PERSPECTIVE

Application of iron oxide anoparticles in neuronal tissue engineering

Since the introduction of nanotechnology, nanoscale materials have developed rapidly and have been applied in various fields including in the pharmaceutical industry, medicine, and tissue engineering. Among a variety of nanomaterials, magnetic iron oxide nanoparticles (IONPs) have been intensively investigated for numerous *in vivo* applications such as gene and drug delivery, diagnostics, cell labeling and sorting. Compared to nanoparticles made from other materials, IONPs have several additional unique applications due to their magnetic properties, for example, magnetic cell separation, magnetic resonance imaging (MRI) and X-ray contrast agents, tumor hyperthermia, and in the targeting of bioactive agents immobilized on magnetic materials with the presence of an external magnetic field (Gupta and Gupta, 2005). IONPs are considered biocompatible and biodegradable (reviewed by Wang et al. (2009)). Indeed, some IONPs preparations have been approved for clinical applications by the Food and Drug Administration (FDA) and/or the European Commission (EC) and are commercially available, such as Lumirem (as a contrast agent for MRI for the gastrointestinal tract) and Feraheme (for the treatment of iron deficiency anemia) (Cortajarena et al., 2014).

In recent years, we have developed implants for the treatment of paraplegic patients from acute and chronic spinal cord injuries (SCI), and for the reconstruction of peripheral nerves following a severe segment loss. Here we describe two possible applications of IONPs in neuronal tissue engineering: As a component in fibrin hydrogel scaffold, giving it magnetic properties, and as a vehicle for stabilization and transportation of neurotrophic factors conjugated to IONPs.

Among a variety of methods for preparation of IONPs, which are described in the scientific literature, we have used two methods for our purposes. In the first method, gelatin coated IONPs of narrow size distribution, with an average dry diameter of about 20 nm, were synthesized by nucleation of iron oxide onto gelatin nuclei, followed by stepwise growth of thin layers of iron oxide films onto the gelatin-iron oxide nuclei (Ziv-Polat et al., 2012). In the second method, dextran coated IONPs of narrow size distribution and with an average dry diameter of about 10 nm were prepared by co-precipitation of Fe^{2+} and Fe^{3+} ions in a saturated dextran solution by the addition of ammonium hydroxide (Ziv-Polat et al., 2014).

Magnetic fibrin hydrogel scaffold: Scaffolds for neuronal tissue engineering are designed as platforms to support the three-dimensional (3D) growth of neuronal cells and regenerated nerve fibers. Broad arrays of synthetic and natural polymers have been investigated as scaffolds for tissue engineering, among them fibrin hydrogels showed great potential (Ahmed et al., 2008). Fibrin hydrogels (or fibrin glue) are made by interaction between two blood coagulation components: fibrinogen and thrombin, which when combined form a clot. In human plasma the half-life of thrombin is a few seconds, due to tight control by various inhibitors and components of the blood vessel wall. In order to provide thrombin with long-term protection from its natural inhibitors, it was conjugated physically to IONPs (Ziv et al., 2009). Indeed, we have illustrated that appropriate conjugation of thrombin to IONPs preserved its clotting activity, or even improved it, stabilized the thrombin against its major inhibitor antithrombin III and prolonged its storage stability. In addition, *in vivo* studies with incisional wounds on rat's skin indicated that the thrombin-conjugated nanoparticles enhanced the healing process significantly better than free thrombin and compared to untreated wounds (controls) (Ziv-Polat et al., 2010). On the bases of these results, it was decided to use the thrombin-conjugated nanoparticles for the development of a novel magnetic fibrin hydrogel scaffold.

The magnetic fibrin hydrogel scaffold developed was obtained following the combination of three aqueous solutions: thrombin-conjugated iron oxide nanoparticles, fibrinogen, and calcium chloride (Ziv-Polat et al., 2012). The concentrations of each component had an influence on the gelation time, as well as on the mechanical and morphological properties of the fibrin hydrogel. The relative concentrations also influenced the growth pattern of cells cultured in the hydrogel, and its degradation rate due to fibrinolytic enzymes secreted by the cultured cells. Therefore, studies were conducted aiming to determine the optimal relative quantities of each component, to give an appropriate consistency for cell growth, together with sufficient degradation times (Shahar et al., 2015). The formed scaffolds are magnetic, transparent and provide a 3D environment for growth of various cells (**Figure 1A–C**). Magnetic fibrin scaffolds, with or without incorporated cells for transplantation, can be implanted either as a coagulated intraluminal filler in a biodegradable conduit (such as chitosan) or injected as a liquid before gelation, without the need of a conduit, to coagulate at the site of injury. In addition, we have demonstrated that this scaffold can be monitored non-invasively by MRI due to its magnetic properties (Skaat et al., 2012).

