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Modulation of macrophage phenotype and protein secretion via heparin-IL-4 functionalized supramolecular elastomers

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ABSTRACT

Hallmark of the *in situ* tissue engineering approach is the use of bioresorbable, synthetic, acellular scaffolds, which are designed to modulate the inflammatory response and actively trigger tissue regeneration by the body itself at the site of implantation. Much research is devoted to the design of synthetic materials modulating the polarization of macrophages, which are essential mediators of the early stages of the inflammatory response. Here, we present a novel method for the functionalization of elastomers based on synthetic peptide chemistry, supramolecular self-assembly, and immobilization of heparin and interleukin 4 (IL-4), which is known to skew the polarization of macrophages into the wound healing “M2” phenotype. Ureido-pyrimidinone (UPy)-modified chain extended polycaprolactone (CE-UPy-PCL) was mixed with a UPy-modified heparin binding peptide (UPy-HBP) to allow for immobilization of heparin, and further functionalization with IL-4 via its heparin binding domain. As a first proof of principle, CE-UPy-PCL and UPy-HBP were premixed in solution, dropcast and exposed to primary human monocyte-derived macrophages, in the presence or absence of IL-4-heparin functionalization. It was demonstrated that the supramolecular IL-4-heparin functionalization effectively promoted macrophage polarization into an anti-inflammatory phenotype, in terms of morphology, immunohistochemistry and cytokine secretion. Moreover, the supramolecular functionalization approach used was successfully translated to 3D electrospun scaffolds for *in situ* tissue engineering purposes, where UPy-HBP retention, and heparin and IL-4 attachment to the supramolecular scaffolds were proven over 7 days. Lastly, human monocyte-derived macrophages were cultured on 3D scaffolds, which, in case of IL-4-heparin functionalization, were proven to promote of an anti-inflammatory environment on protein level. This study presents a novel method in designing a versatile class of functionalized elastomers that effectively harness the anti-inflammatory behavior of macrophages *in vitro*, and as such, may be instrumental for the development of a new class of synthetic materials for *in situ* tissue engineering purposes.

Statement of Significance

Macrophages and their phenotypic and functional plasticity play a pivotal role in metabolic homeostasis and tissue repair. Based on this notion, bioactivated materials modulating macrophage polarization were extensively investigated in the past. Here, we designed immunomodulating, synthetic materials based on supramolecular immobilization of a heparin binding peptide, and further bioactivation with heparin and IL-4, an anti-inflammatory cytokine responsible for M2 activation and polarization. Human monocyte-derived macrophages cultured on heparin-IL-4 bioactivated materials displayed an elongated morphology and an anti-inflammatory phenotype, with downregulation of pro-inflammatory cytokines and

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promotion of anti-inflammatory cytokines over time. This study represents the first step in designing a novel class of synthetic, bioactivated materials that harness the regenerative behavior of host macrophages towards *in situ* tissue regeneration.

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1. Introduction

Acellular synthetic grafts with immunomodulatory capabilities represent a promising, off-the-shelf solution to accelerate cellular infiltration, promote selective recruitment and enhance functional tissue regeneration *in situ* [1]. Recent advances in supramolecular polymers allowed for the development of cell-responsive scaffolds which can interact with their environment and mediate the host response to the biomaterial [2–4]. In a supramolecular approach, bioactive ureido-pyrimidinone (UPy)-modified peptide sequences are mixed in and matched to UPy-polymers via dimerization of self-complementary quadruple hydrogen bonding. Capitalizing on this, we designed a method for the functionalization of supramolecular materials based on the immobilization of heparin, which is known to bind to several growth factors via their heparin binding domain [5–8].

In previous work, heparin was used as a delivery agent for sustained release of growth factors for angiogenic purposes [9–11], or to enhance signaling in nerve regeneration [12]. Several strategies have been investigated for the conjugation of heparin to synthetic materials, such as incorporation in solution prior to electrospinning [13], covalent immobilization on electrospun structures *a posteriori* via a polyethylene glycol (PEG) linker [14,15], and end-point immobilization [16,17] on plasma-treated surfaces [18]. Interestingly, strategies based on the synthesis of peptides with high affinities for heparin were introduced [19,20], which allowed for the use of heparin as base polymer for the nucleation of hydrogel networks with improved binding efficiency and local retention of heparin due to the peptide presence [20]. Taking inspiration from those studies, we designed a UPy-modified heparin binding peptide (UPy-HBP) which was used for the functionalization of a degradable, supramolecular elastomer suitable for a broad variety of tissue engineering applications, e.g. cardiovascular tissue engineering [3,4]. After processing by dropcast or electrospinning, the supramolecular elastomers were functionalized with heparin and interleukin-4 (IL-4), which was previously shown to bind to heparin [7,21] (Fig. 1).

At the cellular level, IL-4 is known to influence the early stages of the inflammatory response upon implantation of synthetic materials, by modulating the activation and polarization of macrophages. Macrophages can acquire several phenotypes, with pro-inflammatory “M1” or anti-inflammatory “M2” macrophages being the extremes of a broad range of phenotypes [22], changing in response to growth factors and external cues such as cytokines. Particularly, IL-4 was shown to induce anti-inflammatory macrophages, amplifying their response via IL-4 induced secretion of anti-inflammatory cytokines, e.g. interleukin-10 (IL-10), transforming growth factor- β 1 (TGF- β 1) and matrix metalloproteinase-9 (MMP-9) [23,24]. Concurrently, it was demonstrated that IL-4 decreases the pro-inflammatory macrophages activation, encouraging the re-entering of the cell cycle towards an anti-inflammatory and healing environment [22,25–27]. The acknowledgment of anti-inflammatory potential for IL-4 paved the way to the development of IL-4 delivery systems for the modulation macrophage polarization in several tissue engineering applications [28–31]. In those studies, IL-4 was either entrapped into hydrogel based materials [28,29], biotinylated and attached to biotinylated, decellularized scaffolds using streptavidin as a linker [30], or linked via electro-

static interactions to non-degradable, layer-by-layer deposited synthetic meshes [31]. However, IL-4 functionalization of degradable elastomers has not yet been achieved.

The aim of the present study was manifold: (1) to demonstrate the feasibility of the IL-4 functionalization of supramolecular materials via the synthesis and use of a UPy-modified heparin binding peptide; (2) to investigate the immunomodulatory properties of the IL-4 and heparin functionalized materials in terms of macrophage polarization and production of pro- and anti-inflammatory cytokines; and (3) to translate the proposed approach to the processing of synthetic materials for cardiovascular tissue engineering purposes.

