Differentiation of neural-type cells on multi-scale ordered collagen-silica bionanocomposites

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Supplementary Materials

1. Materials and Methods

1. Collagen extraction and purification

Type I collagen was extracted from young Wistar rat tails as previously described[1] and obtained as solutions in 500 mM acetic acid. Rat tails were kindly provided by UFR Life Sciences at Paris Diderot University, where all animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Paris Diderot University and approved by the Animal Ethics Committee of AFAQ. Sample purity was assessed by SDS-PAGE electrophoresis. Collagen concentration was determined by titrating the amount of hydroxyproline.

2. Synthesis of fluorescent silica particles

50 µL of Alexa 488 (Invitrogen) at 1 mg.mL^{-1} in dimethylsulfoxide (DMSO, VWR) was conjugated to 1.2 µL of aminopropyltriethoxysilane (APTES 99%, Sigma-Aldrich) in 149 µL of DMSO and left under stirring for 5 h. Stöber silica particles were synthesized using 100 mL absolute ethanol (VWR, GPR RectaPur), 5.5 mL ammonium hydroxide solution (NH_4OH 25%, Carlo Erba), 4 mL tetraethyl orthosilicate (TEOS 98%, Sigma Aldrich) and 200 µL of Alexa-APTES. TEOS and Alexa-APTES were successively added to the solution dropwise, and the sol was stirred overnight at RT. Ethanol and ammonia were removed by centrifugation and SiNPs were washed twice with absolute ethanol through a ultrasonic redispersion-centrifugation process (10 000 rpm for 5 min) before drying under vacuum at 30°C. These particles were dispersed in 300 mL absolute ethanol with 2.4 mL of ultra-pure water and 3 mL of NH_4OH. 6 mL of TEOS were added dropwise and the mixture was kept under stirring overnight. SiNPs were centrifuged and washed twice with absolute ethanol through a ultrasonic redispersion-centrifugation process (10 000 rpm for 5 min) before drying under vacuum at 30°C.

3. Synthesis of functionalized SiNPs

Sulfonate- and amine-bearing particles were prepared using 3-mercaptotrimethoxysilane (MPTMS, 95%, Sigma-Aldrich) and APTES, respectively, according to previously reported procedures.[2] Bi-functional particles were functionalized with amine groups and thiol groups from a stoichiometric mixture of the two silanes. Typically, 0.77 g of SiNPs was dispersed in a mixture of 77 mL absolute ethanol and 1.7 mL NH_4OH before rapid addition of 0.375 mL APTES and 0.298 mL MPTMS (2.1 mmol.g^{-1} silica). The mixture was stirred for 2 hours at RT. Subsequently, the reaction mixture was heated at 80°C until evaporation of the two thirds of the volume. The mixture was left to cool down to RT and was subsequently washed two times with absolute ethanol (10 000 rpm for 5 min). Finally, oxidation of thiol groups led to sulfonic acid functionalized particles. In a typical reaction, 0.6 g of thiol-modified particles was suspended in 30 mL hydrogen peroxide (H_2O_2 35%, Sigma Aldrich) under stirring at RT for 48 hours. Hydrogen peroxide was removed by centrifugation (12 000 rpm for 15 min) before addition of 25 mL sulfuric acid (H_2SO_4 96%, Carlo Erba) and stirring for 2 hours at RT. Water was carefully added to the mixture in an ice bath because of exothermic reaction, and the mixture was centrifuged (15 000 rpm for 15 min). The particles were then washed twice with water and twice with absolute ethanol through a ultrasonic redispersion-centrifugation process (10 000 rpm for 5 min) before drying under vacuum at 30°C.

4. Peptide coupling between amine groups on SiNPs and IKVAV peptide

The IKVAV peptide was synthesized on a rink Amide resin using the classical Fmoc-strategy on a 0.2 millimolar scale. For each coupling, a solution containing 4 equivalents of Fmoc protected amino acids, hydroxybenzotriazole hydrate (HOBT.H_2O, 97%, Sigma Aldrich) and N,N'-Diisopropylcarbodiimide (DIC,

Sigma Aldrich) dissolved in dimethylformamide (DMF, Sigma-Aldrich) was added to the resin. After 30 minutes, the success of the coupling was checked by Kaiser tests. A solution of 20% piperidine in N-methyl-2-pyrrolidone (NMP) was used for deprotection. The resin was allowed to shake for 1 minute, and then 15 minutes with each time a fresh solution of piperidine in order to get a complete deprotection. Several (5) washings with NMP were done after couplings and deprotections. 4 equivalents of glutaric anhydride (95%, Sigma-Aldrich) used as a spacer were finally coupled. The peptide was cleaved from the resin with deprotection of all protective groups with a mixture of TFA/TIS/H_2O 95:2.5:2.5 v:v:v. The resin was shaken for 2 hours and the filtrate recovered. The solvent were removed and the peptide was precipitated in diethyl ether. The solid was recovered by centrifugation and washed 2 times in diethyl ether. Water was added and the solution was lyophilized until dryness.

A tert-butyloxycarbonyl (Boc) protective group was added on the amine of the lysine side chain to avoid side reaction during the coupling procedure. 100 mg of unprotected IKVAV was added to 10 mL of 1:1 H_20/tetrahydrofuran (THF) mixture. One equivalent of sodium bicarbonate ($NaHCO_3$) was then added to get a basic solution (if not it was adjusted with a diluted solution of $NaHCO_3$ to pH 8), before the addition of one equivalent of di-*tert*-butyl dicarbonate. The mixture was stirred at room temperature (RT) overnight and monitored by TLC (stained with ninhydrine to reveal unreacted amines). The THF was removed and the peptide was extracted 3 times with ethyl acetate. The organic layers were combined and dried with sodium sulfate, before filtration and concentration. Purity was checked by HPLC.

