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Authors: Maryam Sharifiaghdam, Elnaz Shaabani, Zeynab Sharifiaghdam, Herlinde De Keersmaecker, Bart Lucas, Joris Lammens, Hossein Ghanbari, Ladan Teimoori-Toolabi, Chris Vervaet, Thomas De Beer, Reza Faridi-Majidi, Stefaan C. De Smedt, Kevin Braeckmans, Juan C. Fraire

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ARTICLE

Macrophage reprogramming into a pro-healing phenotype by siRNA delivered with LBL assembled nanocomplexes for wound healing applications

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Maryam Sharifiaghdam^{a,b}, Elnaz Shaabani^{a,b}, Zeynab Sharifiaghdam^c, Herlinde De Keersmaecker^{a,d}, Bart Lucas^a, Joris Lammens^e, Hossein Ghanbari^b, Ladan Teimoori-Toolabi^f, Chris Vervae^g, Thomas De Beer^g, Reza Faridi-Majidi^{h*}, Stefaan C. De Smedt^a, Kevin Braeckmans^{a,d*}, Juan C. Fraire^a

Excessive inflammatory responses in wounds are characterized by the presence of high levels of pro-inflammatory M1 macrophages rather than pro-healing M2 macrophages, which leads to delayed wound healing. Macrophage reprogramming from the M1 to M2 phenotype through knockdown of interferon regulatory factor 5 (irf5) has emerged as a possible therapeutic strategy. While downregulation of irf5 could be achieved by siRNA, it very much depends on successful intracellular delivery by suitable siRNA carriers. Here, we report on highly stable selenium-based layer-by-layer (LBL) nanocomplexes (NCs) for siRNA delivery with polyethyleneimine (PEI-LBL-NCs) as the final polymer layer. PEI-LBL-NCs showed good protection of siRNA with only 40% siRNA release in buffer of pH=8.5 after 72 h or in simulated wound fluid after 4h. PEI-LBL-NCs also proved to be able to transfect RAW 264.7 cells with irf5-siRNA, resulting in successful reprogramming to the M2 phenotype as evidenced by a 3.4 and 2.6 times decrease in NOS-2 and TNF- α mRNA expression levels, respectively. Moreover, irf5-siRNA transfected cells exhibited a 2.5 times increase of the healing mediator Arg-1 and a 64% increase in expression of the M2 cell surface marker CD206⁺. Incubation of fibroblast cells with conditioned medium isolated from irf5-siRNA transfected RAW 264.7 cells resulted in accelerated wound healing in an in vitro scratch assay. These results show that irf5-siRNA loaded PEI-LBL-NCs are a promising therapeutic approach to tune macrophage polarization for improved wound healing.

Introduction

Wound healing, under healthy physiological conditions, is a highly ordered biological process, that mainly includes 4 stages: hemostasis, inflammation, proliferation, and remodeling.^{1,2} The inflammatory response plays a crucial role in the initial steps of wound repair, with macrophages being the primary cells involved in the healing process.³ Macrophages are highly plastic cells, whose phenotype evolves during the different stages of wound healing. The M1 phenotype is pro-inflammatory, while the M2 phenotype is anti-inflammatory and pro-healing.^{4,5} Excessive inflammatory responses during wound

healing, that can be caused by chronic diseases like diabetes, are characterized by the presence of high levels of M1 macrophages at the expense of M2 macrophages, which leads to delayed wound healing.⁶

A transition from the M1 to M2 phenotype is needed to switch from an inflammation to a proliferation state in the wound healing process.⁷ Unfortunately, the normal cascade of events involved in this phenotypic polarization can be disturbed in diabetic conditions as a consequence of hyperglycemia and the presence of end-products of advanced glycation that affect normal operation of the mediators in the jack/stat signalling pathway.⁸ One strategy that could be used to overcome this problem is downregulation of the intracellular mRNA sequences controlling M1 phenotype pathways. It has been demonstrated that small interfering RNA (siRNA) mediated

^a Laboratory of General Biochemistry and Physical Pharmacy, Faculty of Pharmacy, Ghent University, Ghent, B-9000, Belgium Address here. ^b Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran. ^c Department of Physiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran. ^d Center for Advanced Light Microscopy, Ghent University, 9000 Ghent, Belgium. ^e Laboratory of Pharmaceutical Technology, Department of Pharmaceutics, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium. ^f Molecular Medicine Department, Pasteur Institute of Iran, Tehran, Iran. ^g Laboratory of Pharmaceutical Process Analytical Technology (LPPAT), Department of Pharmaceutical Analysis, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium.

knockdown of interferon regulatory factor 5 (irf5), the transcription factor which governs macrophage polarization to the M1 phenotype, leads to a transition from the M1 to M2 phenotype.⁹⁻¹² Even though this presents an interesting therapeutic avenue, it comes with serious delivery challenges.¹³⁻¹⁵

Getting into the cell by endocytic uptake and subsequent endosomal escape are among the key barriers that must be overcome for successful delivery of siRNA.¹⁶ Due to its negative charge and fairly large size, siRNA molecules cannot efficiently cross the cell's membrane,^{17,18} resulting in poor cell internalization.¹⁹⁻²² In addition, siRNA is prone to rapid degradation, contributing further to low transfection efficiencies when naked siRNA is used. Protection of siRNA and efficient cell internalization can be achieved by the use of nanocarriers, which can shield siRNA from degradation while facilitating intracellular delivery.²³⁻²⁷ A wide variety of carrier materials have been evaluated pre-clinically, including lipid nanoparticles, polyplexes, metal nanoparticles, hydrogels or combinations thereof.²⁸⁻³¹ These delivery carriers are usually endowed with polycationic moieties, which foster ionic complexation with the negatively-charged phosphate backbone of siRNA and aid endosomal escape following endocytic uptake.³² To date, two of the most widely used cationic polymers for *in vitro* delivery of siRNA are chitosan (CS) and polyethyleneimine (PEI).^{33,34} However, we and others have previously shown that forming complexes of siRNA with either of those polymers alone suffers from a lack of colloidal stability and show burst release of siRNA, thus limiting their applications.³⁵⁻³⁸ A wide range of strategies have been suggested to overcome these limitations, including trimethylation, aminoacid-conjugation, thiolation, glycosylation, pegylation, phospholipid-conjugation, aromatic modifications, and crosslinking methods,³⁹⁻⁴⁴ with different rates of success. LBL assembled nano-drug delivery systems have been demonstrated as very promising platforms in drug delivery. LBL assembly comprises the consecutive deposition of complementary/interacting polymers or oppositely charged ions onto a core nanoparticle which serves as a template.⁴⁵ Recently, in the context of cancer treatment, we used a layer-by-layer (LBL) strategy to stably encapsulate siRNA into nanocarriers with excellent colloidal stability.³⁵ Although selenium nanoparticles (SeNPs) have mostly gained attention in the scientific community for their anticancer activity, their antioxidant⁴⁶⁻⁴⁹ and antibacterial activity are increasingly recognized.⁵⁰

Therefore, here we further explored if those advantages of LBL nanoparticles with a Se core could be leveraged to tackle the challenges that are linked to the wound microenvironment, including the presence of negatively charged molecules that can compete with siRNA binding and the presence of other constituents which may lead to e.g. aggregation of the nanocomplex.⁵¹

When considering the use of nanocarriers for diabetic wound healing applications, most of the reported studies have focused on delivering

active compounds, including siRNA,^{52,53} against the expression of inflammation markers such as tumor necrosis factor α (TNF- α). However, such strategies address the inflammation only indirectly,⁵⁴ instead of targeting macrophages as the main cause of chronic inflammation. In this study, we explored the possibility to directly induce macrophage reprogramming from the pro-inflammatory (M1) to the pro-healing (M2) phenotype using the same straightforward Se-based LBL self-assembly approach to deliver irf5-siRNA. In particular, we explored transfection of RAW 264.7 macrophages with irf5-siRNA by using LBL-NCs consisting of CS-coated Se core NPs to which siRNA is complexed and with two different outer layers: chitosan (CS) and polyethyleneimine (PEI). First, we studied the stability and release of siRNA encapsulation in PEI-LBL-NCs and CS-LBL-NCs when incubated in HEPES buffer, DMEM cell culture medium (CCM), or simulated wound fluid (SWF). Next, we explored intracellular uptake, cytosolic siRNA delivery and biological activity of irf5-siRNA loaded LBL-NCs in macrophages. A comparison is provided with commercial transfection reagents jetPRIME and Lipofectamine RNAiMAX. In addition, we evaluated phenotype reprogramming by quantification of mRNA expression of phenotypic factors (irf5, NOS-2, TNF- α and Arg-1). Finally, with an *in vitro* scratch assay it is evaluated if incubation of fibroblast cells in conditioned medium from irf5-siRNA transfected RAW 264.7 cells results in accelerated wound healing.

