Electronic Supplementary Material (ESI) for Biomaterials Science.

Supplementary Data

Supplementary figures

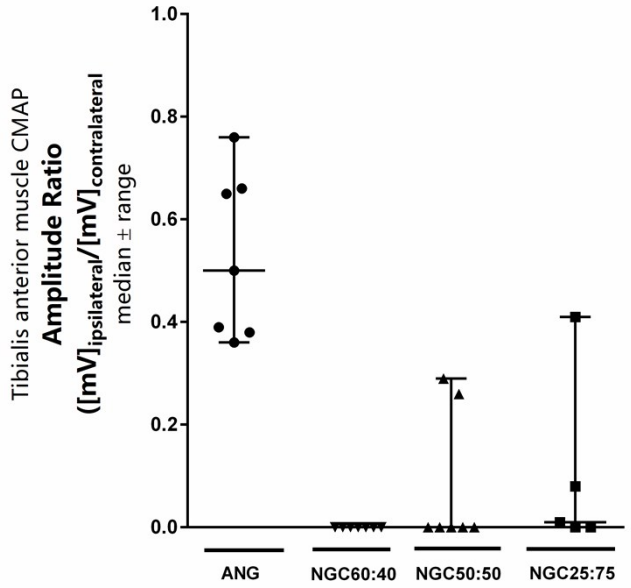


Figure S1 - **Evaluation of functional motor recovery after nerve reconstruction with the studied formulations**. Scatter plot depicting the median and range of the amplitude ratios of evocable compound action potentials recorded 12 weeks after nerve reconstruction. The recordings took place in the tibialis anterior muscle of control group (ANG) and experimental group (NGC 60:40, NGC50:50 and NGC25:75) animals. While all animals of the control group displayed motor recovery, only single animals of the experimental groups did so. Nerve reconstruction with NGC60:40 did not allow for recordable muscle reinnervation in any case.

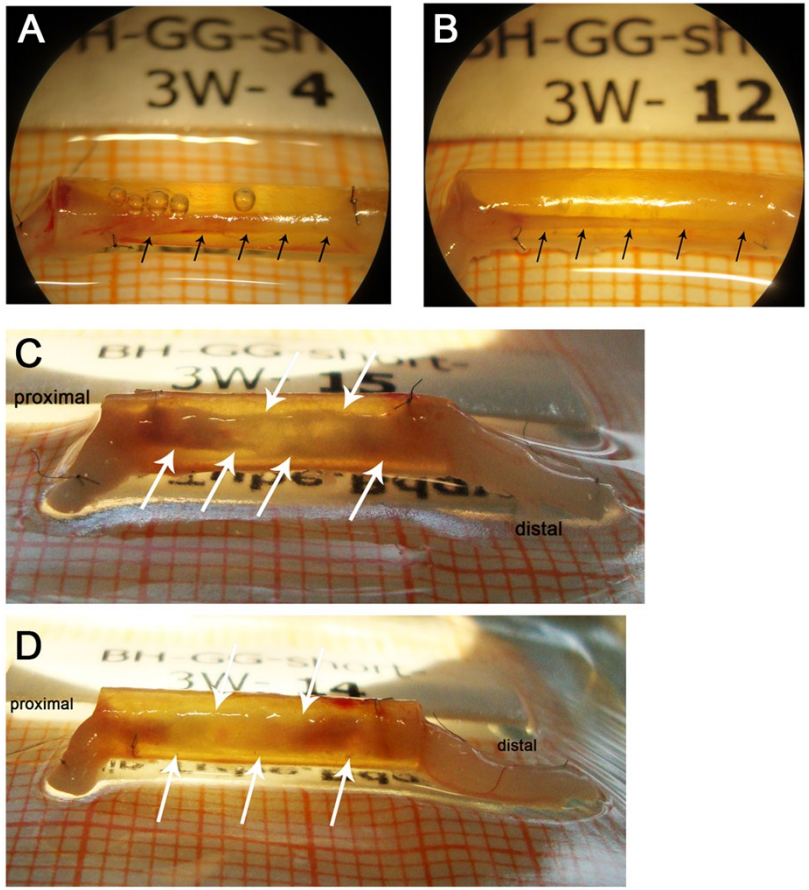


Figure S2 – **Appearance of the studied formulations 3 weeks after reconstruction**. A+B) Regenerated tissue bridge grown through hollow control NGCs. Black arrows point to the tissue cables located inside the NGC. C) Sample from the NGC50:50 group. White arrows point to the border of the infiltrated GG freeze-dried hydrogel. D) Sample from the NGC25:75 group. White arrows point to the border of the infiltrated GG freeze-dried hydrogel. The NGCs are still enclosed by a thin layer of connective tissue that has formed around them. The specimens were placed on glass plates on top of scale-papers with 1 mm intersections.