Neurotropic factors conjugated to iron oxide nanoparticles: Neurotrophic factors are added to neuronal cultures in order to enhance nerve fiber regeneration, neuronal cell growth, and maturation. The main disadvantage of the free neuronal growth factors is their short half-life of just a few minutes due to enzymatic degradation and other adverse elements. For example, the half-life time of basal fibroblast growth factor (FGF-2), brain derived neurotrophic factor (BDNF), and β-nerve growth factor (βNGF) in blood are 1.5–3, 10, and 30 minutes, respectively (reviewed in Ziv-Polat et al., 2014). In order to increase their stability and to prolong their activity, several neurotrophic factors including βNGF, FGF-2 and glial cell-derived neurotrophic factor (GDNF) have been covalently conjugated to IONPs (Ziv-Polat et al., 2014). The stability of the free versus conjugated neurotrophic factors was examined in various concentrations of fetal calf serum, as well as in neuronal cultures, and in culture medium (containing 10 % serum) stored at 37°C. Results revealed that all the conjugated growth factors were significantly more stable than the free ones under all the examined conditions (**Figure 2**). Additional experiments examined the biological activity of aged growth factors, which were pre-incubated in culture medium containing 10% serum at 37°C for several weeks. For example, the results for free versus conjugated GDNF showed that after 2 weeks of pre-incubation, the aged free GDNF no longer increased nu-

