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Neural differentiation on synthetic scaffold materials

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The potential of stem cells to differentiate into a variety of subgroups of neural cells makes stem cell differentiation and transplantation a promising candidate for neurodegenerative disorder therapies. However, selective differentiation of stem cells to neurons while preventing glial scar formation is a complex process. Mimicking the natural environment of neural tissue is pivotal, thus various synthetic materials have been developed for this purpose. The synthetic scaffolds can direct stem cells into a neural lineage by including extracellular factors that act on cell fate, which are mainly soluble signals, extracellular matrix proteins and physical factors (*e.g.* elasticity and topography). This article reviews synthetic materials developed for neural regeneration in terms of their extracellular matrix mimicking properties. Functionalization of synthetic materials by addition of bioactive chemical groups and adjustment of physical properties such as topography, electroactivity and elasticity are discussed.

1. Introduction

Neurodegeneration is the progressive loss of structure and function of nerve cells. Degeneration is a common phenomenon in many neurodegenerative diseases and nerve injury. Neurodegeneration generally occurs due to the accumulation of misfolded aggregated proteins in some parts of the aging brain and, as a result, cell death and inflammatory damage occur in those areas in the brain.[1] Clinically, there are different types of neurodegeneration in different neurodegenerative diseases. Therefore, there are some differences in the proximal triggers and pathological markers such as Lewy bodies in Parkinson's disease, plaques and tangles in Alzheimer's disease, demyelination in multiple sclerosis and motor-neuron death in ALS. On the other hand, despite different triggering factors, these diseases share some overlapping downstream and secondary pathways such as neuroinflammation. Adult central nervous system cells have poor regeneration capacity, so any damage to the central nervous system might be permanent. Lost cells cannot be replaced with new functional ones, and remaining nerve cells cannot make new connections after injury due to the inhibitory properties of the extracellular matrix.[2] Besides neurodegenerative disorders,

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traumatic injuries such as spinal cord injuries have destructive effects on motor, sensory, and autonomic functions. It generally causes a permanent loss of sensation below the site of the injury. In the case of peripheral nerve injuries, cells in the peripheral nervous system have a regeneration capacity unlike the ones in the central nervous system.

Fully restoring the functional capacities of neurons after damage due to traumatic injuries or neurodegenerative disorders is still not possible. Although conventional therapies provide neural regeneration up to a certain level, they are not as efficient as desired. When the neural regeneration term is used, it refers to several different mechanisms to restore the functions of the degenerated neural tissue. It either occurs by the generation of new neurons from the progenitor cells residing nearby the damaged area, the generation of new synapses by the neurons that survive after the damage or by the repair of the axons/myelin sheets around the axons to prevent secondary cell loss after injury. It is more effective to combine these strategies in order to achieve functional regeneration of the nervous system. However, due to some intrinsic characteristics of the nervous system such as the unproliferative nature of neurons, the presence of progenitors in very localized areas in low numbers along with the upregulation of inhibitory elements upon injury leading to glial scar formation, the regeneration capacity of the central nervous system (CNS) is quite limited. This limited capacity of regeneration is aimed to be improved by materials designed for stem cell culture, differentiation and transplantation, which are called 'scaffolds'.

Stem cells are promising candidates for the treatment of neurological disorders since they can differentiate into neural cells when induced appropriately. Their differentiation can be enhanced by using bioactive materials, which can also be used as vehicles for cell transplantation to the damage site. In previous studies, various synthetic materials have been analyzed as scaffolds *in vitro* and *in vivo* for their potential to induce neural differentiation and nerve regeneration.[3–6] This review is focused on synthetic materials developed for neural regeneration including polymeric materials and self-assembled systems, and their modifications to support neural differentiation.

Natural cues for directing cell fate consist of different chemical, physical and biological signals in the extracellular matrix (ECM), neighboring cell interactions, soluble factors such as chemokines, growth factors and hormones, and the inherited potency of the cell.[7] Combination of these factors and their interactions affects the ultimate cellular differentiation mechanisms (Fig. 1). Understanding this complex environment and mimicking it as efficiently as possible will enable the directing of cell differentiation in a desired manner. One way of mimicking the natural environment of cells *in vitro* is to supply soluble factors in the culture media. This approach is relatively simple but costly since it requires a high concentration of soluble factors. Also it is not very efficient since it does not mimic the mechanical properties of the cells' natural environment. Cells are embedded in tissues in a three-dimensional (3D) manner and they can migrate or grow extensions such as axons and dendrites of neural cells. Physical properties of the 3D network including stiffness, roughness and pore size are also important and should be considered while designing a synthetic system that mimics the native tissue. As conventional *in vitro* cell culture surfaces cannot provide an appropriate environment to cells, 3D scaffolds combined with soluble signals or bioactive signals

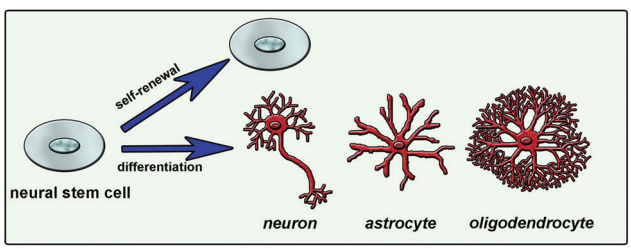

Fig. 1 Neural stem cell fate determination is mainly guided by extracellular matrix molecules, soluble factors and cell-to-cell interactions.

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tethered to the surface are used to mimic the natural neural niche.

Natural extracellular matrix proteins or synthetic scaffolds have been used for developing 3D scaffolds to mimic the biological, chemical and mechanical properties of natural matrix of the cells. Although natural proteins are biocompatible, it is not easy to functionalize these scaffolds with desired bioactive groups, or to manipulate their mechanical properties such as stiffness. Thus, these scaffolds might not be sufficient to direct the differentiation of cells especially into complex cells like neurons.[8–12] On the other hand, synthetic materials can be synthesized with desired functionalities such as specific bioactive groups, stiffness and roughness in order to mimic the natural environment of cells.

2. Approaches in the design of synthetic materials for neural differentiation

A variety of different methods can be used to develop scaffold materials with desired properties, including the support of cell survival and the induction of differentiation into desired cell types. Besides changing the material type, nerve regeneration studies have also focused on designing scaffolds with incorporated bioactivity for the induction of neurogenesis. One approach to induce differentiation is by adding soluble inducers while culturing cells on non-bioactive scaffolds. In this approach, a scaffold is a better environment for cell survival compared to a tissue culture plate. However, as the scaffold itself does not include any signal for differentiation, differentiation is induced solely by the soluble inducers added to the growth media. Soluble inducers commonly used for neural differentiation include neurotrophic factors (nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) *etc.*) and retinoic acid (RA).

Another approach is by embedding the soluble inducers mentioned above into the scaffold in order to provide a gradual release or by tethering these inducers to the surface of the scaffold through functional groups, in order to provide spatial organization to cells. In the case of gradual release, the scaffold is not bioactive but has a role in differentiation by releasing inducers at desired concentrations over a longer time period. When tethering the inducers to the surface, the scaffold is bioactive, however its bioactivity is not directly related to the differentiation process. Bioactive groups on the material's surface are presented so that they bind to the soluble inducers and present them similar to the extracellular matrix components (such as heparan sulfate proteoglycans) presenting growth factors to the cells. Soluble factors can also be immobilized on material surfaces directly by covalent attachment without the use of the growth factor affine proteoglycans.[13]

Functionalization of the surface with bioactive signals is a method used for differentiation induction directed by scaffold. Bioactive groups can be either chemical groups inspired by the natural environment of cells in a specific tissue, like the brain,

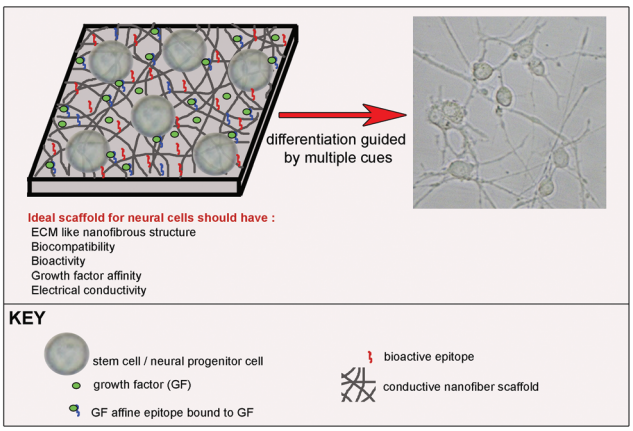

Fig. 2 Bioactive scaffold design for neural differentiation of stem cells.

or functional groups of natural inducers such as peptides found in neural differentiation-inducing proteins. Besides the addition of bioactive signals, scaffolds can also be functionalized by tuning their mechanical properties and physical morphology in order to support neural cell survival and differentiation. Since they present bioactive groups, these scaffolds can be used alone for the induction of differentiation, depending on the complexity of the final cell type and potency of the starting cell for differentiation into desired cell phenotypes (Fig. 2). Such a scaffold can induce differentiation into different neuronal subtypes. However, it might not be sufficient for maturation of the induced cells into functional cells. Additional inducers can be added to the culture medium to promote maturation or the scaffold can be functionalized with multiple bioactive groups to overcome the maturation problem.