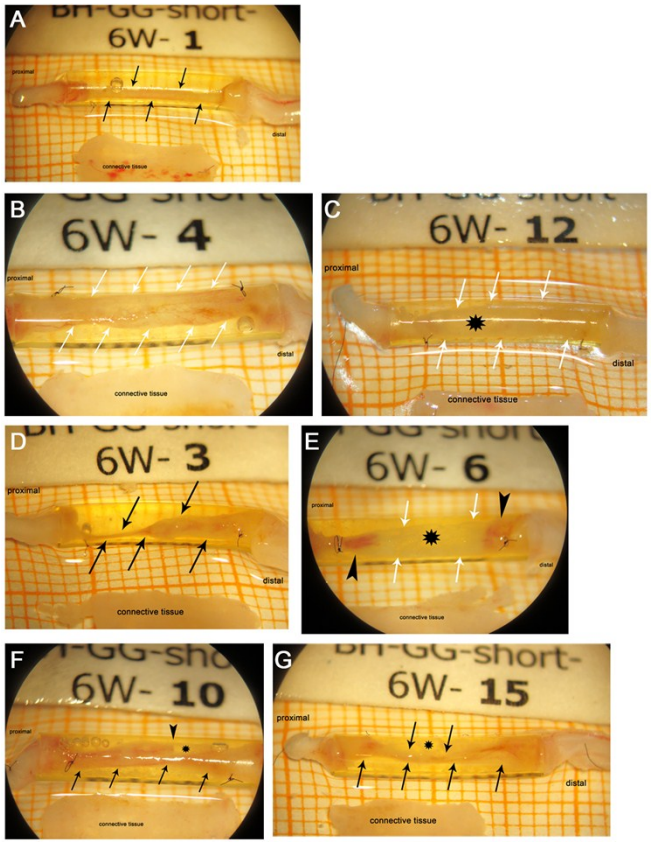


Figure S3 - **Appearance of the studied formulations through the microsurgery microscope 6 weeks after reconstruction**. A) Regenerated nerve tissue grown through a hollow control NGC. Black arrows delineate the tissue bridge that has formed between the nerve ends. B+C) Samples from the NGC60:40 group. There is no presence of ingrown tissue within the GG residues indicated by the asterisk and delineated by white arrows. D+E) Samples from the NGC50:50 group. Black arrows in D) delineate ingrown tissue in the proximal part of the NGC, which is thin and free of surrounding GG residues. White arrows in E) point to the border of an empty GG freeze-dried hydrogel scaffold. The asterisk marks the GG material, while the black arrows signal the infiltrated blood supply in close vicinity to the nerve ends. F+G) Samples from the NGC25:75 group. Black arrows indicate the presence of ingrown tissue. Arrowheads point to a border of the tissue and asterisks indicate residues of freeze-dried GG hydrogel scaffold. Eventually, small vessels can be seen along or inside the GG freeze-dried hydrogel scaffolds. The connective tissue that has formed around the NGCs has been removed. The specimens were placed on glass plates on top of scale-papers with 1 mm intersections.

