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Interfacing cells with microengineered scaffolds for neural tissue reconstruction

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Highlights

- Recent advances in the strategies for neural tissue engineering are presented
- Engineered neural microenvironments are built by top-down or bottom-up techniques.
- Top-down approaches rely mostly on optical/soft lithography and 3D printing.
- Bottom-up strategies involve self-assembly of particles, hydrogels or even cells.
- Guiding neuro-implants allow functional brain recovery and 3D tissue reconstruction.

Abstract

The development of cellular microenvironments suitable for neural tissue engineering purposes involves a plethora of research fields ranging from cell biology to biochemistry, neurosciences, physics, nanotechnology, mechanobiology. In the last two decades, this multi-disciplinary activity has led to the emergence of numerous strategies to create architectures capable of reproducing the topological, biochemical and mechanical properties of the extracellular matrix present in the central (CNS) and peripheral nervous system (PNS). Some of these approaches have succeeded in

inducing the functional recovery of damaged areas in the CNS and the PNS to address the current lack of effective medical treatments for this type of injury. In this review, we analyze recent developments in the realization of two-dimensional and three-dimensional neuronal scaffolds following either *top-down* or *bottom-up* approaches. After providing an overview of the different fabrication techniques employed for tailoring the biomaterials, we draw on specific examples to describe the major features of the developed approaches. We then conclude with prospective proof of concept studies on *guiding* scaffolds and regenerative models on *macro-scale* brain implants targeting neural regeneration.

Introduction

Degenerative, cerebro-vascular and traumatic diseases of the central (CNS) and peripheral (PNS) nervous systems are major public health problems and have increased substantially over the past 25 years (GBD 2015 Neurological Disorders Collaborator Group, 2017). Among them, traumatic and non-traumatic brain injuries, such as stroke (J. Wang et al., 2014) and spinal cord injuries (Kabu, Gao, Kwon, & Labhasetwar, 2015), and neurodegenerative diseases including Alzheimer (Duncan & Valenzuela, 2017), Parkinson (Kikuchi et al., 2017) and Huntington (Connor, 2018) are the most common causes of morbidity and handicap worldwide. Therapeutic strategies aiming to repair damaged regions of the nervous system ideally should support tissue regeneration, but also induce a healing process necessary to restore functions linked to motor, sensory and cognitive processes (Gu, 2015). To date, a straightforward and successful treatment for CNS and PNS injuries has not yet been identified.

In the last decades, cell-delivery strategies have been explored extensively in order to promote tissue regeneration in the injured areas of the nervous system and, thus, to induce functional

recovery. Having a good source of cells is not always sufficient to achieve a successful regenerative strategy. Following the continuous blooming of sophisticated fabrication technologies for tissue engineering, in recent years, the development of strategies mimicking microenvironments in our body, with the accurate functional, topological and mechanical cues, has grown exponentially and has provided architectures more relevant for the brain.

The current review provides an exhaustive recapitulation of the existing methodologies to conceive biomaterials and cell-based architectures aiming at regenerative strategies for neural tissue engineering. Here, two ‘philosophies’ behind the development of the bio-artifacts for tissue regeneration are explored: top-down (TD) and bottom-up (BU) approaches. In the first one, the final architecture is obtained *via* a series of well-controlled fabrication steps (such as optical lithography, soft lithography, nanoimprint, 3D printing, bio-printing). The second approach relies mainly on self-assembly mechanisms for the generation of biomimetic microenvironments (such as micro-beads self-assembly, graphene/graphene oxide sheets/fibers, electrospinning, hydrogel self-assembly). Major technological and biological advantages and limitations of the two strategies are discussed within the framework of two-dimensional (2D) and tri-dimensional (3D) neuronal scaffolding. Additionally, a review of techniques based solely on cells forming tissue-like constructs (i.e. neurospheroids/organoids) and based on *in vivo* brain implants is provided (Figure 1).

1. Cell-based guiding strategies in CNS and PNS injuries

Neural regenerative medicine involves therapies that promote tissue repair and, finally, functional recovery after nerve tissue (CNS and PNS) injury. Currently, hopes rely on cell-based therapies, administered during the sub-acute or chronic phase, to sustain tissue reconstruction and

counteract cell loss (Steinberg et al., 2016). The control of neuronal outgrowth and the formation of restored synapses supported by a network of astrocytes, assumes then a paramount importance in order to ensure adequate regeneration and functional recovery after injury. This is particularly evident in spinal cord injury (SCI), where the successful regeneration of neuropil and the related locomotor recovery were associated to the presence of aligned immature astrocytes (Hofstetter et al., 2002). The guiding role of activated astrocytes, radially aligned around the lesion where brain plasticity supports regeneration, is mediated by the release of trophic factors and has also been recently emphasized for transplanted embryonic neurons after aspiration of motor cortex (Gaillard & Jaber, 2007; Saha, Peron, Murray, Jaber, & Gaillard, 2013). However, even if astrocytes help in attracting transplanted stem cells, they are unable, alone, to provide an adequate structure and to restore the initial brain architecture. Importantly, spinal cord and brain tissue do not hold the same capacity to regenerate as other tissues or PNS. Damage to the CNS or the PNS typically results in the formation of a glial scar and establishment of severe inflammation, leading often to the permanent loss of cognitive and motor functions (Huang et al., 2014). Together with outgrowth, maturation of neurons and myelination of such, provided by oligodendrocytes or Schwann cells, is also crucial for efficient neural conduction after injury. Thus, tissue regeneration after CNS and PNS injury includes enhancing neurogenesis, gliogenesis and angiogenesis, promoting cell connection and restoring neural circuits. A therapeutic strategy targeting all these events would have clinical success.

