Review paper

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Application of nanotubes and nanofibres in nerve repair. A review

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Abstract

Nanoscience is the science of small particles of materials on a nanometre scale in at least one dimension. Nanomaterials can interact with tissues at the molecular level with a very high degree of functional specificity and control. A large group of nanomaterials includes nanotubes, nanofibres, liposomes, nanoparticles, polymeric micelles, nanogels and dendrimers. Such materials can be tailored to react with specific biological systems at a molecular or even supra-molecular level and respond to the cell environment while minimizing undesired side effects. Neuron injuries lead to complex cellular and molecular interactions at the lesion site in an effort to repair the damaged tissue and to regenerate the axon for reconnection with its target organ. Strategies to enhance and stimulate regeneration use various nerve conduits and synthetic guidance devices. A promising strategy for treatment of neuronal injuries is to support and promote axonal growth by means of nanotubes and nanofibres. Nanotubes can be produced from various materials, such as carbon, synthetic polymers, DNA, proteins, lipids, silicon and glass. Carbon nanotubes are not biodegradable and can be used as implants. Moreover, they serve as an extracellular scaffold to guide directed axonal growth. In the review we summarize the results of nanotube and nanofibre application in nerve repair after injury.

Key words: *nanotubes, nanofibres, nerve injury, nerve repair.*

Current advances in nanotechnology have led to the development of a new field of research – nanoscience. It is the science dealing with small particles of materials on a nanometre scale in at least one dimension (1-100 nm) [46]. The main goal of nanotechnology is the development and application of nanomaterials that display unique physical, chemical and functional properties not shown by bulk materials. Nanomaterials can interact with tissues at the molecular level with a very high degree of functional specificity and control [45].

The physicist Richard Feynman was the pioneer of nanotechnology. In 1960 he recognized the potential of molecules at the nanometre scale and suggested that they possess unique physical properties. A large group of nanomaterials consists of nanotubes, nanofibres, liposomes, nanoparticles, polymeric micelles, nanogels and dendrimers. Such materials can be tailored to react with specific biological systems at a molecular or even supra-molecular level [13].

Neuron injuries lead to complex cellular and molecular reactions at the lesion site in an effort to repair the damaged tissue and to regenerate the axon for reconnection with its target organ [19]. Damage of the peripheral nerve leads not only to degradation of the myelin sheath, but also to degeneration of motoneuron bodies. Knakiewicz *et al*. [20] showed that after injury of the ventral branches of spinal

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nerves C5-C6-C7-C8-Th1 in rabbits some neurons of spinal cord anterior horns died and this process depended on the time after the damage.

Neurons in the CNS are sensitive to various pathologies such as ischaemia. Ischaemia can induce alterations in neuron structure and, in the area of the damage, angiogenesis, necrosis and glial reaction [41].

These changes in the neuron body are associated with alterations in expression of various genes and cytokines such as p53, p38, c-jun, INK, cyclin-dependent kinase 5 and caspase 3 and they correlate with the severity of the nerve damage [15].

Unsuccessful results of neuronal regeneration after injury are influenced by various factors, such as inflammatory cell activation and production of molecules inhibiting regrowth and leading to secondary injury [12]. There are numerous barriers that must be overcome in order to achieve axonal regeneration after injury in the nervous system: scar tissue, gaps in nervous tissue formed during phagocytosis of dying cells, several factors that inhibit axon growth in the mature CNS of mammals, and a failure of many adult neurons to initiate axonal extension [1,10,17]. Strategies to overcome the inhibitory factors in regeneration use various nerve conduits and synthetic guidance devices. A tubular conduit, made of degradable or non-degradable compounds, can guide and facilitate peripheral nerve regeneration. Various conduits have been fabricated for bridging nerve gaps after injury, and both natural and synthetic materials have been used [37]. The main characteristic of these materials is a longitudinal organization mimicking the natural structure of the nerve pathway within the brain and spinal cord. They are designed to serve as conduits for axonal elongation and to constrain the direction of regenerative outgrowth. Moreover, they should be able to direct regenerating axons to reconnect with their target neurons and enhance functional restoration of the nerve [3]. Many experiments have been performed to study functional recovery after injury in animal models [6,31,52].