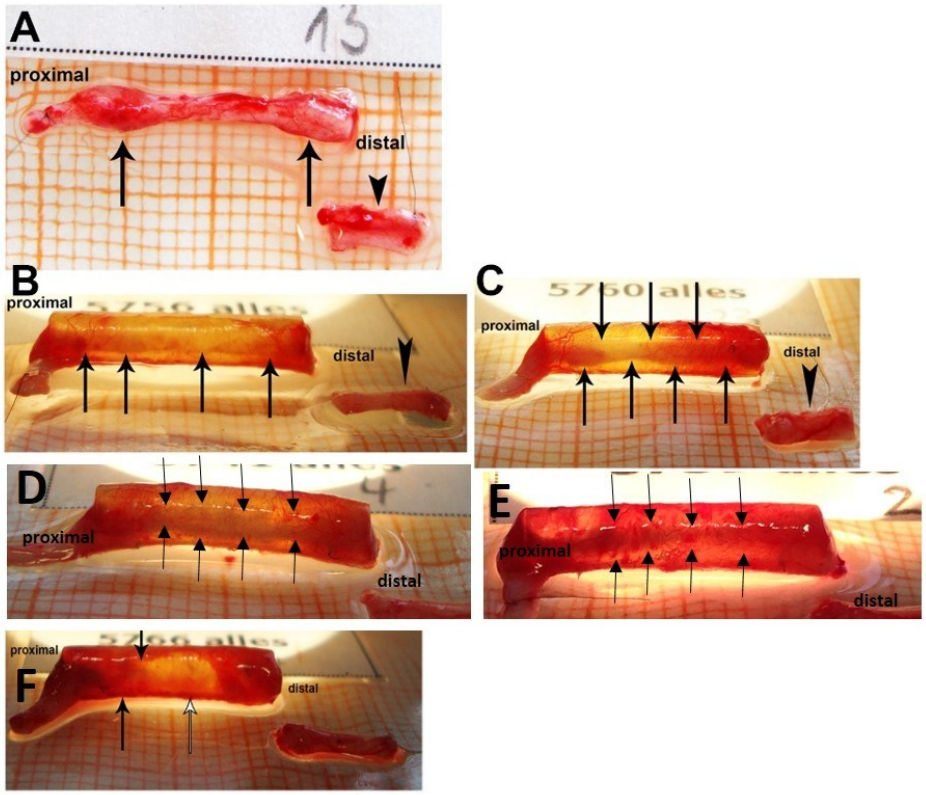


Figure S4 - Aspects of nerve implants with electrodiagnostically proven muscle reinnervation at the moment of explantation after 12 weeks in vivo (view through the microsurgery microscope). The connective tissue that has formed around the NGCs has not been removed. Black arrow heads point to the distal nerve segment further processed for nerve morphometry. The specimens have been placed on a glass plate on top of scale-paper with 1 mm intersections. A) Autologous nerve graft, black arrows point to the suture line. B-C) Samples from the NGC50:50 group. Black arrows indicate the presence of tissue most likely grown through Gellan Gum scaffolds. Both specimens were affiliated with a positive evaluation for functional recovery. D-F) Samples from the NGC25:75 group: Black arrows indicate the presence of tissue most likely grown partially through and partially along the Gellan Gum hydrogel matrix. White arrow in F) points to a whitish area not clearly indicating if the GG scaffolds contains ingrown tissue or not. Samples shown in D and F were affiliated with positive evaluation for functional recovery while sample shown in E was not.

Supplementary tables

Table S1 - Micro-CT analysis of freeze-dried hydrogels for *in vitro* assays: Quantification of mean pore size, trabecular thickness and porosity from n=3 samples each.

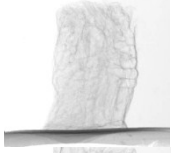
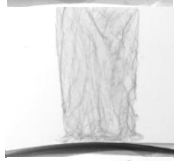
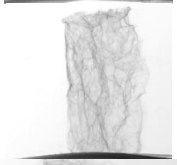
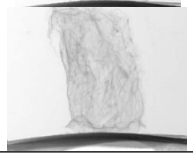
Freeze-dried hydrogel formulation	*Mean Pore Size (µm)*	*Trabecular Thickness (µm)*	*Porosity (%)*	*Image µ-CT*
H60:40	265.5 ± 2.9	58.1 ± 2.1	80.5 ± 2.5	
H50:50	339.0 ± 75.9	65.8 ± 4.7	81.5 ± 8.2	
H25:75	299.2 ± 25.5	65.4 ± 2.5	81.2 ± 1.4	
H0:100	230.6 ± 3.2	61.8 ± 0.78	79.2 ± 1.7	

Table S2 - Micro-CT analysis of nerve guidance conduits with different gellan gum freeze-dried hydrogels as fillers, similar to those prepared for evaluation *in vivo*: Quantification of mean pore size, trabecular thickness and porosity from n=3 samples each.

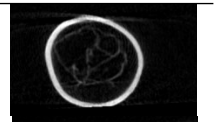
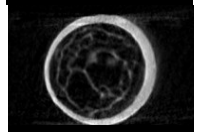
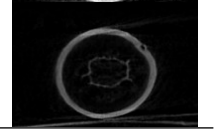
Nerve guidance conduit, NGC (Freeze-dried hydrogel filling chitosan tube)	*Mean Pore Size (µm)*	*Trabecular Thickness (µm)*	*Porosity (%)*	*Image µ-CT*
NGC60:40	377.8 ± 50.8	98.0 ± 13.5	82.8 ± 5.2	
NGC 50:50	321.6 ± 89.4	109.0 ± 4.9	73.8 ± 7.3	
NGC 25:75	360.7 ± 36.0	121.4 ± 13.4	79.4 ± 3.3	

Table S3 - Nerve morphometry of distal nerve segments. Table 3 shows the results from the morphometrical analysis of distal nerve segments that specifically originate from functionally recovered nerves. Data are given in mean ± SD for healthy nerve samples, AUTOG and NGC25:75, while for NGC50:50 (only 2 animals per group) the single values are presented

Formulation	Animals (Code number)	Macroscopic evaluation (1)	H&E (1)	NF200	ED1 (2)	Neo-vascularization