Peptide coupling was performed between carboxylate acid on IKVAV and amine groups at the surface of SiNPs. 33.4 mg of SiNPs ($5{,}1.10^{-4}$ mmol) were dispersed in a mixture of 140 µL DMSO and 34 µL DMF, with 4 equivalents of IKVAV, HOBT.H_2O and hexafluorophosphate benzotriazole tetramethyl uranium (HBTU, 98%, Iris Biotech GmbH) and 8 equivalents of N,N-diisopropylethylamine (DIEA, 99.5%, Sigma-Aldrich). The mixture was stirred for 40 min and subsequently washed twice with DMSO and twice with dichloromethane (DCM, Carlo Erba) through a ultrasonic redispersion-centrifugation process (10 000 rpm for 5 min) before drying under vacuum. To deprotect the amine from the BOC group, an acidic treatment with trifluoroacetic acid (TFA, Sigma Aldrich) was performed under sonication for 20 min. SiNP-IKVAV were washed three times with absolute ethanol through a ultrasonic redispersion-centrifugation process (10 000 rpm for 5 min) before drying under vacuum.

5. Particle characterization

Zeta-potential measurements were performed on a Malvern Zetasizer Nano spectrometer using DTS1060C cells. SiNPs were dispersed at a concentration of 0.5 $g.L^{-1}$ in 100 mM KCl buffer at different pHs adjusted with NaOH and HCl at RT.

For Transmission Electron Microscopy (TEM) observations, a drop of sample in aqueous solution was deposited on carbon-coated copper grids (300 mesh). After 3 minutes, the excess liquid was blotted with filter paper. TEM was performed at RT using a Tecnai spirit G2 electron microscope operating at 120 kV and the images were recorded on a Gatan Orius CCD camera.

6. Preparation and characterization of the composite materials

Collagen solutions in acetic acid were concentrated using centrifugal filtration units (Vivaspin® Sartorius, 100 kDa cutoff) spun at 2500 rpm, at a temperature of 10°C, until reaching the desired final concentration (15 $mg.mL^{-1}$). SiNPs were weighted, diluted in acetic acid 500 mM (450 $mg.mL^{-1}$) and put

under sonication for 5 min. Solutions were mechanically mixed together, poured in a 1 mL syringe and degassed by centrifugation at 1500 rpm for 10 min at 10°C, except for two conditions in order to avoid bubbles into the filament: 1000 rpm for 10 min at 10°C for SiNP-SO3- and 1000 rpm for 10 min followed by 1500 rpm for 5 min at 10°C for SiNP-IKVAV+SiNP-SO_3^-. The 1 mL syringe, filled with the composite solution, was mounted with a 23-gauge (inner diameter of 390 µm) blunt stainless-steel needle, and loaded vertically. A motorized stage was used to bring down a rod in contact with the syringe piston and to impose its speed. The extruded fluid velocity was 25 $\mu m.s^{-1}$ ie 0.44 $\mu L.s^{-1}$. The collecting cuvette was filled with a fibrillogenesis buffer PBS 5x (pH 7.4). At the blunt needle exit, formation of collagen-based filaments was monitored by an optical through-hole mounting. Prior to their use, the filaments were left for swelling stabilization in fibrillogenesis buffer at RT for 2 weeks, under gentle agitation to prevent them from sticking together. The initial collagen and SiNP concentrations were fixed at 15 $mg.mL^{-1}$ and 450 $mg.mL^{-1}$ respectively, which ended up in the final syringe with concentrations of 13.5 $mg.mL^{-1}$ and 45 $mg.mL^{-1}$ respectively.

7. Cell experiments

PC12 cells derive from a rat tumour of chromaffin cells, which are of neural crest lineage. This justifies the use of an adrenal medulla tumour for nerve growth studies. PC12 neural type cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 5% horse serum, 100 $\mu g.mL^{-1}$ penicillin, 100 $\mu g.mL^{-1}$ streptomycin and 1% glutamax at 37°C in a humidified atmosphere of 5% CO_2.[3,4] Recombinant FGF1 (50 $ng.mL^{-1}$, R&D Systems, 232-FA) was added to the culture medium in presence of heparin (10 $\mu g.mL^{-1}$) for inducing differentiation. The cells were grown in 75 mm^2 flasks (BD Falcon). All culture reagents were purchased from Gibco unless specified.

Filaments were immersed in cell culture medium for 3 h and washed 3 times with culture medium. The filaments were casted in a 48 well plate. PC12 cells were seeded at a low density (50 000 cells per well) in order to minimize cell–cell contacts. Cells were incubated on the filaments at 37°C and 5% CO_2 for 48 h. Then the cell culture medium was changed, and FGF was added to the culture medium in presence of heparin (10 $\mu g.mL^{-1}$). Cell culture medium was then changed every 3 days, with addition of FGF (in presence of heparin).

Cell adhesion was determined by counting the nuclei. For each condition, at least 500 nuclei were counted reported in unit area. For the quantitative assessment of neuronal differentiation (measurement of the number of neurites with a length larger than the cell body size, neurite length and alignment along the filament main axis) at least 5 different filaments were analyzed.

Alamar blue assay was used to assess the metabolic activity of adhering cells after transfer of filaments in clean plates dedicated to Alamar blue assay. Aliquots of 20 µL of stock Alamar blue solution (5 $mg.mL^{-1}$) were diluted 10 times and another 10 times when added to each well containing 200 µL of medium (0.05% final solution) and incubated with the cells for 3 h. Following incubation, the medium was removed and diluted five times. The absorbance was determined on a UV-visible spectrophotometer (Uvikon XL Secomam, BioServ) at 570 and 600 nm. A subtraction analysis of the dual wavelength was performed (D570 to D600) to increase accuracy of the measurement.

8. Polarized light microsocpy

For optical imaging, filaments were put in PBS 5x between a glass slide and a coverslip. Polarized light microscopy was performed using a transmission Nikon Eclipse E600 Pol, equipped with crossed polarizers, a waveplate and a Nikon DXM 1200CCD camera.