Cell-based approaches hold much promise as effective therapies to restore CNS and PNS function after injury in humans (Detante, Moisan, Hommel, & Jaillard, 2017; George & Steinberg, 2015; Kalladka et al., 2016; Steinberg et al., 2016). Cell therapy can potentially favor tissue repair, integration and regeneration, but an optimal cell source has not yet been found. Tissue repair can

occur through direct replacement of damaged cells, or via production of growth factors and cytokines that stimulate endogenous neurogenesis, gliogenesis and angiogenesis. It is of paramount importance to integrate these strategies with supporting biocompatible materials, to assess their effective compatibility, as shown in two pioneer studies by our group (Bédurier et al., 2012; Chalard et al., 2018). This feature is the first guarantor of correct cell development, reconnection and *in vivo* effectiveness, as demonstrated later by our group (Davoust et al., 2017).

Cell-based approaches have been used in preclinical models of CNS and PNS injury, and some have been translated to the clinic. They consist in the engraftment of one or more cell types directly in the injury site: 1/ neural progenitor stem cells (able to differentiate into the 3 main brain lineages: neurons, glial cells and oligodendrocytes); 2/ mesenchymal stromal cells (MSCs), able to easily differentiate into several cell lineages (endothelial cells, adipocytes, myocytes, chondrocytes, osteoblasts), but very limited number of neurons; 3/ induced pluripotent stem cells (iPSCs, generated by somatic cells of the patient, i.e. fibroblasts); 4/ embryonic stem cells (pluripotent stem cells harvested from the human blastocyst with high proliferation capacity) that can be differentiated through genetic modification, soluble factors or mechanical forces into cell lines; 5/ oligodendrocyte precursors cells or Schwann cells (the latter harvested from biopsy of a peripheral nerve and able to release neurotrophic factors promoting neurite outgrowth, especially in the PNS); 6/ endothelial cells (constitutive of the vessel walls in the neural tissue); 7/ neural cell lines (Assinck, Duncan, Hilton, Plemel, & Tetzlaff, 2017; Kalladka et al., 2016; Steinberg et al., 2016; Tabakow et al., 2013). Beside this, crucial issues related to immunogenic, inflammatory reactions and engraftment rejection by the host, do limit xenografts and even allografts. In this context, autologous procedures would bear a significant advantage; however, nerve tissue can only be taken

from a limited number of anatomical sites and may result in a loss of site-based nerve function (Emgård et al., 2003; Ingulli, 2010).

A valuable source of nerve tissue is the gastrointestinal tract, which contains a highly organized network of nerves, called the enteric nervous system (ENS). The ENS is a large division of the PNS and is referred to as the '*second brain*' for its morphological and functional analogies with the CNS (Furness, 2012). The ENS contains adult neurons and glial cells (siblings of glia in the CNS), and enteric neural precursor cells. The latter are the renewing cell population within the ENS (Furness, 2012). Recent evidence demonstrates the presence of robust adult neuro-genesis in the adult ENS, with a remarkable rate of neural turnover (Kulkarni et al., 2017). This has a great advantage compared to the limited neurogenesis in the CNS, and may drive profound biological and clinical implications. To date, ENS-derived cells have been used as cell-therapy for gut neuropathies (Goldstein, Hofstra, & Burns, 2013; Mckeown, Stamp, Hao, & Young, 2013; Obermayr, Hotta, Enomoto, & Young, 2013) to replace lost cells and obtain functional recovery. Transplanted ENS-derived cells have also been demonstrated to integrate within the lesioned chick spinal cord (Jevans, McCann, Thapar, & Burns, 2018), in injured adult brain (Belkind-Gerson et al., 2016) and for SCI, with promising results (Gómez et al., 2018). In addition, transplanted ENS-derived cells can be autologous, with no potential risk of immune-mediated engraftment rejection. Moreover, ENS-derived cells do not require substantial modifications before their engraftment, which is a crucial advantage compared to iPSCs.

So far, the clinical efficacy of cell-based strategies for brain/spinal cord repair has been limited considerably by the poor effective integration, survival rate and differentiation of the grafted cells. Concerning this matter, the crosstalk between grafted cells and surrounding microenvironment, mainly constituted by extracellular matrix (ECM), is a key factor for the establishment of a new

and functional neuronal network. The presence of a biomimetic infrastructure (*scaffold*) able to host and support neurons, and to guide the development of axon and neurite connections, would promote efficient integration and differentiation of the new-grafted cells within the damaged nerve tissue. This strategy would also help the arrangement of correct connections, avoiding the formation of aberrant neuronal pathways.

2. Design roadways and fabrication principles for building neural scaffolds

2.1. Top-down and bottom up strategies

The realization of engineered architectures using *top-down methodologies* relies on a precise sequence of fabrication steps where the final object is obtained by shaping a pristine material through the removal of extraneous parts. Among these techniques, we can find optical and electron beam lithography, soft lithography, microcontact printing, nanoimprint lithography as well as several additive manufacturing approaches. Historically, *optical lithography (OL)* (Berkowski, Plunkett, Yu, & Moore, 2005) is the typical example of this family of processes to realize 2D devices. Briefly, after choosing the targeted material and developing an optical mask that contains transparent and opaque regions reproducing the desired features, the sample is coated by a photosensitive solution (i.e. photo-resist). An ultraviolet (UV) light source is then projected onto the sample through the optical mask where only the regions underneath the transparent areas are exposed. The exposure to light provokes a chemical change in the photoresist that becomes soluble (if positive) or insoluble (if negative) in a chemical developer. The uncovered areas of the sample can then be shaped according to the final design by adding other materials or by removing the original material (i.e. etching). Typical examples of OL in the development of micro-engineered surfaces for cell culture involve the creation of Si micropillars whose geometry is used as a cue to