2.1. Use of synthetic scaffolds in combination with soluble inducers

Nonbioactive synthetic scaffolds can be used in combination with soluble inducers. These scaffolds combined with soluble inducers are not actively involved in the differentiation process; however, they support differentiation by providing a 3D environment for cells that is more similar to their natural environment when compared to two-dimensional (2D) tissue culture surfaces. Neurons can have outgrowths in all directions within 3D scaffolds, thus 3D scaffolds are more accurate representations of *in vivo* tissue architecture, compared to 2D scaffolds. They provide *in vivo*-like cell–cell interactions which increases cellular survival and leads to more realistic gene expression and cellular behavior. 2D cultures have less compatibility with *in vivo* systems. For example, it has been shown that dopaminergic neurons isolated from an embryonic brain display longer viability in 3D systems when compared to monolayer 2D cultures.[14] On the other hand, nutrient deprivation in 3D scaffolds causes more severe alterations in the expression of specific genes, cell proliferation and viability as well as productivity. Also, it was shown that mechanical injuries cause a more severe response in cells grown in 3D neural

cultures than 2D scaffolds even when they are subjected to the same strain and strain rate.[15]

These scaffolds do not have bioactive groups and they usually do not induce differentiation by themselves, except for when stiffness is used to control differentiation pathway.[8–12] Hence, defining an optimal cocktail of soluble inducers such as growth factors plays an essential role in the success of differentiation; however, it is costly and complex. Inducers are often chosen by considering the extracellular signals that play a role in neural cell survival and differentiation in the central nervous system. This approach is beneficial in terms of the ease of material synthesis as the material is not further modified and it is sufficient that the material does not interfere with cell survival. However, the lack of bioactivity makes the use of additional inducers, such as growth factors, inevitable. The selection of soluble inducers and their concentrations should be optimized to achieve the differentiation of cells cultured on nonbioactive scaffolds. Several different materials, mostly polymers, are used for the production of such scaffolds, examples of which are given below.

Nanofibrous poly-L-lactic acid (PLLA) scaffolds with high porosity were used for differentiation of neural stem cells (NSCs) and found to support neurite outgrowth.[16] Poly(ethylene-*co*-vinyl alcohol) (EVAL) membranes are another type of polymeric scaffold used for neural cell culture. Rat NSCs cultured on these scaffolds differentiated into neurons and astrocytes in the presence of basic fibroblast growth factor (bFGF). However, polyvinyl alcohol (PVA) scaffolds used in the same study did not support cell viability.[17] Although they do not contain bioactive signals, such scaffolds provide an initial environment for cells to produce their own microenvironment. NSCs encapsulated in a biodegradable polyethylene glycol (PEG) hydrogel in the presence of bFGF were observed to produce fibronectin, an indication of the production of a suitable microenvironment. These cells later differentiated into neurons and astrocytes, as demonstrated by immunohistochemistry and western blot analyses. Differentiated neurons were found to express synaptic protein synaptophysin and they were responsive to the neurotransmitter GABA (Fig. 3) indicating the functionality of *de novo* differentiated neurons.[18] Human bone marrow mesenchymal stem cells (MSCs) cultured on polyesters of 3-hydroxyalkanoate scaffolds in the presence of neural induction media (serum-free DMEM containing bFGF, IBMX, INDO and insulin) differentiated into neural cells with a better efficiency when compared to those grown on polylactic acid (PLA) scaffolds. In comparison, 3D scaffolds supported differentiation better than 2D polymer films and smaller pore sizes resulted in more effective differentiation, while scaffolds with larger pore sizes lead to a promotion of proliferation.[19] In addition to MSCs, NSCs were also found to effectively differentiate into neural cells on polyhydroxyalkanoates (PHA) with better efficiency when cultured on 3D scaffolds rather than 2D films of the same polymers. Among the three different PHA scaffolds used, poly(3-hydroxybutyrate) (PHB), copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate (P3HB4HB) and copolymer of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx), PHBHHx was found to support neural differentiation of NSCs with better efficiency as demonstrated by higher levels of β-III tubulin expression by western blot and immunostaining.[20] Microspheres composed of copolymers of PHA were also used for neural cell culture. PC-12 cells, primary cortical neurons (CN), and neuronal progenitor cells (NPC) were cultured on poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) microspheres along with the soluble growth factor, BDNF. PHBV microspheres supported the growth and proliferation of PC-12 cells and the differentiation of NPCs into neurons. However, the level of maturation of differentiated NPCs was lower when compared to the full maturation of CNs.[21]

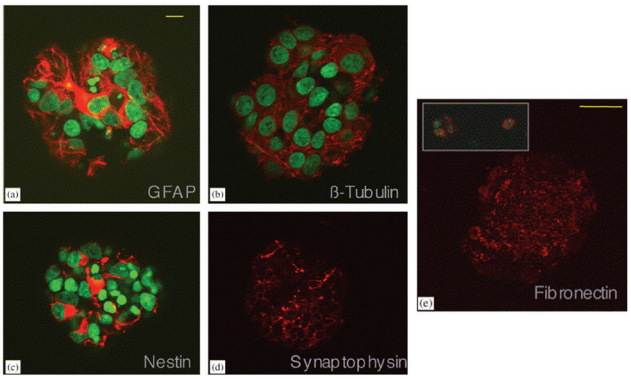

Fig. 3 Differentiation of NSCs into astrocytes (a) and neurons (b) in PEG hydrogels. Expression of nestin by undifferentiated NSCs (c), synaptophysin by differentiated neurons (d) and fibronectin by neural cells (e) are also shown. Scale bar represents 10 μm (a–d) and 50 μm (e). Reprinted with permission from ref. 18.

Another nonbioactive polymeric scaffold for neural regeneration was fabricated by ink-jet microdispensing PLA/polycaprolactone (PCL) in 80/20 proportions. In this study, genetically modified human embryonic kidney cells (EcR-293 cells), which can mimic Schwann cells by secreting NGF upon induction, were utilized. A PLA/PCL scaffold was found to support cell adhesion and cell growth.[22] Another genetically modified cell line used for neural differentiation was NSCs which were altered to express TrkC and neurotrophin-3 (NT-3). These cells differentiated on macroporous poly(lactic-*co*-glycolic acid) (PLGA) scaffolds without the use of additional soluble inducers. TrkC and NT-3 expression by NSCs favored neuronal differentiation over astrocyte and oligodendrocyte differentiation.[23]

Polymeric scaffolds fabricated by electrospinning have also been used for neural differentiation of stem cells. Polyurethane (PU) scaffolds with high porosity produced by electrospinning technique were used for the differentiation of human embryonic stem cells (hESCs) into neurons. The hESCs cultured on PU scaffolds were induced with a neural induction medium composed of Neuronal A basal medium supplemented with N2 and B27 supplements along with epidermal growth factor (EGF) and bFGF. Differentiated cells were positive for β-III tubulin, an early neural marker, MAP2ab, a mature neuron marker, and tyrosine hydroxylase (TH), a dopaminergic cell

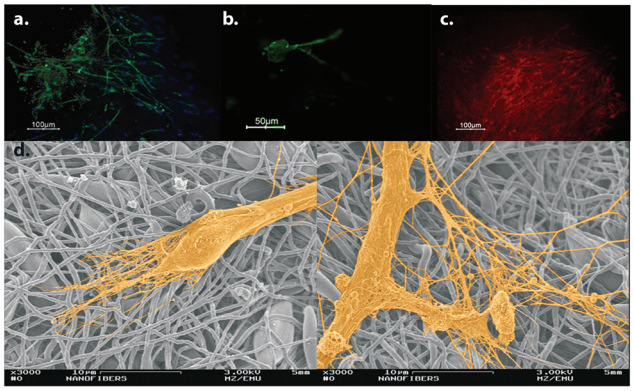

Fig. 4 Neural differentiation of hESCs on electrospun PU scaffolds. Differentiated cells express β-III tubulin (a), MAP2ab (b) and TH (c). (d) SEM micrographs of differentiated cells are demonstrated. Reprinted with permission from ref. 24.

marker. Differentiated cells did not express glial fibrillary acidic protein (GFAP), indicating neural differentiation in the absence of astrocytic differentiation. The morphology of the differentiated cells, as demonstrated by scanning electron microscopy (SEM) images, also resembled neuronal morphology[24] (Fig. 4).

Soluble factors can be used in combination with extracellular matrix proteins for a more efficient induction of neural differentiation. Poly(lactic-*co*-glycolic acid) (PLGA)/poly(L-lactic acid) (PLLA) polymer scaffolds with pore sizes between 250–500 μm coated with fibronectin or Matrigel™ were used for neural differentiation of hESC by adding RA into the growth media. The PLGA/PLLA scaffold provided a biodegradable 3D matrix and fibronectin/Matrigel™ coating which, along with RA, served as neural differentiation inducers. The hESCs grown on these scaffolds formed rosette-like structures with a similar morphology to an embryonic neural tube and expressed nestin and β-III tubulin. These structures were also found to express neurofilament (NF) by RNA analysis.[25]

Encapsulating differentiation inducer proteins in scaffolds provides sustained release over a long period depending on the scaffold material. Synthetic materials are commonly used to entrap neural inducer proteins to provide a gradual release for supporting neural differentiation. In one study, PLGA conduits and NT-3 were combined to generate NT-3-loaded PLGA carriers and this scaffold was used to co-culture NSCs and Schwann cells. Sustained release of NT-3 from the scaffold lasted up to 4 weeks. Immunoreactivity against MAP2 showed the differentiation of NSCs into neurons. In addition, synaptic structures and myelin sheaths were detected in the co-culture by double-immunostaining and electron microscopy analyses. Synapses between cells were excitable and capable of releasing synaptic vesicles under depolarization conditions. These results indicated a positive effect of NT-3 release from PLGA on the differentiation of NSCs into neurons, the development of synaptic connections and the myelination of neurites.[26]

NGF-encapsulating scaffolds are also commonly used in neural cell culture. A biodegradable electrospun copolymer of ε-caprolactone and ethyl ethylene phosphate (PCLEEP) with encapsulated NGF was used for the culture of PC-12 cells. Sustained release of NGF from the scaffold was observed for 3 months, resulting in neurite outgrowth of PC-12 cells.[27] Sustained release of NGF from PCL was obtained for 28 days by using PCL-bovine serum albumin (BSA)-NGF nanofibers that were also fabricated by electrospinning. The incorporation of BSA into fibers enhanced the sustained release and homogeneous distribution of NGF when compared to PCL fibers without BSA incorporation.[28] Hydrogels of polymers are also used for sustained release of neural inducers. NGF-loaded lysine-incorporated poly(2-hydroxyethylmethacrylate) [p(HEMA)] hydrogels resulted in the slow release of NGF due to the positive charge of the hydrogel provided by the lysine moieties. Dorsal root ganglion (DRG) neurons cultured in these hydrogels extended much longer neurites when compared to the soluble NGF-treated cells.[29] The p(HEMA) microporous gels with a gradient of immobilized NGF were used for PC-12 neurite outgrowth assay. In this study, neurites grew in the direction of higher concentration of NGF in the gradient.[30]