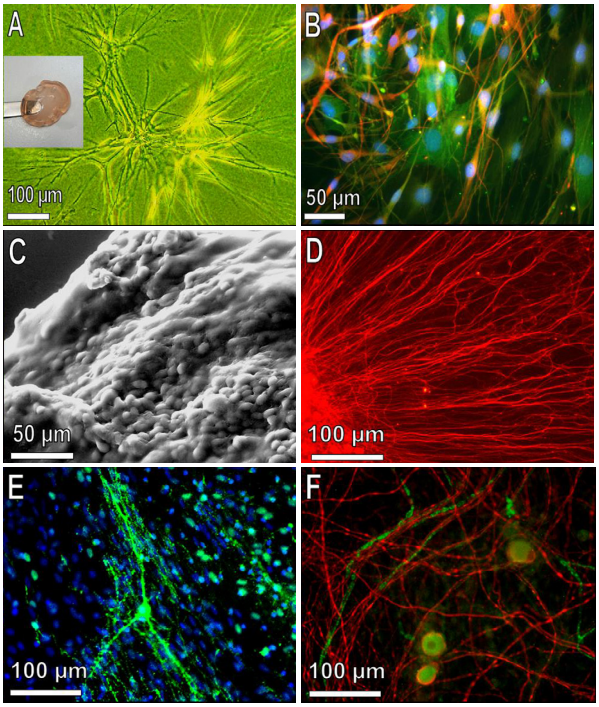

Figure 1 Three-dimensional cultures of nasal olfactory mucosa (NOM) cells in a magnetic fibrin hydrogel scaffold (A–C), and the effect of neurotrophic factors-conjugated to iron oxide nanoparticles (IONPs) on dorsal root ganglia (DRG) and spinal cord cultures (D–F).
(A) Phase-contrast microscopy of NOM cells growing in a 3D pattern within the magnetic fibrin hydrogel. Insert box: macroscopic picture of transparent fibrin hydrogel clot, made in a 24-well culture plate and taken out after coagulation. The NOM cells were mixed with the hydrogel components before the gelation. (B) Immunofluorescent staining of an NOM cell culture exposed to basic fibroblast growth factor 2 conjugated to IONPs. The cell culture is composed of olfactory ensheathing glial cells (red–anti S100) and neuronal cells (green–anti NF). The cell nuclei are stained blue with DAPI. (C) Environmental scanning electron microscope (ESEM) image of NOM cells grown in a 3D pattern in the magnetic fibrin hydrogel. The relative humidity in the ESEM when the photo was taken was 60%. (D) Immunofluorescent staining, with anti NF antibody, of early neuronal fibers sprouting from a DRG explant in an organotypic culture exposed to nerve growth factor (NGF)-conjugated to IONPs, already 24 hours after seeding. (E) Immunofluorescent staining with anti synaptophisin of spinal cord culture exposed to GDNF-conjugated to IONPs. In green–numerous synapses on a single spinal cord neuron. In blue–cell nuclei. (F) Immunofluorescent staining of a myelinated DRG culture 12 days after seeding in gel enriched with GDNF-conjugated to IONPs. Non myelinated nerve fibers appears in red (anti NF) and myelinated fibers appears in green (anti myelin basic protein). DAPI: 4′,6-Diamidino-2-phenylindole; NF: neurofilament; GDNF: glial cell-derived neurotrophic factor.

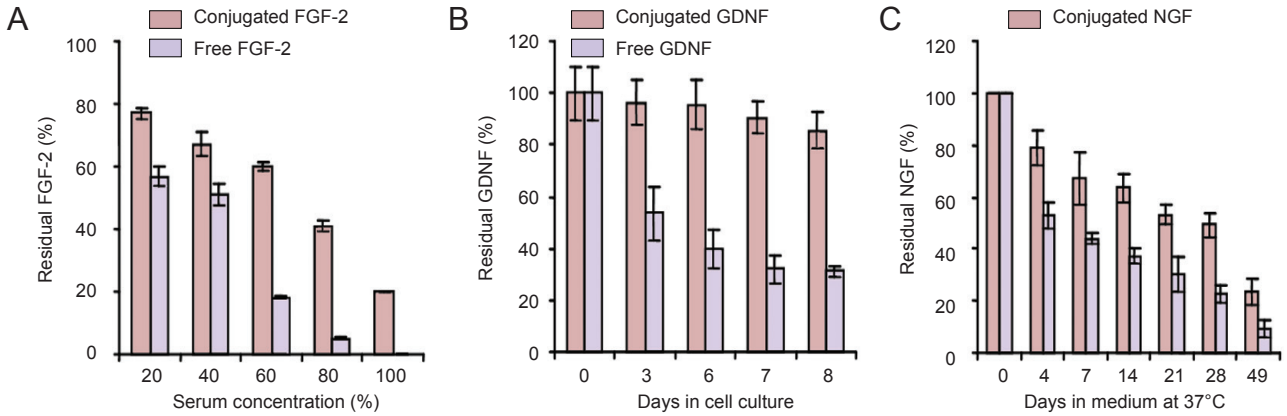

Figure 2 Representative graphs illustrating the increased stability of various neurotrophic factors following their conjugation to iron oxide nanoparticles.
(A) Stability in serum: Free *versus* conjugated-FGF-2 (10 ng/mL, final concentration) were incubated with various concentrations of fetal calf serum at 37°C. Following 3 days of incubation, the residual concentrations of the factors were measured, using an appropriate ELISA kit. (B) Stability in tissue culture: Free *versus* conjugated-GDNF (10 ng/mL) were added once to dissociated DRG cell cultures at the beginning of the experiment. The culture medium was not changed during the experiment and aliquots from it were collected at different days post cultivation. The residual concentration of factors in the aliquots was measured as described above. (C) Stability in culture medium, incubated in 37°C: Free versus conjugated-βNGF (10 ng/mL) were added to culture medium containing 10% serum and placed at 37°C. Aliquots were collected after different points of time, and the concentration of the residual factor in the samples was measured by ELISA. *Similar results were obtained for all free *versus* conjugated factors under all the examined conditions. FGF-2: basic fibroblast growth factor 2; GDNF: glial cell-derived neurotrophic factor; βNGF: β-nerve growth factor; DRG: dorsal root ganglia.

meric neurite outgrowth from cultured dorsal root ganglia when compared to control conditions. In contrast, the aged GDNF-conjugated IONPs maintained its neurite outgrowth inductive activity which was still significantly increased over control conditions (Morano et al., 2014).

