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Carol A. Rivera Martinez [a1]
Jorge Almodovar [a1] [a2]

[a1] Bioengineering Program, University of Puerto Rico Mayaguez, Call Box 9000, Mayagüez, Puerto Rico 00681-9000, USA

[a2] Department of Chemical Engineering, University of Puerto Rico Mayagüez, Call Box 9000, Mayaguez, Puerto Rico 00681-9000, USA

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Carol A. Rivera Martinez [a1]
Jorge Almodovar [a1] [a2]

[a1] Bioengineering Program, University of Puerto Rico Mayaguez, Call Box 9000, Mayagüez, Puerto Rico 00681-9000, USA

[a2] Department of Chemical Engineering, University of Puerto Rico Mayagüez, Call Box 9000, Mayaguez, Puerto Rico 00681-9000, USA

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Carol A. Rivera Martinez [a1]
Jorge Almodovar [a1] [a2]

[a1] Bioengineering Program, University of Puerto Rico Mayaguez, Call Box 9000, Mayagüez, Puerto Rico 00681-9000, USA

[a2] Department of Chemical Engineering, University of Puerto Rico Mayagüez, Call Box 9000, Mayaguez, Puerto Rico 00681-9000, USA

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suturing site due to the fragility of the scaffold.[118]

Axonal regeneration is highly desired,[46] and work contributes through the bioengineered LN-coated chitosan multi-walled conduit containing BMSCs was tested in vivo to serve as a bridge between a 10 mm long gap in the sciatic nerve of Sprague–Dawley rats. The chitosan component has been very attractive due to its biocompatible and biodegradable properties.[119, 120] An overview of the chitosan film preparation consisted of lyophilized chitosan, re-dissolved on 2% acetic acid and placed on coverslip to allow the solvent to evaporate. The chitosan film was modified with plasma treatment to increase the hydrophilicity, followed by LN covalently bonded by surface loading. Three groups were tested: empty silicone conduit, LN-modified chitosan scaffolds in silicone conduit (multi-walled) and LN-modified chitosan scaffold with BMSCs in silicone conduit (multi-walled plus BMSC). The BMSC resulted to be key element during in vivo experiment to down-regulated the extent of inflammation, reducing hyperplasia, and for proper neuronal growth [Figs. 2(a)–2(c)]. This is also corroborated with recovery index for 74.31 ± 26.51% for the conduit of BMSC, 17.31 ± 5.89% for the chitosan group and 31.52 ± 10.43% for the empty group.[46]

Figure 2. Multi-walled chitosan channel inner silicone conduit (a); the connective tissue-like surrounding the outer tube of the laminin (LN) -conjugated chitosan scaffolds and nerve regeneration along with the outer wall of the conduit (b); nerve grow back through the inner channels of the BMSC-treated chitosan scaffold conduit (c). The arrow indicated a regenerated nerve. Reprinted from Hsu et al.[46] Copyright (2013), with permission from Elsevier.

Chitosan was also the focus of the work by Meyer et al.,[121] in which the main concern was the capacity of artificial nerve guides to reconstruct critical length of sciatic nerve defects (15 mm) in vivo using healthy Wistar and diabetic Goto–Kakizaki rats. The diabetic rats were included to evaluate if the effect is the same under a relevant increase of blood glucose level that resembles type 2 diabetes patients, the above is done as a control because diabetic animals are less sensitive to nerve injury and they have a slow regeneration capacity.[122] The innovation is the introduction of the chitosan film to chitosan nerve tubes in order to achieve recovery of a critical defect length of 15 mm, since it was proven that the hollow chitosan tubes were capable to repair defects of 10 mm.[123] The chitosan film promoted SC attachment and supported DRG neurite outgrowth in vitro,[124] and this motivated combining the fine-tuned chitosan nerve guides (CNGs) tubes with the chitosan film. A novel outcome in diabetic rats is the presence of substantial regenerative matrix after 56 days of surgery, due to an increased number of activated SC, which attracts re-growing axons. Improvement on functional and morphological results of nerve regeneration, compared with hollow CNG, states that the role of chitosan of modifying an existing product introduces a new generation of medical devices for peripheral nerve reconstruction.