2. Materials and methods

2.1. Heparin binding peptide synthesis

The UPy-HBP peptide (GLRKKLGKA) (Fig. 1, Table 1) was manually synthesized via Fmoc solid phase peptide synthesis (SPPS) on an Fmoc-Rink-Amide resin (Novabiochem) at a 400 μ mol scale. The resin was allowed to swell for 15 min in N-methylpyrrolidone (NMP). Fmoc deprotection was done with 5 mL 20% (v/v) piperidine in NMP and incubated for 5 min, twice. Amino acids (4 eq.) (Novabiochem) were activated with 2-(1H-Benzotriazole-1-yl)-1,1,3,3-Tetramethyluronium hexafluorophosphate (HBTU) (3.9 eq.) in NMP and alkylated with N,N-diisopropylethylamine (DIPEA) (8 eq.) in a total volume of 12 mL. Amino acids were coupled once for 30 min. The final amino acid (Phe) was Boc-protected. All steps were performed under constant agitation and with washing steps with NMP (4 mL) in between. After the peptide coupling, UPy-COOH was coupled at the N-terminus while the peptide was still on the resin using PyBOP and DIPEA in DMF overnight. In a final step, the UPy-peptide was cleaved from the resin using 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS) and 2.5% H₂O for 3 h at room temperature. The cleaved UPy-peptide was precipitated in ice-cold diethylether, centrifuged (2000 rpm, 10 min) and dissolved in MeCN/H₂O (1:6, v/v %). The cleaved UPy-peptide was purified using a preparative LC-ESI-MS system, equipped with a reverse phase C18 column. After filtration, the UPy-peptide was purified using a linear gradient of 30–35% MeCN/H₂O with 0.1% TFA and a flow of 20 mL·min⁻¹. The final purity of the UPy-peptide was determined with an analytical LCMS using a 5–70 MeCN gradient in H₂O with 0.1% formic acid (FA) in a final yield of 42%. After lyophilization, the purified UPy-peptide was stored at –30 °C.

2.2. Supramolecular films: dropcast

Chain extended UPy-polycaprolactone (CE-UPy-PCL; Suprapolix BV, Eindhoven, the Netherlands, Mn = 14.7 kg/mol), Mw = 36.8 (kg/mol) (Fig. 1) and CE-UPy-PCL pre-mixed with UPy-HBP (CE-UPy-PCL+UPy-HBP) were dissolved in hexafluoro-2-propanol (HFIP) at 20 mg/mL and stirred overnight at room temperature. 70 μ L of solution were dropcast on 0.7 cm² chamberslides (Nunc Lab-Tek II Chamber slide system, ThermoScientific, US). The HFIP was evaporated overnight and residual solvent removed in vacuo at 40 °C overnight. The dropcast films were UV-sterilized for 1 h and incubated with serum free medium overnight. Human

Fig. 1. Chemical structures of the chain extended UPy-polycaprolactone (CE-UPy-PCL (A) and the UPy-HBP (B). Schematic representation of the processing and IL-4 and heparin functionalization of dropcast or electrospun CE-UPy-PCL+UPy-HBP (C). The solutions of CE-UPy-PCL and UPy-HBP were premixed before processing into drop cast films and/or electrospun scaffolds. The heparin-IL-4 functionalization is obtained via incubation of the pre-mixed solution of heparin and IL-4 onto the CE-UPy-PCL+UPy-HBP material.

Table 1

Coupling order of amino acids during synthesis of the UPy-heparin binding peptides, from C-terminus to N-terminus. Protecting groups of sidegroups are: *tert*-butoxycarbonyl (Boc), trityl (Trt), and 2,2,4,6,7-pentametyldihydrobenzofuran-5-sulfonyl (Pbf).

Name	Sequence	Coupling order of synthesis
UPy-HBP	GLRKKLGKA	Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Gly-OH

¹H NMR (400 MHz, DMSO-*d*₆): δ 11.56 (s, 1H), 9.70 (s, 1H), 8.17–7.00 (m, 15H), 5.75 (s, br, 2H), 4.34–4.30 (q, 1H), 4.24–4.14 (m, 6H), 4.04–4.02 (t, 2H), 3.93 (s, 2H), 3.85–3.65 (2d, 2H), 3.61–3.51 (m, 22H), 3.15–3.08 (m, 4H), 2.96–2.91 (m, 6H), 2.76–2.67 (m, 6H), 2.10 (s, 3H), 1.71–1.21 (m, 47H), 0.89–0.82 (m, 12H). ESI-MS (positive mode): *m/z* calcd. for [M+H]⁺ 1164.4; found: 583.1 [M+2H]²⁺. HPLC: 5.30 min, *m/z*: 583.08 [M+2H]²⁺, 1164.67 [M+H]⁺, 1186.58 [M+Na]⁺.

recombinant IL-4 (Peprotech, Rocky Hill, NJ) was diluted in 20 ng/mL of heparin sodium salt (Sigma-Aldrich, St. Louis, MO) in Hank's balanced salt solution (HBSS) at two different concentrations: 0.2 ng/mL and 20 ng/mL. IL-4 and heparin were mixed on a rollerbank for 30 min to allow pre-complexation. Dropcast films were incubated with the IL-4-heparin solutions overnight at 37 °C. Prior to cell seeding, the construct were washed twice with HBSS to remove unbound heparin and IL-4.

2.3. Dropcast characterization: atomic force microscopy, liquid chromatography-mass spectrometry and water contact angle measurements

The supramolecular morphology of the CE-UPy-PCL upon addition of the UPy-HBP was investigated via atomic force microscopy (AFM). AFM samples were prepared by dropcasting 70 µL of CE-UPy-PCL or CE-UPy-PCL+UPy-HBP (0.5 mol%, 1 mol%) solution (50 mg/mL in HFIP) on 13 mm glass cover slips. AFM images were recorded using a Digital Instrument Multimode Nanoscope IV using silicon cantilever tips (PPPNCR-50, 204–497 kHz, 10–130 N/m) operating in the tapping regime with a scan rate of 1 Hz. Images were further analysed using Gwyddion 2.43. The root mean square roughness was calculated for an area of 100 µm².

The release of UPy-HBP from the dropcast CE-UPy-PCL+UPy-HBP was measured via analytical reversed phase high pressure liquid chromatography-mass spectrometry (LC-MS) on a system consisting of the following components: Shimadzu SCL-10A VP system controller, Shimadzu LC-10AD VP liquid chromatography pumps (with an Alltima C18 3u (50 mm × 2.1 mm) reversed phase column and gradients of water-acetonitrile supplemented with 0.1% (v/v) formic acid), a Shimadzu DGU 20A3 prominence degasser, a Thermo Finnigan surveyor auto sampler, a Thermo Finnigan surveyor detector and Thermo Scientific LCQ Fleet. A standard curve was prepared by dissolving the UPy-HBP in Milli-Q, supplemented

Table 2

Markers selected for immunostainings and macrophage phenotype investigation.