9. Multiphoton microscopy

In situ SHG/2PEF images of silica-collagen hybrid biocomposites were acquired at three different positions for each sample to check for the sample structure homogeneity. We used a custom-built laser-scanning multiphoton microscope as previously described.[5] Excitation was provided by a femtosecond titanium–sapphire laser (Mai-Tai, Spectra-Physics) tuned to 860 nm, scanned in the XY directions using galvanometric mirrors and focused using a 25× objective lens (XLPLN25XWMP2, Olympus), with a resolution of typically 0.4 µm (lateral) × 1.2 µm (axial). SHG and 2PEF signals were detected in the forward direction, using appropriate spectral filters. We observed a limited photobleaching of the stained NP in our excitation conditions (10-15 mW at the focus), while SHG was not affected as expected for such a non-resonant signal.

For 2PEF imaging, the mean 2PEF signal per pixel was very low for sample not containing stained SiNPs, i.e. collagen filaments (0.2 Photons), while the 2PEF signal per pixel was much higher for collagen+SiNP-IKVAV filaments (1.6 Photons, using the very same conditions for all the images). This confirms the signal assignment to the presence of particles dispersed homogeneously in the wire, as is particularly the case for the collagen+SiNP-IKVAV filaments.

For SHG imaging, we used either circular polarization in order to image all structures independently of their orientation in the image plane, or a set of linear polarizations with different orientations in order to perform polarization-resolved measurements. All polarization-resolved images were acquired at 36 excitation angles θ regularly spaced between 0° and 360°, using 200 kHz acquisition rate and 300×300 nm² pixel size. 2x2 binning was applied before pSHG processing. pSHG images were processed as already described[5] to provide two images: (i) the average of all images acquired with the set of linear polarizations, that is similar to an SHG image acquired with circular polarization; (ii) a map of the main orientation of collagen in the image plane. The latter pSHG image is displayed using the HSV Look-up-table, where H (Hue) is the orientation displayed in the insert, and V (brightness) selects pixels where the pSHG processing is satisfactory (R2>0.5: (V=R2)$\in$[0.5;1] and V=0 for R2<0.5).

10. Fluorescence microscopy

For fluorescence imaging, cells were fixed with 4% paraformaldehyde in PBS and 1 mM $CaCl_2$ for 30 min at RT. For immunostaining, fixed samples were first permeabilized with 0.1% Triton X-100 in PBS (20 min, RT). Actin filaments were fluorescently labeled with AlexaFluor-488-conjugated phalloidin (Life Technologies; 165 nM, 1 h at RT) for visualization. Cell nuclei were stained with DAPI (Life Technologies; 300 nM, 10 min at RT). Samples were analyzed using a AxioImager D.1, Zeiss microscope. Cell morphology was quantified from phalloidin stained fluorescent images acquired by a 10x objective from randomly selected regions on the coverslip (at least 500 cells analyzed on each filament). Acquired images were analyzed using ImageJ software.

11. Statistical analysis

Statistical analysis was performed using Graphpad Prism v.6 software. Analysis were performed using a Mann-Whitney non parametric test; each condition on six sixplicates, except when specified. Values in graphs are the mean ± standard error of mean (SD).

2. PC12 cells on filaments with varying collagen and SiNP concentrations

No significant difference between collagen and collagen-SiNP filaments in terms of neurite per cell and neurite length were observed with 27 mg.mL^{-1} collagen filaments ([SiNP] = 0.45 and 4.5 mg.ml^{-1}, Fig.S1,S2). We decided to decrease collagen concentration to 13.5 mg.mL^{-1} to amplify the effect of incorporated SiNPs. Again, no significant variation were observed with [SiNP] = 0.45 and 4.5 mg.mL^{-1} (Fig.S3,S4). We therefore increased [SiNP] concentration to 45 mg.mL^{-1} and fixed collagen concentration to 13.5 mg.mL^{-1}.

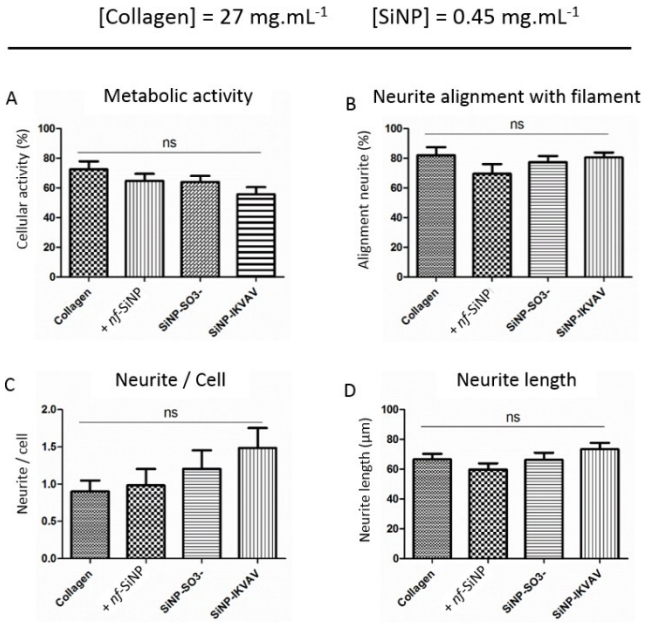

Fig. S1. PC12s after 10 days of culture on collagen-SiNP filaments ([Collagen] = 27 mg.mL^{-1}, [SiNP] = 0.45 mg.mL^{-1}). (A) Metabolic activity, (B) alignment along the filament, (C) number of neurites per cell and (D) neurite length. The column represents mean ± standard error of mean (SD) (* $p < 0.05$, ** $p < 0.01$; calculated against the pure collagen filaments, using one-tailed Wilcoxon-Mann-Whitney non parametric test; at least 5 filaments analyzed).