affect the orientation and growth of neuronal cells (Dowell-Mesfin et al., 2004; Limongi et al., 2013). The equivalent of OL at much higher levels of single feature resolution is *electron beam lithography* (EBL) where electrons are used instead of UV light. Often OL is used as an intermediate step in *soft lithography* (SL) approaches (Rogers & Nuzzo, 2005) in order to create master molds (usually made by silicon or stiff photoresists) featuring the negative geometry of the desired 2D design. The mold is then covered (pressed) by (on) the desired material (often Poly(dimethylsiloxane) –PDMS- or other soft polymers) that, after a curing step, can be peeled off assuming the final topology whose features can be employed to guide cell alignment (L. Vaysse, Beduer, Sol, Vieu, & Loubinoux, 2015). Often the PDMS molds can be also used as a stamp to pattern molecules or other compounds on a 2D surface, what is called *microcontact printing* (μ CP) (Kumar & Whitesides, 1993). When the size of the features to be reproduced is in the sub-micrometric range, it is necessary to resort to EBL and etching to create the master molds. These are then employed either within thermoplastic *nanoimprint lithography* (Chou, Krauss, & Renstrom, 1996) (NIL), where the material is heated before molding, or UV-NIL, where a photosensitive materials is cured after molding. Grooves and ridges with sub-micrometric feature resolution obtained with this technique proved to be an efficient tool for axonal guidance (Johansson, Carlberg, Danielsen, Montelius, & Kanje, 2006). Finally, another intriguing molding process for 2D scaffold fabrication recently reported the ability to create *neurotemplated scaffolds* created by a process of silicification of living neurons that played an important role on neurite length of neural cells grafted on them (B. J. Kim et al., 2018).

All these techniques target the realization of 2D architectures, nevertheless, during the last two decades the use of *additive manufacturing* (AM) processes experienced an exponential interest from both academy and industry, especially in the biomedical field, for the realization of devices

with more biomimetic 3D geometries. The simplest AM technology is the one based on *fused deposition modeling* (FDM) where the 3D object is obtained, layer by layer, by extruding a thermoplastic material through the nozzle of a moving head whose displacement is controlled remotely by a computer. Some recent advances showed also how cells could be incorporated inside bioinks to be extruded under mild conditions in order to obtain cell-laden architectures, what is called *bioprinting* (Jia et al., 2016), although this technique is not able to create free-standing 3D elements in absence of sacrificial materials. Nonetheless, although in both techniques the different elements of the architecture (cells and/or biomaterials) are structured and located in a deterministic way by moving a nozzle over a surface and piling different layers (that from a microfabrication point of view lies in the top-down category), we also acknowledge that the biological community includes the modular combination of cells/biomaterials in the bottom-up category (Mandrycky, Wang, Kim, & Kim, 2016). For this reason, we can place FDM/Bioprinting at the interface between the two classifications. An alternative 3D bio-printing methodology to FDM is based on the ejection of small droplets (1–100 picoliters; 10–50 μm diameter), instead of large filaments, what is called *inkjet bioprinting* (K. H. and S. L. and L. T. and S. V. V. and L. G. and A. Ovsianikov, 2016). The technique relies either on acoustic waves produced by piezoelectric crystals to force the liquid in small amounts through the nozzle, or by vaporizing the bioink around a heating element that expels the droplets out from the printing head. More recently, an increasing interest has been devoted toward *light assisted 3D fabrication techniques* (Guvendiren, Molde, Soares, & Kohn, 2016) such as stereolithography (SLA) (Accardo, Courson, Riesco, Raimbault, & Malaquin, 2018), digital light projection (DLP) and two-photon lithography (2PL) (Accardo et al., 2017; Accardo, Blatché, et al., 2018a). With these techniques, the 3D object is realized by photopolymerizing (i.e. solidifying) the liquid photosensitive material by using either a layer-by-

layer approach from a series of transverse-plane image slices, or a direct laser writing. These techniques can reach micrometric resolution, comparable with cell-sizes and are able to build extremely accurate 3D architectures meanwhile there is a continuous quest for materials capable to feature at the same time biocompatibility and photosensitive properties. Recent examples showed indeed how it is possible to integrate biocompatible hydrogels such as PEGDA into free-standing architectures favoring the development of ramified 3D neuronal networks (Accardo, Blatché, et al., 2018b). Finally, it is also worth mentioning that scaffolds featuring 3D geometries can also be obtained without exploiting additive manufacturing technologies but “cheaper” 3D molding techniques (at the cost of a lower level of precision in the architecture geometry). It is for instance the case of the *porogen leaching method*, where after designing the structure of a template material (such as pore size, shape, overall porosity, interconnectivity) and incorporating a polymer solution, it is possible to create porous 3D scaffolds by removing the template materials using a chemical solvent (Martínez-Ramos et al., 2015, 2012).

While top-down approaches rely on “sculpting” a block of raw material towards a precise geometry using a predefined series of micro-fabrication steps, *bottom-up methodologies* are mostly based on the self-assembly of small building blocks. The most used approaches for building 2D cellular scaffolds following this strategy are based on the *self-assembly* (Thiruvengadathan et al., 2013) of micro-particles and/or other kinds of nanostructures such as graphene/graphene oxide sheets/ribbons (El Merhie et al., 2018; Guo, Qiu, Liu, & Liu, 2017). The term ‘self-assembly’ implies that a structure builds itself from modular construction units through a fine tuning of their interactions inside a liquid solution where they have been dispersed. This requires to control finely the size, shape and surface properties (e.g. electrostatic, wetting, chemical functionalization, thermal) of the small (micron or sub-micron scale) building blocks in order to tune their attractive

and repulsive long range and short-range forces and allow them to assemble spontaneously into specific macro-scale architectures. An example of this approach reported how self-assembled silica nano-beads can affect the neuritogenesis and intracellular cytoskeletal dynamics of hippocampal neurons via nanotopography-mediated filopodial activities (Kang et al., 2012). Another interesting 2D strategy is the use of *plasma reactive ion roughening* (Tamada & Ikada, 1993) processes that allow to integrate random roughness and surface chemistry modifications and can be used as a tool to enhance cell adhesion and proliferation (Accardo, Shalabaeva, & La Rocca, 2018; Cesca et al., 2014).

