Gold Nanoparticle-Decorated Scaffolds Promote Neuronal Differentiation and Maturation

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ABSTRACT: Engineered 3D neuronal networks are considered a promising approach for repairing the damaged spinal cord. However, the lack of a technological platform encouraging axonal elongation over branching may jeopardize the success of such treatment. To address this issue we have decorated gold nanoparticles on the surface of electrospun nanofiber scaffolds, characterized the composite material, and investigated their effect on the differentiation, maturation, and morphogenesis of primary neurons and on an immature neuronal cell line. We have shown that the nanocomposite scaffolds have encouraged a longer outgrowth of the neurites, as judged by the total length of the branching trees and the length and total distance of neurites. Moreover, neurons grown on the nanocomposite scaffolds had less neurites originating out of the soma and lower number of branches. Taken together, these results indicate that neurons cultivated on the gold nanoparticle scaffolds prefer axonal elongation over forming complex branching trees. We envision that such cellular constructs may be useful in the future as implantable cellular devices for repairing damaged neuronal tissues, such as the spinal cord.

KEYWORDS: Axonal elongation, gold nanoparticles, nanocomposite scaffold, neuronal tissue engineering

Injury to the central nervous system (CNS) including the brain and spinal cord, resulting from physical trauma, neurodegenerative diseases, or ischemia, is one of the leading causes of death and chronic disability in humans. Post injury, neurotoxicity, formation of free oxygen radicals, and tissue inflammation often cause secondary damage, which leads to the expansion of the initial injured zone and death of neural tissue in injury, neurotoxicity, formation of free oxygen radicals, and anisotropic contraction force. Anisotropic contraction of a matrix may lead to cell death or to the assembly of nonfunctional neuronal circuits.

The use of engineered 3D scaffolds for repairing neural damage is considered to be a promising approach in neural tissue engineering. These artificial scaffolds provide topographical, biochemical, and mechanical cues for proper organization and tissue formation, mimicking the native neuronal extracellular matrix (ECM). Various scaffolds, including electrospun nanofiber scaffolds, have been examined as candidate platforms for neuronal differentiation and growth, promoting cell–cell and cell–matrix interactions and altering growth strategy.

Recently, we and others have demonstrated that the neuronal pattern of growth may be affected by nanometric topography. In response to these nanometric cues, the motile growth cone at the tip of the axonal branch is fixed to the extracellular translocations in the substrate via adhesion receptors. For example, metal nanoparticles, serving as anchoring sites for the small filopodial projections on 2D surfaces, were shown to improve neurite–substrate interactions, leading to controlled growth of the neuronal processes. In other studies, carbon nanotubes were used to affect the morphology of cultured cells and even to alter their electrical activity. The combination of well-defined and specific nanoscale cues with 3D scaffolds may provide an advantageous supporting microenvironment and therefore a more relevant therapeutic approach.

In a series of studies we have previously shown that gold nanowires and nanoparticles embedded within various biomimetic scaffolds promote the assembly of cardiac cells into elongated and aligned tissues, able to generate a strong, anisotropic contraction force. Here, we sought to investigate the effects of these matrices on other electrogenic...
Three-dimensional composite scaffolds were fabricated using electrospinning, decorated with gold nanoparticles (AuNPs) and characterized for particle size, distribution, and topographical properties. Next, the effect of the designed cues on neuronal growth and maturation was investigated (Figure 1).

Fibers with diameters of several hundreds of nanometers have been previously shown to promote beneficial effects in neurons, including proliferation and differentiation in neuronal stem cells. Therefore, we have fabricated electrospun fiber scaffolds from polycaprolactone (PCL)-gelatin with an average fiber diameter of 260 ± 70 nm (Figure 2A and Supplementary Figure 1). The fiber diameter may be controlled by changing the electrospinning parameters, such as polymer concentration, applied voltage, velocity of polymer injection, etc. In addition, scaffold’s porosity may be further tuned by coelectrospinning of the scaffold’s polymer with a sacrificial polymer that later on can be dissolved during cell seeding with culture medium. While PCL provided mechanical strength to the fibers, the bioinductive gelatin contributed to the fibers’ biological activity, promoting cell adhesion and spreading. We next sought to incorporate AuNPs to the electrospun fiber scaffolds by evaporation of gold (Figure 2B–D). The functional groups in the gelatin were utilized as binding sites for the evaporated gold NPs. Next, EDX analysis was performed in order to confirm the AuNP presence (Figure 2C). Environmental scanning electron microscope (ESEM) images revealed c.a. 10 nm islands of AuNPs (dark) on the surface of the fibers (light). Scale bar = 5 nm. (F, G) Roughness of the pristine (F) and AuNP fibers (G) as imaged by AFM.