Graphical abstract. Macrophage reprogramming into a pro-healing phenotype by siRNA delivered with LBL assembled nanocomplexes for wound healing applications

Experimental Section

Materials. Na_2SeO_3 and Chitosan (CS, low molecular weight, degree of deacetylation: 80%) were purchased from Sigma-Aldrich. L-Glutamine, Penicillin/Streptomycin solution (5000 IU/mL penicillin and 5000 $\mu\text{g}/\text{mL}$ streptomycin) (P/S), Fetal Bovine Serum (FBS), Trypan Blue, 0.25% Trypsin-EDTA, and Dulbecco's phosphate-buffered saline (DPBS) were supplied by Gibco BRL (Merelbeke, Belgium). CellTiter-Glo[®] Luminescent Cell Viability Assay was purchased from Promega (Leiden, Netherlands). Hoechst 33342 was purchased from Molecular Probes (Erembodegem, Belgium). Lipofectamine RNAiMAX reagent was purchased from Invitrogen. JetPEI (jetPRIME[®]) was purchased from Polyplus-transfection[®] (Illkirch, France). For uptake experiments, siCTRL duplexes were labeled with Alexa Fluor 647 dye at the 5' end of the sense strand (si-AF647) (Eurogentec, Seraing, Belgium). For endosomal escape experiments, red-labeled fluorescent oligonucleotides (AF647 ONs) were obtained from Eurogentec Seraing, Belgium. Predesigned Dicer-Substrate irf5-siRNA (DsiRNA), TriFECTa[®] RNAi Kit was purchased from Integrated DNA Technologies, Leuven, Belgium.

Preparation of LBL-NCs

Core nanoparticle synthesis: Se@CS NPs. Se nanoparticles stabilized with chitosan were prepared as previously reported³⁵. Briefly, 4 mL

of 200 mM vitamin C (ascorbic acid) solution was added dropwise into 5.83 mg Na_2SeO_3 dissolved in 40 mL of 0.2% (w/v) chitosan solution under magnetic stirring 900 RPM for half an hour at room temperature. The stabilized Se@CS NPs were collected by centrifugation at 21,000 g for 60 min, allowing to discard unreacted reagents and soluble remaining products of the reaction. The collected NPs were dispersed in RNase-free water and stored for further use. DLS/Zeta potential characterization were performed to confirm synthesis of the core nanoparticles.

Layer-by-Layer nanocomplex synthesis on Se@CS core NPs. LBL-NCs were synthesized in two steps as follows. First, siRNA was surface loaded on the Se@CS core at the previously reported optimal ratio 1:40 (w/w) of siRNA to Se atoms under constant stirring at 400 RPM for 60 min.³⁵ Secondly, a final polymeric layer was applied by adding a 0.5% (w/v) of PEI or 0.5% (w/v) CS solution to the Se@CS:siRNA dispersions under continuous stirring for another 60 min. The resulting PEI-LBL-NCs or CS-LBL-NCs were centrifuged again at 21,000 g for 60 min to remove all unreacted materials, after which the collected pellet was resuspended in RNase-free water and stored for further use.

Characterization of PEI-LBL-NCs and CS-LBL-NCs

Hydrodynamic diameter and surface charge of PEI-LBL-NCs and CS-LBL-NCs were determined using a Zetasizer Nano (Malvern, Worcestershire, UK) in each step of the synthesis.

siRNA entrapment in the PEI-LBL-NCs and CS-LBL-NCs was confirmed by agarose gel electrophoresis. For this, a 2% agarose gel was prepared by dissolving agarose (UltraPure Agarose, Invitrogen, Erembodegem, Belgium) in 100 mL TBE buffer (98 mM Tris, 88 mM Boric acid, 2 mM Na₂EDTA with pH 8). GelRed (Biotium, Hayward, CA) was added to TBE buffer for siRNA detection. 5 µL of Gel Loading Buffer (Ambion, Merelbeke, Belgium) was added per 20 µL sample before running for 20 min at 100 V. A total volume of 20 µL per line was pipetted and naked siRNA was added as a control. A Kodak digital camera (Kodak EDAS 120, Rochester, NY) was used to take images of the gel under UV light (Bio-Rad UV transilluminator 2000, California, USA).

The molecular structure of the layers of the PEI-LBL-NCs and CS-LBL-NCs were analyzed using Fourier Transform InfraRed spectroscopy (FTIR). First, the NCs were lyophilized using a temperature-controlled steel shelf. The shelf temperature was subsequently decreased to -40 °C at 1°C/min. Next, the drying chamber was pulled vacuum (100 µbar). Subsequently, the shelf temperature was increased to -25 °C for primary drying and was kept constant for 24h. Finally, secondary drying was achieved by increasing the shelf temperature to 20 °C at 0.1 °C/min. After 10 h of secondary drying the chamber was vented with dry nitrogen gas. After lyophilization, the dried samples were pressed against a diamond crystal to obtain uniform pellets, whose spectra were recorded with an ATR-FTIR Nicolet IS5 spectrophotometer (ThermoFisher, Waltham, USA) over a range of 4000 to 500 cm⁻¹ with a resolution of 4 cm⁻¹ on ATR-diamond crystal.

siRNA Release from PEI-LBL-NCs and CS-LBL-NCs. *In vitro* release studies were performed to determine the degree of siRNA release from the LBL-NCs at different time points (0, 4, 24, 48 and 72 h) after synthesis. In these experiments, PEI-LBL-NCs and CS-LBL-NCs were incubated in microtubes containing HEPES buffer at pH 7.4 and 8.5, and were gently shaken at 37°C. At specific time points (0, 4, 24, 48 and 72 h), the supernatant of the samples was taken from one of the microtubes after centrifuging at 21,000 g for 60 min, and the concentration of siRNA in the supernatant was measured using a NanoDrop at a wavelength of 260 nm to determine the amount of released siRNA.^{55,56}

Study of PEI-LBL-NCs and CS-LBL-NC dissociation. The decomplexation of siRNA from the LBL-NCs was studied by agarose gel electrophoresis. Besides PEI-LBL-NCs and CS-LBL-NCs, we also evaluated in a comparative way jetPRIME-NCs and Lipofectamine RNAiMAX-NCs, as these are popular commercial transfection reagents. jetPRIME-NCs and Lipofectamine RNAiMAX-NCs were prepared following the protocols provided by the manufacturer. Next, all prepared samples were diluted to 160 nM of siRNA concentration with HEPES buffer (pH 7.4, 10 mM), DMEM cell culture medium (CCM), or simulated wound fluid (SWF). SWF was composed of 2% BSA, 0.02 M calcium chloride, 0.4 M sodium chloride, and 0.08 M tris(hydroxyl) aminomethane in deionized water, with the pH adjusted to 8.5.⁵⁷ Incubation in HEPES buffer, CCM, and SWF was carried at room temperature for 4h. In addition, to force siRNA dissociation from the NCs as a positive control, 2% sodium dodecyl sulfate (SDS) was added to PEI-LBL-NCs, CS-LBL-NCs, and jetPRIME-NCs, while RNAiMAX-NCs was incubated with dextran sulfate (DS) 0.1%, all at room temperature for 15 min. Dissociation was analyzed by gel electrophoresis, as described before.

Complementary to gel electrophoresis, dissociation of fluorescently labeled siRNA from LBL-NCs in the above mentioned fluids was also studied by Fluorescence Fluctuation Spectroscopy (FFS). Fluorescence fluctuation time traces were obtained by focusing a 640 nm laser line 50 µm above the glass bottom through a water immersion objective lens (60x Plan Apo VC, N.A. 1.2, Nikon, Japan) in the various samples that were dispensed in a 96-well plate (Item No.: 655892, Grainer Bio-one, Frickenhausen, Germany). The fluorescence signal was recorded by a photon counting instrument (PicoHarp 300, PicoQuant) coupled to a Nikon C2 confocal microscope. Time traces were obtained by the recording of the fluorescence fluctuations during 60 s, from which the fraction of released siRNA is calculated as reported before.⁵⁸

Cytotoxicity studies. RAW 264.7 (ATCC® TIB-71™) cells were cultured in 75 cm² flasks in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS, 4 mM L-Glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate and 100 µg/mL P/S in a humidified incubator at 37°C with 5% CO₂ atmosphere. Based on previously reported protocols, in all experiments, cells were cultured in CCM containing lipopolysaccharides (LPS, 100 ng/mL, from Escherichia coli O111:B4) for 24h to polarize them into M1 macrophages.⁵⁹ Polarized cells were seeded in 96-well plates at 2×10⁵ cells/mL and were allowed to attach

overnight. On the next day, cells were treated with PEI-LBL-NCs and CS-LBL-NCs (ratio 1:10 (V/V), LBL-NCs to CCM) for 4h at 37°C in CCM. Next, cells were washed with PBS and incubated in fresh CCM for an additional 24h. On the next day, after replacing the CCM with 100 μ L of fresh medium, 100 μ L of CellTiter-Glo® reagent was added in each well and mixed for 20 minutes on an orbital shaker (Rotamax 120, Heidolph, Germany) to induce cell lysis. To let the luminescent signal stabilize, the mixture was further incubated at room temperature for an additional 10 minutes. Next, 100 μ L suspension of each well was transferred to white 96 well plates and the luminescence signal was recorded by a GloMax™ 96 Luminometer (Promega). Wells containing CellTiter-Glo without cells were included to determine the background luminescence.

Intracellular uptake. Uptake experiments were performed by seeding 2.5×10^5 RAW 264.7 polarized cells/mL, in 96-well plates which were allowed to attach overnight. The next day, PEI-LBL-NCs and CS-LBL-NCs loaded with AF647 siRNA were prepared as described above. Cells were incubated with PEI-LBL-NCs and CS-LBL-NCs (4–32 nM siRNA concentration) for 4h at 37°C, after which they were washed and detached from the well plates using trypsin. After 5 minutes incubation, cells were neutralized with complete CCM, and transferred to U-Bottom 96-well plates (Greiner Bio-One GmbH, Vilvoorde, Belgium) and centrifuged (5 min, 500 g). Collected cell pellets were resuspended in flow buffer (DPBS-, 0.1% Sodium Azide, 1% Bovine Serum Albumin) for flow cytometry analysis (CytoFLEX, Beckman Coulter, Krefeld, Germany).