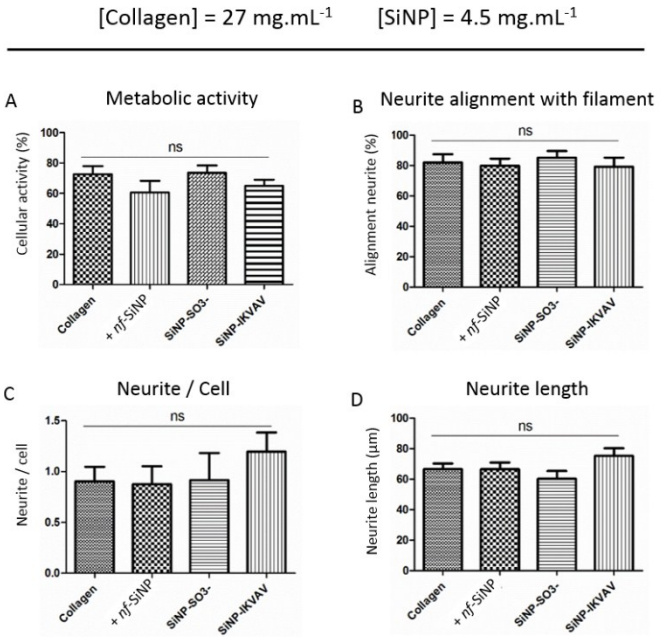

Fig. S2. PC12s after 10 days of culture on collagen-SiNP filaments ([Collagen] = 27 mg.mL^{-1}, [SiNP] = 4.5 mg.mL^{-1}). (A) Metabolic activity, (B) alignment along the filament, (C) number of neurites per cell and (D) neurite length. The column represents mean ± standard error of mean (SD) (* $p < 0.05$, ** $p < 0.01$; calculated against the pure collagen filaments, using one-tailed Wilcoxon-Mann-Whitney non parametric test; at least 5 filaments analyzed).

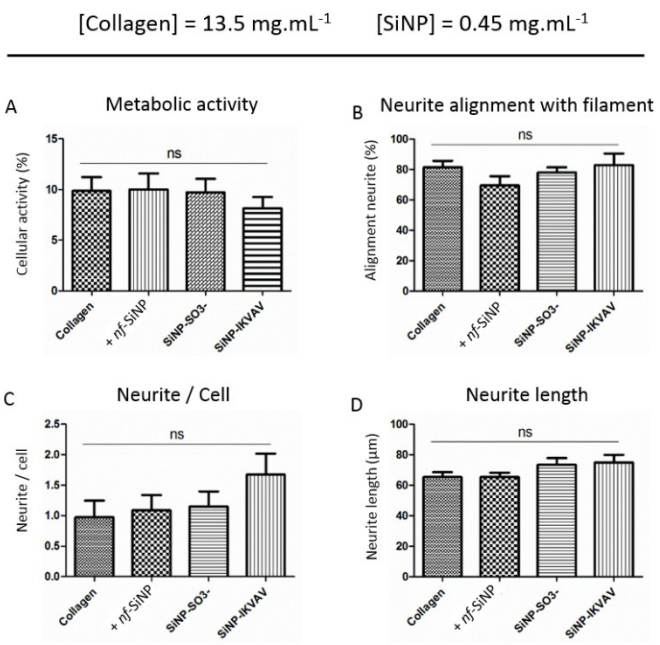

Fig. S3. PC12s after 10 days of culture on collagen-SiNP filaments ([Collagen] = 13.5 mg.mL^{-1}, [SiNP] = 0.45 mg.mL^{-1}). (A) Metabolic activity, (B) alignment along the filament, (C) number of neurites per cell and (D) neurite length. The column represents mean ± standard error of mean (SD) (* $p < 0.05$, ** $p < 0.01$; calculated against the pure collagen filaments, using one-tailed Wilcoxon-Mann-Whitney non parametric test; at least 5 filaments analyzed).

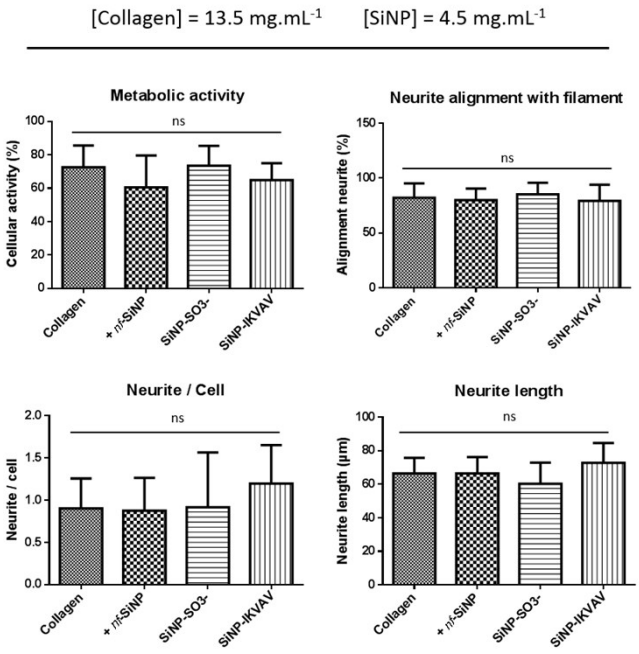

Fig. S4. PC12s after 10 days of culture on collagen-SiNP filaments ([Collagen] = 13.5 mg.mL^{-1}, [SiNP] = 4.5 mg.mL^{-1}). (A) Metabolic activity, (B) alignment along the filament, (C) number of neurites per cell and (D) neurite length. The column represents mean ± standard error of mean (SD) (* $p < 0.05$, ** $p < 0.01$; calculated against the pure collagen filaments, using one-tailed Wilcoxon-Mann-Whitney non parametric test; at least 5 filaments analyzed).