The sustained release of another neuronal induction factor, RA, was also obtained by encapsulating RA within aligned PCL nanofibers. MSC neuronal fate was affected by both nanofiber topography and controlled RA release. Without RA release, nanofiber topography was not sufficient to induce synaptophysin expression from MSCs, emphasizing the importance of the combined effect of topography and sustained release.[31]

Chemical conjugation of growth factors to electrospun nanofibers is also effective in inducing neural differentiation, even more effective than physical adsorption, which leads to burst release, as shown by Cho *et al.* In this study, amine-terminated PEG-poly(ε-caprolactone) conjugates were electrospun to obtain random and aligned nanofibers. NGF was chemically conjugated to free amine ends of PEG on the surface of the fibers. MSCs seeded on these NGF-conjugated scaffolds transdifferentiated into neural cells after 7 days, as evidenced by the expression of both immature (nestin and β-III tubulin) and mature (MAP-2) neural markers by RT-PCR and immunostaining analysis. The expression of neural markers was at the highest level on NGF-conjugated aligned scaffolds when compared to random fibers and NGF-adsorbed fibers.[32]

2.2. Physical, chemical or biological functionalization of scaffolds for promotion of neural differentiation

Chemically, physically and biologically functionalized scaffolds can hold several characteristics of the natural environment of cells at the same time. Bioactivation can be achieved through modulating the scaffold by addition of small chemical groups inspired by specific chemicals found in different tissues (*e.g.* phosphate in bone) as well as by presenting short peptide sequences on the surface of the scaffold that are functional domains of inducer proteins. Physical properties of tissues including elasticity, stiffness, roughness and electrical conductivity are other important parameters that should be considered for scaffold functionalization. In this section, different approaches of scaffold functionalization for neural cell culture are explained in detail.

2.2.1. Modification of scaffold to provide control over substrate stiffness. When designing a scaffold for neural differentiation, the mechanical properties of the scaffold should be designed to be optimal, near to that of brain tissue, which is below 1 kPa.[33] Cell differentiation caused by tissue elasticity is proposed to be driven by myosin-II motors and the same mechanism can be effective for cell responses to scaffold stiffness.[34] Scaffolds produced by using this approach are beneficial in that they provide similar mechanical signals to cells as those cultured in their natural environment. The mechanical stimulation of stem cells by culturing in such a scaffold can be directed into desired cell fates. However, the process of producing such scaffolds requires some extra care in order not to interfere with cell viability. Scaffolds with adaptable stiffness can be produced by adjusting the level of crosslinking agents. Most of the crosslinking agents are cytotoxic by themselves, so an additional step is required to get rid of remaining agents after crosslinking of the scaffold to avoid a cytotoxicity problem.

In a pioneering study, polyacrylamide gels with elasticities between 0.1–1 kPa were coated with collagen I and used for the direct induction of neural differentiation of hMSCs without a requirement for any soluble factor. The hMSCs obtained a neuronal morphology (Fig. 5) with the expression of a wide array of neural markers, including commitment (nestin), early differentiation (β-III tubulin), and mature neural cell markers (NF and MAP2) as demonstrated by immunofluorescence, western blot and microarray analyses. After three weeks of culture on these soft substrates, hMSCs committed to neural cell fate irreversibly even under the influence of myogenic and osteogenic inducers.[9]

Laminin-coated methacrylamide chitosan (MAC) hydrogels with different stiffness values were also used for analysis of stem cell behavior with respect to changing stiffness. The proliferation and differentiation of NSCs were found to be promoted on the softest scaffolds with elasticities less than 1 kPa (Fig. 6).[10] MAC hydrogels with a similar stiffness to brain tissue were also functionalized by IFN-γ and resulted in neural differentiation of NSCs more effectively than brain-derived neurotrophic factor (BDNF)-treated NSCs.[35] Polydimethylsiloxane (PDMS) substrates with differing stiffness produced by using varying proportions of cross-linking agents were also used for NSC culture. In this study, astrocyte differentiation occurred at the highest level on soft substrates while oligodendrocyte differentiation rate (induced by addition of thyroid hormone) was found to be independent of substrate stiffness. The number of differentiated neurons was also observed to be independent of stiffness, while maturation of these differentiated neurons was highly dependent on the degree of stiffness. Neurite length and expression of synaptic proteins were promoted on scaffolds with stiffness values near to that of brain tissue (Fig. 7).[12]

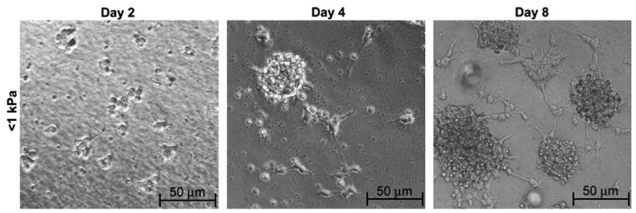

Fig. 6 Differentiation of NSCs in neurobasal media on soft MAC hydrogel of <1 kPa substrate stiffness over 8 days of culture. Reprinted with permission from ref. 10.

2.2.2. Electrically conductive scaffolds. Since neurons are electrically excitable cells, electrical conductivity is an important physical property to enhance neural cell activity.[36] Providing electrical conductivity allows electrical stimulation of the cells cultured within these scaffolds and this might be useful in terms of eliciting action potential by cells and improving synaptic connections. Electrical conductivity can be incorporated into synthetic scaffolds by using conductive materials during synthesis. Aligned nanofiber scaffolds formed by electrospun PLLA blended with carbon nanotubes (CNT) were constructed for this purpose. Both the conductivity of CNT and the alignment of the fibers were found to promote neural differentiation of mouse embryonic stem cells as shown by a higher expression of mature neuronal markers MAP-2 and neuron specific enolase (NSE).[37] Electrically active electrospun fibers were also produced from blends of poly(lactide-*co*-ε-caprolactone) (PLCL) and a conductive polymer, polyaniline

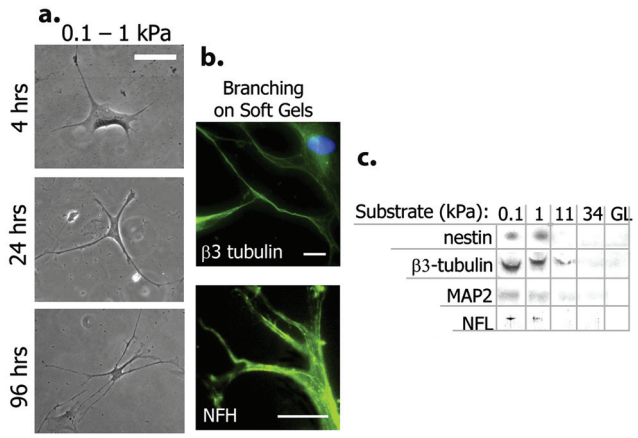

Fig. 5 Neural differentiation of hMSCs on soft polyacrylamide gel scaffolds. (a) Change in cell morphology when cultured on the substrate with low stiffness. Note the neural projection-like branches. (b) Expression of neural markers β-III tubulin and NF by differentiated cells. (c) Western blot analysis of neural markers for cells grown on PA gels with different elasticities. Glass (GL) surface was used as control. Reprinted with permission from ref. 9.

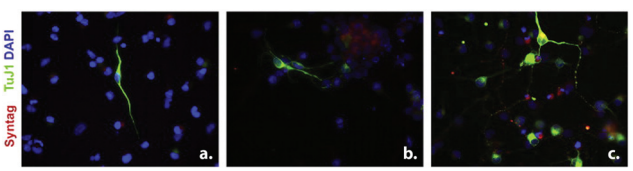

Fig. 7 Differentiation of NSCs into neurons on tissue culture plate (a) and PDMS substrates with changing stiffness; 750 kPa (b) and 12 kPa (c). Reprinted with permission from ref. 12.

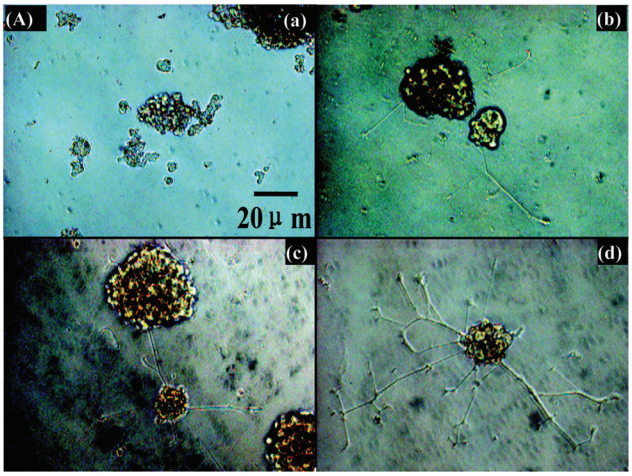

Fig. 8 Neurite extension of PC-12 cells on electroactive surfaces. Tissue culture plate without (a), and with NGF (b), electroactive ATQD-RGD without (c) and with NGF (d) (50 ng mL^{-1}). Reprinted with permission from ref. 39.

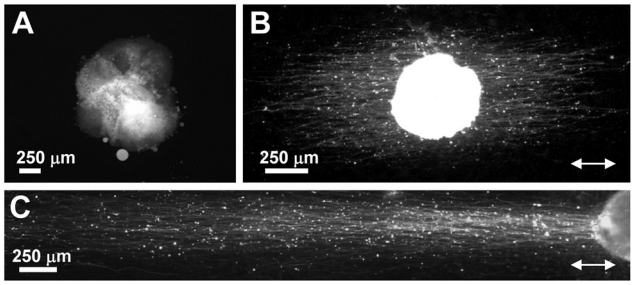

Fig. 9 Neurite outgrowth from DRG cells on PLLA nanofibers demonstrated by neurofilament staining. DRG cells on (A) random nanofibers, (B) aligned nanofibers, and (C) aligned nanofibers immobilized with bFGF. Reprinted with permission from ref. 40.