For each sample, the forward and side scatter (respectively FSC and SSC), as well as red fluorescence (excitation at 638 nm and detection with a 660/20 nm band pass filter) were measured for 60 s at a flow rate of 60 μ L/min using a CytoFLEX flow cytometer. Finally, data analysis was performed using the FlowJo software package (Tree Star Inc., Ashland, OR, USA), for quantifying the percentage of positive cells (uptake (%)) and the relative mean fluorescence intensity per cell (rMFI). For selected siRNA concentrations intracellular uptake was compared with commercial jetPRIME-NCs and RNAiMAX-NCs.

Uptake of NCs was also studied by confocal microscopy. A Nikon A1R HD confocal laser scanning microscope (Nikon Benelux, Brussels, Belgium) was used, equipped with a laser box (LU-N4 LASER UNIT 405/488/561/640, Nikon Benelux, Brussels Belgium) and detector box (A1-DUG-2 GaAsP Multi Detector Unit, GaAsP PMT for 488 and

561 and Multi-Alkali PMT for 640 and 405 nm). Images were recorded with a 20x air objective lens (CFI plan Apo VC 20X, NA 0.75, WD 1000 μ m) (Nikon, Japan) using the NIS Elements software (Nikon, Japan). Briefly, RAW 264.7 polarized cells were seeded in 35 mm CELLview glass bottom microscopy dishes (Greiner Bio-One, Vilvoorde, Belgium) at a density of 2.5×10^5 cells per mL and were allowed to settle overnight. After removal of CCM, the cells were treated for 4h with NCs, or naked AF647 siRNA as a carrier-free control. Cell imaging was performed after 4h of treatment at 37°C. Before confocal imaging, cells were washed and stained with Hoechst 33342 (1 mg/mL in H₂O; 1000x diluted). After 20 min incubation with Hoechst, cells were washed 3 times with PBS. Stained cells were provided with full CCM and put on the microscope with incubator conditions to enable live-cell imaging (5% CO₂, 100% humidity, and 37 °C). For visualization, the 408 and 640 nm laser lines were applied to excite the Hoechst-labeled nuclei and the AF647 siRNA, respectively. Images were analyzed using the ImageJ software (FIJI, <https://fiji.sc/>).

Visualization and quantification of endosomal escape. Endosomal escape was evaluated by a dequenching assay as described before.^{36,60} For this, all carriers evaluated were prepared using AF647 ONs (red-labeled fluorescent oligonucleotides): PEI-LBL-NCs, CS-LBL-NCs, JetPRIME-NCs, RNAiMAX-NCs. The freshly prepared carriers and free AF647 ONs as a negative control, were added to the cells (2×10^5 cells per mL seeded in 35 mm CELLview microscopy dishes) for 4h at 37°C, after which they were washed and incubated overnight at 37 °C and 5% CO₂. After 24h, cell nuclei were stained with Hoechst 33342 and handled as previously described. For visualization, the 408 and 640 nm laser lines were applied to excite the Hoechst-labeled nuclei, and the AF647 ONs, respectively. Upon endosomal escape, free AF647 ONs will be released into the cytosol and they will accumulate in the nucleus. Data analysis was performed using ImageJ software to determine the total cell number and number of cells with AF647 ON-positive nuclei. The ratio of both gives the percentage of cells in which at least one endosomal escape event occurred. Nuclei were detected in the blue channel by thresholding (applying the same offset values for every image), and intensity analysis of the nuclear fluorescence signal in the red channel was performed. At least 150 cells in a minimum of 20 images were included for all conditions tested.

Transfection efficiency analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR). To evaluate irf-5 siRNA induced

knockdown and its biological effects, we performed quantitative reverse transcription polymerase chain reaction (qRT-PCR). In this regard, polarized cells with LPS were plated in 24-well plates at a density of 2×10^5 cells/mL. Cells were treated with the NCs for 4h as explained before. After 24h of treatment, RNA was extracted as follows: total RNA was extracted using Aurum™ Total RNA Mini Kit (Biorad, Hercules, CA, USA). RNA concentration was assessed using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Rockford, IL, USA). The conversion of approximately 100 ng of RNA to cDNA was performed by the manufacturer's instructions with an iScript cDNA synthesis kit (Biorad, Hercules, CA, USA) in Hard-Shell® 384-Well PCR Plates (Biorad, Hercules, CA, USA). The protocol of cDNA synthesis was finished by incubation in the CFX384 Touch Deep Well Real-Time PCR System (Biorad, Hercules, CA, USA), using the recommended protocol by the supplier: priming for 5 min at 25°C, reverse transcription for 20 min at 46°C, RT inactivation for 1 min 95°C. Real-time PCR was performed using iTaq™ Universal SYBR® Green Supermix (Biorad, Hercules, CA, USA) in a total reaction mixture volume of 10 μ L, composed of: 5 μ L SYBR® Green Supermix, 4.5 μ L cDNA and 0.5 μ L of primer (100 nM). We included 10-fold serial dilution of mixed cDNA for each primer. All DNA primers were purchased from Integrated DNA Technologies (Leuven, Belgium). The following primer sequences were used: Arg-1: Primer 1: GAATGGAAGAGTCAGTGTGGT, Primer 2: AGTGTGATGTCAGTGTGAGC, iNos-2: Primer 1: GACTGAGCTGTTAGAGACACTT, Primer 2: CACTTCTGCTCCAAATCCAAC, irf-5: Primer 1: ATCTACGAGGTCTGCTCAA, Primer 2: CAGGCTCTGTGATGCTCAG, TNF- α : Primer 1: AGACCCTCACACTCAGATCA, Primer 2: TCTTTGAGATCCATGCCGTTG, Gapdh: Primer 1: AATGGTGAAGGTCGGTGTG, Primer 2: GTGGAGTCATACTGGAACATGTAG. Analyses were performed as follows: polymerase activation and DNA denaturation at 95°C for 30 sec, then denaturation at 95°C for 5 sec, followed by gradient annealing/extension and plate read 56.5-60°C, 30 sec. The denaturing/annealing process was repeated over 40 cycles, melt curve analysis was set 65-95°C, 0.5°C increments at 5 sec/step. Relative gene expression was determined by comparing the C_t value (the "cycle threshold", defined as the number of cycles required for the fluorescent signal to cross the threshold) of the gene of interest to that of the Gapdh housekeeping gene. The results were analyzed by CFX Maestro™ Software.

Measurement of surface markers expression. To evaluate the expression of cell surface markers for M1 or M2 phenotypes, RAW 264.7 cells treated with PEI-LBL-NCs were surface-stained with a monoclonal antibody specific for CD206 (M2 marker) and CD86 (M1 marker). Flow analysis was performed 24h after the addition of PEI-LBL-NCs loaded with irf5-siRNA or Ctrl-siRNA. Next, cells were collected and washed with PBS, incubated with Fc receptor blocker (CD16/32) to block non-specific Fc binding (BD Biosciences, Erembodegem, Belgium), and surface stained for CD206 (APC anti-mouse CD206 (MMR) Antibody, Clone: C068C2, Biolegend, San Diego, CA) and CD86 (FITC Rat Anti-Mouse CD86 Clone GL1 (RUO), BD Biosciences, Erembodegem, Belgium) for 30 min at 4°C. After additional washing steps, the cells were analyzed using a CytoFLEX flow cytometer and analysis was performed using FlowJo software.

In vitro wound healing analysis. A scratch assay was performed to examine the potential *in vitro* wound healing effect of irf5-siRNA loaded PEI-LBL-NCs. This experiment is based on two steps: (1) Treatment of RAW 264.7 cells with PEI-LBL-NCs and collecting secreted factors after 24h, which will refer to as conditioned CCM (C-CCM) and (2) Treatment of NIH-3T3 cells with C-CCM.

First step: LPS treated RAW 264.7 cells were seeded in a 12-well plate at 2×10^5 cell/mL. On the next day, cells were treated with PEI-LBL-NCs loaded with irf-5-siRNA and Ctrl-siRNA for 4h, including the LPS group as a control. After 4h incubation, cells were washed and fresh CCM was added to each well. Next, cells were incubated at 37°C with 5% CO₂ atmosphere for 24h. On the next day, the C-CCM was collected containing secreted factors from the RAW 264.7 cells. Those samples were centrifuged at 12,000 g for 10 minutes to remove any particles and cell debris, subsequently filter-sterilized through a 0.22 μ m syringe filter (Millipore, Billerica, MA, USA), and then stored at -80 °C until further use in the second step of the experiment.

Second step: NIH-3T3 cells (embryo derived fibroblast cells, ATCC-CRL-1658) were cultured in DMEM CCM supplemented with 10% Calf serum, 4 mM L-Glutamine, 4500 mg/L glucose, and 100 μ g/mL P/S in a humidified incubator at 37°C with 5% CO₂ atmosphere. 75,000 cell/mL NIH-3T3 fibroblasts were seeded in 6-well cell culture plates and incubated in standard cell culture conditions. After obtaining a confluent monolayer of cells, a scratch was gently created along a straight line in the cell culture using a 200 μ L pipette tip. After that, cells were washed with PBS to remove cell debris and then

replenished with fresh CCM or C-CCM diluted in fresh CCS at 1:1 (v/v) for 24h.

Wound closure was analyzed at 0 and 24h. Cells were photographed by transmitted light microscopy, and wound closure was analyzed by Fiji software by calculating the ratio between wound surface area (= area without cells) after 24h and 0 h (i.e. before the addition of different media).

Statistical analysis. All statistical analyses were performed using GraphPad software (La Jolla, CA, USA), and propagation of errors was applied when necessary. One-way ANOVA or the Student's t-test was used for comparing conditions. All results are reported as mean $\pm$ standard deviation and differences with a *** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$ were considered significant.