3. Characterization of the filaments by bright field, fluorescence and polarized light microscopies

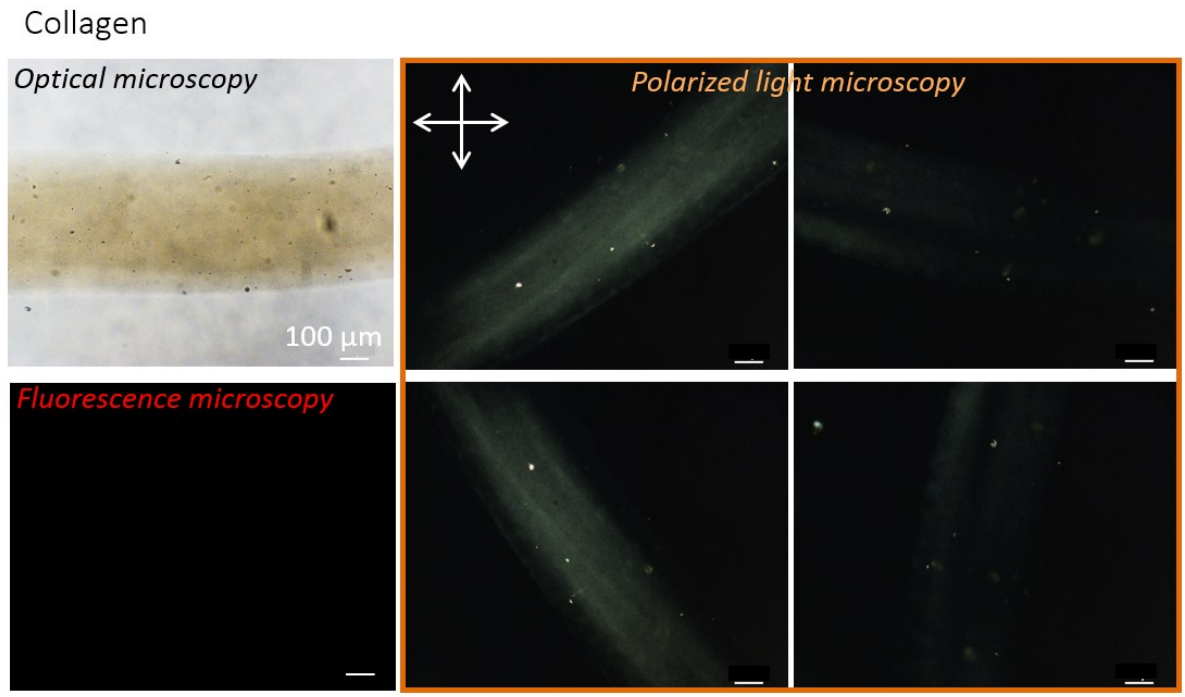

Fig. S5. Collagen filament ([Collagen] = 13.5 mg.mL^{-1}) observed by bright field, fluorescence and polarized light microscopies. PLM imaging was performed by taking 4 serial images with the sample oriented *versus* the cross polarizers (see white arrows) twice at extinction and at maximal transmission (every 45°). Scale bars 100 µm.

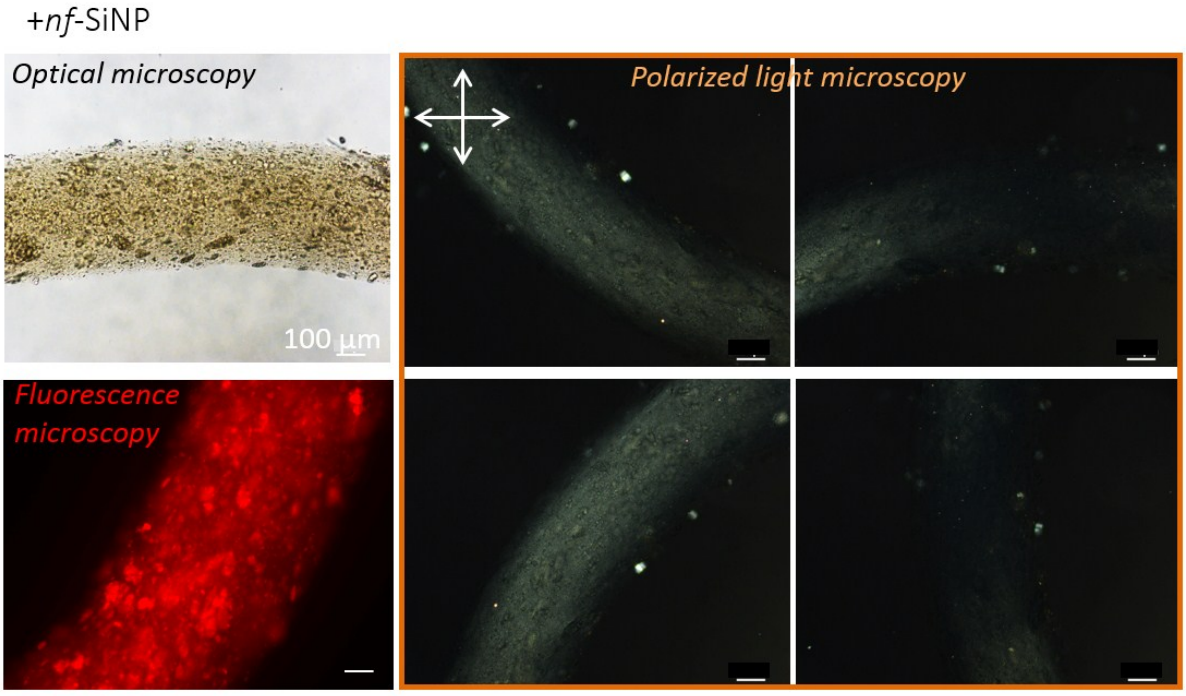

Fig. S6. Collagen + *nf*-SiNP filament ([Collagen] = 13.5 mg.mL^{-1}; [SiNP] = 45 mg.ml^{-1}) observed by bright field, fluorescence and polarized light microscopies. PLM imaging was performed by taking 4 serial images with the sample oriented *versus* the cross polarizers (see white arrows) twice at extinction and at maximal transmission (every 45°). Scale bars 100 µm.

