spinal cord hemisection model. Neurite growth (pan-neurofilament) and the infiltration of astrocytes (GFAP), T lymphocytes (CD3) and macrophages (CD68) (Supplementary data 4) were quantified in the lesions 4 weeks following the injection of alginate supplemented with fibrinogen compared to non-treated operated animals. Injection of alginate:fibrinogen hydrogel did not significantly impact the spinal cord tissue. It did elicit a CD3 response, although low, (3 times more CD3 staining; the same level of CD68 staining) compared with the control, probably due to the human origin of the fibrinogen. Alginate:fibrinogen hydrogel did not significantly affected neurofilament, and GFAP stainings (Figure 3). Then, while the alginate:fibrinogen did not support by itself spinal cord regeneration, it was well tolerated and was thus considered fit to locally deliver VEGF to the spinal cord.

Influence of formulation on the VEGF release profile

The *in vitro* release of VEGF, either in its free form or encapsulated in chitosan nanoparticles or PLGA microspheres, was measured.

Different release profiles were observed for the different VEGF formulations incorporated into alginate (Figure 4). The VEGF release from nanoparticles was slow but linear and allowed the delivery of a constant amount of VEGF over time ($\pm 8\%$ / 5 days). A total of 45% of the VEGF encapsulated in microspheres was released within 14 days, and then the release reached a plateau (less than 3%/5 days). Free VEGF was rapidly released (85% after 2 weeks; 18% after 1 hour). The burst release

was higher for the microspheres (13% after 1 hour) than for the nanoparticles (2%). Faster release was expected when free VEGF was incorporated into hydrogels.

Influence of VEGF delivery on spinal cord regeneration

Because VEGF has a neurotrophic effect [16, 17, 32-34] in addition to its well-known angiogenic activity, it may be able to stimulate spinal cord regeneration. The influence of local VEGF delivery on angiogenesis and neurite growth around and in the lesion was studied in a rat spinal cord hemisection model. VEGF was incorporated into alginate:fibrinogen hydrogels and injected into spinal cord lesions. To provide fast VEGF release (within 2 weeks) and VEGF sustained release at the site of spinal cord injury, both free VEGF and VEGF nanoparticles were incorporated into alginate:fibrinogen (1 μ g VEGF each) before injection. VEGF microspheres were not selected for the *in vivo* experiment due to a very slow VEGF release.

VEGF delivery stimulated endothelial cell infiltration into the lesion site (Supplementary data 5). 2- and 4-times more staining for endothelial cells was found in lesions injected with VEGF-loaded hydrogels compared to lesions injected with alginate:fibrinogen alone and to untreated operated animals, respectively ($p < 0.001$) (Figure 5).

In addition to stimulating angiogenesis, local VEGF delivery from alginate:fibrinogen hydrogels supported neurite growth in and around the lesion. Indeed, greater β III tubulin and GAP43 staining was observed in the lesions of animals injected with VEGF-loaded hydrogels than in the lesions of animals treated with alginate alone and of untreated operated animals (Figure 6a, 6b and 6c).

Animals implanted with VEGF-loaded hydrogels exhibited 1.25-times (non significant, $p = 0.1636$) and 1.4-times ($p < 0.05$) more β III tubulin staining than animals

implanted with alginate:fibrinogen alone or control animals, respectively (Figure 6d). The β III tubulin staining observed at the lesion could be attributed to neuronal cells, differentiating neurons, dead cells or even to debris that were not cleared at the time of analysis. The staining of growing neurites (GAP43) was significantly higher for VEGF-loaded hydrogels than for hydrogels alone (1.5-fold) and the control (4.4-fold) (Figure 6e). The higher GAP43 immuno-reactivity could be attributed to spinal cord plasticity.

Influence of VEGF on rat functional recovery after spinal cord injury

The evolution of each rat's walking pattern was followed over time using Catwalk™ to determine if the observed plasticity would induce some kind of functional improvement.

The injection of VEGF-loaded alginate hydrogels did not improve the functional outcome of rats (Figure 7). Similar paw intensities were recorded for all conditions. Two weeks post-injury, all the surviving rats were able to support their weight.

Discussion

Our objective was to study the regenerative potential of an injectable system that gellifies in the spinal cord lesion and delivers VEGF locally to spinal cord injuries. We found that the local delivery of VEGF to the injured spinal cord stimulated angiogenesis and spinal cord plasticity. However, it did not impact the functional recovery of the treated animals.