Figure 1. Schematic overview of the study. Polymeric nanofibers from PCL/gelatin are fabricated by electrospinning (A). Gold nanoparticles (10 nm) are evaporated on the fibers (B) to create nanocomposite scaffolds (C). Neuronal cells seeded within the nanocomposite scaffolds grow elongated axons and form 3D networks (D).

Figure 2. AuNPs electrospun fiber scaffolds. (A) Fibrous scaffolds with average fiber diameter of 260 nm were fabricated by electrospinning of PCL and gelatin. Scale bar = 10 μm. (B) AuNPs were evaporated on the fibers to create the nanocomposite scaffolds. (C) EDX analysis confirming the existence of AuNPs on the fibers. (D) ESEM image of AuNPs on the surface of the fibers. Scale bar = 500 nm. (E) TEM image of the fibers revealed c.a. 10 nm islands of AuNPs (dark) on the surface of the fibers (light). Scale bar = 5 nm. (F, G) Roughness of the pristine (F) and AuNP fibers (G) as imaged by AFM.
shown, the c.a. 10 nm particles were dispersed on the fibers, creating gold islands rather than a continuous layer of the metal (Figure 2E). This allows the cells to interact with the PCL-gelatin fibers and the AuNPs, providing both biological motifs and topographical and electrical cues. AFM results provided further evidence for modification of the surface of the fibers with nanoscale roughness providing topographic cues (Figure 2F,G).

Next we sought to evaluate the effect of the engineered topography on cell morphology. Primary neurons from the medicinal leech were seeded on the 3D fibrous scaffolds with AuNPs or on pristine scaffolds, by a single droplet. As this model allows a low density culture of neurons, it enables to perform morphological analyses of neuronal processes at the single cell level. Since metallic nanoparticles may harm cells, we have initially shown that the nanocomposite scaffolds are not toxic (Supplementary Figure 2). To evaluate neuronal growth within the scaffolds the cell-seeded constructs were immunostained for α-tubulin on day 6. Confocal images revealed that neurons grown on the pristine scaffolds developed short neurites which were incorporated close to the cell soma (Figure 3A). In contrast, neurons grown on the AuNP scaffolds had longer and thinner neurites with dendritic trees spreading further from the cell soma (Figure 3B). Movies of Z-stack images of the primary neurons revealed outgrowth of the neurites in the 3D space, implying that the extensions have penetrated into the fibrous scaffold and interacted with the fibers (Supplementary Movies 1 and 2). In most cases, the short outgrowth of neurites on pristine scaffolds led to no network formation, as compared to neurites growing on AuNPs (Figure 3C,D and Supplementary Table 1). To further evaluate the interactions of the neurons with the fibrous scaffolds, the cell constructs were fixed and imaged using SEM. As shown, the soma adhered to the top layer of the scaffold, while the neurites were not limited to one level, penetrating into the mesh, extending, and adhering to fibers on different layers (Figure 3E and F).

During cell dissociation procedure the neurons lose their original morphology. After seeding, an outgrowth process starts, where neurites originate from the soma, extend and branch, developing into a complex dendritic tree. Quick morphogenesis, including neurite initiation and extension toward target sites may be essential for effective regeneration of neuronal tissue post trauma. To quantify the effect of AuNPs scaffolds on the neuronal outgrowth process, several morphometric parameters, at the single cell level, were measured. As shown in Figure 4A, neurons on AuNPs scaffolds developed into significantly longer branching trees \((p = 0.008)\). Neurons on AuNPs scaffolds also developed to a significantly longer distance (Figure 4B; \(p < 0.0001\)). Moreover, the length of the longest neurite in each neuron indicated that the AuNPs scaffold may encourage a longer outgrowth (Figure 4C; \(p < 0.0001\)). On the other hand, neurons on the AuNPs scaffold had less neurites originating out of the soma (Figure 4D; \(p = 0.007\)) and significantly lower total number of branches (Figure 4E; \(p < 0.0001\)). These morphometric parameters may indicate

![Figure 3. Primary neuron growth on the scaffolds. Confocal microscopy images of single neurons cultivated on pristine (A) or AuNPs (B) scaffolds and stained against α-tubulin. Bar = 100 μm. (C, D) Neuronal networks formed on pristine (C) or AuNPs (D) scaffolds. Bar = 50 μm. E. SEM images of neurons growing on AuNPs scaffolds. Bar = 10 μm. (F) Neurite growth cones extending minor processes between the nanofibers. Bar = 3 μm.](image)

![Figure 4. Morphometric analyses of primary neuron growth on pristine and AuNPs scaffolds. (A) Average total branching tree length. (B) Average growth radius. (C) Average longest neurite length. (D) Average number of neurites originating from the soma. (E) Average number of branching points. At least five cell-seeded scaffolds were analyzed in each group.](image)
on the tendency to prefer elongation over forming complex branching trees in the AuNP scaffolds. Overall, the morphometric analyses revealed a distinct neuronal behavior on the different scaffolds, indicating that AuNP scaffolds are more suitable to promote axon elongation, therefore, more appropriate for bridging over long gaps.