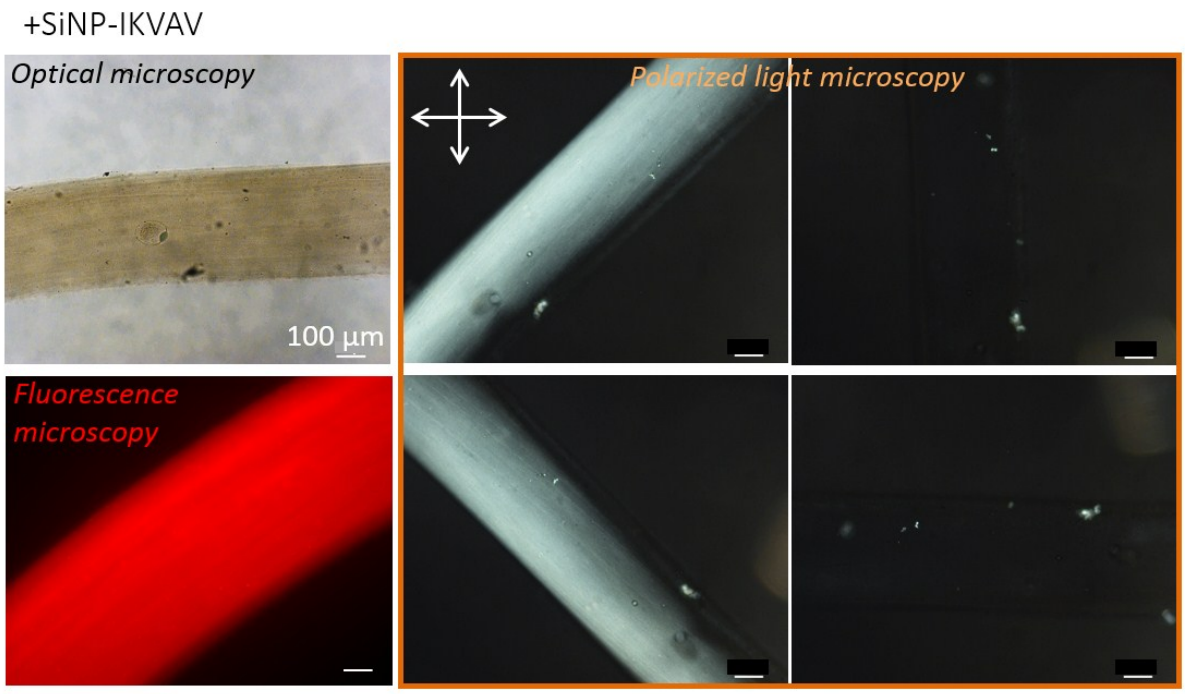

Fig. S7. Collagen + SiNP-IKVAV filament ([Collagen] = 13.5 mg.mL^{-1}; [SiNP] = 45 mg.ml^{-1}) observed by bright field, fluorescence and polarized light microscopies. PLM imaging was performed by taking 4 serial images with the sample oriented *versus* the cross polarizers (see white arrows) twice at extinction and at maximal transmission (every 45°). Scale bars 100 µm.

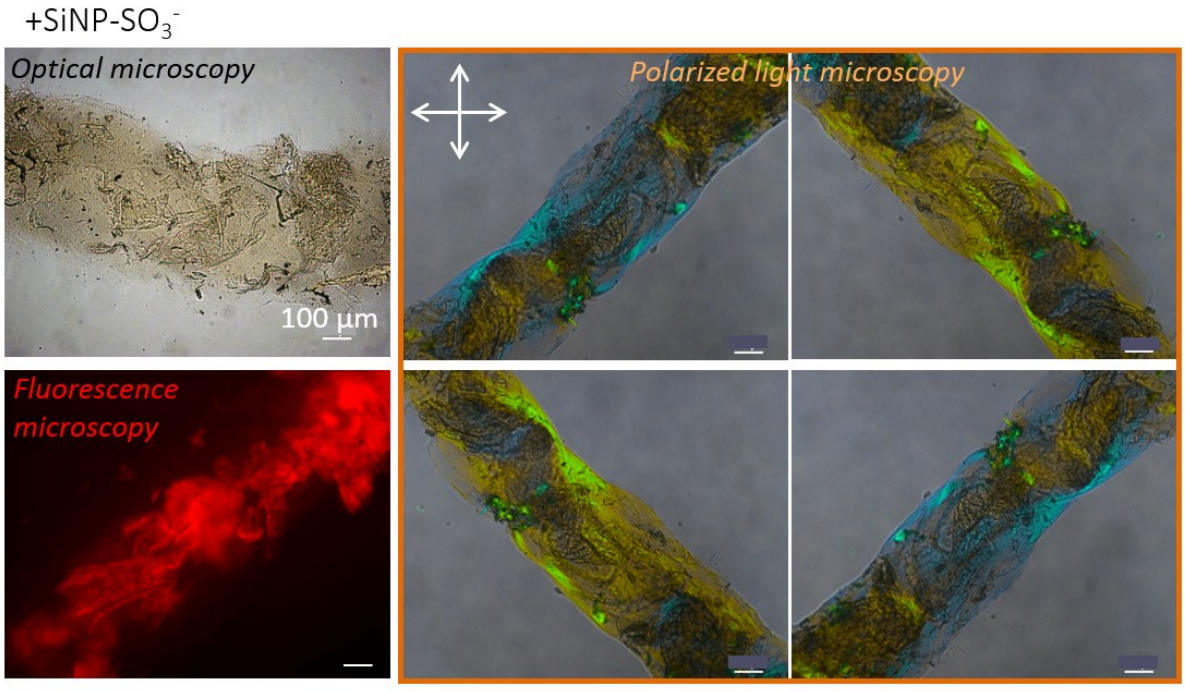

Fig. S8. Collagen + SiNP-SO_3^- filament ([Collagen] = 13.5 mg.mL^{-1}; [SiNP] = 45 mg.ml^{-1}) observed by bright field, fluorescence and polarized light microscopies. PLM imaging was performed by taking 4 serial images with the sample oriented *versus* the cross polarizers (see white arrows) twice at extinction and at maximal transmission (a waveplate was added in this case to improve contrast). Scale bars 100 µm.