The biological activity of free versus conjugated neurotrophic factors was tested in cultures of adult nasal olfactory mucosa (NOM) cells (Skaat et al., 2011; Ziv-Polat et al., 2012) and cultures of fetal spinal cord (SC) and dorsal root ganglia (DRG) (Ziv-Polat et al., 2014). NOM cell cultures were prepared from adult Luis inbred rats and were intended for autologous transplantation into spinal cord injuries. The cells were cultured either in magnetic fibrin scaffolds or in NVR-Gel (composed mainly of hyaluronic acid and laminin). Both scaffolds were enriched with either free or conjugated-FGF-2. Results revealed that the conjugated factor significantly enhanced the growth and neuronal differentiation of the NOM cells in both scaffolds, compared to the same or even a 5-fold concentration of the free FGF-2. The NOM cells, which were cultured in the magnetic fibrin hydrogel,

exhibited a 3D growth pattern and early differentiation into tapered bipolar nerve cells and ensheathing cells. It was also demonstrated, through the use of FGF-2 conjugated to fluorescently labeled IONPs and through the Prussian blue iron staining, that the conjugated FGF-2 was internalized by the NOM cells, entrapped mainly in the lysosomes (Skaat et al., 2011). The amount of the IONPs in the cells was decreased with time, probably due to the gradual degradation of the nanoparticles in the lysosomes. According to scientific literature the degraded iron might be incorporated into body's iron storage in the form of ferritin or transferrin or in red blood cells hemoglobin (reviewed by Wang et al. (2009)).

The organotypic SC and DRG cultures were prepared from rat fetuses (day 15 of pregnancy) and were intended for *in vitro* studies on peripheral nerve regeneration. Free or conjugated βNGF, GDNF and FGF-2 were added (10 ng/mL, of each factor separately) either to NVR-Gel or to the magnetic fibrin scaffolds and subsequently to the nutrient medium at each feeding. Three parameters were examined: 1) Intensity and early sprouting of DRG nerve fibers. 2) Formation of cell networks and synapses in established SC cultures. 3) Early onset of myelin and its progression in DRG cultures. Results revealed that all three conjugated neurotrophic factors enhanced early sprouting of nerve fibers from DRG slices compared to the corresponding free factors. However, the most efficient sprouting, from almost all DRG explants, was observed in cultures exposed to conjugated-βNGF (**Figure 1D**). In SC cultures, the conjugated GDNF enhanced the formation of ramified nerve fiber networks with many synapses on each neuron (**Figure 1E**), compared to cultures exposed to other free or conjugated factors. The most significant result was that conjugated GDNF accelerated the onset and progression of myelin in DRG cultures considerably earlier than the free GDNF and the other free and conjugated factors (Ziv-Polat et al., 2014) (**Figure 1F**). This is probably due to covalent binding of the GDNF to the IONPs, which increased its stability and prolonged its biological activity.

Another advantage of the IONPs is that they can be easily seen by transmission electron microscopy (TEM) due to the absorption of the transmitted electrons by the iron atoms. This feature can be used to track various bioactive materials conjugated to IONPs in cultures and to study their mechanism of action. For example, TEM analysis of myelinated DRG cultures exposed to conjugated-GDNF showed that IONPs of size ranging 10–15 nm were localized in the DRG axons as well as between the myelin lamella formed by Schwann cells. Also, no damage to both types of cells was observed following the exposure to the IONPs. These results indicated that GDNF-conjugated IONPs accelerated the onset and progression of myelination by the activation of both DRG neurons and Schwann cells (Ziv-Polat et al., 2014).

Conclusions: Here we described the use of thrombin conjugated IONPs in the development of a novel magnetic fibrin hydrogel scaffold. We also showed that the covalent binding of neurotrophic factors to IONPs enhanced and prolonged the beneficial activity of the conjugated factors in both neuronal and NOM cultures.

These *in vitro* findings were used as a basis for *in vivo* experiments for the reconstruction of peripheral nerves. Indeed, our preliminary studies on regeneration of rat sciatic nerve after a severe segment loss showed positive results for both our developed magnetic fibrin hydrogel scaffold and the neurotrophic factors conjugated to IONPs. Results of *in vivo* studies have been submitted for publication.

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