One of the main challenges of neuronal tissue engineering is cell differentiation and maturation. As nanomaterials and nanotopographies have been shown to affect neuronal development, we next sought to assess whether the AuNPs can affect the differentiation of immature cells. It was previously shown that PC12 cells, cultured on collagen-coated substrates with the addition of nerve growth factor (NGF), differentiate into neuron-like cells with properties of mature neurons with excitable membranes. We hypothesized that the microenvironment within our composite scaffolds may encourage the cells to acquire morphological features of mature neurons. We further speculated that the combination of gelatin and AuNPs could be used as anchoring sites for the cells and thus, replace the essential collagen coating. PC12 cells were seeded on the fibrous scaffolds with or without 10 nm AuNPs. β-NGF was added to the growth medium to initiate the differentiation stage and neuronal growth, and development was evaluated on day 6. As shown in Figure 5A, single neurons cultured on the pristine scaffolds exhibited rounded morphology with limited-sized neurite extensions. On the contrary, PC12 cells cultured on the AuNP scaffolds revealed extended neurites with dendritic trees spread farther from the cell soma (Figure 5B). Z-stack imaging revealed neurite outgrowth in-between the fibers (Supplementary Movie 3). Furthermore, while in the pristine scaffolds the neurons could not extend long neurites and therefore did not form neuronal networks (Figure 5C, and Supplementary Table 1); the neurons on the AuNP scaffolds were able to reach adjacent cells and formed basic neuronal networks (Figure 5D).

As 2D substrates are the main cultivation system for PC12 cells and collagen coating is considered essential for the differentiation of these cells, we have compared the 3D cell growth on scaffolds, with or without AuNPs and without additional collagen coating, to the growth on such surfaces. Morphometric analyses of confocal images revealed that the neurons on the AuNP scaffolds exhibited neuronal growth strategy similar to the primary neurons (as shown in Figure 4). The average total branching tree length, average growth radius, and the average length of longest neurite were higher in the AuNP scaffolds (Figure 5E–G). Furthermore, the neuronal morphology in the AuNP scaffolds resembled the morphology on the 2D collagen-treated surfaces, indicating on the same differentiation level and emphasizing the importance of the AuNPs for 3D neuronal tissue engineering. Interestingly, the average number of branching points in cells grown in AuNP scaffolds was significantly lower than the number seen in pristine scaffolds and on the 2D collagen-treated surfaces (Figure 5H). Moreover, coating of the AuNP scaffolds with collagen enhanced the NPs effect in the examined parameters (Supplementary Figure 3). Over all, as in the primary culture, the ability to grow longer neurites with less branched dendritic tree points-out on the potential of the composite biomaterial to bridge over longer gaps, as seen in spinal cord injuries and may provide a platform for effective tissue regeneration.

In summary, we have demonstrated the beneficial effect of AuNPs on the differentiation, growth, and maturation of neurons on 3D biomaterial scaffolds. The nanocomposite material is composed of PCL-gelatin nanofibers, providing mechanical support for the cultured cells. Decoration of the fibers with 10 nm AuNPs provided additional topographical and anchoring sites for superior morphogenesis. Morphometric analyses of primary and neuronal cell line behavior on nanocomposite electrospun fibers revealed elaborated neuronal growth and axonal elongation, leading to more complex neuronal networks.

Future studies should focus on determining the exact role of the AuNPs in neuronal tissue engineering. For example, investigating whether these nanoparticles promote axon elongation and higher expression of neuronal markers due to topographical cues or due to their conductivity, which allows better transfer of an electrical signal, as seen with cardiomyocytes. Another interesting study would be investigating the effect of nanoparticle size and shape on neuronal tissue assembly. Finally, we envision that the reported
may be useful in the future as implantable cellular devices for repairing damaged neuronal tissues, such as the spinal cord.

**REFERENCES**


(21) Ferran, A.; Faraci, P.; Cecchini, M.; Beltram, F. Biomaterials 2010, 31 (9), 2565–73.


(23) Xie, C.; Hanson, L.; Xie, W.; Lin, Z.; Cui, B.; Cui, Y. Nano Lett. 2010, 10 (10), 4020–4.