Poly lactic-based co-polymers (PLLA) has also been used to develop multifunctional films due to its biodegradability, low toxicity, and functional end groups.[125, 126] The word multifunctional applies since the film possess

Carol A. Rivera Martinez [a1]
Jorge Almodovar [a1] [a2]

[a1] Bioengineering Program, University of Puerto Rico Mayaguez, Call Box 9000, Mayagüez, Puerto Rico 00681-9000, USA

[a2] Department of Chemical Engineering, University of Puerto Rico Mayagüez, Call Box 9000, Mayaguez, Puerto Rico 00681-9000, USA

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[a2] Department of Chemical Engineering, University of Puerto Rico Mayagüez, Call Box 9000, Mayaguez, Puerto Rico 00681-9000, USA

surface micropatterns for topographical cues along with surface gradients of NGF, which it is released in a controlled manner using nanoparticles. The multifunctional films were tested in vitro using PC-12 cells as a model of peripheral nerve regeneration. An overview for the engineering of the multifunctional films consisted on a dry-phase inversion technique to develop the porous topography that allowed the distribution within the film of the micro particles CPEG:CPH (composed of random copolymers: CPTEG, CPH) that controlled the release of the GFs. The surface gradients were developed using longitudinal micropatterns through ester–amine interactions and physical self-assembly. Figure 3 shows a scanning electron microscopy (SEM) image of the topography of the multifunctional films (A); and the bioactive release of *β*-NGF promoting neurite extension (B). Neurite extension was quantified through the device outgrowth module software used for the collection of cellular images in two different *β*-NGF conditions: *β*-NGF released from microparticles and soluble *β*-NGF directly added to PC-12 cells in culture media. The film promoted guided neurite extension of cells of 10 µm total per cell in 2 days.[119]

Figure 3. (a) SEM images of porous, OVA encapsulated 50–50: CPTEG-CPH particles incorporated in patterned PLLA films; (b) Bioactivity of β-NGF released from 50 : 50 CPTEG: CPH microparticles (125 µg/mL) on the neurite extension of PC-12 cells. PC12 cell density: 2×10^4 cells/cm^2. Incubation time: 2 days. Neurites of the cells in every condition were immunostained with βIII-tubulin along with Cy3 secondary (shown in red), and DAPI for nuclei (shown in blue). Reprinted from Uz et al.[126] Copyright (2016), with permission from Elsevier.

Similarly, Torigoe et al., used a film model to study the regenerative effects of hyaluronan tetrasaccharide (HA4) on axons applied to common peroneal nerves cut from mice. Although HA is considered as a regenerative inhibitory agent in the CNS, HA can be depolymerized to specific forms of oligosaccharides containing different properties. For example, HA4, has been shown to decrease the death of K562 bone marrow cells and, increase the growth of PC-12 cells.[127] The proximal end of the nerve was maintained in vivo between two 3×4 mm^2 sheet of fluorine resin films sewn together and accompanied by the addition of a few drops of Ringer's Solution, thus observing the growth of regenerating axons. For the purposes of this investigation, instead of the Ringer's solution, HA4 drops were applied in the surface of the film at concentrations of 10, 100, or 1000 µg/mL and the neurite behavior was evaluated at 6 h, 2, 3 and 4 days later. In all groups (HA4 10, 100, 1000, and control) after 6 h, terminal bulbs could be seen at the proximal tip of the injured nerve, but only in the HA4 100 and HA4 1000 groups were extensions of terminal sprouts observed and on day 2 the regenerating axons were spread over the film. HA4 100 was cited as promoting increased axon length with a growth rate of 268 µm/day, approximately three times faster than controls. From day 3, the appearance of SCs occurs, which accelerate the growth of regenerating axons. At this stage, the growth of HA4 100 group was equal to that of the control and it was possible to determine that the SCs were distributed along the axons. This improvement in regeneration using HA4 is due to the reduction of the time of onset of terminal sprouting and the accelerated growth of axons, which manifests itself in response to an increase in the metabolism of neurons.[127]

