NGF–loaded PLGA microparticles for advanced multifunctional regenerative electrodes

G.Giudetti, J. del Valle Macià, X. Navarro Acebes, S. Micera

Abstract— **Nerve guide conduits are currently the elective device for peripheral nerve reconstruction applications, as nerve autograft often is hampered by procedure invasiveness and limited nerve availability. Many technological improvements have been approached to enhance nerve regeneration driven by these devices, whose main drawbacks are often disordered sprouting and ineffective axon guidance. Among the adopted solutions to overcome these problems, embedding of extracellular matrix (ECM) proteins and neurotrophic factors (NF) in nerve conduits has been a promising one. Using free NFs, however suffers from different drawbacks mainly due to diffusion, degradation and local concentration boosting. As part of a wider EU-funded program for next gen regenerative electrodes, we developed NGF-loaded PLGA microparticles to use them immersed in a gel biomatrix that is being embedded in nerve conduits before implant, and allow for timed-controlled delivery instead of an initial concentration boost. Here we report the technological steps for the synthesis and initial testing with mouse dorsal root ganglia (DRG) explants, towards their full integration with a complex three-dimensional biomatrix into next-gen regeneration electrodes.**

I. Introduction

Nerve guide conduits (NGCs) have been widely used to repair transected nerves. Filling NGCs with neurotrophic factors [1] or providing them with biocompatible scaffolds [2] has been reported to enhance regeneration. However free neurotrophic factors can be rapidly degraded [3], can diffuse outside the internal lumen or get diluted after liquid infiltration resulting in sub-optimal concentrations and poor regeneration outcomes.

Our objective was then to test NGF encapsulated in Poly-Lactic Co-Glycolic acid (PLGA) microspheres (MPs) for in vitro improvement of neurite outgrowth, towards applications in *in vivo* nerve regeneration studies. This work is part of a EU funded program (FP7-NMP MERIDIAN, no.280778) aimed, (among other objectives) at the development of next-gen regeneration interfaces, as an improvement to current state of the art silicon nerve regeneration conduits. The envisioned device will in fact be comprised of an external biocompatible cylindrical wall, divided in two chambers by a planar electrode to provide recording/stimulation capabilities (see Fig.1): the conduit will be embedded with a biocompatible hydrogel biomatrix consisting of oriented polymeric fibers to drive axon guidance, thanks to local sustained release of neurotrophic factors. Embedding different mixes of neurotrophic factors-loaded microparticles will endow this device with the capability of selective axon regrowth, that will allow control on specific regeneration of sensory or motor neurons.

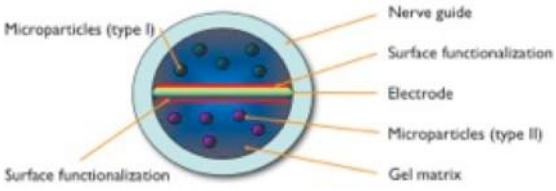

Figure 1. final advanced regenerative electrode endowed with separation capability for sensory and motor neurons.

Specifically, Nerve growth factor (NGF) has neurotrophic effects on sensory neurons, prevents cell death and improves myelination. Therefore, a local and sustained release of NGF performed by microspheres made of poly-lactic-*co*-glycolic acid (PLGA) would enhance peripheral nerve regeneration in NGCs, in comparison with single bridging

II. Protocols and Testing

A. PLGA microparticles fabrication

The chosen material for the microparticles was Poly-lactic Co-Glycolic acid (PLGA), a biocompatible and biodegradable co-polymer widely use for controlled release studies [4]. To synthesize the PLGA microparticles, a Water-in-oil-in-water (WoW) protocol was followed, as already described in [5], with modifications to adapt to the laboratory equipment. Briefly, 30-35 mg Poly-Lactic Co-Glycolic acid (50:50), are dissolved in 1ml DCM. Low molecular weight PLGA (Mw 24,000-38,000) has been chosen as it is demonstrated having better encapsulation efficiency [6]. The first emulsion is created with 200ul aqueous solution of the molecule of interest (usually at a concentration of 10 μg/ml) with an immersion homogenizer. This water-in– oil emulsion is then poured in 7ml PVA 5% aqueous solution to create the second emulsion. After 40-50' on the magnetic stirrer the double emulsion is poured in 40 ml PVA 0.1% aqueous solution to allow for solidification and solvent evaporation over 16h on the magnetic stirrer. The microparticles are washed with distilled water twice by centrifugation at 4650

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RCF at 4°C and finally resuspended in an aqueous solution added with penicillin/streptomicyn, to control possible downstream bacterial contamination.