Further, as for top-down strategies, also bottom up techniques have experienced exciting developments concerning 3D fabrication approaches during the last years. In such field, two very well established techniques are *hydrogel self-assembly* (Fitremann et al., 2017; Kopeček & Yang, 2012) and *electrospinning* (Hasan et al., 2014). The first one relies on the synergistic combination of synthetic supramolecular compounds, macromolecules, proteins and or peptide domains that leads to the spontaneous organization of 3D hierarchical architectures that swell but do not dissolve in water and, according to their nature, can be degradable or non-degradable in a living organism (Hunt, Chen, Van Veen, & Bryan, 2014). One of the main advantages of this approach is the ability to promote the formation of 3D neuronal networks providing at the same time the possibility to be injected, therefore enabling minimally invasive delivery (Bédurier et al., 2015). The second one involves the use of electrostatic forces to extrude micrometric and sub-micrometric thin fibers of viscoelastic polymer solutions with aligned features. This strategy finds applications for recovering SCI (Schaub, Johnson, Cooper, & Gilbert, 2016).

Finally, *scaffold-free approaches* are a family of bottom-up tissue self-assembly techniques that can lead to the formation of spheroids/organoids (i.e. multicellular 3D tissue-like architectures

resulting from spontaneous cell assembly and organization). Spheroids have been shown to exhibit improved biological properties with regard to regenerative capacity, since they facilitate intense cell–cell interactions and resemble other physiological conditions of complex tissues with 3D architecture (Laschke & Menger, 2017). In organoids, higher order self-assembly structures compared to spheroids, stem cells self-organize through cell sorting and spatially defined differentiation to resemble organ cell types, structures and functions, and need a matrix for their generation and organization (Lancaster et al., 2013; Zhuang, Sun, An, Chua, & Chew, 2018).

2.2. Biomaterials porosity, stiffness and cell mechanical properties

Besides the nature of the topology (2D or 3D) employed in the scaffold, two other key parameters that are directly linked to the ability of controlling the adhesion, proliferation and differentiation of cells in engineered microenvironments, regardless of the top-down or bottom-up adopted strategy, are the *porosity* and the *stiffness* of the biomaterial. Porosity (Loh & Choong, 2013), which is defined as the ratio of volume of pores to the total volume of the material under consideration, is a crucial parameter to let the cells infiltrate easily within the material, allow the perfusion of nutrients and facilitate the vascularization of the restored tissue. According to the specific application (e.g. in vitro drug screening models, tissue engineering) and to the type of cells to be accommodated, the pore size of the employed scaffold must be accurately tuned by exploiting the most suitable fabrication approach (e.g. salt leaching, gas forming, phase separation, 3D printing). Recently it has been shown how enhanced porosity coupled to specific geometric orientation of the developed architectures favored cell body inclusion within the scaffold as well as directional neurite outgrowth (D. Kim, Kim, Lee, & Yoon, 2017). The porosity of the architecture is also directly correlated to other paramount parameters of the engineered architecture, which are its structural stability and mechanical properties. Indeed, a too enhanced

porosity can sensitively influence the stiffness of the scaffold and lead to collapse either during the cell culture or during the implantation. Further, cells are able to recognize the mechanical properties of a material over which they proliferate and these properties direct the motion through a phenomenon called durotaxis (Harland, Walcott, & Sun, 2011). The stiffness of a biomaterial, defined as its property to resist deformation under an applied force, and notably its Young's Modulus, plays a fundamental role not only in cell migration but also in cell differentiation and neurite length, as demonstrated for human mesenchymal stem cells (hMSCs). These cells were able to differentiate into neurons or glial cells in presence of softer (1 kPa) or stiffer (10 kPa) materials, respectively (Her et al., 2013). Also, neural progenitor cells were shown to differentiate into neurons with longer neurite length in presence of hydrogels featuring a stiffness (400 Pa) close to the one of brain tissue (≈ 600 Pa) (Tarus et al., 2016).

2.3. Biodegradability and implantability

Another important feature of cell scaffolds is their ability to dissolve after a certain amount of time without the release of toxic or inflammatory byproducts, what is called *biodegradability*. This characteristic strictly depends on the physical and biochemical properties of the material employed for scaffold fabrication. Biodegradability assumes particular relevance for tissue engineering applications where, ideally, the supporting role of the implant should gradually fade away, mostly through hydrolytic or enzymatic degradation pathways, after fulfilling its scope, sometimes releasing very slowly products that act as fuel for cells like PLA hydrolyzed in lactate (Álvarez et al., 2014). Most of the materials employed for *in-vivo* brain and spinal cord regeneration are polymer-based, therefore it is important to classify them into two types, namely natural polymers and synthetic polymers (Hsu, Hung, & Chen, 2016; Sensharma, Madhumathi, Jayant, & Jaiswal, 2017). Naturally-derived polymers (such as collagen, hyaluronic acid, benzyl ester of hyaluronan,

gelatin, chitosan and alginate) show a native biocompatibility and degradability, nevertheless they are still affected by several drawbacks, such as the limited sources of available material and the batch-to-batch variability. On the other hand, synthetic polymeric materials (such as polylactic acid-PLA, polycaprolactone-PCL, poly(ethylene glycol) diacrylate-PEGDA, Poly(dimethylsiloxane)-PDMS) can ensure a good level of reproducibility, as well an easy tunability of their mechanical properties. However, except PLA and PLGA (Ai et al., 2013) they do not often hold optimal degradability/biocompatibility, for which additional biochemical functionalization is required. Interestingly, recent advances showed how hybrid approaches can lead to more efficient biomaterials, such as gelatin methacrylate (GelMA) (Yue et al., 2015) where free amine groups of the gelatin are substituted with methacrylate anhydride while preserving the arginine-glycine-aspartic acid (RGD) sequences that promote cell attachment.