(PAni). PC-12 cells were cultured on these electrically active fibers and their viability, differentiation, and morphologies of neurite extensions were analyzed. The PLCL-PAni blends enhanced NGF-induced neurite outgrowth by PC-12 cells. Growth-associated protein 43 (GAP-43) and β-III tubulin expressions were also found to be higher in cells cultured on these nanofibers.[38]

In another study, glass surfaces coated with the electroactive silsesquioxane precursor *N*-(4-aminophenyl)-*N*′-(4′-(3-triethoxysilyl-propyl-ureido) phenyl-1,4-quinonenediimine) (ATQD) were covalently modified with cyclic RGD peptide. PC-12 cells were reported to extend neurites on these surfaces even in the absence of NGF. Addition of NGF further enhanced the level of neurite extension (Fig. 8).[39]

2.2.3. Effect of substrate topography. Surface geometry, topography and alignment are other physical parameters that affect neural differentiation. Modifying surfaces with a certain geometry or topography or providing alignment in the surface enhances cell orientation and improves the polarity, which is important in neural cell development. Besides, orienting themselves on the scaffold, neurons can also orient their neurites in the direction of alignments on the surface. Such an alignment is especially beneficial when considering such materials for regeneration of peripheral nerves whose alignments are naturally guided by Schwann cells. Aligned nanofiber substrates with immobilized signal proteins are found to induce neurite extension in several studies.

Aligned PLLA nanofibers produced by electrospinning technique modified by bFGF and laminin immobilization *via* heparin interaction could efficiently induce neurite extension from DRG. Neurites of DRG cells were extended in the direction of alignment, which has considerable importance for nerve bridging in clinical applications (Fig. 9).[40] Aligned PLCL nanofibers also induced DRG neurite alignment through the direction of fibers and coating PLCL fibers with multi-walled carbon nanotubes was found to lead to extension of much longer neurites. CNTs used in this study were ionically modified. This coating increased the hydrophilicity of the PLCL fibers, which was attributed to longer neurite production. In addition, the CNT coating provided electrical conductivity to the non-conducting PLCL fibers, making the environment more suitable for neural cells.[41] Much longer neurites were produced by PC-12 cells cultured on CNT-coated PLCL nanofibers compared to the uncoated PLCL fibers, similar to the response of DRG cells.[42]

Embryonic stem cells (ESCs) seeded on aligned electrospun PCL fibers exhibited neurite growth in the same direction as the alignment, similar to DRG cells on PLLA and PLCL fibers, after retinoic acid treatment (Fig. 10). In addition, the number of differentiated astrocytes was less on aligned PCL fibers compared to cells cultured on random PCL fibers.[43] Nanoscale ridge-groove patterns of polyurethane acrylate (PUA) fabricated by UV-assisted capillary force lithography were also used to direct selective neural differentiation of ESCs without the use of any soluble inducers. ESCs seeded on aligned PUA substrates differentiated into neural cells that express neural markers Tuj1, HuC/D and MAP2, but not GFAP, indicating the absence of astrocytes. Cells cultured on flat PUA surfaces didn't express any of these neural markers. Some of the cells on the ridge-groove patterns even differentiated into serotonergic and GABAergic neurons as demonstrated by the expression of serotonin and GABA.[44]

Aligned electrospun nanofibers were also used for NSC differentiation. The direction of NSC elongation and neurite outgrowth was found to be parallel to the direction of PLLA fibers for aligned scaffolds (Fig. 11). Also, the rate of NSC differentiation was higher for PLLA nanofibers than that for microfibers, indicating that the aligned nanofibrous PLLA scaffold could have more potential use in neural tissue engineering than microscale aligned fibers.[45] Effects of PLLA nanofibers might vary with respect to different properties of these fibers such as fiber diameter or density. For example, when DRG cells were cultured on highly aligned PLLA electrospun fibers, the direction and extent of neurite extension and Schwann cell migration from DRG explants was found to be influenced significantly by fiber diameter. The length of neurites was shorter on small diameter fibers (293 nm) when

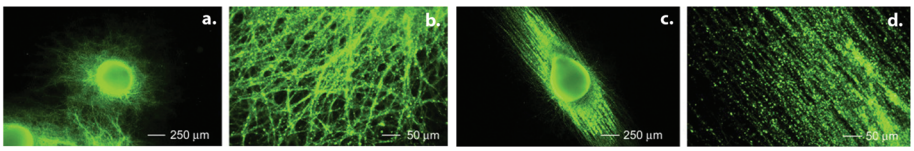

Fig. 10 Neural differentiation of ESCs on random (a,b) and aligned (c,d) PCL fibers. Immunohistochemistry is performed for a neural cell marker, Tuj1. Reprinted with permission from ref. 43.

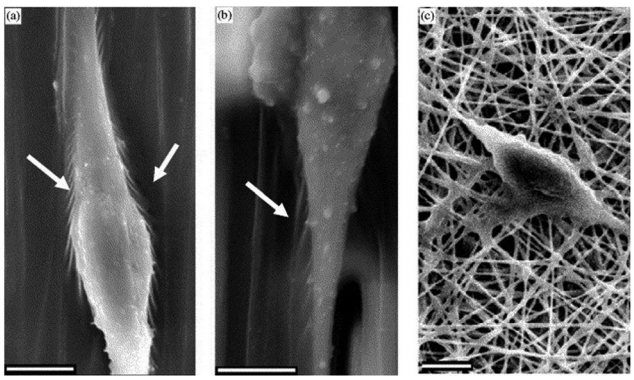

Fig. 11 SEM images of NSCs differentiated on aligned nanofibers (a), microfibers (b) and random nanofibers (c) of PCL. Arrows indicate sites of interaction between cells and the scaffold. Reprinted with permission from ref. 45.

compared to the large diameter ones (1325 nm).[46] In another study, increasing the PLLA fiber density was found to be correlated to an increase in neurite density, without affecting the length of the extending neurites (Fig. 12).[47] The effect of topography on NSC differentiation has also been demonstrated with several other polymeric fibers as scaffolds. NSCs cultured on aligned poly(ε-caprolactone)/gelatin scaffolds exhibited neurite outgrowth parallel to the fiber direction. In this study, gelatin was found to further promote differentiation in addition to scaffold alignment.[48]

Neuritogenesis and major neurite (axon) formation was demonstrated to be accelerated in primary motor neurons cultured on electrospun PLLA nanofibers (0.6–0.8 μm diameter) when compared to PLLA films and glass substrates. However, there was no difference between random and aligned fibers in the acceleration of neurite formation, and the minor neurite density and neurites were shorter in cells grown on fibers.[49]

Besides the aligned polymeric fibers, nanogratings were also used to produce aligned surfaces for stem cell differentiation. Aligned PDMS nanogratings lead to neural differentiation of hMSCs, even in the absence of any soluble factor, as evidenced by upregulation of the neural markers nestin, β-III tubulin, MAP2 and synaptophysin. Cells seeded on flat PDMS showed significantly lower expression of these neural markers, while the addition of retinoic acid increased their expression levels. Also, nanogratings were found to induce neural differentiation better than micropatterned PDMS substrates.[50] Micropatterned PDMS surfaces coated with poly-L-lysine (PLL) and laminin can also serve as effective neurite guidance surfaces for NSCs but the channel width should be properly defined for proper alignment and adequate neuron density. Smaller micropatterns were found to provide a perfect alignment, but hindered neurite development. On the other hand, larger micropatterns provided higher neuron density but neurites escaped out of the microchannel.[51] Precisely-sized micropatterned poly(methyl methacrylate) (PMMA) grooved scaffolds were used for mature astrocyte differentiation from radial glia-like cells (RGLC) *in vitro* without adding any soluble or substrate-adsorbed biochemical factors. RGLC were highly organized and the cells aligned along a 2 μm-patterned PMMA line. They expressed both nestin and Pax6, and generated different intermediate progenitors. These micropatterned surfaces also supported and directed axonal growth and neuronal migration.[52]

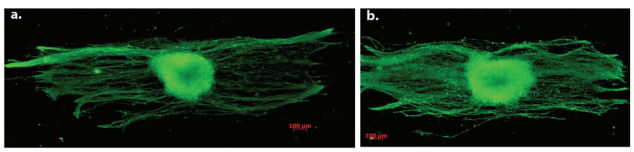

Fig. 12 Effect of fiber density on the number of neurites produced by DRG cells cultured on **a.** low density, and **b.** high density aligned electrospun PLLA scaffolds for five days. Immunohistochemistry is performed for neurofilament. Reproduced with permission from ref. 47.