To test the correct encapsulation of molecules, fluorescent FITC-BSA was used as tracking dye (Fig. 2), and its fluorescence into the microspheres was checked with an inverted fluorescence microscope (Eclipse Ti; Nikon Instruments, Japan) equipped with a cooled CCD camera (DS-Fi1C; Nikon Instruments, Japan) and suitable fluorescence filters.

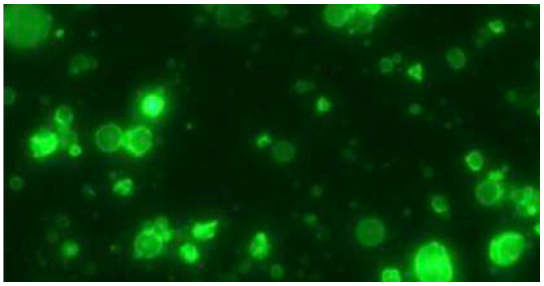

Figure 2. FITC-BSA-filled PLGA microspheres imaged in aqueous suspension under fluorescent light. Fluorescence is clearly limited to the volume of the microspheres.

The surface morphology of the microspheres (Fig. 3) was checked using a scanning electron microscope (EVO™MA10 Scanning Electron Microscopy, ZEISS, Germany) with an acceleration voltage of 10 kV. SEM imaging permitted rapid assessment of particle dispersity and allowed a quick modification of the synthesis protocol to obtain a more evenly distributed microsphere size. Specifically, a consistent change was observed using non-hydrolyzed PVA instead of hydrolyzed PVA while creating the second emulsion in the protocol. Non-hydrolyzed PVA has higher viscosity and reasonably hampers coalescing of the microdroplets formed during the first homogenization phase, thus permitting smaller and evenly distributed sizes.

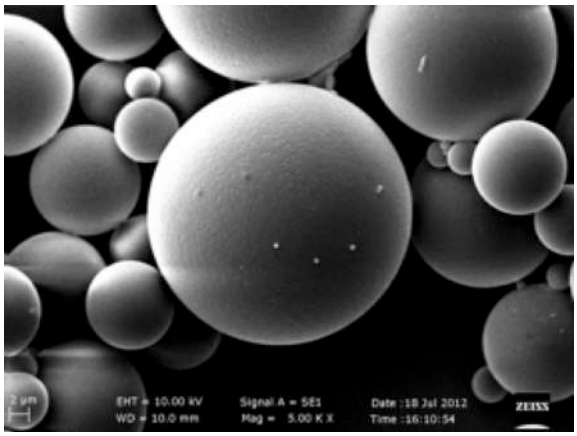

Figure 3. SEM micrograph of PLGA microspheres synthetized in non-hydrolyzed PVA and clearly showing a rough surface.

Microparticles produced with this modified protocol have a mean size of 6-8 µm and range between 1 and 20 µm diameter (Fig. 4): this is a desirable size range, as nanometric spheres would likely be endocythosed by cells and bigger microspheres would be difficult to handle due to quick precipitation after suspension. To assess mean particle size of produced batches, as Dynamic Light Scattering (DLS) was not feasible due the micrometric size of the particles, an optical microscopy-based method was implemented. Briefly, microparticles are imaged immediately after synthesis in 5µl distilled water on a glass coverslip, and snapshots of different fields are taken in visible light. Particle size is evaluated via software treatment and analysis with the Fiji software [7]. Fig. 4 shows the sequential steps of the software pipeline, and the summarization of the distribution of the measured diameters of the microspheres.