Furthermore, taking into consideration that scaffolds must be used for *in-vivo* trials, the *implantability* of the developed structure is of paramount importance. This means that the implanted scaffold needs to be easily integrated within the host tissue, without damaging neither itself, nor the cellular network nor the host tissue. With this aim, hydrogel scaffolds obtained by using bottom-up strategies, that can be directly injected at the damage location (Gopalakrishnan, Shankarappa, & Rajanikant, 2018), have to date a clear advantage over scaffolds fabricated by top-down approaches. However, recent advancements concerning top-down strategies in the field of shape-memory materials may start to fill this gap (Montgomery et al., 2017).

2.4. Biochemical functionalization and cells

In order to enhance the biocompatible features of the materials employed for neural repair approaches, several *biochemical functionalization* strategies can be adopted either by coating the scaffold with specific biomolecules (post-fabrication) or by integrating them directly inside the

material (pre-fabrication). In the first approach, the surface modification of the materials can include bioactive molecules such as ECM proteins (e.g. laminin, fibronectin, vitronectin) (Millaruelo, Nieto-Sampedro, & Cotman, 1988), growth factors (nerve growth factor-NGF, brain derived neurotrophic factor-BDNF, glial derived neurotrophic factor-GDNF) (Liu et al., 2014), arginine-glycine-aspartic acid (RGD) groups for cell adhesion (Tarus et al., 2016) or synthetic compounds such as polylysine (Y. H. Kim, Baek, Han, Chung, & Jung, 2011) to favor neural cell adhesion, differentiation and proliferation. Nevertheless, bulk modification of the materials is often more advantageous as the bio-compounds are integrated directly inside the biomaterial, and not only on the surface, therefore allowing cells to always benefit from their presence even in case of partial degradation of the scaffold. Furthermore, the choice of the *cells* to be seeded on the scaffold (or even within it) is a crucial step in the field of neural tissue engineering, as they represent the building blocks around which the new tissue will be generated. Cells employed in regenerative medicine, and in particular for neural tissue engineering (Sensharma et al., 2017), can be of three types: autologous (harvested from the patient), allogenic (coming from a donor of the same species) and xenogenic (transplantation of cells between different species). In all cases, great attention must be devoted to the amount of cells that can be harvested and amplified, and to possible immune response of the host tissue.

3. *In vivo* proof of concept studies on guiding neuro-implants

The term neuro-implant refers to scaffold for neural tissue reconstruction. This section and the following will cover only neuro-implants inserted in the brain. Even though some experimental models are far from fully recapitulating the pathology and the clinical situation, they evidence great advances in brain tissue reconstruction by demonstrating key efficient milestones (TABLE 1).

Guiding scaffolds with preferred orientations aim at replacing brain lesioned structures with a complex architecture, like the six-layer cortex with orthogonal connections, or at creating a bridge between brain areas that result disconnected by a large lesion. Cortical thickness is around 2 mm and long-distance bundles of fibers have a length of minimum 4 mm. In rodent studies, the size of oriented neuro-implants is around 2-4 mm of length for lesion averaging 50-100 mm³ of volume (Vaysse et al., 2015) and are thus defined as *macro-scale implants*. More often, the proposed structures are formed by aligned directional or orthogonal channels, with the aim to optimize reconnections along the three-dimensional axes.

The Australian consortium (T.-Y. Wang, Forsythe, Nisbet, & Parish, 2012) has demonstrated that a thin electrospun PCL (poly(ϵ -caprolactone) roll-like scaffold, with or without functionalization with BDNF-mimetic molecule, was able to redirect subventricular zone (SVZ) neural progenitors from the rostral migratory pathway to the injured cortex, a structure difficult to repopulate. A possible limit of the study is that the lesion's size was small (4 mm³) since it corresponded to the fresh injury when the scaffold was inserted with a 21G needle. Thus, this process probably does not occur in case of big lesions, which affect the SVZ or in case of large lesions needing extensive tissue reconstruction.

Infiltration or colonization of scaffolds by several types of cells (as discussed above) have been largely demonstrated with a broad series of materials (PLA, PCL, PDMS, acrylate, Matrigel). In these materials, the size of pores/channels usually ranges from 40 μ m to 450 μ m (Davoust et al., 2017; Martínez-Ramos et al., 2012; Wong, Krebsbach, & Hollister, 2008). Sometimes, the material itself allows cell infiltration because of its intrinsic porosity (Fon et al., 2014; Nisbet et al., 2009; Wang et al., 2012; Xu et al., 2018). Such pore size may allow complete development of the neuro-glio-vascular unit because cells may optimally interact. Indeed, a capillary is less than 50 μ m far

from any neuron in the brain, which seems to be the maximum distance for appropriate oxygen and glucose supply in a functional environment.

In another elegant study, in order to regenerate a cortical area that had been just removed, a gradient of semaphorin (Sema)3A was created in a glass tube filled with Matrigel[®] and subsequently inserted in the fresh lesion. The system was able to attract endogenous stem cells, which organized along a dorso-ventral gradient and differentiated; the final result was a sophisticated architecture (Z. Xu et al., 2018). The same strategy might be applied to regenerate areas with various degrees of cell densities, and not necessarily a cortex. However, the non-controlled composition of Matrigel may hinder the translation of this strategy to humans.

4. Regenerative medicine studies with guiding neuro-implants

Some pre-clinical studies using animal models of stroke or brain trauma have tried to implant scaffolds several days after the insult into large damaged areas needing *macro-scale implants*, thus reproducing a situation closer to the human pathology (TABLE 2).

Vascularization is the first prerequisite for tissue regeneration and originates from perilesional areas. This paramount process does not seem to be hindered by the implant of biomaterials (Álvarez et al., 2014) nor organoids (Lancaster et al., 2013), since vessel-like endothelial structures resembling the physiological vascularization were observed through very narrow channels (Martínez-Ramos et al., 2015, 2012). Very interestingly, scaffold-induced regeneration of nerve tissue was accompanied by appropriate perfusion, as shown *in vivo* with SPECT imaging -Single photon emission computed tomography (Le Fric et al., 2017).