A promising strategy for treatment of neuronal injuries is to support and promote axonal growth by the use of nanometre-scale materials, especially nanotubes and nanofibres. They mimic tubular structures that appear in nature, such as microtubules, ion channels and axons. Nanotubes can be produced from various materials, such as carbon, synthetic polymers, DNA, proteins, lipids, silicon and glass. Techniques of their fabrication include templating of nanotubes on porous templates, on electrospun nanofibres of degradable polymers and using self-assembled nanofibres of peptide molecules. These methods allow for the production of different nanotube designs for various purposes [13]. Nanotubes have larger inner volumes (relative to the dimensions of the tube) that can be filled with any desired biochemical substances. This property creates the possibility of loading the inside of a nanotube with various biochemical loads [24].

Carbon nanotubes were discovered by Sumio Iijima in 1991 [16]. They are composed of carbon atoms arranged in structures similar to graphite, with five-membered or seven-membered rings [2]. They and related carbon spheres belong to a broader class of carbon allotropes named fullerenes [13]. Carbon nanotubes have excellent properties which have made them attractive for application: small size, flexibility, strength, inertness, electrical conductivity and ease of combination with various biological compounds [23]. Carbon nanotubes are not biodegradable and can be used as implants. Moreover, they serve as an extracellular scaffold to guide directed axonal growth and can regulate neurite branches [25].

Nanotubes

Mattson *et al.* [26] reported the first application of carbon nanotubes in neuroscience research. They used multi-walled carbon nanotubes coated with a biochemical compound (4-hydroxynonenal) for growth of embryonic neurons of a rat brain. The authors observed that on unmodified nanotubes neurons extended only one or two neurites with only a few branches. However, neurons growing on nanotubes coated with a bioactive molecule developed multiple neurites with extensive branching. The study confirmed the effectiveness of using nanotubes as substrates for neuronal growth.

Walsh *et al.* [50] examined whether substrates with a nanometre-scale surface coated with dural meningeal cells influence the outgrowth of neurites of dorsal root ganglion neurons. Meningeal cells were isolated from the cranial meninges of rats by peeling from the surfaces of the cerebral cortices. Dorsal root ganglion (DRG) neurons were isolated at postnatal day 1 from adult rats. Neurons were plated on meningeal monolayers and cultured. Dorsal root ganglion behaviour on the substrates was analysed by examining the length of neuronal outgrowth using

beta III-tubulin by means of an epifluorescence microscope equipped with a camera. The digital images were analysed to determine both orientation of neurons and their length. The authors found that neurites growing on meningeal cell monolayers had greater length than in the control group and were directed parallel to the underlying surface. They suggested that nanometre-scale materials can be used to improve the alignment of meningeal cells at the biomaterial surface sufficiently to influence the length and direction of regenerating neurons. The authors stated that such a technique may be a new approach for improving bridging materials for nerve repair after injuries.

Nakayama *et al.* [33] investigated the regeneration of peripheral nerves in bioabsorbable polymer nanotubes implanted at the site of nerve injury. These tubes were filled with fibrin gel and implanted into a nerve gap after transection of a rat sciatic nerve. The authors found remyelination of the injured structure in the middle parts of the tubes, but no regeneration in the tubes without fibrin gel (control group). Thus they concluded that use of fibrin gel as filling material enhanced sciatic nerve regeneration in rats and the polymer tubes were effective for nerve regeneration. Some experiments confirmed that addition of nerve growth factors to nanotubes may enhance the process of nerve regeneration.

Matsumoto *et al.* [25] reported the first study on neurite outgrowth of embryonic chick dorsal root ganglion using carbon nanotubes coated with nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). They showed that neurotrophins coating the carbon nanotubes promoted neurite outgrowth in the same manner as soluble NGF and BDNF. The authors revealed that neurotrophin-coated carbon nanotubes can stimulate neurite outgrowth of chicken dorsal root ganglion neurons.