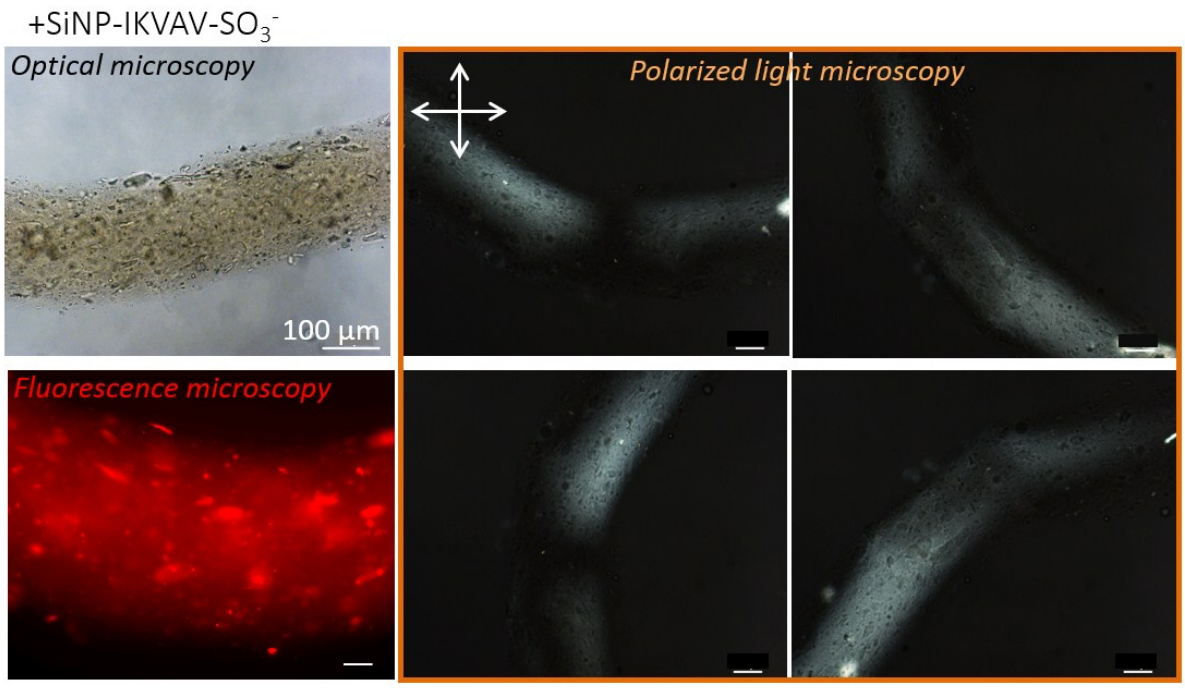

Fig. S9. Collagen + SiNP-IKVAV-SO_3^- filament ([Collagen] = 13.5 mg.mL^{-1}; [SiNP] = 45 mg.ml^{-1}) observed by bright field, fluorescence and polarized light microscopies. PLM imaging was performed by taking 4 serial images with the sample oriented *versus* the cross polarizers (see white arrows) twice at extinction and at maximal transmission (every 45°). Scale bars 100 µm.

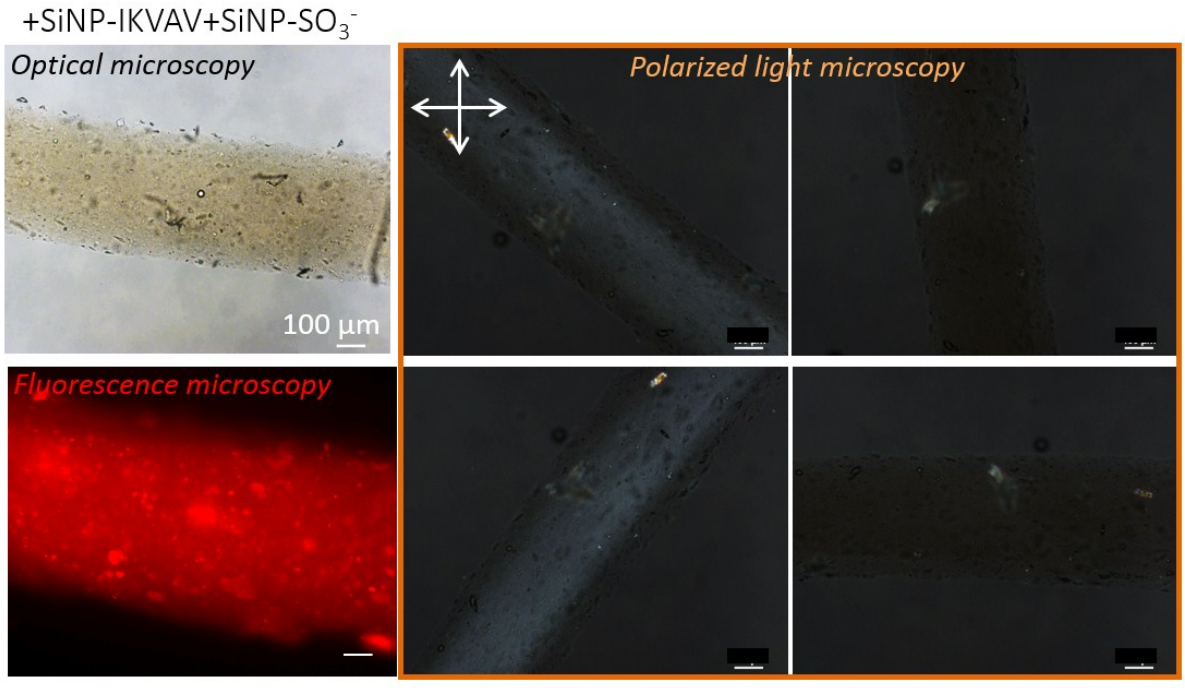

Fig. S10. Collagen + SiNP-IKVAV + SiNP-SO_3^- filament ([Collagen] = 13.5 mg.mL^{-1}; [SiNP] = 45 mg.ml^{-1}) observed by bright field, fluorescence and polarized light microscopies. PLM imaging was performed by taking 4 serial images with the sample oriented *versus* the cross polarizers (see white arrows) twice at extinction and at maximal transmission (every 45°). Scale bars 100 µm.