We have previously discussed about inflammation during nerve injury. This phenomenon may be affected by the implant of biomaterials. Indeed, PCL is known for being slightly inflammatory

(Fon et al., 2014) whereas PDMS scaffolds reduced signs of inflammation (Davoust et al., 2017). Moreover, PCL scaffolds coated with graphene showed a decrease of astrocyte infiltration (Zhou et al., 2016) and the ones engineered with the BDNF-mimetic molecule caused increase of microglial cells number (Fon et al., 2014). The same applies for the glial scar evolution after injury. Materials that become partly or fully encapsulated in a fibrous scar will not allow cells to infiltrate the scaffold in good amount and will be avoided, like in the case of PCL channel scaffolds (Nisbet et al., 2009; Wong et al., 2008). Conversely, insertion of materials (TABLES 1 and 2) had no effect on glial scar or rather tended to render the scar thinner (Davoust et al., 2017; Zhou et al., 2016). Surprisingly, and conversely to in vitro studies, aligned versus random PCL fibers was sometimes found to be a hinder to neurite penetration (Fon et al., 2014; Nisbet et al., 2009), whereas aligned PLA fibers implanted dorso-ventrally were a clear for mimicking cortical architecture (Álvarez et al., 2014).

The advantage of 3D orthogonal guiding channels, compared to mono-directional channels, has been observed for glial and neuronal cell colonization (Wong et al., 2008). However, one or bi-directional perpendicular organization was unable to fully reconstruct cortical tissue far from the SVZ stem cell niche, compared to tissues adjacent to it (Davoust et al., 2017; Martínez-Ramos et al., 2012; Vaysse et al., 2015). Further, the process leading to the complete tissue reconstruction and to mature neurons may take several months (Davoust et al., 2017; Vaysse et al., 2015) depending, among other factors, on the speed of biomaterial's degradation once implanted. Too rapidly degradable hydrogels (< 1-2 months) lead to poor tissue reconstruction and brain atrophy (Bible et al., 2009). On the contrary, non-degradable materials take space serving for consolidation and maintain of structures in the injured area but they may act as barriers between live tissues (Bédurier et al., 2012; Davoust et al., 2017). In theory, semi-flexible biocompatible materials like

PDMS can be removed from neuronal spheroids networks (Kato-Negishi, Tsuda, Onoe, & Takeuchi, 2010), but, in the case of neuro-implants, this will disrupt the neovascularisation around the scaffolds and the fragile reconstructed tissue. Thus, the optimal material would be the one with very slowly degradable characteristics such as PLA partially degraded but still present at 15 months (Álvarez et al., 2014).

As for the best material for scaffold fabrication, physical properties have been demonstrated to be determinant in printing tissue and many materials with features close to brain stiffness or even slightly stiffer are valid candidates. However, it has to be mentioned that implanted materials may release products with non-negligible toxicity. This is the case, for example, of ethyl- or hydroxyethyl acrylate, whose carcinogenic effects need to be fully explored before continuing the tests (“IARC Monographs on the Evaluation of Carcinogenic Risks to Humans – IARC,” n.d.). An additional open question remains about the fate of time-evolution of scaffold materials after implantation. PCL fibers coated with electroactive graphene were able to attract neuroblasts but unable to sustain their full differentiation into neurons (Zhou et al., 2016). It is thus not clear whether chemical features of materials, like the formation of degradation products or the intrinsic physical characteristics in electroactive materials, may prevent the physiological cell evolution. When the outcome of the implants made with PDMS, PCL, PLA, ethyl-acrylate or HE-acrylate was evaluated in the long-term studies, i.e. 2 or 15 months after the implantation, mature neurons could be observed (Bédurier et al., 2012; Davoust et al., 2017; Fon et al., 2014; Martínez-Ramos et al., 2012). Importantly, one study performed with scaffolds made with dorso-ventral aligned electrospun PLA nanofibers demonstrated long-distance reconnections of differentiated endogenous neuronal cells, which were embedded on the fibers (Álvarez et al., 2014). Motor recovery after CNS injury relies on key motor tracts, and the corpus callosum between both

primary motor cortices of each hemisphere is one of them. In the study of Alvarez and coll., the cells that had migrated to the injured cortex were able to emit projections onto the other hemisphere motor areas passing along the corpus callosum. Since animals were new-born, and the neurogenesis was thus highly active, such important result needs to be reproduced and confirmed in brain-damaged adult animals.

3D scaffolds have been tested to regenerate brain tissues, alone or in the presence of neural stem cells grafted on them. Only a few studies compared both strategies. In a couple of investigations performed by our groups, it was clear that the presence of progenitor cells on the scaffolds was a significant advantage for tissue regeneration after a corticostriatal lesion in rats (Bédurier et al., 2012; Davoust et al., 2017).

A final key point to discuss about is the real efficacy of the neuro-implants in promoting functional recovery. To our knowledge, to date only our group has demonstrated in two separate studies with preclinical models of stroke, the functionality of oriented neuro-implants. In our evaluations, the forelimb strength of the rat forepaw recovered better in animal receiving the biomaterial (PDMS) *plus* the cells (human neural stem cells), compared to the ones receiving the implants alone or stem cells alone (Bédurier et al., 2012; Davoust et al., 2017).