Surface topography also affects the resting membrane potential and voltage-gated calcium channel responsiveness, which are important parameters in the determination of the functionality of a differentiated neural cell. Neural progenitor cells grown on polystyrene microbead arrayed substrates exerted more negative resting membrane potentials and higher voltage-gated calcium channel responsiveness when compared to cells grown on flat polystyrene surfaces, indicating that surface roughness can direct the differentiation of stem cells into more functional neural cells.[53]

Besides topography, surface geometry was also found to have profound effects on cellular behaviors including adhesion, proliferation and differentiation. Poly(ε-caprolactone) nanowires fabricated by a solvent free nanoscale template technique were found to support PC-12 cell adhesion, proliferation and viability better when compared to smooth PCL surfaces. Cells were found to interact with PCL nanowires *via* their lamellopodia and filopodia, as evidenced by SEM imaging. Neural differentiation was evidenced by the presence of neurite extensions as well as NF and TH expression evidenced by immunofluorescence analysis, while neurite extension was not observed on a smooth PCL surface.[54]

2.2.4. Chemical modifications of the scaffolds. The incorporation of specific chemical groups into scaffolds in order to mimic the abundance of some chemical moieties in the extracellular environment is another important modification for neural differentiation. Inspired by the abundance of certain chemical groups in specific tissues, substrates can be functionalized for the induction of NSC differentiation. Glass surfaces activated by hydroxyl (–OH), sulfonic ($–SO_3H$), amino ($–NH_2$), carboxyl (–COOH), mercapto (–SH) and methyl ($–CH_3$) groups were used for NSC culture to deduce if any of these groups leads differentiation into a specific neural cell subtype. $–SO_3H$ functionalized surfaces induced more oligodendrocytic differentiation, while NSCs grown on –COOH functionalized surfaces were more prone to differentiate into astrocytes. Neuronal differentiation could take place only on amino-functionalized surfaces, however, the differentiation efficiency was low (Fig. 13).[55]

Another method of functionalizing polymeric scaffolds is through mimicking the chemical structure of key proteins in neural differentiation. Acetylcholine-like polymers were synthesized by click chemistry and free radical polymerization from a bioactive unit mimicking acetylcholine, dimethylaminomethyl methacrylate (DMAEMA) and a bioinert unit, poly(ethylene glycol) monomethyl ether-glycidyl methacrylate (MePEG-GMA). Polymeric surfaces consisting of 1 : 60 (mol/mol) of MePEG-GMA to DMAEMA were found to support adhesion, normal morphology and neuronal outgrowth of hippocampal neurons effectively.[56]

The introduction of specific chemical groups on polymeric scaffolds can also be used for modification of surface tension, which in turn was found to affect neurite outgrowth. Embryonic cortical neurons that were cultured on electrospun PLLA and PLGA fibers treated with KOH to change surface tension grew longer neurites on the surfaces with the lowest tension, which were also the most hydrophobic scaffolds used in the study.[57] Chemical heterogeneity of the surface, which leads to surface disorder and the formation of local gradients in the surface free-energy, is another parameter that was found to have a role in neuritogenesis. PC-12 cells seeded on a self-assembled monolayer of alkylsiloxanes, which were highly disordered with high levels of chemical heterogeneity, could extend neurites within 48 h even without NGF treatment.[58]

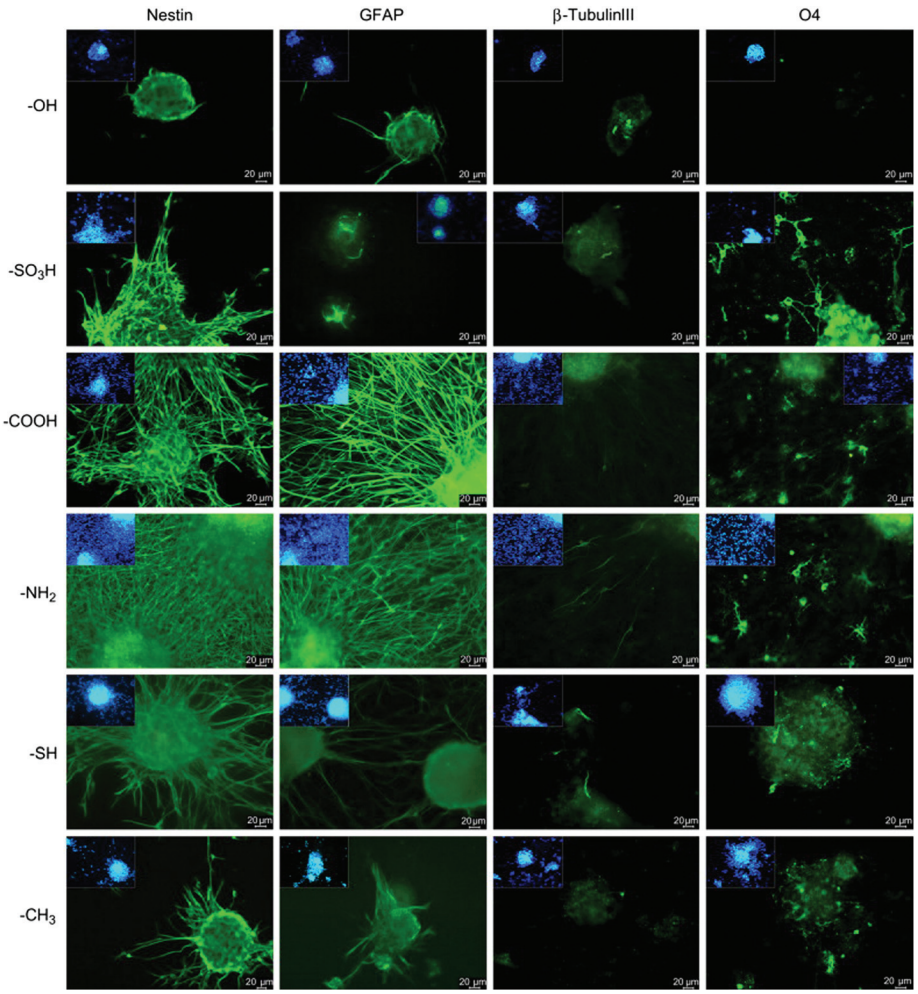

Fig. 13 Differentiation of NSCs on chemically functionalized glass substrates as demonstrated by expression of markers for astrocytes (GFAP), neurons (β-III tubulin) and oligodendrocytes (O4). Undifferentiated NSCs are shown by nestin expression. Reprinted with permission from ref. 55.

2.2.5. ECM proteins for scaffold biofunctionalization. Extracellular matrix proteins that are known to affect neural cell adhesion and differentiation can also be used for modification of scaffolds to incorporate bioactivity. As these proteins are active role players in the differentiation of cells in their native environment, their use in scaffolds is quite advantageous in terms of improving differentiation efficiency. However, since these proteins are isolated from animals, their use in scaffolds limits their clinical use.

In order to find an optimal combination of inducer molecules and biomaterials for NSC differentiation, a combinatorial protein display was carried out to screen mixtures of a variety of biomaterials with different soluble signals. Natural and synthetic matrices (fibronectin (FN), laminin (LN), PLL, RGD, IKVAV, PEI; poly(ethyleneimine)) containing different growth factors (EGF, NGF, CNTF, NT-3) were immobilized onto gold surfaces by photo-assisted patterning of an alkanethiol self-assembled monolayer as spots. NSCs were grown on these spots, each with different combinations of matrices and growth factors, and their differentiation was analyzed by immunohistochemistry. This study revealed FN/CNTF and RGD/CNTF to be the most efficient inducers of astroglial differentiation and LN/NGF, FN/NGF, RGD/NGF, FN/NT-3 and RGD/NT-3 as the most effective neuronal differentiation inducers among the studied combinations.[59]

In another study, methylcellulose (MC) scaffolds functionalized with laminin-1 (MC-*x*-LN1) were used for culturing primary murine neurospheres. MC-*x*-LN1 was found to enhance both NSC survival and maturation. In addition, lower levels of apoptotic activity were observed on MC-*x*-LN1 when compared to unmodified MC controls. The expression of neuronal and oligodendrocyte precursor markers showed a higher differentiation level on laminin-functionalized scaffolds when compared to cells on unmodified MC surfaces.[60]

Aerogels are also an important class of materials with tunable chemical, physical, and surface properties. Polyurea crosslinked silica aerogels (PCSA) were coated with PLL, basement membrane extract (BME), or laminin-1 and used for a culture of DRG neurons. Interactions of DRG neurons were tracked on PCSA and PLL, BME, and laminin-coated aerogels. In this study, laminin was found to be the most effective surface treatment for the attachment and growth of DRG neurons on the PCSA surface.[61]

Polymeric scaffolds can also be functionalized by producing fibers of a mixture of a polymer and an ECM protein by electrospinning technique. Electrospun blended PLLA/laminin fibers promoted neural differentiation of PC-12 cells in the presence of NGF significantly more efficiently than the unblended PLLA nanofibers.[62] PLCL/collagen fibrous scaffolds fabricated by electrospinning were used for neural differentiation of MSCs. Neural induction was performed gradually, first culturing MSCs in pretreatment media composed of β-mercaptoethanol, EGF, and bFGF; and then in the neural induction media consisting of N2 supplement, β-mercaptoethanol, insulin, EGF, NGF, and BDNF. MSCs on PCL/collagen fibers gained a neuronal phenotype with multipolar

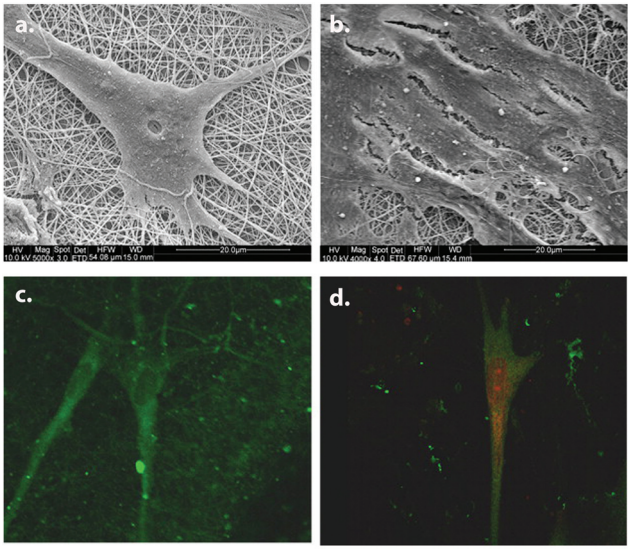

Fig. 14 Neural differentiation of MSCs on PLCL/collagen scaffolds. SEM images of differentiated (a) and undifferentiated (b) MSCs. Neurofilament (green) and nestin (red) expressions of differentiated MSCs are shown in (c) and (d) respectively. Reprinted with permission from ref. 63.

elongations along with the expression of neural markers NF and nestin (Fig. 14).[63]

2.2.6. ECM-derived short peptides for scaffold biofunctionalization. The presentation of short peptides that are functional domains of signal proteins on the scaffold surface is another approach for the induction of neural differentiation. This approach provides the differentiation-inducing properties of ECM proteins as these scaffolds present peptides that interact with cell surface receptors similarly to native ECM proteins. Besides, it also holds the advantage of producing completely synthetic yet bioactive scaffolds without the risk of pathogenic contaminations caused by the use of animal-derived proteins.