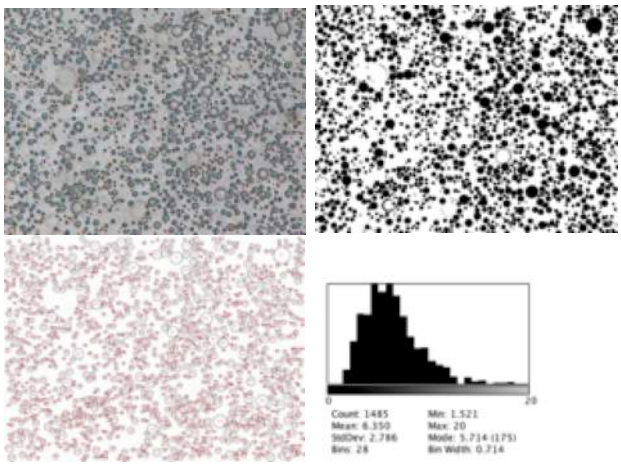

Figure 4. Original 16 bit image (top left), binarized image (top right), artifact removal, particle count and evaluation of particle diameter -Feret diameter- based on pixel scaling (bottom left), resulting in a diameter distribution and relative statistics (bottom right).

B. In vitro testing of microparticles on neurite outgrowth

Testing different neurotrophins other than NGF is currently under process, but preliminary results give proof of concept that the microparticles could improve motor and sensory axon regeneration. *In vitro* cultures of organotypic dorsal root ganglia (DRG) were performed to test whether microparticles with encapsulated NGF improve neurite outgrowth in comparison with free NGF. DRG were cultured in a 3D collagen matrix as previously described [8] under pre-conditioned medium during 0, 1 or 2 weeks with microparticles with PBS (MPPBS), free NGF and microparticles with NGF (MPNGF). Cultures with fluorescent FITC-BSA encapsulated were also used as control. Neurites were labeled with immunohistochemistry and the Neurite-J plugin for Image-J was used to quantify the length of the longest neurite and the number of axons at increasing distances from the DRG. MP themselves do not interfere with nerve regeneration (Fig. 5). On the other hand, preliminary data show that NGF encapsulated in MP increases the maximum neurite length after 1 and 2 weeks of pre-conditioning in comparison with free NGF. Finally, while both NGF and MPNGF cultures increase the amount of sensory neurites at 0 weeks of treatment, only encapsulated NGF increases the amount of sensory axons after 1 week of treatment with differences even with the free

NGF cultures. After 2 weeks of treatment, MPNGF DRG show a tendency to present more growing axons.

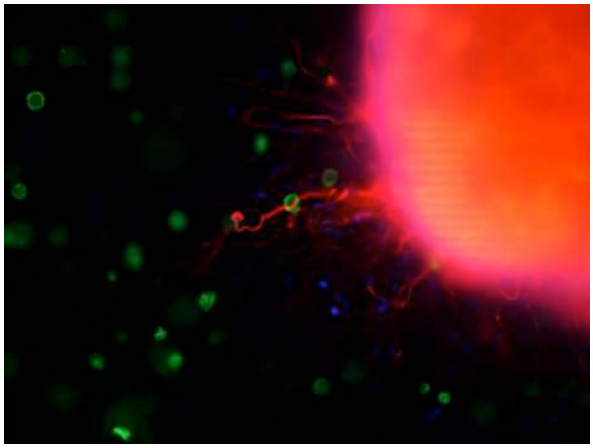

Figure 5. Neurite outgrowth from sensory neurons in a 3D collagen matrix. DRG explant after two days in vitro with RT97, a marker for phosphorylated neurofilaments (red), BSA-FITC microparticles are seen in green, nuclei (blue) are stained with DAPI.

III. Conclusions

An integrated PLGA microparticle synthesis and characterization protocol has been implemented, allowing also for quick modifications needed for encapsulation of different neurotrophins. The PLGA MPs themselves do not interfere with nerve regeneration. On the other hand, NGF encapsulated in MP increases neurite growth of DRG after 1 and 2 weeks of pre-conditioning in a collagen matrix, in comparison with free NGF. NGF encapsulation in microspheres definitely boosts neurite outgrowth of DRG slices *in vitro*. This work lays a solid technological basis to implement local controlled neurotrophic-factor release in innovative regenerative electrodes and will allow future development of regenerative systems capable of discriminating regrowing neurons in terms of their sensorial or motor function.

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