Group N = 3	Marker	Cell	Function	Primary antibody	Secondary antibody
A	CD86	MCSF-treated macrophages	Pan macrophage	Rabbit anti-human IgG (Abcam)	Donkey anti-rabbit IgG A488 (Invitrogen)
	CCR7	IFN- γ treated macrophages	Pro-inflammatory	Goat anti-human IgG (Novus Biologicals)	Donkey anti-goat IgG A555 (Invitrogen)
	CD206	IL-4 treated macrophages	Anti-inflammatory	Mouse anti-human IgG1 (AbD-serotec)	Donkey anti-mouse IgG A647 (Jackson IR)
B	CD86	MCSF-treated macrophages	Pan macrophage	Rabbit anti-human IgG (Abcam)	Donkey anti-rabbit IgG A488 (Invitrogen)
	CD64	IFN- γ treated macrophages	Pro-inflammatory	Mouse anti-human IgG1, k (Abcam)	Goat anti-mouse IgG1 A647 (Molecular probes)
	CD163	MCSF/IL-10/IL-4 treated macrophages	Anti-inflammatory	Mouse anti-human IgG2a (LS BioSciences)	Goat anti-mouse IgG2a A555 (Molecular probes)

Table 3

Proteins analyzed via Multiplex ELISA.

Protein	Symbol	Function
Interleukin 10	IL-10	Anti-inflammatory factor; Inhibitor of TNF- α , IFN- γ , IL-1 β .
Transforming growth factor β 1	TGF- β 1	Anti-inflammatory factor; downregulation of inflammatory cytokine in monocytes and macrophages; MMP9-dependent activation.
Matrix Metalloproteinase 9	MMP-9	Anti-inflammatory factor, involved in ECM breakdown and remodelling.
Monocyte chemoattractant protein 1	MCP-1	Pro-inflammatory factor; chemotactic for monocytes and undifferentiated macrophages.
Tumor necrosis factor α	TNF- α	Pro-inflammatory factor; regulator of apoptotic cell death.
Interleukin 6	IL-6	Pro-inflammatory factor.

with 0.1% (v/v) formic acid, at the following concentrations: 50, 10, 5, 1, 0.5, 0.2 and 0.1 μ g/mL. The total ion count for the four most abundant hydrogen-adducts of the UPy-HBP was calculated with the genesis algorithm. Dropcast samples were incubated with 500 μ L of Milli-Q for 7 days at 37 °C. The solution was supplemented with 0.1% formic acid prior to measuring with LC-MS.

Possible changes in the wettability levels of CE-UPy-PCL due to the mixing with UPy-HBP were investigated performing water contact angle measurements (WCA). Dropcast samples were prepared by pipetting 70 μ L of CE-UPy-PCL or CE-UPy-PCL-HBP (0.5 mol%, 1 mol%, 2 mol%, 4 mol%) solution (50 mg/mL in HFIP) on 13 mm glass cover slips. Samples were analysed with a contact angle system OCA30 from DataPhysics using the SCA20 software. 5 μ L drop of deionized water was centred on top of each dropcast film, and images were captured at a rate of 25 frames per second. Contact angles were determined at the polymer-air-water interface 60 s after the drop's placement. Five samples were measured per condition and results were presented as the mean $\pm$ standard error of the mean (SEM).

2.4. Monocytes isolation and seeding in dropcast films

Human peripheral blood buffy coats were obtained from three healthy donors under informed consent (Sanquin, Nijmegen, The Netherlands). Buffy coats were diluted in 0.6% (w/v) sodium citrate in PBS, and the peripheral mononuclear cell fraction (hPBMCs) was isolated using density gradient centrifugation on *iso*-osmotic medium (Lymphoprep, Axis-Shield, Oslo, Norway). Afterwards, monocytes were isolated with a double step density gradient centrifugation, as reported elsewhere [32]. Briefly, the hPBMCs suspension was layered on a high density hyper-osmotic solution (10% (v/v) 1.6 M NaCl; 41.5% (v/v) water; 48.5% (v/v) Percoll by Sigma-Aldrich) and centrifuged to allow for monocytes separation from lymphocytes. Subsequently, the isolated fraction was layered on a low density *iso*-osmotic solution (10% (v/v) 1.5 M NaCl; 48.5% (v/v) water; 41.5% (v/v) Percoll by Sigma-Aldrich) and centrifuged to separate monocytes from platelets and dead cells. The monocyte

layer was collected, repetitively washed, and resuspended in RPMI medium supplemented with 10% (v/v) FBS and 1% (v/v) Pen/Strept. The purity of the isolated monocyte fraction was assessed with flow cytometry. Cell populations were quantified by gating the lymphocyte and monocyte clouds in the forward scatter-side scatter plots, which combined formed the total hPBMC gate. The monocyte fraction was normalized to the total hPBMCs gate. The monocyte fraction before and after isolation was measured, and the efficiency of the isolation procedure calculated. Data analysis was performed using the Guava Express Pro software package, combined with FCS Express (De Novo Software, Los Angeles, CA). Isolated monocytes were seeded on IL-4-heparin functionalized films and their relative controls at a seeding density of 1.4×10^6 cells/cm², with and without 100 ng/mL of macrophage colony stimulating factor (MCSF; Peprotech, Rocky Hill, NJ) to induce monocyte-to-macrophage differentiation, and medium, with or without MCSF, was refreshed every 2 days.

2.5. Immunofluorescence for cell seeded dropcast films

At day 3 and day 7 of culture, macrophage morphology was observed with light microscopy and via actin staining (TRITC-Phalloidin, dilution 1/200, Sigma Aldrich). Moreover, cell phenotype was determined via immunofluorescence analysis using the antibodies listed in Table 2.

Formaldehyde-fixed samples were washed in PBS and permeabilized in 0.5% Triton X-100 in PBS (Merck Serono). Non-specific binding was blocked by incubation with 10% (w/v) bovine serum albumin (Sigma-Aldrich) in PBS. Cells were incubated overnight at 4°C with primary antibodies (1:100 v/v) in 1% bovine serum albumin in PBS (Table 2). The films were then washed and incubated for 60 min with the secondary antibodies (1:300 v/v) (Table 2), and subsequently stained with 4',6-diamidino-2-phenyl indole (DAPI, Sigma-Aldrich). After washing steps, the stained constructs were directly visualised with an inverted fluorescent microscope (Zeiss Axiovert 200 M, Carl Zeiss Light Microscopy, Göttingen Germany).

2.6. Protein production for cell seeded dropcast films: multiplex ELISA

Based on previous literature, a panel of relevant pro-inflammatory and anti-inflammatory cytokines was selected (Table 3), and the individual secretion levels quantified via multiplex ELISA.