Conclusions

Studies assessing long-term functional recovery in animals, combined with complex *in vivo* experiments to assess biomaterial behavior, need to merge the competences of engineer and biologist researchers, which render this kind of evaluations rather delicate. Considering that so far too few brain repair studies have been realized *in vivo*, we do not know yet the right structure, mechanical gradients, spatial confinement and stiffness for neural populations, as well as the best

cell population, to achieve a satisfactory biomimetic reconstruction. It is clear that, after implantation, the scaffold engrafted with cells makes contacts with the peri-lesioned areas, evolving in a highly complex manner. This is due to the interaction between the 3D material and the host environment, which have different adhesion and guidance pathways together with a variable stiffness and spatial organization. Efficient regeneration of neural tissue relies on the correct orchestration of these time-dependent processes. To answer this need, nowadays, we face 4D rather than 3D bioengineering. Some basic mechanisms (adhesion, migration, mechanosensing) are so far deciphered, but real modelling attempts are still dramatically lacking. Additionally, crucial key points are still unanswered in pre-clinical models: long-term bio-evolution of the neuro-implants, related processes like long-term follow-up (more than 3 months), network formation or myelination, have never been assessed in *in vivo* studies.

To date, in-depth evaluation of long-distance reconnections between areas disconnected by the lesion lacks. Before identifying the best experimental strategy that can evolve into a therapeutic option, implantable scaffolds need to favour neuronal and glial differentiation and to restore lost functions. So far, only a small number of studies in rodents have provided such evidence (Bédurier et al., 2012; Davoust et al., 2017). Moreover, primate safety and efficacy studies will also be a necessary step. Finally, from a technological point of view, it will be probably necessary to envision new hybrid approaches, merging *bottom-up* and *top-down* strategies, in order to reach the optimal synergy between the advantages of scaffold-free and scaffold-based tissue engineering techniques (A. Ovsianikov, Khademhosseini, & Mironov, 2018).

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Figure 1. Overview of the current strategies employed to obtain neuronal scaffolds and brain-implants. The main approaches are categorized into 2D Scaffolds, 3D scaffolds and Tissue-like constructs. Each strategy is then further discussed in terms of the underlying engineering methodology based on top-down (TD), bottom-up (BU) or cell biology-based models. TD methodologies imply shaping of materials via microfabrication processes such as optical lithography and additive manufacturing while BU methodologies relies on self-assembly of particles, hydrogels or even cells. All the figures are reproduced with permission from Ref. Accardo et al., 2018b,c; Álvarez et al., 2014; Bédrier et al., 2015; Guo et al., 2017; Jeong et al., 2015; Kang et al., 2012; Kato-Negishi et al., 2010; Lancaster et al., 2013; Limongi et al., 2013; Mansour et al., 2018; L. Vaysse et al., 2015.

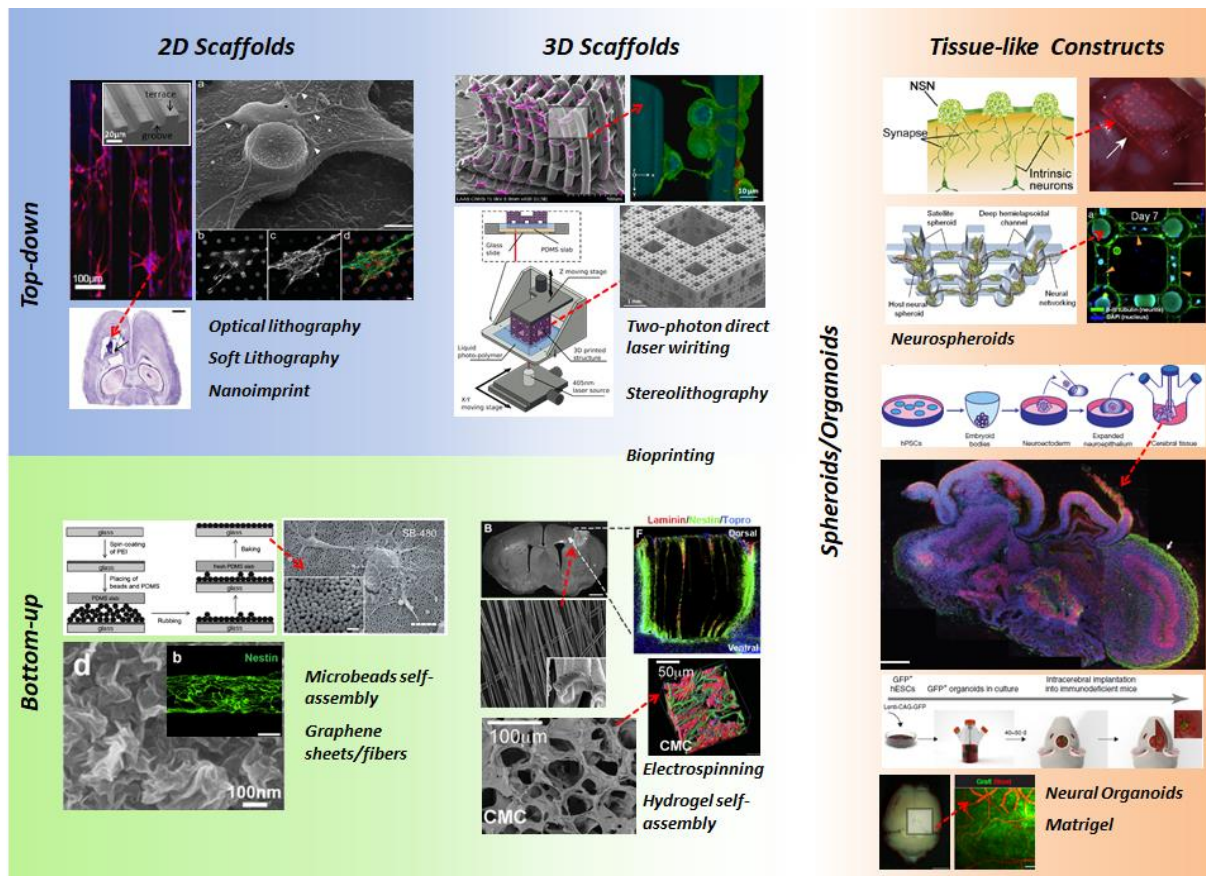

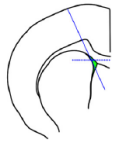
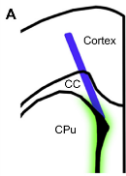
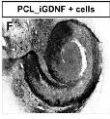


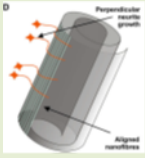
Table 1. Proof of concept: *in vivo* studies.