Group	Number of samples analyzed	Total axon number	Nerve fiber density [number/mm^2]	Axon diameter [µm]	Fiber diameter [µm]	Myelin thickness [µm]	g-ratio
Healthy nerve	3/3	9351 ± 2433	13723 ± 2059	5.45 ± 0.97	7.56 ± 0.97	1.06 ± 0.25	0.71 ± 0.06
AUTOG	7/7	14882 ± 4335	33023 ± 6945	2.28 ± 0.23	3.23 ± 0.33	0.48 ± 0.12	0.69 ± 0.05
NGC50:50	2/7	8081 14434	18198 24549	2.56 2.16	3.37 3.14	0.40 0.49	0.74 0.67
NGC25:75	3/5	14181 ± 8905	20922 ± 11952	2.13 ± 0.21↖	2.88 ± 0.33↖	0.38 ± 0.06↖	0.72 ± 0.02

Table S4 - 3 weeks' time point general evaluation

						(3)
NGC60:40	1	+	-	-	-	x
	8	+	+	-	+	x
	9	-	+	-	-	-
	16	+	x	x	x	+
NGC50:50	2	+	+	+	-	x
	7	+	-	-	-	x
	10	++	+	-	+	+
	15	+	x	x	x	-
NGC25:75	3	++	++	++	+	x
	6	-	-	-	-	x
	11	++	x	x	x	++
	14	+	x	x	x	+

[1](-) No tissue within the NGC; (+) Strips of tissue mainly between the NGC wall and the GG; (++) Tissue along the entire NGC, but fragmented; (+++) complete nerve bridge

[2]In the case of ED1-immunopositive activated macrophages, (-) indicates very few visible red fluorescent cells, (+) indicates an intermediate and (++) a more intense immune response with increasing numbers of red fluorescent cells detected

[3]For neovascularization, (-) indicates no visible blood vessels and (+) refers to visible blood vessels

[4](x) Samples not analyzed since NGCs were macroscopically looking totally empty or purulent

Table S5 - 6 weeks' time point general evaluation

Formulation	Animals (Code number)	Macroscopic evaluation	H&E	NF200	ED1	Neo-vascularization (when applicable, (when applicable, + or - only)
NGC60:40	4	++	+	+	-	+
	5	-	x	x	X	x
	12	+	+	+	-	+
	13	-	x	x	x	x
NGC50:50	3	+	++	+	+	+
	6	+	-	-	-	+
	11	-	x	x	X	x
	14	-	x	x	x	x
NGC25:75	2	++	+++	++	-	+
	7	++	-	-	-	+
	10	+++	+++	+++	+	x
	15	+++	++	+	-	x

[1](-) No tissue within the NGC; (+) Strips of tissue mainly between the NGC wall and the GG; (++) Tissue along the entire NGC, but fragmented; (+++) complete nerve bridge

[2]In the case of ED1-immunopositive activated macrophages, (-) indicates very few visible red fluorescent cells, (+) indicates an intermediate and (++) a more intense immune response with increasing numbers of red fluorescent cells detected

[3]For neovascularization, (-) indicates no visible blood vessels and (+) refers to visible blood vessels

[4](x) Samples not analyzed since NGCs were macroscopically looking totally empty or purulent

Table S6- 12 weeks' time point general evaluation. All positive controls, consisting of nerve autotransplants, resulted in electrodiagnostically proven functional recovery and a complete nerve bridge mainly consisting of NF200-immunopositive axons.

Formulation	Animals (Code number)	Macroscopic evaluation[1]	H&E	NF200	ED1[2]	Electrodiagnostic recordings (+ or – only)	Neo-vascularization (when applicable, + or - only)[3]
NGC60:40	5742	-	-	-	-	-	X
	5745	+	-	-	-	-	X
	5749	++	+	+	+	-	+
	5753	-				-	X
	5757	+++	+	+	+	-	+
	5761	-				-	X
	5765	-	-	-	-	-	X
NGC50:50	5740	+	++	+	+	-	+
	5743	-	x	x	x	-	X
	5748	++	++	+	-	-	X
	5752	+	+	+	-	-	+
	5756	+++	x	x	x	+	X
	5760	+++	+	++	+	+	X
	5764	++	x	x	x	-	X
NGC25:75	5739	+++	+++	++	-	-	X
	5741	+++	++	++	+	+	+
	5755	-	X[5]	x	x	-	X
	5763	+	++	++	-	+	+
	5766	-	x	x	x	+	X

[1](-) No tissue within the NGC; (+) Strips of tissue mainly between the NGC wall and the GG; (++) Tissue along the entire NGC, but fragmented; (+++) complete nerve bridge

[2]In the case of ED1-immunopositive activated macrophages, (-) indicates very few visible red fluorescent cells, (+) indicates an intermediate and (++) a more intense immune response with increasing numbers of red fluorescent cells detected

[3]For neovascularization, (-) indicates no visible blood vessels and (+) refers to visible blood vessels

[4](x) Samples not analyzed since NGCs were macroscopically looking totally empty or purulent