Particularly, at day 3 and day 7 of culture, the supernatants of monocytes cultured with or without MCSF (Supplementary materials) were collected and protein secretion levels analysed using an in-house developed and validated multiplex immunoassay based on Luminex technology, at the Multiplex core facility of the Laboratory for Translational Immunology of the University Medical Center Utrecht, The Netherlands. Samples were incubated with antibody-conjugated MagPlex microspheres (BioRad, Hercules, CA) for 1 h at room temperature with continuous shaking, followed by 1 h incubation with biotinylated antibodies, and 10 min incubation with phycoerythrin-conjugated streptavidin diluted in high performance ELISA (HPE) buffer (Sanquin). Acquisition was performed using a FLEXMAP 3D system controlled with xPONENT 4.1 software (Luminex, Austin, TX). Data was analyzed by 5-parameter curve fitting using BioPlex Manager software (version 6.1.1, Biorad). Cytokines measurements were corrected for the amount of attached cells in each experimental group. Cells were counted with ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA), counting the DAPI stained nuclei on a minimum of 5 microscope images at 20X magnification per group. The normalized concentrations were plotted as scatter dot plots, with each dot representing the normalized single measurement, with the mean of the different donors (Supplementary Materials, 1.4), or as percentages of average secretion over the three donors. Moreover, in order to obtain a relative M2/M1 index, the percentage of the mean production per cytokine was calculated, followed by dividing the mean percentage of M2 cytokines (IL-10, TGF- β , MMP-9) by the mean percentage of M1 cytokines (IL-6, TNF- α , MCP-1) per sample [33].

2.7. Supramolecular scaffolds: electrospinning

To produce 3D microporous scaffolds, CE-UPy-PCL, either with or without increasing concentrations of UPy-HBP (i.e. 0.5 mol%, 1 mol%, 2 mol%), was dissolved in chloroform (CHCl₃; Sigma) and Hexafluoro-2-propanol (HFIP; Sigma) (15% w/v polymer in CHCl₃/HFIP 80/20% v/v). After being stirred overnight at room temperature, the polymer solution was used to fabricate electrospun meshes on an IME technologies EC-CLI equipment (IME Technologies, Geldrop, The Netherlands). The polymer solution was dispensed via a syringe pump (PHD 22/2000, Harvard Apparatus, Holliston, MA) to a moving 14 G needle placed at 17 cm distance from a rotating cylindrical mandrel wrapped with aluminum foil (diameter, 25 mm; rotation speed, 100 rpm). The solution flowed at 35 μ L/min in presence of 18 kV voltage, at constant temperature of 23 °C and 30% relative humidity. The voltage of the collector was set to 0 kV for CE-UPy-PCL and –1 kV for CE-UPy-PCL+UPy-HBP. The resulting fibre diameter was measured with scanning electron microscopy (SEM, Quanta 600F, FEI, Eindhoven, The Netherlands), and averaged over 10 fibres per image (250X magnification), with 5 images in total per scaffold. The overall thickness of the electrospun sheet was measured at 10 different locations with a high-power digital microscope (VHX-500FE, Keyence, Mechelen, Belgium). Results are presented as mean $\pm$ standard deviation.

2.8. Characterization of functionalized supramolecular scaffolds: LC-MS and heparin and IL-4 binding assays

To measure the possible release of UPy-HBP over time from electrospun CE-UPy-PCL+UPy-HBP, circular scaffolds (diameter, 8

mm) were punched out of the electrospun meshes and incubated in Milli-Q for 7 days. The amount of UPy-HBP released was measured via LC-MS, as described for dropcast films in Section 2.2.

In order to indirectly assess the functionality of the UPy-HBP after electrospinning and the possibility for heparin immobilization, a heparin binding assay was performed on CE-UPy-PCL+UPy-HBP with increasing concentrations of UPy-HBP (0.5 mol%, 1 mol%, 2 mol%), and results compared to pristine CE-UPy-PCL. Scaffolds were secured to the bottom of a black 96-wells plate (MicroFluor, ThermoScientific). 20 ng/mL of Heparin-fluorescein isothiocyanate (Heparin-FITC) in PBS was loaded onto the scaffolds, and the well plate incubated overnight at 37 °C. Afterwards, the supernatant was collected and used for indirect assessment of bound heparin-FITC via fluorescent analysis, using a plate reader (Fluoroskan Ascent FL, ThermoScientific) at wavelengths $\lambda_{\text{ex}}/\lambda_{\text{em}}$ of 485 nm/538 nm.

CE-UPy-PCL+UPy-HBP (0.5 mol%) scaffolds functionalized with 0.2 ng/mL of IL-4 in 20 ng/mL of heparin in HBSS were tested for IL-4 retention over time in static conditions and under flow. Briefly, the IL-4, heparin and HBSS were first mixed on a rollerbank for 30 min to allow for pre-complexing. Scaffolds strips of 1.5 mm $\times$ 0.7 mm were cut out of the electrospun sheets and incubated with the IL-4-heparin solution overnight. The unbound IL-4 concentration was measured at different time points, i.e. 10 min, 30 min, 1 h, 1 day, 3 days and 7 days for both static and flow conditions, using an IL-4 specific ELISA kit (Human IL-4 ELISA kit, BioSource International, Camarillo, CA), according to the manufacturer's instructions. For the flow experiments, the functionalized scaffolds were mounted into a parallel-plate flow chamber, within an in-house developed mesofluidics setup previously described (Smits et al., 2012). Serum free medium was added to the syringes of a Fluidic Unit (ibidi GmbH, Martinsried, Germany) with a total volume of 10 mL per sample. Serum free medium was flushed along the scaffold in a pulsatile flow using a modified pressure pump (ibidi GmbH). Flow conditions were set at a frequency of 1 Hz, with peak shear stress and peak pressure of approximately 1.6 Pa and 100 mm Hg, respectively, resembling typical hemodynamic conditions of small-diameter arteries [34]. Medium samples were collected during flow via in-line injection ports (ibidi GmbH) on the outlet port of the flow chambers at the above-mentioned time points.

2.9. Monocytes isolation and seeding in supramolecular scaffolds

Human monocytes were isolated from human peripheral blood buffy coats of the three healthy donors as described in Section 2.4. Circular scaffolds with a diameter of 8 mm were punched out of the CE-UPy-PCL and the CE-UPy-PCL+UPy-HBP electrospun meshes and fixed to the bottom of a sterile, membrane-free Transwell insert (Corning Transwell polyester membrane cell culture inserts, 6.5 mm Transwell with 3.0 μ m pore polyester membrane insert, Corning, Sigma-Aldrich) with a custom made sterile polyether ether ketone (PEEK) ring. The obtained constructs were sterilized in UV for 5 mins on both sides, inserted in 12-well plates and incubated overnight at 37 °C with different solutions. Particularly, CE-UPy-PCL+UPy-HBP scaffolds were incubated either with HBSS (n = 5 per donor), 20 ng/mL of heparin in HBSS (n = 5 per donor), or 0.2 ng/mL of IL-4 in 20 ng/mL of heparin in HBSS (n = 5 per donor). In addition, CE-UPy-PCL+UPy-HBP scaffolds were incubated with 0.2 ng/mL of IL-4 in HBSS solution (n = 5 per donor) to account for possible aspecific bindings of IL-4 to the CE-UPy-PCL+UPy-HBP. Prior to cell seeding, the constructs were washed twice with HBSS to remove unbound heparin and IL-4. 300 μ L of a monocyte suspension containing 2.5×10^6 monocytes were pipetted on top of each scaffold, and incubated at 37 °C for 15 mins to allow for cell adhesion. Subsequently, scaffolds were centrifuged at 150 G for 10 mins to allow for cell infiltration. After centrifugation, scaffolds

Fig. 2. AFM phase images of dropcast CE-UPy-PCL, CE-UPy-PCL + 0.5 mol% UPy-HBP, and CE-UPy-PCL + 1 mol % (scale bar 100 nm). Contrast levels of regions of interest for CE-UPy-PCL+UPy-HBP were adjusted for better visualization of nanofibre formation.