Nanoscaffolds

Carbon nanotubes and their derivatives can be used as scaffolds for neuronal growth. Scaffolds may promote regeneration of injured neurons and provide a marked improvement over traditional nerve grafts in their ability to overcome degenerative processes and restore some nerve function [5]. They have the potential to improve the specificity of materials for various neural-engineering applications and guidance for axonal regeneration after injuries [43]. A biologically compatible scaffold should deliver proper substrates for cell growth, survival and differentiation. It should be derived from biological materials, have a controlled rate of biodegradation, promote cell-substrate interactions, integrate with the environment in vivo, and be compatible with physiological conditions without cytotoxicity or an immune response [10]. The scaffolds are usually fabricated from biomaterials and may be seeded with committed tissue-specific cells or non-committed stem cells. Then the cell load is grown in a specific environment in the presence of growth factors and cytokines that allow them to differentiate. Nanomodification of scaffolds can minimize the immune response, and induce and enhance fast regeneration of tissues [38].

Silva *et al.* [44] designed a nanofibre scaffold composed of peptide amphiphile molecules which self-assembled into a network. The surface of nanofibres consisting of the active peptide sequence isoleucine-lysine-valine-alanine-valine (IKVAV) was designed to engage in cell signalling by acting as ligands for cell surface receptors. The authors encapsulated neural progenitor cells and neural retinal cells in the nanofibre scaffold. They mixed cell culture suspensions with peptide amphiphile solutions, trapping the cells in the interior of the gel. After 1 and 7 days, 30% and 50% respectively of the neural progenitor cells expressed beta III tubulin, a marker of mature neurons. The authors also described a complete absence of astrocyte development, less than 1% and 5% at 1 and 7 days respectively in vitro. Similar results were obtained with the use of retinal cells. This nanofibre system may be used for limiting the effects of reactive gliosis and glial scar formation after nerve injury.

Scaffolds can also be created by using biocompatible polymers, such as poly-l-lactic acid (PLLA), poly-lactide-co-glycolide (PLGA) and polycaprolactone (PLC). Yang *et al.* [53] studied the efficacy of a nanoscaffold made of PLLA for neurite outgrowth in vitro using neural stem cells. They showed that the direction of neural stem cells and their neurite outgrowth was parallel to the direction of PLLA fibres in the nanoscaffold. The rate of differentiation of neural stem cells was significantly higher for PLLA nanofibres than that of microfibres. The authors concluded that a nanofibrous PLLA scaffold could be used for nerve regeneration as a potential cell carrier.

Panseri *et al.* [37] studied the effectiveness of nanotubes made of biodegradable polymers (PLGA/PCL) in supporting regeneration of rat sciatic nerve in vivo.

The animals were randomly assigned to 3 groups: 1 ($n = 5$) – with transection of the sciatic nerve, 2 ($n = 5$) – with removal of a small segment of the sciatic nerve in order to leave a 10-mm gap between the transected stumps, 3 ($n = 40$) – with implantation of a nerve conduit filled with saline solution following neurotmesis. After transection the nerve stumps in groups 1 and 2 were left unrepaired. Functional reconnection of the sciatic nerve stumps was demonstrated by the neurolabelling method and the presence of muscle action potentials following electrical stimulations proximally to the former gap. The authors observed that 4 months after injury, the sciatic nerve stumps failed to reconnect in groups 1 and 2. However, in 70.6% of animals from group 3 nanotubes induced nerve regeneration and functional reconnection. The authors concluded that nanotube nerve conduits are promising scaffolds for stimulating and guiding peripheral nerve regeneration in an animal model of sciatic nerve transection. Moreover, these tubes can be filled with various substances, such as collagen, fibrin, and neurotrophic factors, which may enhance and facilitate nerve outgrowth after injury.

Valmikinathan *et al*. [49] studied the role of a nanofibrous PLGA spiral scaffold in neural regeneration. They showed that this nanoscaffold promoted cultured Schwann cell attachment and proliferation and also mimicked the extracellular matrix in vitro. The authors proposed that this type of nanoscaffold can be potentially used in nerve regeneration.

Koh *et al*. [21] studied the efficacy of a nano-structured scaffold coupled with laminin in promotion of axonal outgrowth in PC12 cells. Incorporation of laminin allowed it to mimic the extracellular matrix structure and create a biomimetic scaffold. Such modification of nanofibres was able to enhance axonal growth.

Self-assembling peptide nanofibre scaffolds

The need for tissue repair has encouraged the creation of biomaterial scaffolds that can be used to fill the gaps that develop as a result of injury. Self-assembling peptide nanofibre scaffolds (SAPNS) are a promising option for enhancing neuronal regeneration after injury. They have many benefits over other biomaterials: a minimal risk of carrying pathogens, a three-dimensional environment for cell growth and migration, and excellent physiological properties with minimal cytotoxicity due to SAPNS composition of naturally occurring amino acids. This kind of scaffold is also associated with no inflammation or immune response after transplantation into animals.