In such a study, functional domains of ECM proteins were produced as a fusion protein to the *Escherichia coli* outer membrane protein, OmpA, and attached to gold-coated surfaces. These bioactive surfaces effectively induced neurite extension and branching of neurites in PC-12 cells, as well as differentiation of NSCs.[64] Differentiation of fetal NSCs was promoted on superporous p(HEMA-AEMA) hydrogels modified with laminin-derived IKVAV, when compared to unmodified p(HEMA-AEMA). NSCs cultured on IKVAV-p(HEMA-AEMA) scaffolds expressed higher levels of β-III tubulin, NF and synaptophysin.[65] Polyamide electrospun nanofibers covalently attached to neuroactive tenascin C-derived peptides were found to promote neuritogenesis and neurite extension in primary neurons isolated from several different regions of brain, when compared to those cultured on unmodified polyamide scaffolds and PLL-coated glass cover slips.[66]

In another study, poly(HEMA-*co*-AEMA) nerve guidance channels were produced by a fiber templating technique and modified with laminin-derived peptides (YIGSR and IKVAV). Primary chick DRG cells cultured in these channels could effectively extend neurites. There was no statistical difference

in terms of neurite length between the cells in these channels and those on a PLL/laminin positive control surface, indicating that laminin-derived epitopes are as efficient as laminin itself in the induction of neurite outgrowth.[67]

Self-assembled peptide scaffold systems contain functional domains found in ECM proteins or other neural differentiation inducer proteins, which bind to cell surface receptors. Peptide amphiphile (PA) nanofibers with cell specific signals can be produced by synthesis of the epitope of a signal protein and linking this peptide to an alkyl tail along with hydrophobic amino acids. PA molecules form nanofibers in aqueous solutions by self-assembly through charge neutralization and hydrogen bonding. These nanofibers present epitopes on their surface, directly available to cell surface receptors. PA nanofibers with laminin epitope (IKVAV) were found to induce selective differentiation of neural progenitor cells into neurons while suppressing astrocyte differentiation.[68] PA nanofibers can present multiple epitopes at the same time. PA nanofibers, formed from laminin-derived IKVAV-PAs along with growth factor affine heparan sulfate-mimicking PAs, stimulated PC-12 differentiation cooperatively. Neurite outgrowth was promoted when cells were cultured on scaffolds presenting both functional groups. In addition, cells were found to extend neurites on these scaffolds even in the presence of inhibitory chondroitin sulfate proteoglycans.[69] Heparan sulfate-mimicking PAs were found to have affinity towards NGF, providing an increase in the local concentration of NGF in close vicinity of cells. More importantly, NGF does not lose its bioactivity after interaction with these PA nanofibers, which was evident from its inductive effect on neurite outgrowth (Fig. 15).[70]

The PA nanofibers were also used in a spinal cord injury (SCI) model in mice. IKVAV-PA injection led to a reduction in astrogliosis and cell death while increasing the number of oligodendroglia at the site of injury. IKVAV peptide alone was not able to promote recovery, which showed that both the nanofiber structure of the PA and the IKVAV sequence were required.[71] The anatomical basis of the behavioural recovery coming from the injection of IKVAV-PA was analyzed in a separate study and the major factor for this improvement was found to be the increased density of serotonergic fibers close to the lesion.[72]

The mechanical properties of PA nanofiber scaffolds can also be modified by using different proportions of signal incorporated PAs and non-bioactive but mechanically more stable molecules. Such an approach leads to the formation of more stable and stiffer gels.[73] In addition, their gelation kinetics can be modified by changing the amino acid sequences that are important for structural properties of the fibers. By including more hydrophilic and bulky amino acids, gelation can be slowed down without disrupting bioactivity, which can be important for *in vivo* applications such as injections.[74]

The PA gels can also be used for efficient delivery of biological molecules to the treatment site. One such example is the delivery of Sonic hedgehog (SHH) *via* monodomain gels containing aligned PA nanofibers. SHH protein has important roles in peripheral nerve regeneration. SHH signalling was inhibited in rats and their cavernous nerve was crushed leading peripheral nerve damage to form the animal model in this study. SHH was then delivered to the crushed cavernous nerve by the PA gel and promoted regeneration, suppressed penile apoptosis and improved erectile function. Such a treatment might be crucial in regeneration of the cavernous nerve in prostatectomy and diabetic patients where SHH levels are decreased.[75] A hybrid matrix approach with the combination of type I collagen and peptide amphiphile nanofibers was also shown to support neuronal survival, morphogenesis, and fine control over dendrite and axon growth of Purkinje cells (PC). While collagen provided a favorable mechanical support, the laminin epitope concentration was adjusted to control the matrix bioactivity by using PAs. Therefore, this system enabled the adjustment of laminin epitope density to control bioactivity without affecting its structural integrity.[76]

Self-assembled peptide nanofibers produced from alternating basic, hydrophobic and acidic amino acids (*e.g.*, RADA16) are also used for neural cell culture. They were initially reported to support the adhesion, neurite extension and synapse formation of PC-12 cells as well as primary neural cells.[77] These scaffolds better enhanced neural differentiation when they were functionalized with epitopes of ECM proteins (laminin, collagen, and fibronectin) and bone marrow homing peptides (BMHP1, and BMHP2).[78] RADA scaffolds were also synthesized by incorporating an FGL motif, a synthetic FGF receptor derived from a neural cell adhesion molecule (NCAM). DRG neurons cultured on these scaffolds extended much longer neurites when compared to bare (RADA)16 scaffolds, and the number of cells extending the neurites was higher in FGL-incorporated scaffolds.[79] When (RADA)16 scaffolds were mixed with laminin for biofunctionalization, NSC differentiation was enhanced in 3D culture.[80] (RADA)16-I scaffolds were also successful in axonal regeneration of an *in vivo* acute optic tract injury, and provided visual recovery.[81]

Peptide nanofibers can also be used as delivery vehicles for the sustained delivery of cytokines. (RADA)16 scaffolds modified by the addition of negatively or positively charged amino acids were used for sustained delivery of vascular endothelial growth factor, bFGF, and BDNF. Positively charged growth factors could be released more readily from positively charged scaffolds, while they were released slowly from negatively charged scaffolds.[82] Glycine spacers were shown to increase

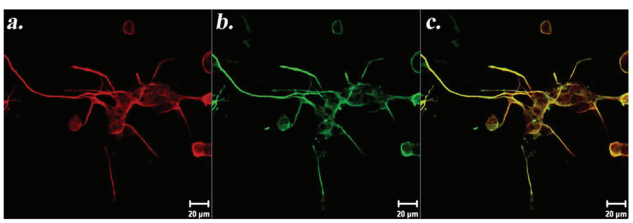

Fig. 15 Immunostaining of PC-12 cells against β-III tubulin (a) and Syn1 (b) on NGF-treated heparan sulfate-mimicking nanofibers. Panel (c) shows merged images of β-III tubulin and Syn1 on the same cells. Reprinted with permission from ref. 70.

both the stability and functional motif exposure of the nanofibers. Nanofibers with more glycine residues incorporated between the self-assembling sequence (RADA)16-I and the bioactive sequence (PFSSTKT, derived from BMHP1) supported NSCs adhesion, proliferation and differentiation more effectively than those with shorter spacers.[83]

Short peptide epitopes derived from ECM components were also used to produce peptide-modified silica thin gels for neural differentiation induction. Embryonic carcinoma stem cells were cultured on laminin-derived YIGSR, fibronectin-derived RGD and tenascin-derived NID peptide-modified surfaces in the presence of retinoic acid. RGD/YIGSR-modified substrates induced longer neurite formation, while the RGD/YIGSR/NID substrate resulted in an increased number of neurites per field.[84]

Mussel adhesive protein-inspired immobilization strategies are also useful in the attachment of bioactive signals to organic and inorganic materials for the modification of scaffolds. Growth factors and adhesion peptides containing amine and thiol groups were immobilized onto the polydopamine (PD)-coated polymer substrates for NSCs differentiation. ECM protein-derived adhesion peptides (fibronectin [RGD] and laminin [YIGSR]) and neurotrophic factors (NGF and glial cell line-derived neurotrophic (GDNF)) were immobilized by using this strategy. These scaffolds promoted differentiation and proliferation of human fetal brain-derived NSCs and human induced pluripotent stem cell-derived NSCs. Enhancement of neuronal differentiation of human fetal NSCs in PS-PD substrates was revealed by immunostaining and qRT-PCR analyses of Tuj1. Immunostaining and qRT-PCR of astrocyte marker GFAP demonstrated that immobilization of GDNF, C(K)GGYIGSR, and KGGRGD enhanced glial differentiation (astrocyte lineage) of human NSCs on the PS-PD substrates.[85]

Nanofiber-like viruses can also be used for the presentation of cell surface receptor binding epitopes on the scaffold surface. M13 phages were genetically engineered to express IKVAV and RGD peptides as their coat proteins at high density and they were drop-cast in order to produce aligned nanofibrous scaffolds. NSCs could easily differentiate into neurons on these viral scaffolds and produced neurites in the same direction as the alignment of the viral nanofibers.[86]