Table 4

Contact angle (Θ) at the air-water-material interface of CE-UPy-PCL and CE-UPy-PCL with increasing concentrations of UPy-HBP.

UPy-HBP (mol%) in CE-UPy-PCL	Water contact angle [Θ] in degrees [$^\circ$]
0	68.70 $\pm$ 0.67
0.5	66.86 $\pm$ 1.79
1	67.20 $\pm$ 1.23
2	69.40 $\pm$ 1.27
4	70.74 $\pm$ 1.02

were transferred to new 12-well plates containing 1.5 mL of fresh medium and incubated up to 7 days at 37 $^\circ$ C, and medium was refreshed every 2 days.

2.10. Protein secretion in supramolecular scaffolds

After 3 or 7 days of culture, the supernatant was collected, snap frozen and stored at -80°C until analysis. Secretion levels of pro- and anti-inflammatory cytokines (Table 3) were quantified via multiplex ELISA, as described in Section 2.6. Protein concentrations in the supernatant were corrected for the DNA amount of the corresponding scaffolds. To quantify the DNA content, scaffolds were sacrificed, snap frozen, and lyophilized overnight. After measuring the dry weight, the scaffolds were snap frozen again and dismembrated 3 times 30 s at 3000 rpm with a microdismembrator (Sartorius, Göttingen, Germany). A digestion buffer containing 100 mM phosphate buffer (500 mM), 5 mM L-cystein, 5 mM EDTA and miliQ, supplemented with 140 $\mu\text{g}/\text{mL}$ of papain from papaya latex (Sigma) was prepared. The dismembrated scaffold were digested in 500 μL of the digestion buffer overnight at 60 $^\circ\text{C}$. Ultimately, samples were centrifuged for 10 mins at 4 $^\circ\text{C}$, and the DNA content in the supernatant measured using the Qubit[®] dsDNA BR (Broad-Range) Assay Kit (ThermoFisher Scientific). The DNA amount was normalized for the dry weight of the scaffolds.

2.11. Statistical analysis

For the multiplex ELISA analysis in 2D and 3D, statistical analysis was performed with the software GraphPad Prism 5, using *t*-test, one-way or two-way ANOVA and a *post hoc* Bonferroni's multiple comparison test. Cells of three different donors were used. For each donor and for each condition, experiments were

performed in triplicate for dropcast films and quintuplicate for electrospun scaffolds. Data are presented as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. Dropcast characterization: atomic force microscopy, liquid chromatography-mass spectrometry and water contact angle measurements

AFM images of CE-UPy-PCL show truncated fibre-like structures attributed to the hard phase formed by UPy aggregation (Fig. 2). Notably, upon introduction of 0.5 mol% and 1 mol% of UPy-HBP in CE-UPy-PCL, large regions of phase separation were visible, and small regions of the fibre-like structures similar to those formed in the pristine polymer film. Addition of the UPy-HBP did not result in differences in surface roughness (Supplementary materials, Fig. A1). Importantly, LC-MS analysis revealed that, up to 7 days, the released mass of UPy-HBP from the CE-UPy-PCL +UPy-HBP was lower than 1.5% of the total amount of UPy-HBP initially incorporated into the dropcast films (Supplementary material, Section 1.1). Furthermore, the presence of UPy-HBP at the surface did not induce changes in the water contact angle of CE-UPy-PCL substrates (Table 4).

3.2. Cell characterization and phenotype assessment on dropcast films: immunostaining

Flow cytometry analysis revealed >93% monocyte purity after isolation of hPBMCs from 3 different donors (data not shown). After three days of culture, the monocyte-derived macrophages adhered onto the dropcast films, with mainly rounded cells for control groups (pristine CE-UPy-PCL, CE-UPy-PCL+UPy-HBP, heparin-functionalized CE-UPy-PCL+UPy-HBP, or IL-4 incubated CE-UPy-PCL+UPy-HBP) and a substantial fraction of cells exhibiting elongated, spindle-like morphology for CE-UPy-PCL+UPy-HBP films functionalized with either 0.2 ng/mL (Fig. 3) or 20 ng/mL of IL-4 in heparin (data not shown), regardless of the MCSF treatment. However, treatment of cells with MCSF resulted in higher cell attachment (Figs. A6 and A7).

Immunofluorescence analysis revealed that at day 3, regardless of the functionalization, seeded cells expressed predominantly pro-inflammatory markers (CCR7⁺, CD64⁺), while, at day 7, a switch towards markers of the anti-inflammatory phenotype was

Fig. 3. Actin staining of monocyte-derived macrophages cultured with MCSF (100 ng/mL) for 3 days (left) and 7 days (right), on dropcast CE-UPy-PCL+UPy-HBP, heparin functionalized CE-UPy-PCL+UPy-HBP (20 ng/mL of heparin in HBSS), CE-UPy-PCL+UPy-HBP incubated with IL-4 (0.2 ng/mL in HBSS), and CE-UPy-PCL+UPy-HBP functionalized with heparin and IL-4 (0.2 ng/mL of IL-4 in 20 ng/mL of heparin in HBSS). White arrows indicate the elongated morphology characteristic of anti-inflammatory macrophages (scale bar 50 μ m).

Fig. 4. Macrophage distribution and polarization in response to CE-UPy-PCL and IL-4/heparin functionalized CE-UPy-PCL+UPy-HBP (0.2 ng/mL of IL-4 in heparin) over time (day 3, day 7). Monocyte-derived, MCSF-treated (100 ng/mL) macrophages were stained for two different combinations of antibody: CD86/CCR7/CD206 (A) and CD86/CD64/CD163 (B) (scale bar 50 μ m).

detected, with enhanced expression of CD206 and CD163, and a downregulation of the pro-inflammatory markers (CCR7⁺, CD64⁺) for cells cultured on functionalized films, for both 0.2 ng/mL (Fig. 4) and 20 ng/mL of IL-4 in heparin (data not shown).