In this study, we choose an alginate hydrogel that gellifies *in situ* when co-injected with a calcium chloride solution to locally deliver VEGF. Injectable hydrogels present several advantages like the prevention of further damages to the injected tissues [2]

and the adaptation of the implant to the shape of the defect. We examined the influence of the hydrogel concentration and supplementation with fibrinogen on the hydrogel moduli. G' moduli decreased with alginate concentration in a non-linear way and fibrinogen addition only influenced the 1% alginate hydrogel modulus. Hydrogel stiffness has been shown to influence neurite growth. Scoot *et al.* observed that neurite growth was significantly more enhanced on hydrogels with a G' modulus of approximately 70 Pa than on hydrogels with a higher modulus (400 and 900 Pa) [35]. Additionally, the spinal cord modulus is approximately 200 Pa [36]. Then, the 0.5 and 0.25 % alginate hydrogels (130 and 30 Pa, respectively) might be more adapted to spinal cord regeneration than the 1% alginate hydrogels. In addition, the 1% alginate solution gelifies immediately when in contact with the 50 mM calcium chloride solution and clogs the needle before it is possible to inject it. 4 weeks post-injection, residues of the 0.5% alginate hydrogel were detected in the lesion while none were seen for the 0.25% alginate hydrogel. Then, a 0.5% (w/v) solution was then preferred to 1% and 0.25% alginate.

Influence of 0.5% alginate hydrogel on cell proliferation and survival was tested *in vitro*. Although alginates have been widely used in tissue engineering applications [37, 38], cell proliferation on alginate is controversial. Indeed, it has been reported that attachment-dependent cells are unable to specifically interact with alginate, most likely due to its highly hydrophilic nature [39]. In addition, mammalian cells do not have receptors for alginate, limiting the adhesion of these cells to these hydrogels [40]. However, alginate hydrogels have been shown to support the survival and proliferation of adipose-derived stem cells [41], but stem cells have been reported to produce their own extracellular matrix [42]. In our study, moderate NIH-3T3 cell proliferation and no SH-SY5Y cell proliferation was observed on alginate. It can be

hypothesized that the cell origin influences cell adhesion to alginate. Lawson *et al.* showed that in contrast to rat cells, human cells do not readily attach to or proliferate on alginates [43]. When type I collagen was added no significant improvement in human cell adherence was found, as we observed when alginate was supplemented with fibrinogen. This might have been due, as observed in with fibrinogen, to the fast release of the added protein, limiting its action on cell proliferation over time.

Since our system was intended for injection in neuronal tissues, the influence of alginate and its supplementation with fibrinogen on neurite growth was assessed *ex vivo* on DRG cultures. Neurite growth was inexistent on alginate alone but was stimulated by the addition of fibrinogen. We hypothesized that, unlike the cells that were cultured for the MTS test, no medium was added to the apical compartment of the DGR culture. These results are consistent with the literature showing that alginate *per se* does not support robust neuronal survival and outgrowth [31] and inhibits the metabolic activity of olfactory ensheathing cells (OECs), Schwann cells (SCs) and bone marrow-derived MSCs in culture, in addition to inhibiting DRG neurite outgrowth [44]. However, this behavior was improved after enriching alginate with fibronectin or cell adhesive peptides [44]. Recently, Matyash *et al.* showed that neurite outgrowth on alginate can be achieved without modification with extracellular matrix components by preparing soft hydrogels with sub-stoichiometric concentrations of Ca^{2+} , Ba^{2+} or Sr^{2+} [45]. Based on these results, alginate hydrogel was supplemented with fibrinogen for the following *in vivo* study.

Before to be used as a vehicle to deliver VEGF, reaction of spinal cord to alginate:fibrinogen hydrogel injection was evaluated. The 0.5% MVG alginate:fibrinogen hydrogel did not stimulate neurite growth by itself. Only a slight lymphocyte reaction was observed, probably due to the human origin of fibrinogen.

VEGF, like most growth factors, has a short half-life and is sensitive to enzymatic degradation. To ensure efficiency and a substantial amount of VEGF at the lesion site, VEGF was encapsulated in nanoparticles or in microspheres before being incorporated in alginate:fibrinogen hydrogel. The formulation was expected to influence the release kinetic of VEGF, as well as the incorporation in a hydrogel. Burst release was higher for microspheres than nanoparticles, but remained equivalent to the one observed by Formiga et al. [25] when measuring VEGF release from microspheres in suspension. It is probably due to VEGF adsorption at the microsphere surface. Release of free VEGF, as expected was the fastest. VEGF release was slightly slower when the microspheres were incorporated into alginate than when directly incubated in PBS [25]. This difference can be explained by the hindered diffusion of VEGF, interactions between alginate and VEGF, or slower microsphere degradation due to the lower water availability in the hydrogel; most likely, a combination of these factors was involved. The slow release from chitosan/dextran sulfate (CS/DS) nanoparticles could be explained by VEGF binding to dextran sulfate, which would slow down its diffusion through the hydrogel [24]. In addition, compared to the release from microspheres that occurs mainly by diffusion, electrostatic interactions within the CS/DS/VEGF complexes slow down the VEGF release until the interactions disappear.