3. Identification methods for *de novo* differentiated neurons

The methods used in the evaluation of the effect of culture conditions (*i.e.* scaffold or medium components) are critical for the assessment of the effectiveness of the biomaterials. Analyses of already differentiated neurons or differentiation of neural progenitor cells are simpler due to the limited cell fates and end with more reliable results, however, analysis of the differentiation of stem cells from other tissues requires more effort. Use of more than one method should be preferred in order to avoid unreliable results. For instance, although the distinct morphology of neural cells is useful to get an idea about the identity of the differentiated cell, it might be misleading if used alone. Cellular toxicity is known to cause cell body shrinkage along with some extensions that might be confusing due to its resemblance to neural cell morphology.[87] Hence, morphological observations should be supported with the expression of lineage-specific genes and functional tests to verify that the differentiated cell is able to conduct action potentials. Several methods are used for the analysis of different aspects of neural differentiation. Morphological observations are mainly based on optical microscopy images that are useful in terms of neurite outgrowth analysis. A variety of softwares are available for neurite outgrowth-based measurements from optical microscopy images. Among these softwares, Image J is the most commonly used one. To evaluate the effect of scaffolds on neurite outgrowth, the total length of neurites per image,[12,28,29,39–43,45–47,49,51,56,57,62,64,66,67,69,70,76,77,79,83,86] mean number of neurites extended by a single cell,[38,49,58,64] number of branches in a specified area,[64] and percentage of cells extending neurites[27,28,30,38,49,60,66,69,79] are commonly measured by using Image J. The imaging of cells with electron microscopy is also useful in morphological analysis of differentiated cells as it provides more detailed images at higher magnifications. Growth cone morphology and the morphology of the extended neurites can be easily observed in SEM images.[16,19,20,24,39,45,48,53,54,61–63,69,78,86] Morphology of synapses can be analyzed in fine detail by transmission electron microscopy imaging.[26]

Since morphological observations alone are not sufficient to define the differentiation status, they can be supported with molecular information such as the expression of marker genes. There are various lineage-specific marker genes defined for neural progenitor cells, neurons, astrocytes and oligodendrocytes, and some of these even specify the subtypes of neurons.[88] Table 3 gives a list of the most commonly used marker genes and analysis methods. Expression of these genes can be analyzed both at the RNA level (by microarray and RT-PCR methods) and protein level (by western blot, immunostaining and ELISA methods). It is important to check protein levels, even if mRNA levels rise indicating differentiation. mRNA levels, although informative, might be misleading in some cases where gene expression levels at the transcriptional and translational levels differ.[89,90]

By using morphological observations along with expression analysis of various marker genes, a differentiated cell's identity can be defined. However, in order to verify whether a neuron is capable of conducting action potentials and making synapses like its natural counterparts, additional tests are required to verify its functionality. Electrophysiological recordings of single cells by patch clamp is quite useful for such an analysis.[76] Commercially available calcium-binding dyes can also be used to analyze stimulation based on calcium ion flux in differentiated neurons. Stimulation by neurotransmitters or high extracellular K^+ ion concentrations leading to depolarization results in an increase in the intracellular calcium ion levels. Fluorescence upon binding of intracellular calcium to

Table 1 Synthetic materials used as substrates for neural cell culture and differentiation

Substrate	Cells used	Ref.
Acetylcholine-like polymers	Hippocampal neurons	56
Glass	PC-12, NSC	39, 55
Gold	PC-12, NSC	64
M13 phages (nanofiber-like viruses)	NSC	86
Methacrylamide chitosan hydrogels	NSC	10, 35
Methylcellulose	Primary murine neurospheres	60
Peptide amphiphile nanofibers	Neural progenitor cells, PC-12, Purkinje cells	68–76
Peptide nanofibers (RADA16)	PC-12, NSC, DRG	77–83
Polyacrylamide gels	MSC	9
Polyamide nanofibers	Primary neurons from brain	66
Polycaprolactone (PCL) fibers	ESC, MSC, EcR-293 cells	22, 31, 43
Polydimethylsiloxane (PDMS)	NSC, MSC	12, 50, 51
Poly(ethylene-*co*-vinyl alcohol)	NSC	17
Polyethylene glycol (PEG)	NSC	18, 56
Polyhydroxyalkanoates (PHA)	MSC, NSC	19, 21
Poly(lactic-*co*-glycolic acid) (PLGA) fibers	NSC, ESC, cortical neurons	23, 25, 26, 57
Poly(lactide-*co*-ε-caprolactone) (PLCL)	PC-12, DRG, MSC	38, 41, 42, 63
Poly-L-lactic acid (PLLA)	ESC, MSC, NSC, DRG, cortical neurons	16, 25, 37, 38, 40–42, 45, 46, 57, 63
Polydopamine substrate	NSC	85
Poly(methyl methacrylate) (PMMA)	Radial glia-like cells	52
Poly(2-hydroxyethylmethacrylate) (p(HEMA))	DRG cells, PC-12, NSC	37, 65, 67
Polyurea cross-linked silica aerogels	DRG	61
Polyurethane scaffolds	ESC	24, 29, 44, 61
Poly(ε-caprolactone)	PC-12, MSC, NSC	27, 28, 32, 48
Silica thin gels	Embryonic carcinoma cells	84

Table 2 Functionalization of synthetic substrates for improved neural cell culture and differentiation

Substrate	Functionalization	Cells used	Result	Ref.
Modification for providing control over substrate stiffness				
Polyacrylamide gels	Stiffness in 0.1–1 kPa range, coating with collagen I	MSC	Neuronal morphology along with neural marker expressions	9
Methacrylamide chitosan hydrogels	Stiffness modification and coating with laminin	NSC	Proliferation and differentiation is promoted on softest scaffolds	10
Methacrylamide chitosan hydrogels	Stiffness modification and functionalization with IFN-γ	NSC	Neural differentiation	35
PDMS	Stiffness modification	NSC	Differentiation into astrocytes and maturation of differentiated neurons highest on softest substrates	12
Providing electrical conductivity by modifications				
PLLA	Electrospinning blending with carbon nanotubes	ESC	Neural differentiation is promoted	37
PLCL	Electrospinning blending with PAni	PC-12	Neurite outgrowth is enhanced	38
Glass	Silsesquioxane precursor ATQD covalently attached to RGD peptide	PC-12	Neurite outgrowth even in the absence of NGF	39
Providing alignment in substrate				
PLLA fibers	Aligned fibers	NSC, DRG	NSC differentiation. Neurite extension in the direction of alignment, both for NSCs and DRGs	45, 46
	Aligned fibers modified with bFGF and laminin immobilization by heparin interaction	DRG	Neurite extension in the direction of alignment	40
PLCL nanofibers	Aligned fibers coated with carbon nanotubes	DRG	Longer neurites growing in the direction of alignment	41
PCL fibers	Aligned fibers	ESC	After RA treatment, lower number of astrocytes on aligned fibers along with neurite extension in the direction of alignment	43
Polyurethane acrylate	Nanoscale ridge-groove patterns	ESC	Selective neural differentiation even in the absence of any soluble factor	44
Poly(ε-caprolactone)	Aligned fibers produced by electrospinning blending with gelatin	NSC	Differentiation is promoted, neurite extension in the direction of alignment	48
PDMS	Aligned nanogratings	MSC	Upregulation of neural markers	50
	Aligned channels by micropatterning and coating with poly-L-lysine and laminin		Alignment of neurites differs with channel width	51

Table 2 *(Contd.)*

Substrate	Functionalization	Cells used	Result	Ref.
PMMA	Microgrooved patterns	Radial glia-like cells	Cell alignment along with directed axonal growth	52
PA nanofibers	Aligned PA nanofibers releasing SHH	*In vivo* study for cavernous nerve regeneration	Cavernous nerve regeneration, suppression in penile apoptosis and improvement in erectile function	75
Chemical modifications				
Glass	Introduction of chemical groups; –OH, $–SO_3H$, $–NH_2$, –SH, –COOH, $–CH_3$	NSCs	Differentiation to oligodendrocytes on $–SO_3H$-modified surface, astrocytes on –COOH-modified surface; neurons only on $–NH_2$-modified surface	55
Acetylcholine like polymers	Synthesized by using acetylcholine mimicking dimethylaminomethyl methacrylate	Hippocampal neurons	Cells adhere, preserve normal morphology and extend neurites	56
PLLA or PLGA fibers	Modification of surface tension by KOH treatment	Cortical neurons	Longer neurites on surfaces with lowest tension	57
Use of ECM proteins for biofunctionalization				
Methylcellulose	Laminin-1 functionalization	Primary murine neurospheres	NSC survival and maturation	60
PLLA fibers	PLLA/laminin fibers produced by electrospinning blending	PC-12	Neural differentiation is enhanced	62
PLCL	PLCL/collagen fibers produced by electrospinning blending	MSC	Neuronal phenotype and expression of NF and nestin	63
Polyurea cross-linked silica aerogels	Poly-L-lysine/basement membrane extract/ laminin-1 coating	DRG	Better attachment and growth of DRG on laminin-coated surface	61
Peptide nanofibers (RADA16)	Mixing with laminin	NSC	Differentiation is improved in 3D culture	80
Use of ECM-derived short peptides for biofunctionalization				
Gold	ECM proteins fused to *E. coli* outer membrane protein	PC-12, NSC	Neurite outgrowth and branching	64
Peptide amphiphile (PA) nanofibers	Laminin-derived epitope (IKVAV) incorporation	Neural progenitor cells	Selective differentiation into neurons, suppression of astrocyte differentiation	68
PA nanofibers	IKVAV incorporation	*In vivo* study for SCI treatment	Reduction in astrogliosis and cell death, increase in oligodendroglia cell number at injury site	71, 72
PA nanofibers	Heparan sulfate-mimicking epitope incorporation	PC-12	Neurite outgrowth provided by NGF affinity of nanofibers	70
PA nanofibers	IKVAV incorporation along with heparan sulfate-mimicking epitopes	PC-12	Neurite outgrowth is promoted by cooperative effect of the two epitopes	69
PA nanofibers	Mixing collagen I with PAs carrying laminin-derived epitopes to produce a hybrid matrix	Purkinje cells	Improved mechanical support and adjustment of laminin epitope density is provided for control over dendrite and axon growth	76
Peptide nanofibers (RADA16)	Epitopes derived from laminin, collagen, fibronectin and bone marrow homing peptides are incorporated	NSC	Neural differentiation is enhanced	78
Peptide nanofibers (RADA16)	FGL motif (a synthetic FGF receptor derived from NCAM) is incorporated	DRG	Higher percentage of cells extend neurites and neurites are much longer	79
Silica thin gels	Modified with laminin (YIGSR)/ fibronectin (RGD)/tenascin (NID)-derived epitopes	Embryonic carcinoma cells	Longer neurite outgrowth on RGD/YIGSR, increased number of neurites on RGD/ YIGSR/NID	84
PS	Immobilization of fibronectin (RGD), laminin (YIGSR)-derived peptides and NGF, GDNF on polydopamine-coated PS	NSC	Enhancement in differentiation	85
M13 phages (nanofiber-like viruses)	Genetic engineering for expression of IKVAV and RGD epitopes on phage surface	NSC	Neural differentiation and outgrowth of neurites in the same direction of phage alignment	86
Polyamide nanofibers	Covalently attached Tenascin–C-derived peptides	Primary neurons from brain	Neurite outgrowth	66
p(HEMA-*co*-AEMA) nerve guidance channels	Modified with laminin-derived peptides (YIGSR and IKVAV)	DRG cells, NSC	Neurite outgrowth of DRG, differentiation of NSC	65, 67