3.3. Protein production for cell seeded dropcast films

Overall, the presence of the UPy-HBP and heparin did not affect cytokine production per se, as demonstrated by the comparable

concentrations detected for cells cultured on bare CE-UPy-PCL and CE-UPy-PCL+UPy-HBP or heparin-incubated CE-UPy-PCL and CE-UPy-PCL+UPy-HBP, respectively (Fig. 5, Fig. A4). However, when looking at the % of average secretion, heparin incubated CE-UPy-PCL+UPy-HBP constructs significantly downregulated the production of IL-6 and MMP-9 when compared to heparin incubated CE-UPy-PCL films (Fig. 4). CE-UPy-PCL+UPy-HBP constructs functionalized with different doses of IL-4 in heparin, i.e. 0.2 ng/mL and 20 ng/mL, revealed time dependent immunomodulatory

Fig. 5. Heparin-induced protein secretion. Percentage of average secretion levels of pro- and anti-inflammatory cytokines produced by monocyte-derived macrophage treated with MCSF (100 ng/mL) and cultured for 3 days and up to 7 days on bare or heparin incubated (20 ng/mL) CE-UPy-PCL or CE-UPy-PCL+UPy-HBP for 3 donors, $n = 3$ samples for each donor. Percentages of average protein secretion were normalized for the total amount of attached cells for each group (*, ** denote significant differences of $p < 0.05$ and $p < 0.01$, respectively).

properties (Fig. 6, Fig. A5). Statistically significant downregulation of the pro-inflammatory cytokines IL-6 and MCP-1 was observed for increasing doses of IL-4 in heparin at day 3. At day 7, constructs functionalized with 20 ng/mL of IL-4 in heparin inhibited the production of the pro-inflammatory TNF- α , while no significant differences were detected in IL-6 and MCP-1 secretion for increasing doses of IL-4 in heparin. At day 3, cells seeded on IL-4-heparin functionalized constructs produced low levels of the anti-inflammatory cytokines IL-10 and TGF- β_1 . However, at day 7, increasing doses of IL-4 in heparin resulted in an increase in the anti-inflammatory cytokines IL-10 and MMP-9 and significant upregulation of the production of TGF- β_1 (Fig. 6, Fig. A5).

3.2. 3D supramolecular constructs: electrospinning, LC-MS and functionalization assays

Electrospun meshes with a thickness of $316 \pm 18.3 \mu\text{m}$, and an average fibre diameter of $5.17 \pm 0.4 \mu\text{m}$ were obtained for CE-UPy-PCL and CE-UPy-PCL+UPy-HBP (0.5 mol%). CE-UPy-PCL+UPy-HBP with higher concentrations of UPy-HBP gave comparable results (data not shown). SEM images showed an isotropic network of interconnected fibres, and no relevant changes in fibre morphology due to the presence of the UPy-HBP (Fig. 7A). LC-MS analysis revealed that less than 2% of the initially incorporated UPy-HBP in electrospun CE-UPy-PCL+UPy-HBP was released up to 7 days

Fig. 6. Dose dependent study. Percentage of average secretion of pro- and anti-inflammatory cytokines produced by monocyte-derived macrophage treated with MCSF (100 ng/mL) cultured for 3 days and up to 7 days on CE-UPy-PCL+UPy-HBP films functionalized with increasing doses of IL-4 in heparin, i.e. 0, 0.2 and 20 ng/mL. Concentrations were normalized for the total amount of attached cells for each group (*, **, *** denote significant differences of $p < 0.05$, $p < 0.01$, $p < 0.001$ respectively).

Fig. 7. 3D processing and bioactivation characterization. SEM Images of CE-UPy-PCL (top) and CE-UPy-PCL+UPy-HBP (bottom) electrospun meshes at two different magnifications. Scale bar 500 μm left, 200 μm right (A). Heparin binding assay on the supernatant of electrospun CE-UPy-PCL (0 mol%) and CE-UPy-PCL+UPy-HBP (0.5, 1, 2 mol% of UPy-HBP in CE-UPy-PCL) incubated with 10 $\mu\text{g/mL}$ heparin-FITC (**** denote significant differences of $p < 0.0001$) (B). Attachment of IL-4 to heparin coated CE-UPy-PCL+UPy-HBP (0.5 mol%) scaffolds measured over 7 days in static conditions and under flow (C).

(Supplementary Materials, 1.2). The heparin binding assay revealed a drastic reduction in the intensity of the fluorescent signal in the supernatant of CE-UPy-PCL+UPy-HBP when compared to pristine CE-UPy-PCL, indicating successful immobilization of heparin to the CE-UPy-PCL+UPy-HBP via the UPy-HBP (Fig. 7B). In addition, no significant difference in terms of heparin immobilization was shown when further increasing the concentration of the UPy-HBP from 0.5 mol% to either 1 mol% or 2 mol%. Given the results of the heparin-FITC assay, the IL-4 functionalization was tested for electrospun CE-UPy-PCL pre-mixed with 0.5 mol% of

UPy-HBP only. The IL-4-heparin functionalized electrospun scaffolds displayed excellent bioactivity retention over time, with 82% and 79% of IL-4 retention after 1 week for the static and flow regimes, respectively (Fig. 7C).

3.3. Protein production for cell seeded supramolecular scaffolds

At both day 3 and day 7, heparin functionalization of electrospun CE-UPy-PCL+UPy-HBP resulted in upregulation of the pro-inflammatory cytokine IL-6 and downregulation of the

Fig. 8. Percentage of average secretion of pro- and anti-inflammatory cytokines produced by monocyte-derived macrophage cultured for 3 days and up to 7 days on 3D electrospun CE-UPy-PCL+UPy-HBP films, CE-UPy-PCL+UPy-HBP films functionalized with 20 ng/mL of heparin in HBSS, CE-UPy-PCL+UPy-HBP films incubated with 0.2 ng/mL of IL-4 in HBSS, and CE-UPy-PCL+UPy-HBP films functionalized with 0.2 ng/mL of IL-4 in 20 ng/mL of heparin in HBSS. Concentrations were normalized for the DNA amount of the scaffolds for each group (*, **, *** denote significant differences of $p < 0.05$, $p < 0.01$, $p < 0.001$ respectively).

pro-inflammatory TNF- α and MCP-1 (Fig. 8). Instead, secretion levels of the anti-inflammatory cytokines IL-10, TGF- β_1 and MMP-9 were comparable for CE-UPy-PCL+UPy-HBP and heparin functionalized CE-UPy-PCL+UPy-HBP, at both day 3 and day 7. Cells cultured on IL-4 incubated CE-UPy-PCL+UPy-HBP showed similar trends as for the heparin functionalized scaffolds, when compared to the CE-UPy-PCL+UPy-HBP: upregulation of IL-6, and downregulation of TNF- α and MCP-1 at both day 3 and day 7. In addition, moderate downregulation of the anti-inflammatory IL-10 at day 3, and TGF- β_1 and MMP-9 at both day 3 and day 7 was observed for IL-4 incubated CE-UPy-PCL+UPy-HBP when compared to either heparin functionalized CE-UPy-PCL+UPy-HBP or CE-UPy-PCL+UPy-HBP. IL-4-heparin functionalization resulted in secretion levels of IL-6 which were lower than those of heparin functionalized or IL-4 incubated CE-UPy-PCL+UPy-HBP scaffolds, yet similar to the CE-UPy-PCL+UPy-HBP scaffolds, at both day 3 and day 7. Reversely, secretion levels of the pro-inflammatory TNF- α and MCP-1 for IL-4-heparin functionalized CE-UPy-PCL+UPy-HBP were comparable to both heparin functionalized or IL-4 incubated scaffolds, and significantly lower than those measured for CE-UPy-PCL+UPy-HBP scaffolds, at both day 3 and day 7. Moreover, IL-4-heparin functionalization resulted in upregulation of IL-10 at day 3, which was statistically higher than the concentration measured for IL-4 incubated CE-UPy-PCL+UPy-HBP, and significant overexpression of TGF- β_1 and MMP-9 at both day 3 and day 7 when compared to all the control groups. Lastly, the calculated M2/M1 ratio based on the % of average secretion of anti-inflammatory and pro-inflammatory cytokines was significantly higher for IL-4 heparin functionalized constructs at day 3, when compared to all the other control groups (see Fig. 8).