Results

LBL-NCs synthesis and characterization. The main goal of this work is to design a nanocarrier able to induce macrophage reprogramming from the M1 to M2 phenotype (i.e., pro-inflammatory to anti-inflammatory) through *irf5* knockdown. We chose a straightforward layer-by-layer self-assembly approach to synthesize stable Se based siRNA loaded nanocomplexes (Figure 1A). For this, we firstly synthesized Se core particles coated with chitosan (Se@CS), as previously reported.³⁵ The Se@CS core NPs had a hydrodynamic diameter, measured in RNase-free water, of 208 ± 2 nm with PDI=0.14, and a positive zeta potential of 52.0 ± 0.2 mV, suggesting that chitosan was successfully applied to the nanoparticle's surface (Figure 1B).

In a second step, siRNA was loaded onto the Se@CS core particles, at a ratio of 1:40 (w/w) siRNA to Se atoms, which was previously determined to be an optimal ratio for siRNA complexation on this type of core particles.³⁵ Upon coating with anionic siRNA, the positive surface charge decreased, reaching a value of 34 ± 2 mV. This was accompanied by a decrease in the hydrodynamic diameter from 208 ± 2 nm (Se@CS) to 186 ± 1 nm (Se@CS:siRNA) (Figure 1B), likely due to a condensation of CS upon complexation with siRNA, as has been previously observed.^{36,61}

Next, we applied a final cationic polymeric layer, being either PEI (PEI-LBL-NCs) or chitosan (CS-LBL-NCs). Expectedly this led to an increase in zeta potential to 41 ± 2 and 43 ± 2 mV as well as an increase in hydrodynamic size to 221 ± 2 and 227 ± 1 nm, for PEI-LBL-NCs and CS-LBL-NCs, respectively (Figure 1B). The presence of chitosan in the core Se@CS nanoparticles was previously

demonstrated by Fourier transform infrared spectroscopy (FTIR).⁶² The spectrum showed clear features at 3300–3500 cm^{-1} which correspond to vibrational modes of chitosan, in particular, stretching vibrations of O-H and N-H. Compared to the spectrum of chitosan alone, there was a slight blue shift of the main peaks that it is attributed to the bending vibration of N-H, indicating that selenium mainly interacts with the NH_2 groups of chitosan.⁶²⁻⁶⁴

To further confirm the presence of the 3rd polymeric layer, PEI-LBL-NCs and CS-LBL-NCs were additionally analyzed by FTIR. The characteristic bands of chitosan were clearly noticeable in the FTIR spectrum of CS-LBL-NCs (Figure 1C).⁶⁵ In particular, the broad stretching band of N-H and O-H can be seen from 3,500–2,500 cm^{-1} , as well as representative bands at 1,640 cm^{-1} , 1,563 cm^{-1} , and 1,077 cm^{-1} , which are attributed to C=O stretching (amide I band), N-H deformation (amide II band), and C-O stretching.⁶⁵ The FTIR spectrum of PEI-LBL-NCs also showed characteristic peaks of the PEI polymer at 1,650–1,580 cm^{-1} for N-H bending, 1,310–1,250 cm^{-1} for C-N stretching of aromatic amines, while the peak between 3,000 and 2,800 cm^{-1} is attributed to N-H stretching of the amine groups.⁶⁶ These spectroscopical results further confirm successful deposition of the third and final polymeric layer.

Finally, we checked if siRNA was still present after applying the final polymer layer by gel electrophoresis (Figure 1D). As no siRNA signal was observed from the LBL-NCs nor the supernatant following separation by centrifugation, it could be concluded that siRNA was successfully incorporated in PEI-LBL-NCs and CS-LBL-NCs.

Figure 1. Characterization of PEI-LBL-NCs and CS-LBL-NCs. (A) Schematic representation of the step-by-step synthesis of Layer-by-layer nanocomplexes (LBL-NCs) with chitosan (CS) or Polyethylenimine (PEI) as the outer layer. (B) The hydrodynamic diameter and surface charge of the PEI-LBL-NCs and CS-LBL-NCs for each synthesis step. (C) FTIR spectra of PEI, CS, PEI-LBL-NCs, and CS-LBL-NCs confirming successful capping of the NCs by PEI and CS. (D) siRNA loading in PEI-LBL-NCs and CS-LBL-NCs using a 1:40 mass ratio (siRNA:Se atoms), was evaluated by agarose gel electrophoresis. Particles were separated from supernatant by centrifugation and analysed separately. (E) TEM images of PEI-LBL-NCs.

siRNA release from PEI-LBL-NCs and CS-LBL-NCs in buffers of defined pH and in simulated wound fluid. After successful LBL-NC synthesis and characterization, we proceeded to evaluate the stability of siRNA complexation in conditions that emulate the wound microenvironment. While healthy and intact skin has a slightly acidic pH ranging from 4.0 to 6.0, it shifts towards an alkaline pH of 8.5 or more in wounds.⁶⁷ Thus, siRNA release from LBL-NCs was monitored as a function of time for PEI-LBL-NCs and CS-LBL-NCs in 10 mM HEPES buffer at pH=7.4 and pH=8.5 (Figure 2A-i). The amount of siRNA released from PEI-LBL-NCs and CS-LBL-NCs at specified time points (0, 4, 24, 48 and 72h) was determined by measuring the absorbance of released siRNA in the supernatant at 260 nm. For pH=7.4, CS-LBL-NCs showed a small amount of burst release ($16 \pm 3\%$) in the first 4h, followed by a sustained release up to $28 \pm 6\%$ after 72h. siRNA release in the case of PEI-LBL-NCs was limited to only $2 \pm 1\%$ during the first 4h, reaching up to $15 \pm 6\%$ after 72h (Figure 2A-i). At pH=8.5, CS-LBL-NCs showed almost complete siRNA release already after 4h (Figure 2A-i), which was accompanied by visible aggregation of LBL-NCs after 72h (Figure 2A-ii). As CS has a pKa of 6.5, most of its amine groups will be deprotonated at pH=8.5, which leads to a drastic decrease in the polymer's solubility and ability to electrostatically complex siRNA.⁶⁸ PEI-LBL-NCs, on the other hand, proved to retain siRNA much better at pH 8.5, releasing only $40 \pm 11\%$ after 72h (Figure 2A-i). As PEI has a good buffering capacity throughout a broad pH range (approximately pH=3-10),⁶⁹ it remains positively charged at pH 8.5, thus better retaining the complexed siRNA.

Biological fluids, such as wound fluid, can contain several negatively charged molecules, like albumin, which may destabilize electrostatic binding of siRNA to nanocarriers. To investigate to which extent this is the case for PEI-LBL-NCs and CS-LBL-NCs, they were incubated for 4h in HEPES buffer, Cell Culture Medium (CCM), and simulated wound fluid (SWF). siRNA dissociation was then investigated by agarose gel electrophoresis in comparison with NCs formed with commercial transfection reagents jetPRIME and RNAiMAX. As a positive control for complete dissociation of siRNA from the carriers, dextran sulfate (DS) was added to PEI-LBL-NCs, CS-LBL-NCs and jetPRIME-NCs, while surfactant sodium dodecyl sulfate (SDS) was used to dissociate RNAiMAX-NCs. Incubation in HEPES and CCM led to siRNA release in all cases except for PEI-LBL-NCs (Figure 2B). Bands became even brighter after incubation in SWF, indicative of even more siRNA release from all carriers except PEI-LBL-NCs, even though also in this

case a faint band of released siRNA became noticeable. Note that SWF itself gives a background signal on the agarose gel runs. Electrophoretic runs after the incubation of NCs with DS or SDS resulted in siRNA release from all NCs.

While gel electrophoresis is a valuable first step to obtain qualitative information on siRNA dissociation from the different NCs in various media, Fluorescence Fluctuation Spectroscopy (FFS) can be used to study this more quantitatively.⁵⁸ FFS being a fluorescence based technology, NCs were prepared with AF647 fluorescently labeled siRNA. While naked AF647 siRNA in HEPES buffer (16 nM) had an average intensity of about 200 kHz (photon counts per s) (Figure S3A, red line), upon complexation to NCs the signal's baseline dropped due to disappearance of free siRNA, and bright fluorescence peaks appeared corresponding to NCs which contain multiple siRNA molecules. The ratio of the fluorescence baseline of NCs compared to free siRNA (both corrected for background fluorescence (Figure S3A, black line)) represents the fraction of siRNA that is dissociated from the carriers, which was virtually 0% for PEI-LBL-NCs and between ~20-30% for the other carriers (Figure 2C-i). Upon incubation with CCM for 4h, the baseline of all formulations increased again (Figure S3B), amounting to a dissociation degree of 20%, 48%, 32%, 37% for PEI-LBL-NCs, CS-LBL-NCs, jetPRIME-NCs, and RNAiMAX-NCs, respectively (Figure 2C-ii). In SWF dissociation was even higher (Figure S3C), reaching respectively 40%, 100%, 89%, 91% (Figure 2C-iii), in line with the gel electrophoresis results. Importantly, dissociation from PEI-LBL-NCs was much less as compared to any of the other carriers tested. As a positive control we again performed an incubation with SDS or DS (Figure S3D), resulting in almost complete dissociation of siRNA from the carriers (Figure 2C-iv). Taken together we can conclude from gel electrophoresis and FFS that PEI-LBL-NCs clearly provide the best protection of siRNA in CCM and SWF.