Carol A. Rivera Martinez [a1]
Jorge Almodovar [a1] [a2]

[a1] Bioengineering Program, University of Puerto Rico Mayaguez, Call Box 9000, Mayagüez, Puerto Rico 00681-9000, USA

[a2] Department of Chemical Engineering, University of Puerto Rico Mayagüez, Call Box 9000, Mayaguez, Puerto Rico 00681-9000, USA

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[a1] Bioengineering Program, University of Puerto Rico Mayaguez, Call Box 9000, Mayagüez, Puerto Rico 00681-9000, USA

[a2] Department of Chemical Engineering, University of Puerto Rico Mayagüez, Call Box

itself in response to an increase in the metabolism of neurons.

Three-dimensional Printing

The 3D printing is a novel technique that is transforming science and education and has emerged since the 1980s as an additive manufacturing technology with fundamentals based on stereolithography and is also known as RP (rapid prototyping) or additive manufacturing (AM).[128–130] The 3D bio-printing is the use of 3D printing equipment combined with printing processing of biocompatible materials and cells through layer-by-layer. This technology was introduced in 1993 by Langer and Vancanti, with broad applications on tissue engineering and regenerative medicine fields.[131] In general, the process consists of: imaging, design, selection of materials and cells, and printing of the product.[129] The printing materials are what assigns the classification of the developed scaffolds as: solid, soft, or free scaffold.[128] In the case of 3D scaffolds for peripheral nerve regeneration related with soft tissues and organs, these are classified as soft and free scaffolds.[128]

One of the main advantages of 3D bio-printing technology is the capacity to develop complex structures of custom-tailored shapes and sizes as well as internal shapes, and pores that can be controlled to adjust the diffusion of oxygen, nutrients, and waste of cells.[132, 133] 3D bio-printing technology promises to elaborate structures that foment cellular function, provide mechanical support and generates patient-specific products.[128, 131] As part of current challenges, 3D bio-printing technology involves high working temperatures to achieve melting of materials that compromises scaffolds bioactivity, this is specifically seen on nozzle-based 3D printing technologies as well as FDM (Fused Deposition Modeling) and its variants.[128, 129] Additional drawbacks are present when the equipment involves nozzle technologies that only accepts fluids with low viscosity and low cell density because of the high shear force and issues with clogging. These challenges and limitations are overcome with laser printing, a nozzle-free system, which has a wide range of working viscosity values (1–300 mPa/s) along with high cell density solutions with negligible effect on cell viability during the process.[134] However, laser technologies also have its drawback such as: time consuming because of the necessary low overall flow rate needed for the rapid gelation, and high cost. Upon the many 3D bioprinting strategies the most common are inkjet, micro-extrusion, and laser.[129] 3D bioprinting represents a risen technology for the development of patient-specific products.

The 3D bioprinting uses 3D printing equipment, an example is the use of a commercially inexpensive desktop 3D printer to tailored custom architecture of bio-conduits for peripheral nerve regeneration.[73] The choice of material is crucial on 3D printing technology specially on inkjet bioprinting, since it needs to be printed liquid and then form a solid 3D structure. 3D structure is achieved by the use of materials that can be cross-linked after the deposition by chemical, pH, or ultraviolet mechanisms.[129] This specific material requirement is the reason for the modification of gelatin, which adds properties such as photo cross-linking and radical polymerization. Gelatin methacryloyl (GelMA), is one of the chosen material for the desktop 3D printer application.[73] The developed cryoGelMA, named after the conduit preparation via cryopolymerization, is turned into a bio-conduit by the introduction of multipotent adipose-derived stem cells (ADSCs) to generate local environment to foment the regeneration

9000, Mayaguez, Puerto Rico 00681-9000, USA

Carol A. Rivera Martinez [a1]
Jorge Almodovar [a1] [a2]

[a1] Bioengineering Program, University of Puerto Rico Mayaguez, Call Box 9000, Mayagüez, Puerto Rico 00681-9000, USA