4. Discussion

In this study, we developed IL-4 and heparin functionalized supramolecular materials that show immunomodulatory properties *in vitro*, namely the biasing of macrophage polarization and the modulation of anti- and pro-inflammatory cytokines production over 7 days.

Upon identification of human IL-4 as a heparin-binding cytokine [7,21], IL-4 functionalized 2D and 3D materials were developed using a heparin-based approach. Particularly, a UPy-HBP was mixed with a UPy-modified PCL polymer via supramolecular UPy-UPy interactions. The UPy-HBP modified supramolecular polymer was further functionalized with heparin, which can bind

the UPy-HBP, and IL-4, which is known to bind to heparin via its heparin binding domain. Particularly, IL-4, like several cytokines and growth factors, e.g. interleukin-7 (IL-7), interferon- γ (IFN- γ), and basic fibroblast growth factor (bFGF), has been shown to bind to glycosaminoglycans (GAGs), a group of negatively charged molecules found in all tissue types as components of the extracellular matrix, the cellular membranes and in the secretory granules of many cell types [7,35,36]. The binding of proteins to GAGs involves basic amino acids (histidine, lysine, and arginine) which form electrostatic interactions with the negatively charged sulfate groups of GAGs. Several consensus sequences have been postulated, such as XBBXB, XBBBXXB, XBBBXXBBBXXBB, and TXXBXTBXXBTBB (where X represents hydrophobic or uncharged amino acids, B represents basic amino acids, and T defines a turn). These consensus sequences have been found in more than 20 known heparin-binding proteins [6]. Surprisingly, although IL-4 contains 26 basic amino acids, which are structural components of GAGs-binding sequences, it does not include any of the above mentioned sequences [7,21]. However, Lortat-Jacob et al. demonstrated the binding of IL-4 to GAGs, with higher affinity for N-sulfated GAGs, i.e. heparin and heparan sulfate [7] (the average heparin disaccharide contains ~ 2.7 sulfate groups, whereas heparan sulfate contains ≥ 1 sulfate group per disaccharide [37]).

When focusing on the characterization of the functionalization method proposed from a cellular point of view, we proved the successful immobilization of the heparin-IL-4 complex onto the supramolecular materials via the analysis of cellular morphology after differentiation, cell phenotype and protein secretion, both in 2D dropcast films and 3D electrospun elastomers. Previously, it was demonstrated that macrophage polarization is accompanied by specific changes in cell morphology and phenotype [38] and, particularly, that M2 macrophages exhibit an elongated shape compared to M1 macrophages when polarized *in vitro* [39]. In this study, cell morphological analysis revealed the presence of anti-inflammatory like elongated cells on IL-4-heparin functionalized dropcast films only, while mainly rounded pro-inflammatory like cells were observed for not functionalized dropcast films. Results were supported by immunofluorescent analysis, where, regardless of the functionalization, the expression of pro-inflammatory markers (CCR7, CD64) visible at day 3 was drastically reduced at day 7 on IL-4-heparin functionalized dropcast films only. These results are in line with the IL-4 dictated dampening of the M1 pro-inflammatory macrophages polarization previously proven *in vitro* [22,25–27]. Moreover, despite the fact that some of the differences in secretion levels of pro- and anti-inflammatory cytoki-

nes did not reach statistical significance, protein analysis revealed a trend towards an anti-inflammatory environment at day 7 for IL-4 functionalized constructs only, suggesting a switch from a pro-inflammatory environment to an anti-inflammatory one. These results indicate that the developed IL-4-heparin functionalized construct is an effective tool to mimic the well acknowledged sequential activation of pro- and anti-inflammatory macrophages displayed in the phased natural wound healing response, with predominance of M1 macrophages over M2 macrophages at early stages (1–3 days) of the wound healing process, and slow depletion of M1 and gradual replacement with M2 macrophages at later stages (4–7 days) [40].

The supramolecular approach proposed was successfully translated to electrospun meshes suitable for a broad variety of tissue engineering applications, e.g. cardiovascular tissue engineering [3,4,41,42]. CE-UPy-PCL was premixed with UPy-HBP and electrospun into 3D elastomers, which were validated for UPy-HBP release, and both heparin and IL-4 retention over time. The excellent retention of the UPy-HBP proven by LC-MS analysis, with less than 2% of the UPy-HBP released by electrospun scaffolds over time, allowed for successful immobilization of heparin, as demonstrated by the heparin binding assay performed *in vitro* on electrospun CE-UPy-PCL premixed with UPy-HBP. Notably, these results suggest that the functionality of the UPy-HBP was preserved after processing, namely the application of high voltages during the electrospinning procedure. The retained IL-4 functionalization over 7 days, as proven by the ELISA assay, was confirmed by secretion levels of a selected panel of pro- and anti-inflammatory cytokines analysed for human-derived macrophages cultured up to day 3 or day 7 on functionalized scaffolds. Similarly to the dropcast films, cells cultured on IL-4 heparin functionalized scaffolds showed overall downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines. However, while for dropcast films the downregulation of pro-inflammatory cytokines at day 3 was counteracted by upregulation of anti-inflammatory cytokines only at day 7, in case of functionalized scaffolds the IL-4-heparin upregulation of anti-inflammatory cytokines, and therefore, M2/M1 index, was detectable already at day 3. The observed discrepancy between the 2D and 3D scenarios might be explained by the higher surface area of the electrospun scaffolds, when compared to the dropcast films, and differences in spatial distribution of the heparin-IL-4 complex, i.e. on the surface of the dropcast films or along the fibres of the electrospun scaffolds.