		Year	Guiding Biomaterials	Functionalization	Degradability - Cells	Main biological results
 <p>Gradient hydrogel</p>	Xu et al.	2018	Matrigel		D	Chemo-attraction, cell migration
			in glass cylinder.	Vertical chemotactic Sema3A or Netrin-1 gradient. Sema3A crosslinked on the hydrogel.	ND	Neuronal and glial maturation Tissue reconstruction
	Zhou et al.	2016	Thin roll structure (21G) of electrospun PCL fibers		D	Redirection of the rostral migratory stream to the cortex.
			Infiltrated with self-assembled colloidal electroactive graphene.	Outer layer of graphene or heparin.	D	Reduced glial and increased microglial reaction with graphene. No neuronal maturation with graphene outer layer.
 <p>A Cortex CC CPu</p>	Fon et al.	2014	Aligned PCL microfibers thin roll		D	Enhanced attraction and infiltration inside or outside by neuroblasts, microglia, astrocytes with 'BDNF'. Alignment perpendicular to the fibers. More glial than neuronal infiltration. Few mature neurons.
			incorporated with BDNF-mimetic. Injection with a 21G needle.	30 µg BDNF released in 14d.		PCL induced microglial inflammation normalized by BDNF-mimetic. Normal glial reaction.
 <p>PCL, GDNF + cells</p>	Wang et al.	2012	Roll of random electrospun		D	More proliferation and immature neurons with GDNF or with cells alone.
			GDNF + PCL fibers + cells	vs PCL + cells		Less reactive astroglia.
			vs cells (rat primary neural stem cells).			

Nisbet et al.

2009 Thin roll of aligned or non-aligned PCL fibers. D

Aligned and non-aligned fibers allow neurite infiltration and growth.



Less penetration when fibers were aligned

Preferential growth perpendicular to the direction of PCL fiber alignment.

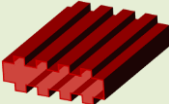
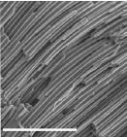
Extent of neurites /alignment.

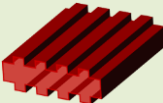

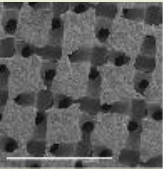
Inflammation peaked at 4-7d and remained for 60d, uninfluenced by fiber alignment.

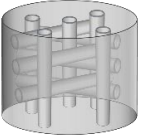
D: degradable; ND: non-degradable

Table 2. Regenerative medicine: *in vivo* studies.

D: degradable; ND: non-degradable

	Year	Guiding Biomaterials		Main biological results	Main functional results	
		Functionalization				
		Degradability - Cells				
	Davoust et al.	2017	2D micropatterned PDMS	ND	Chemo-attraction, cell migration Neuronal and glial differentiation. Guided growth and maturation. Tissue reconstruction.	Forelimb grip strength recovery improved with scaffolds combined with stem cells. Scaffold more efficient than only stem cells.
	Lefriec et al.	2017	Polylysine/lamimin coating. Can be demolded. Human primary adult neural stem cells.		Reduced glial scar.	In vivo vascularization.
	Martinez-Ramos et al.	2015	Scaffold of channels in aligned Ethyl Acrylate or Hydroxy Ethyl Accrylate.	ND	Implantation 1 dpi Cryogenic traumatic brain injury Scaffold vascularization Immature neurons and glia Minimal scar at 8 weeks	

Vaysse et al.	2015	<p>2D micropatterned PDMS</p> <p>Polylysine/laminin coating.</p> <p>Can be demolded.</p> <p>Human hNT2 neural cells.</p>	ND	<p>Chemo-attraction, migration</p> <p>Neuronal and glial differentiation.</p> <p>Guided growth and maturation</p> <p>Tissue reconstruction</p> <p>Small glial scar</p>	<p>Scaffold more efficient on forelimb grip strength recovery when combined with stem cells.</p>	
	Alvarez et al.	2014	<p>Roll of random vs aligned electrospun poly-L/DL lactic acid (PLA70/30). Lactate release.</p> <p>Dorso-ventral vs medio-lateral implantation</p>	D	<p>Guiding aligned fibers.</p> <p>New-born model.</p> <p>Cell attraction, migration,</p> <p>Neuronal differentiation. Better vascularization and tissue regeneration with dorso-ventral implantation.</p>	<p>Long-distance targeted axonal growth targeted on motor regions. Reconnection. Long-term follow-up (15 mo).</p> <p>Functional pre- and post-synaptic machinery.</p>
	Martinez-Ramos et al.	2012	<p>3D aligned or perpendicular channels in Ethyl-Acrylate or Hydroxy-Ethyl Acrylate scaffold</p> <p>+ neural stem cells.</p> <p>Adherence to the brain.</p>	ND	<p>Vascularization.</p> <p>Immature and mature neurons.</p> <p>More colonization when implanted in the SVZ zone vs cortex.</p> <p>Normal glial scar.</p> <p>Moderate inflammation.</p>	
	Wong et al.	2008	<p>3 x 3 mm PCL cylindrical sponge scaffold with 180–250 µm pores.</p>	D	<p>Tissue reconstruction, infiltration and alignment along microgrooves of immature neurons was better with orthogonal channels. No neuronal migration and glial scar for the</p>	

	<p>3D porous or channel (= longitudinal microgrooves) or orthogonal (+ axial microgrooves).</p>	<p>channel scaffold (dorso-ventral orientation). Less inflammation for the channel than cylinder or orthogonal scaffolds.</p>
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D: degradable; ND: non-degradable