the dye can be imaged with confocal microscopy in a time series to obtain a plot of calcium spikes over time.[18,53] Another fluorescent imaging method developed as a functionality test for neurons is based on the release of synaptic vesicles upon depolarization of the cell membrane by high extracellular K^+ ion concentrations. A fluorescent dye, (*N*-3-

Table 3 Lineage-specific markers for identification of nervous system cells

Marker	Function	Ref.	Methods used for analysis of expression
NSC markers			
Nestin	Intermediate filament	91	Immunostaining[12,17,18,20,25,32,43,44,50,52,53,55,59,60,63,65,78,83,85] Western blot[9,52] RT-PCR[10,19,32,37,50]
PAX6	Transcription factor controlling progenitor cell identity and neural fate	92, 93	Western blot[52] RT-PCR[10]
Neuron markers			
β-III tubulin	Microtubule protein specifically expressed in early stages of neural commitment. Recognized by Tuj1 antibody	94	Immunostaining[9,10,12,16,18,20,23–25,31,32,35,38,43,49–53,55,59–61,64,65,68,70,76,78,80,83–86] Western blot[9,18,20,23,26,38] RT-PCR[10,12,19,32,37]
Neurofilament (NF)	Intermediate filament found in axons	95	Immunostaining[9,40,45–47,49,54,57,62,63,65] Western blot[9] RT-PCR[25,50]
Microtubule-associated protein-2 (MAP2)	Provide stability to microtubules in dendrites MAP2ab: specific for mature neurons MAP2c: expressed both in neurons and neural progenitors	96, 97	Immunostaining[21,23,24,26,31,32,37,44,49,50,56,76] Western blot[9] RT-PCR[31,32,37,50]
Tau	Provides stability to microtubules in axons	97	Immunostaining[56]
Neuron-specific enolase (NSE)	Glycolytic enzyme	98, 99	Immunostaining[17] RT-PCR[10,37]
Neuronal nuclear protein (NeuN)	DNA binding nuclear protein found in mature neurons. Recently identified as Fox3 gene product acting as tissue specific splicing factor	100–102	Immunostaining[76]
Doublecortin (DCX)	Microtubule binding protein. Expressed in progenitor cells committed to neuronal; sharply reduced expression after expression of mature neural markers (*i.e.* NeuN)	103	RT-PCR[10]
Synaptophysin	Presynaptic vesicle membrane glycoprotein	104	Immunostaining[18,31,50,65,69,70,76]
Synapsin1	Presynaptic vesicle-associated phosphoprotein	105	Immunostaining[23,26]
Growth-associated protein 43 (GAP43)	Localized in growth cone, GAP43 guides axonal growth and modulates formation of new connections	106	Western blot[38]
Tyrosine hydroxylase (TH)	Rate-limiting enzyme of catecholamine biosynthesis. Essential for dopamine synthesis	107	Immunostaining[24,54,80] RT-PCR[50]
Astrocyte markers			
Glial fibrillary acidic protein (GFAP)	Intermediate filament	108	Immunostaining[10,12,17,18,20,21,23,24,26,31,35,43,44,50–52,55,59,60,68,78,83,85] Western blot[18,26,52] RT-PCR[10,12,19,31,50]
S100B	Calcium binding protein	109	RT-PCR[10]
Oligodendrocyte markers			
Galactosylcerebroside (GalC)	A glycolipid in myelin	110	Immunostaining[10,83]

Table 3 *(Contd.)*

Marker	Function	Ref.	Methods used for analysis of expression
OLIG1	Basic-helix-loop-helix (bHLH) transcription factor required in oligodendrocyte fate specification	111	RT-PCR[10]
Myelin oligodendrocyte glycoprotein (MOG)	A surface marker of oligodendrocytes involved in myelin structure. Expressed late in oligodendrocyte maturation	112,113	RT-PCR[10]
2′,3′-Cyclic-nucleotide 3′-phosphodiesterase (CNPase, identified by RIP antibody)	An enzyme found in myelin	114–116	RT-PCR[10] Immunostaining[10,35,43,55,60,83]
O4	A sulfolipid (sulfatide) found in myelin. Used as a surface marker for oligodendrocytes	117	Immunostaining[43,55,60,83]

triethylammoniumpropyl)-4-(4-(dibutylamino)styryl), is used to stain endocytic synaptic vesicles and track their release upon depolarization.[23,26,77] Resting membrane potentials can also be measured by using a cell permeant potentiometric fluorescent dye, tetramethylrhodamine methyl ester. Intracellular and extracellular fluorescence intensities obtained by confocal imaging can be used to calculate the cells' resting membrane potentials.[53]

In summary, a combination of morphological observations, expression level analysis of lineage specific markers and neuronal activity analysis methods are suggested to be used together in order to correctly identify the *de novo* differentiated neurons.

4. Conclusion

Neural differentiation is a complex task and selective differentiation into a specific subgroup of neural cells is even much harder. Despite the complexity of the process, directing neural cell differentiation is crucial to overcome functional loss of neurons caused by traumatic injuries or neurodegenerative disorders. Since conventional therapies have low rates of success in functional recovery after neural loss, the utilization of bioactive materials is considered as a promising approach for neural regeneration. Biomaterials can be applied in clinics either through the transplantation of *in vitro* differentiated cells into the lesion site; or transplantation of drug/growth factor-loaded biomaterials to direct *in vivo* differentiation of endogenous stem cells. Although biomaterials containing natural materials have the advantage of being biocompatible, they have disadvantages due to immunological reactions and the risk of cross-contamination. Synthetic materials can alternatively be used as the building blocks of scaffolds for tissue engineering as they have the advantage of low immunogenicity. In order to make synthetic materials more biocompatible and bioactive, the mechanical and chemical properties of scaffold materials can be altered, or scaffolds can be modified with biological signals for directing the cell fate determination. Many different approaches and methods are applied for the development of artificial scaffolds to be used in neural regeneration. These include the use of a variety of polymers, alone or in combination either with a different type of polymer or with natural materials, and the use of self-assembling synthetic materials, inspired by nature, such as peptide nanofibers (summarized in Tables 1 and 2). Despite promising results obtained from the application of different scaffolds, comparative studies are required to ensure their efficiency and safety, and to determine optimal materials and methods for neural differentiation, so that they can be used for the treatment of neural injuries and neurodegeneration in clinics.

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ATQD	*N*-(4-Aminophenyl)-*N*′-(4′-(3-triethoxysilyl-propyl-ureido) phenyl-1,4-quinonediimine)
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BME	Basement membrane extract
BMHP	Bone marrow homing peptides
BSA	Bovine serum albumin
CN	Cortical neurons
CNT	Carbon nanotube
DMAEMA	Dimethylaminomethyl methacrylate
DMEM	Dulbecco's Modified Eagle Medium
DRG	Dorsal root ganglion
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
EVAL	Ethylene-*co*-vinyl alcohol
FN	Fibronectin

GABA	γ-Aminobutyric acid
GAP-43	Growth-associated protein 43
GDNF	Glial cell line-derived neurotrophic factor
GF	Growth factor
GFAP	Glial fibrillary acidic protein
hESCs	Human embryonic stem cells
IBMX	3-Isobutyl-1-1-methylxanthine
INDO	Indomethacin
LN	Laminin
MAC	Methacrylamide chitosan
MC	Methylcellulose (MC)
MePEG-GMA	Monomethyl ether-glycidyl methacrylate
MSCs	Mesenchymal stem cells
NCAM	Neural cell adhesion molecule
NF	Neurofilament
NGF	Nerve growth factor
NPC	Neuronal progenitor cells
NSCs	Neural stem cells
NSE	Neuron specific enolase
NT-3	Neurotrophin-3
PA	Peptide amphiphile
P3HB4HB	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
PAni	Polyaniline
PC	Purkinje cells
PCL	Polycaprolactone
PCLEEP	Poly(ε-caprolactone-*co*-ethylethylene phosphate)
PCSA	Polyurea crosslinked silica aerogels
PD	Polydopamine
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PEI	Poly(ethyleneimine)
PHA	polyhydroxyalkanoates
PHB	Poly(3-hydroxybutyrate)
PHBHHx	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
PHBV	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
p(HEMA)	Poly(2-hydroxyethylmethacrylate)
PLA	Polylactic acid
PLCL	Poly(lactide-*co*-ε-caprolactone)
PLGA	Poly(lactic-*co*-glycolic acid)
PMMA	Poly(methyl methacrylate)
PLL	Poly-L-lysine
PLLA	Poly-L-lactic acid
PU	Polyurethane
PUA	Polyurethane acrylate
PVA	Polyvinyl alcohol
RA	Retinoic acid
RGLC	Radial glia-like cells
SCI	Spinal cord injury
SEM	Scanning electron microscopy
TH	Tyrosine hydroxylase

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