VEGF-loaded hydrogel injection in the hemisectioned spinal cord stimulated angiogenesis. The results we obtained were similar to the effect observed with a different VEGF delivery system implanted into the spinal cord that delivered twice the VEGF dose (4 μ g) [46]. Alginate:fibrinogen alone also supported some angiogenesis in the lesion, most likely by offering physical and chemical (fibrinogen) support favorable to cell infiltration. As for the effect of local VEGF delivery on

spinal cord regeneration, VEGF has been recently reported to support the growth of neurites and to improve tissue sparing [47], although these effects are often not maintained in the chronic phase [19]. The positive effects of naringin [48] and simvastatin [49] on spinal cord recovery have been attributed to their influence on VEGF expression, among other mechanisms. The pro-regenerative effects of VEGF observed in this study could be attributed to the direct effect of VEGF on neurogenesis as described by Storkebaum et al. [16] or to an indirect positive effect on angiogenesis, bringing nutrients and oxygen to the lesion but also facilitating the growth of blood vessels as a physical support for neurite growth [17].

The lack of functional recovery observed in our study may partially be explained by suboptimal conditions (dosing, timing). To our knowledge, the optimal VEGF dose leading to functional recovery after spinal cord injury has not yet been described. Van Neerven et al. did not see any improvement after the intrathecal delivery of 4 μg of VEGF distributed over a total of eight daily bolus injection within the first week following injury [20], neither did Widenfalk after intraparenchymal injection of 1 and 20 μg of VEGF [50]. It seems that VEGF has a rather narrow therapeutic range of action on neuroprotection following spinal cord injury [50]. It might then be tricky to determine what would be the right dose and the right timing for its delivery.

In addition, the neurite growth that was observed within the lesion is encouraging but represents only the very first step in the spinal cord healing process. Additional processes that limit spinal cord regeneration, and ultimately functional recovery, need to be addressed; these include the reestablishment of functional connections between the rostral and caudal sides of the lesion. Furthermore, Hawryluk *et al.* demonstrated that other trophic factors were downregulated following spinal cord injury [51], and Lutton *et al.* showed the benefit of combining PDGF and VEGF to reduce secondary

degeneration after spinal cord injury [52]. Then, VEGF alone might not be sufficient to address a problem as complex as spinal cord injury, and alternative drug candidates or combinations of drugs need to be explored.

In conclusion, we demonstrated that alginate hydrogel supplemented with fibrinogen was well tolerated by spinal cord tissue, although it did not act directly on spinal cord regeneration. This hydrogel was then selected to deliver VEGF to injured spinal cords. Free VEGF and VEGF-loaded nanoparticles were incorporated into alginate hydrogels to combine fast release, providing a VEGF boost post-injury, and slower sustained release. This delivery system supported angiogenesis and neurite growth at the lesion site, although no significant functional recovery was observed. Then, the local delivery of VEGF from injectable hydrogels could provide pro-regenerative effects supporting spinal cord plasticity.

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Figure 1: Influence of hydrogel composition on cell adhesion and proliferation.

Metabolic activity following seeding on alginate:fibrinogen hydrogel of fibroblast-like (NIH-3T3 cells) and neuronal-like (SH-SY5Y cells) cells (MTS test) (OD at 492 nm). *** P<0.001, ** P<0.01, * P<0.05, n=5.

Figure 2: Influence of hydrogel composition on *ex vivo* neurite growth.

Neurite growth of explanted newborn rat DRGs on alginate, supplemented or not with fibrinogen, was evaluated 48 hours following seeding. a) alginate and b) alginate:fibrinogen.

Figure 3: Spinal cord reaction to hydrogel injection in a rat spinal cord hemisection model.

Alginate, supplemented with fibrinogen, was injected in hemisected rat spinal cord. Spinal cords were retrieved and analyzed 4 weeks later for T lymphocytes (CD3) and macrophage (CD68) infiltration in the lesion zone, as well as for neurites (neurofilaments) and astrocytes (GFAP). The level of staining in the injury zone (4-6 sections/rat, n=4) was quantified using Frida software, * P<0.05, n=4.

Figure 4: Influence of VEGF formulation on its release from alginate:fibrinogen hydrogel.