Ellis-Behnke *et al*. [9] used SAPNS to repair the transected optic tract in hamsters. The SAPNS was composed of positively and negatively charged L-amino-acids that self-assembled into nanofibres. After transection of the optic tract the SAPNS was injected into the superior colliculus. In the control group saline solution was injected. Axonal regeneration was confirmed by histological and behavioural tests. Histological analysis revealed reconnection of the injured tissue across the lesion after injection of SAPNS in all animals. The authors observed significant repair of the tissue injury that occurred after 2 months. Functional recovery of vision to orient toward a small object was revealed in 75% of hamsters, whereas the controls remained blind. This functional visual recovery was correlated with regeneration of axons at the lesion site.

Guo *et al*. [14] demonstrated that SAPNS could repair the injured spinal cord. They isolated neural stem cells (NPCc) and Schwann cells (SCc), then cultured them with SAPNS and transplanted them into the transected dorsal column of rat spinal cord. A spinal cord dorsal column transection was performed between C6 and C7, followed by the removal of 1 mm of tissue. Then the SAPNS scaffold cultured with NPCc and SCc was transferred into the lesion cavity. In the control group uncultured SAPNS or saline solution was placed into the lesion site. The authors reported that the NPCc and SCc were able to survive and migrate within the scaffold. Moreover, they observed the presence of many blood vessels in the implants that supplied blood for healing and regeneration. The authors observed the growth of axons into the scaffold, indicating that the SAPNS provides a proper environment for cell survival, migration and differentiation and can bridge the lesion site in damaged spinal cord.

Tysseling-Mattiace *et al*. [48] used SAPNS in therapy of spinal cord injury in mice. Nanoscale structures were created by injection of liquid into the extracellular spaces of spinal cord. These cylindrical nanofibres were designed to display the laminin epitope (IKVAV). The authors showed that this method reduced glial scar formation as well as cell death and increased the number of oligodendrocytes at the site of injury. Nanofibres promoted regeneration of descending motor fibres and ascending sensory fibres

across the lesion. These observations indicate that SAPNS displaying neuroactive epitopes on their surfaces can inhibit glial scar formation and promote axon elongation after spinal cord injury in mice. Carbon nanofibres have excellent electric conductivity properties that make them beneficial in use as neural prostheses, but limited evidence on their cytocompatibility currently exists.

Interaction of nanomaterials and cells

In order to determine the biocompatibility of carbon nanotubes as neural implants, McKenzie *et al*. [29] investigated the interaction between astrocytes (glial scar tissue-forming cells) and carbon nanofibres. Carbon fibres were separated into 2 groups: conventional (125-200 nm) and nanoscale (60-100 nm). In each group high surface energy (125-140 mJ/m^2) and low surface energy (25-50 mJ/m^2) were represented. Cultured rat astrocytes were seeded onto fibres for adhesion and proliferation. The authors found that these cells adhered and proliferated on carbon nanofibres that had the largest diameter and the lowest surface energy. However, the authors observed decreased adhesion of astrocytes with increasing percentage of high surface energy in the nanoscaffold. The authors concluded that decreased glial scar tissue formation and positive interaction with neurons should be taken into consideration in estimation of the efficacy of neural implants.

Despite increasing interest in neuroscience nanotechnology, little is known about the electrical interactions between neurons and nanomaterials. Mazzatenta *et al*. [28] demonstrated the presence of an interaction between cultured rat hippocampal neurons and single-wall carbon nanotubes by means of the voltage clamp method and characterized responses evoked via stimulation of these nanotubes. They achieved direct nanotube-neuron interactions by culturing rat hippocampal cells on a film of purified nanotubes. Neurons growing on the surface of nanotubes displayed spontaneous electrical activity. In the current clamp technique they observed a great increase in the average frequency of spontaneous action potentials. The authors reported the possibility to stimulate single and multiple synaptic connections in cultured hippocampal neurons via single-wall carbon nanotubes.