Figure 2. Decomplexation assays. (A-i) *In vitro* release studies were carried out to determine the percentage of siRNA released from the LBL-NCs at different time points (0, 4, 24, 48 and 72h) after synthesis. (A-ii) Image of PEI-LBL-NCs and CS-LBL-NCs incubated in pH=8.5 after 4h and 72h of synthesis. (B) Representative gel showing runs of naked and complexed siRNA after incubation in HEPES buffer (pH 7.4, 10 mM), DMEM cell culture medium (CCM), and simulated wound fluid (SWF, with pH adjusted to 8.5). Additionally, siRNA dissociation from the NCs, PEI-LBL-NCs, CS-LBL-NCs, and jetPRIME-NCs was evaluated after incubation in the presence of sodium dodecyl sulfate (SDS) 2%, and from RNAiMAX-NCs after incubation with dextran sulfate (DS) 0.1% for 15 min. (C) Dissociation degree (%) determined by FFS of AF647 siRNA from NCs incubated 4h in (i) HEPES, (ii) cell culture medium (CCM), (iii) simulated wound fluid (SWF), and (iv) DS/SDS (n=3). Bars represent mean $\pm$ standard deviation, (n = 3, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Cell uptake and cytotoxicity. Next we investigated uptake and cytotoxicity of NCs in RAW 264.7 cells in a dose-dependent manner. RAW 264.7 cells were incubated for 4h with PEI-LBL-NCs and CS-LBL-NCs in a concentration range of 4 to 32 nM siRNA (which is equivalent to 2 to 16 ppm of Se), after which they were washed and supplemented with fresh CCM. After 24h, cell viability was assessed with the CellTiterGlo assay to determine the cell's metabolic activity. Cell viability remained >75% up to a siRNA concentration of 16 nM for both LBL-NCs, gradually decreasing further for higher concentrations (Figure 3A), indicating a dose-dependent relation between NCs concentration and cytotoxicity. This also can be clearly seen in Figure S1 for a range of concentrations of PEI-LBL-NCs and Se@CS core in RAW 264.7 cells. Importantly, toxicity induced by the Se core is substantially less in RAW264.7 and NIH-3T3 cells as compared to H1299 cancer cells (Figure S2).

Intracellular uptake was also measured after 4h incubation with NCs by flow cytometry over the same concentration range, using LBL-NCs prepared with AF647 siRNA (Figure 3B). Both the percentage of positive cells (uptake %) as well as the amount of uptake per cell (rMFI) increased for higher NCs concentrations. However, uptake was markedly higher for PEI-LBL-NCs as compared to CS-LBL-NCs.

Since 16 nM siRNA gave an acceptable cell viability for both LBL-NCs, we compared cytotoxicity and uptake with jetPRIME-NCs, RNAiMAX-NCs and naked siRNA at the same concentration by flow cytometry (Figure 3C and D, respectively) and confocal microscopy (Figure 3E). It was found that JetPRIME-NCs and RNAiMAX-NCs are taken up in an equally large fraction of cells as PEI-LBL-NCs, and even had slightly better cell viability. The amount of AF647 siRNA delivered per cell was the same for PEI-LBL-NCs and jetPRIME-NCs (rMFI around 50%), while RNAiMAX-NCs delivered almost a double amount of siRNA per cell. To which extent this translates into a better transfection efficiency will be investigated in the next section.

Endosomal escape. After uptake, siRNA should gain access to the cell's cytoplasm where it can exert its biological function. This means that, after endocytic uptake, NCs should be able to release the siRNA from the endosomes in which they are trapped before being degraded in acidic endolysosomes.⁷⁰ One way to achieve this is by using cationic polymers with a high buffering capacity in the endosomal pH range, between 4 and 7.5 pH units, that can buffer the protons inside the endolysosomes. This will trigger the so-called

proton sponge effect, which leads to osmotic swelling and bursting of endosomes, thus facilitating escape of cargo molecules to the cytosol.^{71,72} It is indeed known that the endosomal buffering capacity and intracellular nucleic acid delivery of chitosan is significantly lower compared to PEI.^{73,74} Therefore, we investigated to which extent these differences in endosomal buffering translated into differences in endosomal escape efficiency. For this, NCs were prepared using fluorescently labeled AF647 ONs (instead of siRNA) which will accumulate over time in the cell's nucleus if they are successfully released in the cytosol⁷⁰. Therefore, when a cell has a red fluorescent nucleus, it means that at least one endosomal escape event must have happened in that cell. As shown in the confocal images of Figure 4A, accumulation of free ONs in the nucleus of cells after 24h incubation with PEI-LBL-NCs was clearly detectable, but was almost negligible for cells treated with CS-LBL-NCs. This difference can be attributed to a combination of higher uptake efficiency (i.e. more cells that have taken up PEI-LBL-NCs), more NCs per cell (higher rMFI), and higher buffering capacity of PEI. Image analysis revealed that endosomal escape had happened in 52% of the cells treated with PEI-LBL-NCs, while this was only 8% for CS-LBL-NCs (Figure 4B). In comparison, jetPRIME-NCs led to endosomal escape in 57% of the cells, while it was 44% for RNAiMAX-NCs.

Figure 3. Cell viability and uptake of LBL-NCs in RAW 264.7 cells. **(A)** Cell viability, measured by the metabolic CellTiter Glo assay, after 4h incubation with PEI-LBL-NCs and CS-LBL-NCs as a function of NC concentration (expressed as effective siRNA concentration). **(B)** Uptake % and rMFI of PEI-LBL-NCs and CS-LBL-NCs loaded with AF647 siRNA. **(C)** Cell viability after 4h incubation with PEI-LBL-NCs, CS-LBL-NCs, naked siRNA, jetPRIME-NCs, and RNAiMAX-NCs at 16 nM effective siRNA concentration. NTC = non-treated control. **(D)** Corresponding uptake results for NCs prepared with AF647 siRNA. **(E)** Confocal images of RAW 264.7 cells after 4h treatment with different NCs at 16 nM effective siRNA concentration. Nuclei were stained with Hoechst 33342 (blue), while AF647 siRNA is shown in red. The scale bar corresponds to 25 μ m. Bars represent mean $\pm$ standard deviation, (ns = not significant, $n = 3$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Figure 4. Endosomal escape quantification. (A) Representative confocal images of RAW 264.7 cells 24h after incubation with NCs loaded with AF647 ONs. Upon endosomal escape, AF647 ONs will spread toward the cytoplasm and accumulate into the nucleus. AF647 signal is shown in red, while Hoechst stained nuclei can be seen in blue. (B) The percentage of cells in which endosomal escape happened, as quantified by image processing for at least 150 cells for each case. The scale bar corresponds to 25 μ m.

Macrophage phenotypic reprogramming. As a next step we determined the degree of phenotype change from M1 to M2 in RAW 264.7 cells after transfection with *irf5*-siRNA.⁷⁵ First, RAW 264.7 cells were stimulated to the M1 phenotype by LPS (100 ng/mL) during 24h.⁷⁶ Successful induction of the M1 phenotype was confirmed by dysregulation of the mRNA expression level of several markers. Upregulation of Interferon Regulatory Factor 5 (*irf5*) was confirmed as a key regulator of M1 phenotype (Figure 5A,i). In addition, NOS-2 was found to be upregulated (Figure 5A,ii), which is related to the production of NO by M1 macrophages to kill pathogens.⁷⁷ Arg-1 was downregulated (Figure 5A,iii), being linked to collagen biosynthesis and the promotion of tissue healing.⁷⁸ Finally, TNF- α was upregulated (Figure 5A,iv), which is a pro-inflammatory cytokine produced by M1 macrophages during conditions of inflammation.^{78,79} Upon 24h

incubation of those M1 polarized RAW 264.7 cells with *irf5*-siRNA loaded PEI-LBLNCs, *irf5*-mRNA became strongly downregulated compared to transfection with scrambled siRNA (Figure 5A-i). Similarly, NOS-2 and TNF- α became downregulated as well, while Arg-1 was strongly upregulated. Instead, no change in mRNA expression of those markers was seen upon treatment with CS-LBL-NCs. The commercial transfection reagents jetPRIME or RNAiMAX also resulted in significant down- and upregulation of those markers, but to a lesser extent as PEI-LBL-NCs.

These results show that PEI-LBL-NCs perform best to transfect RAW 264.7 macrophages with *irf5*-siRNA. To confirm that the induced transcriptomic changes are indeed related to a repolarization of M1 macrophages to the M2 phenotype, surface signature markers CD86 (M1 macrophage marker) and CD206 (M2 macrophage marker) were

analyzed by flow cytometry after immunofluorescence staining (Figure 5B). Upon LPS stimulation, the percentage of M1 macrophages increased from 7% to 64%. After transfection with irf5-siRNA delivered by PEI-LBL-NCs, the percentage of M1 macrophages decreased again to 3%, while the percentage of M2 cells increased from 1% to 54%. Instead, when cells were transfected with scrambled siRNA the percentage of M1 and M2 macrophages remained unaltered, confirming the specific action of irf5-siRNA.

In vitro wound healing assay. As a final step, we wanted to confirm if decreasing the M1/M2 ratio by irf5-siRNA transfection with PEI-LBL-NCs may be beneficial for wound healing. Therefore, we performed a wound scratch assay with NIH-3T3 cells which were exposed to conditioned cell culture media (C-CCM) isolated from RAW 264.7 cells before and after phenotypic reprogramming with PEI-LBL-NCs. As schematically shown in Figure 6A, LPS treated RAW 264.7 cells were first exposed to PEI-LBL-NCs loaded with irf5-siRNA or scramble Ctrl-siRNA for 4h. After 4h incubation, cells were washed and fresh CCM was added to each well. On the next day, all conditioned CCM (C-CCM) were collected, containing secreted factors from the RAW 264.7 cells. In the second step we performed a macrophage paracrine stimulation assay in NIH-3T3 cells. A wound (scratch) was gently created in the NIH-3T3 monolayer cells using a 200 μ L pipette tip, after which cells were replenished with C-CCM from RAW 264.7 cells (or fresh CCM for the negative control). The wound area was quantified as the cell-free area at time points 0 and 24h.