[a2] Department of Chemical Engineering, University of Puerto Rico Mayagüez, Call Box 9000, Mayaguez, Puerto Rico 00681-9000, USA

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[a1] Bioengineerng Program, University of Puerto Rico Mayaguez, Call Box 9000, Mayagüez, Puerto Rico 00681-9000, USA

[a2] Department of Chemical Engineering, University of Puerto Rico Mayagüez, Call Box 9000, Mayaguez, Puerto Rico 00681-9000, USA

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Carol A. Rivera Martinez [a1]
Jorge Almodovar [a1] [a2]

[a1] Bioengineering Program, University of Puerto Rico Mayaguez, Call Box 9000, Mayagüez, Puerto Rico 00681-9000, USA

[a2] Department of Chemical

of axons, which is possible with the use of 3D printing. The versatility of 3D printing is taken as an advantage to engineer conduits of different geometries such as multichannel or bifurcating and even personalized structures.[25, 131] To validate the conduits, an in vitro test was performed using ADSCs, and in vivo testing was performed using a rat model with a 10 mm sciatic nerve gap. The positive respond of the ASCs to the bio conduits is assured through the deposition and accumulation of extra cellular components along with the expression of NTs such as BDNF and GDNF, all to assure axonal regeneration. It is important to highlight that 3D bioprinting does not alter the properties of the materials. The cryoGelMA conduits meet the standards of an average degradation time frame, which are complete degradation at 2–4 months, along with an inflammation time line of no more than a 3-week period of acute/inflammatory, which will lead to infection.[135] This approach is an example of the potential of 3D printing for the fabrication of a precise structure. The use of a desktop 3D printer using "lock and key" molds, emphasizes the simplicity, flexibility and low-cost application with projections to reconstruct complex personalized nerve conduits to mimic physiological conditions and fomenting cell function to achieve axonal regeneration.

An example of microextrusion, one of the most used techniques of 3D printing, is through the development of a silicone 3D scaffold that under the same printing process offers control over the geometrical, physical, and biochemical cues.[21] The geometry is showcased by the development of a two-branched device to mimic bifurcating nerve, the physical cues via printing patterns (microgrooves), and biochemical cues through the printing of GFs gradient, NGF for sensory path and glial cell derived neurothrophic factor (GDNF) for motor path, on gelatin methacrylate hydrogel droplets that encapsulated the GFs. As one of the advantages of 3D printing, this work uses the original tissue structure as template for the pathway geometry with the use of the SLS (structured light scanning) technique that eventually leads to the 3D nerve model scaffold. The choice of material for the 3D neuro scaffold is silicone mainly because is a neuro compatible material, very stable with promising biodurability, and biocompatible, ideal for 3D printing microextrusion technique.[136] The 3D conduit developed through microextrusion shows a multifunction conduit with physical, biochemical, and doubled branched device. To showcase the 3D printing capacity implementing physical cues, an in vitro testing showed that primary SCG (superior cervical ganglion) neurons followed the physical cues as measured by fast Fourier transform (FFT). In addition, it is shown that the NGF gradient serve as chemoattractant for sensory axons and the GDNF gradient increased migratory velocity of SCs from 5.1 to 12.6 μm/h. The end product consisted of a silicone hollow conduit with gradient patterns of GF hydrogel droplets that serve as biochemical cues, and also physical cues by the addition of microgrooves, which mimics the 3D structure of a real bifurcating nerve, all possible with the use of 3D printing technology, which elevates the versatility of this technique.

As mentioned above, the materials used to perform 3D printing are the ones that classify the type of scaffold, and an example of a free scaffold is one in which is only composed of fibroblast multicellular spheroids (MCSs) developing a biologic tubular structure.[137] Bio 3D printing is characterized by developing a complete biologic tubular structure, in this case using homogeneous fibroblast MCSs. A pre-designed 3D tube-like structure represents the position of the spheroids that are robotically placed into skewers of a 9 × 9 needle array [Figs. 4