Assessment of risk of nanomaterials

Advances in nanotechnology have led to the development of new materials and devices on a nanometre scale for various scientific and therapeutic purposes. The special chemical and physical properties of nanomaterials that make them unique and attractive may be associated with potentially harmful effects on cells and tissues [18]. Nanotubes, because of their surface properties and very small size, may bind and transport toxic chemical compounds as well as being toxic themselves by generating free radicals [1], inducing oxidative stress, and this disadvantage is a major setback for their application in medicine [36].

Seaton *et al*. [42] established potential factors of toxicity of nanoparticles which include length (greater than 15 µm – below it the fibre can be removed by pulmonary macrophages), diameter (less than 3 µm – allows fibres to be inhaled into the gasexchanging part of the lung), insolubility, resistance to dissolution in the lung environment, and sufficient dose of delivery to the target organ.

Patlolla *et al*. [39] found that multi-walled carbon nanotubes added in vitro to normal human dermal fibroblast cells induced massive damage of DNA and apoptosis and were very toxic and harmful at sufficiently high concentrations.

Carbon nanotubes, especially in the form of long, but not short fibres, represent a unique inhalation hazard. They are not completely enclosed by pulmonary macrophages and cannot be effectively removed. Moreover, they are biopersistent and can retain their fibrous shape during residence in the lung environment and thus the long fibre dose accumulates. Such nanotubes can be retained in the pleural mesothelium and initiate inflammation and fibrosis similar to the process produced by asbestos fibres [8]. People exposed to asbestos fibres demonstrate such pleural pathologies as pleural effusion, fibrosis and even mesothelioma [8,27,54].

Mesothelioma is a very aggressive neoplasm with poor prognosis that arises from mesothelial cells of pleural, peritoneal and pericardial cavities [54].

Some cases of mesothelioma arise in the peritoneal cavity, probably as a result of fibre translocation from the pleural cavity [8].

Poland *et al*. [40] revealed that carbon nanotubes, in the form of long fibres, introduced into the abdominal cavity of mice produced inflammation and fibrosis in the peritoneal cavity to the same degree as long asbestos fibres.

Also experimental studies on animals have shown that instillation of multi-walled and single-walled carbon nanotubes can cause pulmonary inflammation,

dose-dependent fibrosis, granulomas and even death [4,32]. Lam *et al*. [22] showed that intratracheal instillation of 0.1 or 0.5 mg of nanotubes into mice caused pulmonary injury that included interstitial and peribronchial inflammation and necrosis. Chou *et al*. [7] in a similar experiment demonstrated a chronic inflammatory response in lungs and the formation of a severe pulmonary granuloma.

Nanotubes' ability to translocate from the site of deposition to another place is very hazardous. Oberdörster *et al*. [35] in studies on animals showed that nanoparticles could be transmitted up the nerves into the cerebrum, cerebellum and olfactory bulb in the brain. Moreover, the authors suggested that depending on particle size, inhaled nanomolecules could be deposited in the nasopharyngeal region during nasal breathing.

We should remember that not only engineered but also incidental contact with nanomaterials can lead to potential health problems. Human skin can be exposed to nanoparticles through application of creams and lotions with special nanoscale compounds used as a sunscreen component or contact with substances during their manufacture [34].

Mortensen *et al*. [30] observed a high level of skin penetration by nanoparticles in UV-exposed mice. Such an effect may be potentially harmful to the skin structure.

In view of the dramatic expansion of nanotechnology, it is essential to establish proper criteria and tests for risk assessment that would protect people working in manufacturing and laboratory sectors against potential health problems [47].

The potential impact of nanomaterials on the environment and health will require the use of special protective monitors for airborne exposure, detectors for waterborne nanomaterials, and sensors measuring exposure and establishing potential hazards [27]. Personnel should treat all new nanoscale materials as potentially hazardous and toxic. Risk management should be an integral part of an occupational safety and health programme, which is based on recognition of the nanomaterial risk, evaluation and measurement of hazard and exposure, and also application of proper control to reduce the risk [51].

Although various applications of nanotubes and nanofibres in neuroscience are in the early stages of development, the unique possibilities offered by these materials for nerve repair, regeneration and neuroprotection are outstanding. Nanotechnology has significant potential for future clinical application in diagnosis and treatment of various disturbances of the central and peripheral nervous systems.

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