The primary goal of this study was to investigate the functionalization method based on supramolecular and heparin chemistry, for the design of a novel class of immunomodulatory materials. Given the necessity to prove the feasibility of the proposed approach, the attention was focused toward a targeted intent: the polarization of macrophages and their cytokine secretions, which have long been identified as crucial mediators of the inflammatory response and, as such, as predictive parameters for the long-term host response to a biomaterial [28,43–46]. Particularly, previous work has proven a correlation between increased M2/M1 ratio in the early phases of the inflammatory response and enhanced long-term tissue regeneration [46]. Along this line, several immunomodulatory approaches revolving around the biasing of macrophage polarization have been developed, which were based, amongst others, on the entrapment and timely elution of IL-4 [28,30,31]. These studies demonstrate the effectiveness of IL-4 and, in general, of the targeting of the macrophage polarization for improved tissue remodelling outcomes. Clearly, the biomaterial-driven immune response elicited *in vivo* is a multi-phased process which encompasses the concerted interaction of several other immune cells than solely macrophages, e.g. neutrophils, mast cells, dendritic cells, and T cells [1]. For example, previously researchers have shown macrophage colocalization

with T cells in cellular aggregates, suggesting a tight communication with the adaptive immune system [47,48]. Particularly, it was demonstrated that subcutaneous implants activate a T helper 2 pathway that guides IL-4-dependent macrophage polarization and shape the immune microenvironment towards improved tissue regeneration [49]. Current studies in our group are focusing on the translation of the proposed method to *in vivo* applications for vascular tissue engineering. The aim of these studies is to investigate the downstream effects of early IL-4-dictated macrophage polarization on the long-term tissue formation and remodeling.

In the *in vivo* scenario, hemodynamic loads such as such as cyclic pressures, strains and shear stresses exerted *in vivo* have regulatory functions in the physiological or pathological functioning of the synthetic grafts implanted. Previous work has demonstrated the importance of flow conditions, and wall shear stress in particular, on the efficacy of graft functionalization [50]. In view of this, in addition to static conditions, the IL-4 retention was tested under dynamic conditions mimicking hemodynamic loads of small-diameter arteries *in vivo* [34]. The IL-4-heparin functionalized electrospun meshes were tested in a mesofluidic system, which was previously developed in our group [51], revealing successful retention of IL-4 up to 7 days under the dynamic regime imposed.

The approach proposed in this study has several other inherent advantages when compared to previously used methods. For example, recently, UPy-SDF1- α derived peptides were developed for convenient incorporation into a UPy-modified polymer scaffold, and tested *in vitro* and *in vivo* as rat abdominal aorta interposition grafts [3]. Interestingly, while the supramolecular bioactivation approach proposed by Muylaert et al. relies on the synthesis and UPy-modification of a specific short peptide sequence mimicking the receptor activating domain of the specific full protein of interest, in the current study, the full protein, i.e. IL-4, was directly premixed with heparin and immobilized onto UPy-HBP modified synthetic materials. Moreover, since the incorporation of the IL-4 and heparin was performed after material processing, the risk of impaired functionality of the functionalized constructs due to the fabrication process was drastically reduced to non-existent, as confirmed by the heparin and IL-4 binding assay performed on the electrospun scaffolds. In addition, the one-time synthesis of the solely UPy-modified heparin binding peptides might allow for straightforward future functionalization with a broader panel of bioactives, i.e. all the chemokines and cytokines displaying a heparin binding domain [5–10,12]. Lastly, the incorporation of heparin might be relevant for additional challenges in the specific field of vascular engineering, i.e. the possibility to generate an elastic network which could mimic the native biomechanical behavior and improve the grafts' functionality. For example, in previous studies investigating elastogenesis *in vitro*, heparin treatment of adult human vascular smooth muscle cells promoted the formation of elastic fibres. Importantly, in silk-based construct, immobilized heparin showed essentially identical biological effects to that of soluble heparin [52], indicating that heparinized vascular grafts may promote elastin formation, in addition to heparin's well-established antithrombotic properties.

There are several limitations to this study. While cellular behaviour was investigated for IL-4 and heparin functionalized electrospun structures, the potential influence of various scaffold microstructures (e.g. various fibre diameters, pore sizes) was not taken into account. Given the complexity of macrophage response to biomaterials, which depends, among others, on topography, mechanical properties, and degradation properties of the scaffold [1], future studies in our group will be performed to investigate the combined effect of bioactivation and the above mentioned factors for fibrous scaffolds. Another limitation of this study was the use of a limited amount of markers and proteins to characterize activated populations of macrophages and the inflammatory

environment, respectively. Antibodies against CCR7 and CD64 were used to identify M1 macrophages, while antibodies against CD206 and CD163 were used to identify M2 macrophages, as they are known to be highly indicative of these classes of polarized macrophages [53,54]. However, the selectivity of some of these markers is still under active source of debate. For instance, even though the mannose receptor CD206 has been long used as the only putative marker for M2 macrophages, it is now well acknowledged the risk of aspecific expression in all the cultured macrophage phenotypes. Moreover, the scavenger receptor CD163 has been selected as a representative marker expressed in regulatory phenotype (“M2b or M2c”) polarized *in vitro* via IL-10 more than IL-4 stimulation [55], although it was long used in combination with CD206 for generic characterization of the M2 family [29,56]. Therefore, the mere expression of these markers should not be used as definitive evidence of macrophage phenotype, and an approach using combinations of markers, and, most importantly, a lack of marker expression should be favoured to ascribe activation outcomes. Importantly, multiple readouts should be used for the characterization of macrophage polarization, e.g. immunostainings, protein assays, as done in this study, and, possibly, gene expression analysis. Unfortunately, in this study, quantitative gene expression analysis supporting these results could not be performed. After disruption of the 2D films, and cell lysates collection, RNA isolation was performed multiple times and with several methods, i.e. RNA binding to silica membranes (Qiagen RNeasy kit, Qiagen, Venlo, The Netherlands), RNA extraction with TRIzol method, RNA isolation via a guanidinium-based lysis buffer and binding to glass fibre filters (RNAqueous™ Total RNA Isolation Kit, ThermoFisher Scientific), or silica-membrane based purification kits specific for whole blood (QIAamp RNA Blood Mini Kit, Qiagen, Venlo, The Netherlands). Unfortunately, none of the attempts gave sufficiently high or pure yield to perform cDNA synthesis and qPCR analysis later on, possibly due to the interference of the negative charges of heparin and the isolated RNA in the silica membrane methods, or general interference of the disrupted synthetic material with all the others extraction methods tested. However, the combined results of immunohistochemistry, cell morphology and, most importantly, protein secretion, are robust functional indicators of macrophage polarization and of the effectiveness of the functionalization method proposed.

5. Conclusions

In this study, we developed IL4-heparin functionalized supramolecular materials showing immunomodulatory properties *in vitro*, which relies on the possibility to bias macrophage polarization and cytokines production over time. The proposed supramolecular approach for IL4 and heparin immobilization was successfully translated to 3D electrospun scaffolds, where bioactivity retention was demonstrated over time, in static conditions and under dynamic conditions mimicking the hemodynamic environment of small-diameter arteries, and immunomodulatory properties confirmed. These bioactivated systems hold a great deal of promise for the future development of truly ‘smart’, cell-responsive multi-functionalized scaffolds which, by mimicking the plethora of signals involved in the complex inflammatory response, can interact with their environment and mediate a favorable host response to the implanted vascular graft *in vivo*.

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Disclosures

The authors report that they have no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.actbio.2018.02.032>.

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