As can be readily appreciated from the microscopy images in Figure 6B, NIH-3T3 cells treated with C-CCM from irf5-siRNA transfected RAW 264.7 cells showed the most complete healing after 24h. This was not the case for NIH-3T3 cells incubated with C-CCM from the RAW 264.7 cells treated with Ctrl-siRNA or LPS alone. Quantification of the cell-covered area confirmed that treatment of cells with C-CCM from irf5-siRNA transfected cells resulted in the most complete wound healing ($98 \pm 7\%$) (Figure 6C).

Finally, as both NIH-3T3 and RAW 264.7 cells are present in the wound microenvironment, we also evaluated to which extent PEI-LBL-NCs are taken up in NIH-3T3 cells and how they affect their viability (Figure 6D). Following 4 h incubation, only 30% of NIH-3T3 cells had taken up PEI-LBL-NCs, while cell viability remained above 80%, suggesting that PEI-LBL-NCs are more likely to accumulate in

RAW 264.7 cells for which we had 83% uptake. This rather unexpected selective uptake in macrophages observed for PEI-LBL-NCs is a promising finding for wound healing applications.

Figure 5. Phenotypic reprogramming of RAW 267.4 cells by transfection with *irf5*-siRNA. (A) The mRNA expression level of (i) *irf5*, (ii) *NOS-2*, (iii) *Arg-1*, and (iv) *TNF-α*, were measured in RAW 267.4 cells activated with LPS using real-time PCR, before and after treatment with NCs loaded with *irf5*-siRNA or scramble Ctrl-siRNA. Results were normalized to the mRNA expression level of *Gapdh*. (B) Representative flow cytometry results showing the CD206⁺ (M2 marker) and CD86⁺ (M1 marker) populations in LPS activated RAW 264.7 cells after treatment with PEI-LBL-NCs loaded with *irf5*-siRNA or Ctrl-siRNA. Bars represent mean $\pm$ standard deviation, (ns = not significant, $n = 3$, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Figure 6. *In vitro* wound healing assay. (A) Schematic representation of the wound healing assay in which the proliferation of NIH-3T3 fibroblasts was monitored after treatment with conditioned cell culture medium (C-CCM), containing secreted factors from macrophage cells (RAW 264.7) stimulated with LPS and treated with PEI-LBL-NCs loaded with irf-5 siRNA or Ctrl-siRNA. (B) Transmission microscopy images of NIH-3T3 cells before (0h) and after treatment with C-CCM from LPS stimulated RAW 264.7 cells, and LPS stimulated RAW 264.7 cells treated with PEI-LBL-NCs loaded with irf-5 siRNA or Ctrl-siRNA, NTC = non-treated control. (C) Quantification of the cell covered area within the scratch was performed by image analysis. (D) Cell viability and uptake of PEI-LBL-NCs in NIH-3T3 cells. Bars represent mean $\pm$ standard deviation, (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Discussion

Proper wound closure relies on an intricate interplay between many cell types, including epithelial, fibroblast, endothelial, and macrophage cells.^{80,81} As macrophages are the primary cells of the inflammatory response, they are crucial regulators of the healing process, being involved in nearly every stage of it.³ They are highly plastic cells, whose phenotype evolves during the different stages of wound healing, from the classical pro-inflammatory (M1) to the anti-inflammatory and pro-healing (M2) activation state.⁴ This bipolar M1/M2 classification is, however, a simplification as macrophages can possess varying degrees of M1 or M2-like qualities.⁵ This phenotypic flexibility allows them to quickly respond to environmental stimuli. For example, in the presence of inflammatory stimuli, macrophages polarize toward the M1 phenotype and release inflammatory cytokines including NO, ROS, TNF- α and an array of cytotoxic molecules that aid in the clearance of invading pathogens and stimulation of the acquired immune response.⁸² They also secrete matrix metalloproteinases (MMPs) in order to break down the extracellular matrix and make room for infiltrating inflammatory cells.^{83,84} As it is shown in scheme 1, when the tissue begins to repair, the healing wound environment promotes polarization toward the M2 phenotype with anti-inflammatory effects, like the production of elevated levels of growth factors to stimulate cellular proliferation, granulation, tissue formation, and angiogenesis.⁸⁵ Unfortunately, this cascade of events can be altered in diabetic conditions due to the effects of hyperglycemia and the presence of end-products of advanced glycation.⁸ This dysregulation leads to an imbalanced inflammatory status with higher levels of pro-inflammatory cytokines, avoiding transition from the inflammatory phase (M1 macrophages) to the regenerative phase (M2 macrophages).⁸⁶ In addition, the accumulation of inflammatory cells in diabetic wounds

leads to an increase of the expression and activity of MMPs compared to normal wounds. This results in poor formation of new connective tissue, thus impairing the healing process.⁸⁷

The interferon regulatory factor (irf) family plays a crucial role in the regulation of metabolic inflammation and in the differentiation of immune cells through toll-like receptors (TLRs), which form part of the larger family of pattern recognition receptors (PRRs) (Scheme 1).⁸⁸ Specifically, as irf5 is involved downstream of the Toll-like receptor (TLR)-MyD88 (myeloid differentiation primary-response protein 88) pathway, it is identified as a key regulator of macrophage polarization.⁸⁹ Higher expression of irf5 leads to long-lasting M1 macrophages that secrete cytokines, chemokines, co-stimulatory molecules (e.g., interleukin 1 (IL-1), IL-6, IL-12, tumor necrosis factor alpha (TNF- α) and oxidative metabolites (e.g., nitric oxide and superoxide).^{85,90} Hence, dysregulation of irf5 expression and/or function has been linked to the pathogenesis of numerous inflammatory diseases, including autoimmune, infectious, cancer, obesity, neuropathic pain, cardiovascular, and metabolic dysfunction.⁹¹ In recent work by Al-Rashed et.al., the authors describe the different irf5-dependent mechanisms by which glucose fluctuations in type 2 diabetes orchestrate M1 macrophage polarization as well as expression of inflammatory cytokines/chemokines and MMP-9 in macrophages, suggesting that irf5 silencing be beneficial to alleviate metabolic inflammation.⁹² Therefore, irf5 has emerged as an attractive target for therapeutic intervention, and one approach to target irf5 is to inhibit the expression of irf5 using siRNA delivery that are specific for sequences in the irf5 mRNA.

To date, systemic delivery of irf5-siRNA loaded in lipidoid nanoparticles has been the most extensively reported strategy for phenotypic reprogramming of macrophages from the M1 to M2

phenotype, such as in the context of spinal cord injury (SCI).^{9,10} Repolarization of macrophages to the M2 phenotype was associated with a decrease in inflammation, attenuation of demyelination and neurofilament loss, and a significant improvement in locomotor function.⁹ In another study, reprogramming of macrophages in muscle tissue damaged by myocardial infarcts leads to a reduction of inflammation and infarct healing.¹⁰ Furthermore, selective suppression of *irf5* in microglia cells with targeted peptide-*irf5*-siRNA complexes resulted in a significant reduction in neuropathic pain in a mouse model¹². All together, these results show that *irf5* suppression can help to control inflammation.

In the particular context of wound healing, it is possible to administer siRNA locally, reducing potential off-target effects which may occur upon systemic delivery.⁹³ But even then a carrier is required that protects the RNA cargo from degradation and enables its transport across cell and endosomal membranes in order to reach the cytoplasm of target cells.^{94,95} Therefore, we selected a recently developed nanocarrier that is based on a layer-by-layer strategy around a selenium core particle, and which already has shown great potential for siRNA delivery in cancerous H1299 cells.³⁵ In particular, we here explored two different outer polymeric layers, PEI (PEI-LBL-NCs) and CS (CS-LBL-NCs). While there were no significant differences in size, zeta potential or siRNA loading, siRNA was better retained in PEI-LBL-NCs when exposed to different pH conditions (pH= 7.4 and 8.5). This can be explained by the fact that PEI has a broader proton buffering capacity from pH 3-10 with amine groups still being protonated even at pH 8.5, so that siRNA can remain electrostatically complexed.^{71,72} Thanks to a strong protection by PEI as the outer layer, 60% of siRNA remained complexed in PEI-LBL-NCs when exposed to simulated wound fluid for 4 h. This was quite different for CS-LBL-NCs, in which case siRNA was almost immediately completely released.

Balancing uptake with cell viability, a concentration of 16 nM siRNA was found to perform well on RAW 264.7 macrophages. At the same effective siRNA concentration, the commercial transfection reagent jetPRIME showed similar uptake and slightly better cell viability. Lipofectamine RNAiMAX resulted in about double the amount of uptake in RAW 264.7 cells, again at slightly better cell viability. However, when looking at their capability to induce endosomal escape, the performance of those commercial reagents was equal

(RNAiMAX) or even slightly worse (jetPRIME) as compared to PEI-LBL-NCs. In agreement with previous reported studies, utilizing pH-responsive PEI as a coating layer resulted in more endosomal escape of siRNA into the cytosolic space.⁹⁶⁻⁹⁸ Of all NCs tested, endosomal escape was by far the least efficient for CS-LBL-NCs, which is likely due to a lack of buffering capacity in the endosomal pH range which is needed to induce endosomal rupture by the proton sponge effect.