Free VEGF, VEGF encapsulated in nanoparticles and in microspheres was incorporated in alginate:fibrinogen hydrogel and its *in vitro* release was quantified by ELISA, n=6.

Figure 5: Influence of VEGF-loaded hydrogels on endothelial cells infiltration.

Alginate:fibrinogen and VEGF-loaded alginate:fibrinogen hydrogels (1µg free VEGF + 1µg VEGF-loaded nanoparticles) were injected in a rat spinal cord hemisection model. Endothelial cell infiltration of the lesion site was quantified 4 weeks later by immunofluorescence (RECA-1), *** P<0.001, n=4. Non-treated operated animals were used as controls.

Figure 6: Pro-regenerative effects of VEGF-loaded hydrogels.

Spinal cords of non-treated operated animals (a), animals injected with alginate:fibrinogen hydrogels (b) or VEGF-loaded alginate hydrogels (c) (1µg free VEGF + 1µg VEGF-loaded nanoparticles) were analyzed 4 weeks post injection by immunofluorescence for neuronal cells (βIII tubulin, red), growing neurites (GAP43, green) and cell nuclei (DAPI, blue). d) Neuronal cells (βIII tubulin) and e) growing neurites (GAP43) at the lesion site were quantified in the injured zone plus 100-130 µm of surrounding tissue (defined by white line), 4-6 sections/condition, n=3, *p<0.05, **p<0.01, *** p<0.001, n=4.

Figure 7: Influence of VEGF-loaded alginate:fibrinogen hydrogel on rat functional evolution- Left hind paw intensity.

Runs were analyzed before and after surgery for 4 weeks (n=7).

Supplementary data 1: Evolution of G' and G'' of 1% alginate hydrogels in function of frequency.

1% alginate hydrogel moduli (Pa), with and without fibrinogen, were recorded in function of frequency (rad/s). G' , ■ G'', ● G' with fibrinogen ▲ G'' with fibrinogen (n=3).

Supplementary Data 2: Impact of alginate concentration on cell infiltration and residence time.

1, 0.5 and 0.25 % alginate hydrogels, supplemented in fibrinogen, were injected in a rat spinal cord hemisection. The rats were euthanized 4 weeks following injection. The spinal cords were stained for neurofilaments (pan-neurofilament), and counterstained by haematoxylin. Hydrogel residues are marked by a star (*).

Supplementary Data 3: In vitro fibrinogen release from alginate hydrogel.

0.5 % alginate hydrogels (300 µl), supplemented with fibrinogen (5 mg/ml), were formed in 48-well plates by addition of CaCl₂ solution (50 mM) (10 min incubation) (n=6). The CaCl₂ solution was removed and the hydrogels were washed with PBS. Then, 500 µl of fresh PBS was added per well and the hydrogels were incubated at 37°C. After 2 hours, 1, 3, 5 and 7 days, the supernatants were collected, centrifuged and their protein content was dosed by BCA (Pierce). The results are either expressed in function of incubation time in days (main graph) or in function of the square route of time in second (Higuchi model; insert).

Supplementary Data 4: Influence of hydrogel injection on spinal cord tissue.

Alginate:fibrinogen treated rats were euthanized 4 weeks following injection and spinal cords were stained for neurofilaments (pan-neurofilament), astrocytes (GFAP), T lymphocytes (CD3) and macrophages (CD68). Non-treated operated animals were used as controls. The level of staining in the injury zone (determined morphologically) (4-6 sections/rat, n=4) was quantified using Frida software (The Johns Hopkins University).

a) Non-treated operated animals- CD3

b) Non-treated operated animals- CD68

c) Non-treated operated animals- PanNeurofilament

d) Non-treated operated animals- GFAP

e) Alginate:fibrinogen- CD3

f) Alginate:fibrinogen - CD68

g) Alginate:fibrinogen - PanNeurofilament

h) Alginate:fibrinogen – GFAP

Supplementary Data 5: Influence of VEGF delivery on angiogenesis

alginate:fibrinogen hydrogels were loaded with 2 µg of VEGF (1 µg free and 1 µg encapsulated in nanoparticles) and injected into the rat spinal cord hemisection model. Rats were euthanized 4 weeks following injection and spinal cords were stained for endothelial cells (RECA-1). Quantification was performed on 0,63 mm² pictures taken in the injured zone (35-40 pictures/condition, 3 rats/condition) using an AxioVision script. a) operated non-treated rats, b) alginate: fibrinogen hydrogel and c) VEGF loaded alginate: fibrinogen hydrogel injected in spinal cord hemisection.