4. Characterization of the filaments by polarization-resolved second harmonic generation microscopy

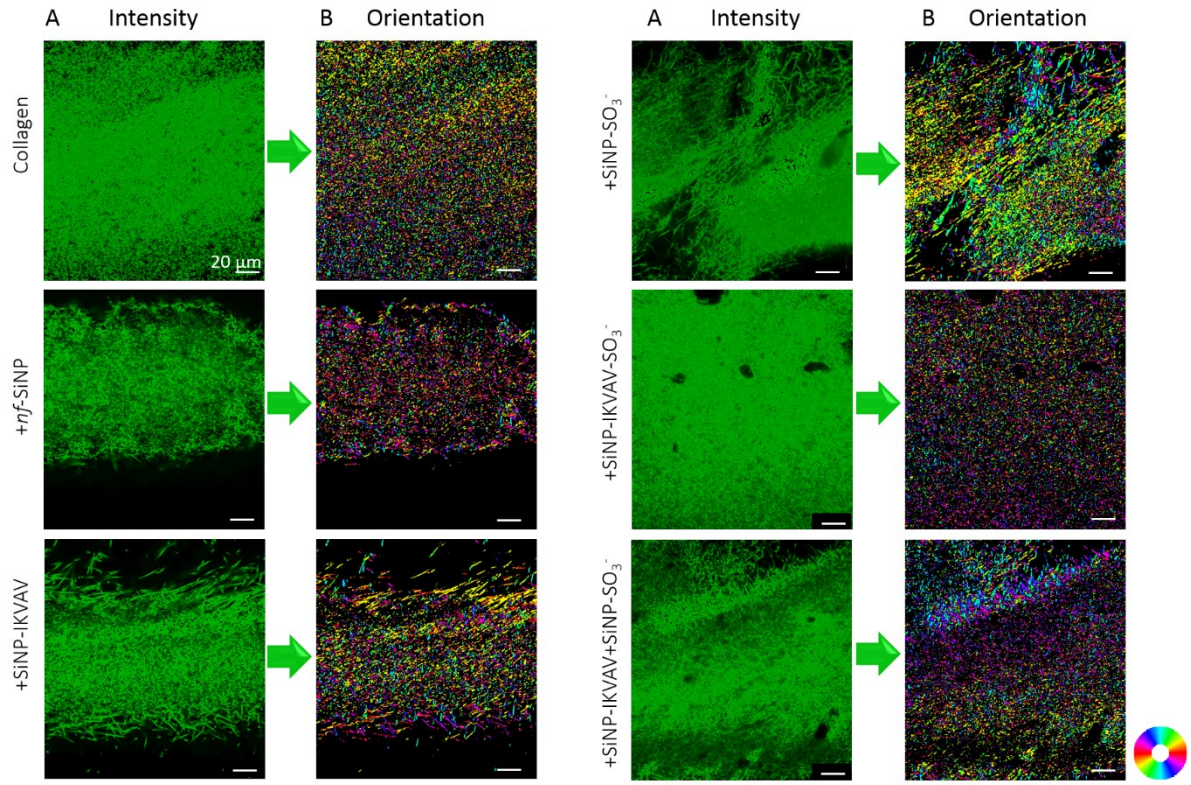

Fig. S11. Polarization resolved SHG(pSHG) imaging (A) averaged intensity and (B) orientation of Collagen-SiNP filaments ([Collagen] = 13.5 mg.mL-1, [particles] = 45 mg.mL-1). The orientation in the pSHG image is displayed on the colored circular scale (on the bottom right line). Scale bars 20 µm.

The structural specificity of SHG images has been further improved by acquiring polarization-resolved images (pSHG), which measures the main orientation of collagen in the imaging plane, in every pixel of the SHG image.[5] Given that each color codes for a given direction, the presence of dots with many different colors throughout the whole image shows that the fibrils of native collagen are small and randomly distributed with respect to one another, with an averaged orientation in the filament axis. Qualitative differences may be noted for the filament incorporating SiNP-SO_3^- that exhibits larger collagen structures, better identified on the representation of orientations.

[1] Gobeaux, F.; Mosser, G.; Anglo, A.; Panine, P.; Davidson, P.; Giraud-Guille, M.-M.; Belamie, E. Fibrillogenesis in Dense Collagen Solutions: A Physicochemical Study. Journal of Molecular Biology 2008, 376 (5), 1509–1522.
[2] Marschall, R.; Bannat, I.; Caro, J.; Wark, M. Proton Conductivity of Sulfonic Acid Functionalised Mesoporous Materials. Microporous and Mesoporous Materials 2007, 99 (1–2), 190–196.
[3] Rodriguez-Enfedaque, A.; Bouleau, S.; Laurent, M.; Courtois, Y.; Mignotte, B.; Vayssière, J.-L.; Renaud, F. FGF1 Nuclear Translocation Is Required for Both Its Neurotrophic Activity and Its P53-Dependent Apoptosis Protection. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 2009, 1793 (11), 1719–1727.
[4] Bouleau, S.; Pârvu-Ferecatu, I.; Rodriguez-Enfedaque, A.; Rincheval, V.; Grimal, H.; Mignotte, B.; Vayssiere, J.-L.; Renaud, F. Fibroblast Growth Factor 1 Inhibits P53-Dependent Apoptosis in PC12 Cells. Apoptosis 2007, 12 (8), 1377–1387.
[5] Teulon, C.; Tidu, A.; Portier, F.; Mosser, G.; Schanne-Klein, M.-C. Probing the 3D structure of cornea-like collagen liquid crystals with polarization-resolved SHG microscopy. Opt. Express 2016, 24 (14), 16084-16098.