Most importantly, from all tested carriers, transfection efficiency with *irf5*-siRNA was clearly superior for PEI-LBL-NCs, as could be seen in LPS stimulated RAW 264.7 macrophages by downregulation of *irf5*, NOS-2 and TNF- α and upregulation of Arg-1. Repolarization of PEI-LBL-NC transfected M1 macrophages to the M2 phenotype was confirmed in our study by a shift in CD 206 (M2) and CD 86 (M1) expression. These findings are consistent with recent data showing that *irf5* ablation inhibits M1 macrophage polarization and suppresses the progression of inflammation.⁹⁹⁻¹⁰¹ It has been shown that CD206 positive macrophages are a paracrine source of hepatocyte growth factor (HGF) in injured muscles, which can help muscle fiber regeneration.¹⁰² Other angiogenic growth factors are also produced by M2 macrophages, such as basic fibroblast growth factor, IGF-1, chemokine (C-C) motif ligand 2, placental growth factor, and vascular growth factor-A (VEGF-A).¹⁰³

Interestingly, with an *in vitro* wound scratch assay on NIH-3T3 fibroblast cells we could confirm that wound healing is accelerated when cells are incubated with conditioned cell medium coming from *irf5*-siRNA transfected RAW 264.7 CD206⁺ macrophages. Combined with the rather unexpected finding that PEI-LBL-NCs are less efficiently taken up by NIH-3T3 fibroblasts as compared to macrophages, this shows that PEI-LBL-NCs are very promising to accelerate wound healing in diabetic patients.

In summary, research in macrophage therapy has shifted from macrophage inhibition and depletion to the promotion of controlled recruitment and modulation.¹⁰⁴ The main purpose of this study was also to highlight the advantages of enforced M1 to M2 macrophage repolarization using the here reported PEI-LBL-NCs for topical diabetic wound healing applications. Our data indicated that the use of LBL design with PEI in the outer layer will lead to a nanocomplex with optimal stability and *irf5*-siRNA protective effect, as well as increase uptake, endosomal escape and transfection efficiency, the end result of which will be inducing macrophages phenotype change from the M1 to M2 phenotype and faster wound healing in an *in vitro* scratch assay (Scheme 1).

Apart from further validation of this concept in *in vivo* studies, it will be of interest to develop systems that allow sustained release of PEI-LBL-NCs, such as advanced wound dressings.¹⁰⁵ This is because in the wound tissue there is a rapid cellular turnover and proliferation of macrophages that will lead to a disappearance of the siRNA gene suppression effect already after one week.¹⁰⁶

Conclusion

Here we reported layer-by-layer nanocarriers loaded with irf5-siRNA to induce macrophage reprogramming from the M1 to M2 phenotype in the context of diabetic wound healing. While using chitosan as the outer layer resulted in low endosomal escape and transfection efficiency, PEI performed much better in terms of stability, uptake, endosomal escape and transfection efficiency. We could demonstrate that irf5-siRNA delivery by PEI-LBL-NCs induced a change in LPS stimulated RAW 264.7 macrophages from the M1 to M2 phenotype. This resulted in faster wound healing in an *in vitro* scratch assay. These results show that PEI-LBL-NCs loaded with irf5-siRNA are a promising effective approach for macrophage reprogramming to a pro-healing M2 phenotype, which is expected to be of benefit for improved wound healing in diabetic patients.

Scheme 1, Macrophage reprogramming into a pro-healing phenotype by irf5-siRNA delivered with layer by layer (LBL) assembled nanocomplexes for wound healing applications. The use of LBL design with PEI in the outer layer will lead to a nanocomplex with optimal stability and irf5-siRNA protection effect, as well as increase uptake, endosomal escape and transfection efficiency. Successful delivery of irf5-siRNA by PEI-LBL-NCs resulted macrophages phenotype change from the M1 to M2 phenotype and faster wound healing in an *in vitro* scratch assay.

Author Contributions

MSh: Conceptualization, Investigation, Writing - Original Draft; **ESh, ZSh, HDK, BL, JL:** Investigation; **HG, LT, ChV, TDB:** Resources; **SDS:** Resources, Funding acquisition; **RFM, KB:** Supervision, Funding acquisition, Review & Editing; **JCF:** Supervision, Writing - Review & Editing.

Conflicts of interest

The authors declare no conflict of interest.

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Supporting Information Available

One additional figure.

References

- Schultz, G. S., Davidson, J. M., Kirsner, R. S., Bornstein, P. & Herman, I. M. Dynamic reciprocity in the wound microenvironment. *Wound Repair and Regeneration* **19**, 134-148 (2011).
- Wear-Maggitti, K. *et al.* Use of topical sRAGE in diabetic wounds increases neovascularization and granulation tissue formation. *Annals of plastic surgery* **52**, 519-521 (2004).
- Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nature reviews immunology* **8**, 958-969 (2008).
- Martinez, F. O. & Gordon, S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime reports* **6** (2014).
- Ogle, M. E., Segar, C. E., Sridhar, S. & Botchwey, E. A. Monocytes and macrophages in tissue repair: Implications for immunoregenerative biomaterial design. *Experimental Biology and Medicine* **241**, 1084-1097 (2016).
- Han, G. & Ceilley, R. Chronic wound healing: a review of current management and treatments. *Advances in therapy* **34**, 599-610 (2017).
- Gan, J. *et al.* Accelerated wound healing in diabetes by reprogramming the macrophages with particle-induced clustering of the mannose receptors. *Biomaterials* **219**, 119340 (2019).
- Peppas, M., Stavroulakis, P. & Raptis, S. A. Advanced glycoxidation products and impaired diabetic wound healing. *Wound Repair and Regeneration* **17**, 461-472 (2009).
- Li, J., Liu, Y., Xu, H. & Fu, Q. Nanoparticle-Delivered IRF5 siRNA Facilitates M1 to M2 Transition, Reduces Demyelination and Neurofilament Loss, and Promotes Functional Recovery After Spinal Cord Injury in Mice. *Inflammation* **39**, 1704-1717, doi:10.1007/s10753-016-0405-4 (2016).
- Courties, G. *et al.* In vivo silencing of the transcription factor IRF5 reprograms the macrophage phenotype and improves infarct healing. *J Am Coll Cardiol* **63**, 1556-1566, doi:10.1016/j.jacc.2013.11.023 (2014).
- Sun, K. *et al.* IRF5 regulates lung macrophages M2 polarization during severe acute pancreatitis in vitro. *World J Gastroenterol* **22**, 9368-9377, doi:10.3748/wjg.v22.i42.9368 (2016).
- Terashima, T. *et al.* Gene therapy for neuropathic pain through siRNA-IRF5 gene delivery with homing peptides to microglia. *Molecular Therapy-Nucleic Acids* **11**, 203-215 (2018).
- Zhou, J., Shum, K.-T., Burnett, J. C. & Rossi, J. J. Nanoparticle-based delivery of RNAi therapeutics: progress and challenges. *Pharmaceuticals* **6**, 85-107 (2013).
- Mulder, G., Jones, R., CEDERHOLM-WILLIAMS, S., Cherry, G. & Ryan, T. Fibrin cuff lysis in chronic venous ulcers treated with a hydrocolloid dressing. *International journal of dermatology* **32**, 304-306 (1993).
- Wang, W., Lu, K.-j., Yu, C.-h., Huang, Q.-l. & Du, Y.-Z. Nano-drug delivery systems in wound treatment and skin regeneration. *Journal of nanobiotechnology* **17**, 1-15 (2019).
- Castanotto, D. & Rossi, J. J. The promises and pitfalls of RNA-interference-based therapeutics. *Nature* **457**, 426-433, doi:10.1038/nature07758 (2009).
- Dong, Y., Siegwart, D. J. & Anderson, D. G. Strategies, design, and chemistry in siRNA delivery systems. *Advanced drug delivery reviews* **144**, 133-147 (2019).
- Lan, B. *et al.* Sustained Delivery of MMP-9 siRNA via Thermosensitive Hydrogel Accelerates Diabetic Wound Healing. (2021).
- Martin, J. R. *et al.* Local delivery of PHD2 siRNA from ROS-degradable scaffolds to promote diabetic wound healing. *Advanced healthcare materials* **5**, 2751-2757 (2016).
- Ren, X. *et al.* An aligned porous electrospun fibrous membrane with controlled drug delivery—an efficient

- strategy to accelerate diabetic wound healing with improved angiogenesis. *Acta biomaterialia* **70**, 140-153 (2018).
- 21 Lo, K. W.-H., Jiang, T., Gagnon, K. A., Nelson, C. & Laurencin, C. T. Small-molecule based musculoskeletal regenerative engineering. *Trends in biotechnology* **32**, 74-81 (2014).
- 22 Tezgel, Ö. *et al.* Collagen scaffold-mediated delivery of NLC/siRNA as wound healing materials. *Journal of Drug Delivery Science and Technology* **55**, 101421 (2020).
- 23 Weng, Y., Xiao, H., Zhang, J., Liang, X.-J. & Huang, Y. RNAi therapeutic and its innovative biotechnological evolution. *Biotechnology advances* **37**, 801-825 (2019).
- 24 Yuan-Yu, H. Approval of the first-ever RNAi therapeutics and its technological development history. *PROGRESS IN BIOCHEMISTRY AND BIOPHYSICS* **46**, 313-322 (2019).
- 25 Zheng, M. *et al.* ROS-responsive polymeric siRNA nanomedicine stabilized by triple interactions for the robust glioblastoma combinational RNAi therapy. *Advanced Materials* **31**, 1903277 (2019).
- 26 Chen, Y. *et al.* A supramolecular co-delivery strategy for combined breast cancer treatment and metastasis prevention. *Chinese Chemical Letters* **31**, 1153-1158 (2020).
- 27 Kim, M., Kim, G., Hwang, D. W. & Lee, M. Delivery of high mobility group box-1 siRNA using brain-targeting exosomes for ischemic stroke therapy. *Journal of biomedical nanotechnology* **15**, 2401-2412 (2019).
- 28 Akinc, A., Thomas, M., Klibanov, A. M. & Langer, R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J Gene Med* **7**, 657-663, doi:10.1002/jgm.696 (2005).
- 29 Castleberry, S., Wang, M. & Hammond, P. T. Nanolayered siRNA dressing for sustained localized knockdown. *ACS Nano* **7**, 5251-5261, doi:10.1021/nn401011n (2013).
- 30 Cordeiro, R. A., Serra, A., Coelho, J. F. J. & Faneca, H. Poly(beta-amino ester)-based gene delivery systems: From discovery to therapeutic applications. *J Control Release* **310**, 155-187, doi:10.1016/j.jconrel.2019.08.024 (2019).
- 31 Lee, S. J., Kim, M. J., Kwon, I. C. & Roberts, T. M. Delivery strategies and potential targets for siRNA in major cancer types. *Adv Drug Deliv Rev* **104**, 2-15, doi:10.1016/j.addr.2016.05.010 (2016).
- 32 Pack, D. W., Hoffman, A. S., Pun, S. & Stayton, P. S. Design and development of polymers for gene delivery. *Nat Rev Drug Discov* **4**, 581-593, doi:10.1038/nrd1775 (2005).
- 33 Mao, S., Sun, W. & Kissel, T. Chitosan-based formulations for delivery of DNA and siRNA. *Adv Drug Deliv Rev* **62**, 12-27, doi:10.1016/j.addr.2009.08.004 (2010).
- 34 Wang, X., Niu, D., Hu, C. & Li, P. Polyethyleneimine-Based Nanocarriers for Gene Delivery. *Curr Pharm Des* **21**, 6140-6156, doi:10.2174/1381612821666151027152907 (2015).
- 35 Shaabani, E. *et al.* Layer by layer assembled chitosan-coated gold nanoparticles for enhanced siRNA delivery and silencing. *International Journal of Molecular Sciences* **22**, 831 (2021).
- 36 Shaabani, E. *et al.* Layer by Layer Assembled Chitosan-Coated Gold Nanoparticles for Enhanced siRNA Delivery and Silencing. *Int J Mol Sci* **22**, 831, doi:10.3390/ijms22020831 (2021).
- 37 Ragelle, H., Vandermeulen, G. & Préat, V. Chitosan-based siRNA delivery systems. *Journal of Controlled Release* **172**, 207-218 (2013).
- González-Domínguez, I., Grimaldi, N., Cervera, L., Ventosa, N. & Gòdia, F. Impact of physicochemical properties of DNA/PEI complexes on transient transfection of mammalian cells. *New biotechnology* **49**, 88-97 (2019).
- Merkel, O. M. *et al.* Stability of siRNA polyplexes from poly(ethylenimine) and poly(ethylenimine)-g-poly(ethylene glycol) under in vivo conditions: effects on pharmacokinetics and biodistribution measured by Fluorescence Fluctuation Spectroscopy and Single Photon Emission Computed Tomography (SPECT) imaging. *J Control Release* **138**, 148-159, doi:10.1016/j.jconrel.2009.05.016 (2009).
- Rampino, A., Borgogna, M., Blasi, P., Bellich, B. & Cesaro, A. Chitosan nanoparticles: preparation, size evolution and stability. *Int J Pharm* **455**, 219-228, doi:10.1016/j.ijpharm.2013.07.034 (2013).
- Lai, W. F. & Lin, M. C. Nucleic acid delivery with chitosan and its derivatives. *J Control Release* **134**, 158-168, doi:10.1016/j.jconrel.2008.11.021 (2009).
- Saranya, N., Moorthi, A., Saravanan, S., Devi, M. P. & Selvamurugan, N. Chitosan and its derivatives for gene delivery. *Int J Biol Macromol* **48**, 234-238, doi:10.1016/j.ijbiomac.2010.11.013 (2011).
- Ewe, A. *et al.* Optimized polyethylenimine (PEI)-based nanoparticles for siRNA delivery, analyzed in vitro and in an ex vivo tumor tissue slice culture model. *Drug Deliv Transl Res* **7**, 206-216, doi:10.1007/s13346-016-0306-y (2017).
- Szymanska, E. & Winnicka, K. Stability of chitosan-a challenge for pharmaceutical and biomedical applications. *Mar Drugs* **13**, 1819-1846, doi:10.3390/md13041819 (2015).
- Zhao, S. *et al.* The future of layer-by-layer assembly: a tribute to ACS nano associate editor Helmuth Mohwald. *ACS nano* **13**, 6151-6169 (2019).
- Al Jahdaly, B. A. *et al.* Selenium nanoparticles synthesized using an eco-friendly method: dye decolorization from aqueous solutions, cell viability, antioxidant, and antibacterial effectiveness. *Journal of materials research and technology* **11**, 85-97 (2021).
- Golmohammadi, R., Najar-Peerayeh, S., Moghadam, T. T. & Hosseini, S. M. J. Synergistic antibacterial activity and wound healing properties of selenium-chitosan-mupirocin nanohybrid system: an in vivo study on rat diabetic staphylococcus aureus wound infection model. *Scientific reports* **10**, 1-10 (2020).
- Biswas, D. P., O'Brien-Simpson, N. M., Reynolds, E. C., O'Connor, A. J. & Tran, P. A. Comparative study of novel in situ decorated porous chitosan-selenium scaffolds and porous chitosan-silver scaffolds towards antimicrobial wound dressing application. *Journal of colloid and interface science* **515**, 78-91 (2018).
- Mao, L. *et al.* In Situ Synthesized Selenium Nanoparticles-Decorated Bacterial Cellulose/Gelatin Hydrogel with Enhanced Antibacterial, Antioxidant, and Anti-Inflammatory Capabilities for Facilitating Skin Wound Healing. *Advanced Healthcare Materials*, 2100402 (2021).
- Filipović, N. *et al.* Comparative study of the antimicrobial activity of selenium nanoparticles with different surface chemistry and structure. *Frontiers in bioengineering and biotechnology* **8**, 1591 (2021).

- Whittam, A. J. *et al.* Challenges and Opportunities in Drug Delivery for Wound Healing. *Adv Wound Care (New Rochelle)* **5**, 79-88, doi:10.1089/wound.2014.0600 (2016).
- Moura, L. I., Dias, A. M., Carvalho, E. & de Sousa, H. C. Recent advances on the development of wound dressings for diabetic foot ulcer treatment--a review. *Acta Biomater* **9**, 7093-7114, doi:10.1016/j.actbio.2013.03.033 (2013).
- Fonder, M. A. *et al.* Treating the chronic wound: A practical approach to the care of nonhealing wounds and wound care dressings. *J Am Acad Dermatol* **58**, 185-206, doi:10.1016/j.jaad.2007.08.048 (2008).
- Kasiewicz, L. N. & Whitehead, K. A. Lipid nanoparticles silence tumor necrosis factor alpha to improve wound healing in diabetic mice. *Bioeng Transl Med* **4**, 75-82, doi:10.1002/btm2.10123 (2019).
- Cetin, M., Atila, A. & Kadioglu, Y. Formulation and in vitro characterization of Eudragit® L100 and Eudragit® L100-PLGA nanoparticles containing diclofenac sodium. *Aaps. Pharmscitech.* **11**, 1250-1256 (2010).
- D'Souza, S. A review of in vitro drug release test methods for nano-sized dosage forms. *J. Adv. Pharm.* **2014** (2014).
- Vu, H. *et al.* A Device to Predict Short-Term Healing Outcome of Chronic Wounds. *Advances in wound care* **9**, 312-324 (2020).
- Zhang, H., De Smedt, S. C. & Remaut, K. Fluorescence Correlation Spectroscopy to find the critical balance between extracellular association and intracellular dissociation of mRNA complexes. *Acta biomaterialia* **75**, 358-370 (2018).
- Lee, C. *et al.* Targeting of M2-like tumor-associated macrophages with a melittin-based pro-apoptotic peptide. *Journal for immunotherapy of cancer* **7**, 1-14 (2019).
- Rehman, Z. U., Hoekstra, D. & Zuhorn, I. S. Mechanism of polyplex-and lipoplex-mediated delivery of nucleic acids: real-time visualization of transient membrane destabilization without endosomal lysis. *ACS nano* **7**, 3767-3777 (2013).
- Maiyo, F. & Singh, M. Polymerized Selenium Nanoparticles for Folate-Receptor-Targeted Delivery of Anti-Luc-siRNA: Potential for Gene Silencing. *Biomedicines* **8**, 76 (2020).
- Sharifiaghdam, M. *et al.* Enhanced siRNA Delivery and Selective Apoptosis Induction in H1299 Cancer Cells by Layer-by-Layer-Assembled Se Nanocomplexes: Toward More Efficient Cancer Therapy. *Frontiers in molecular biosciences* **8** (2021).
- Song, X. *et al.* Effect of molecular weight of chitosan and its oligosaccharides on antitumor activities of chitosan-selenium nanoparticles. *Carbohydrate polymers* **231**, 115689 (2020).
- Song, X., Chen, Y., Sun, H., Liu, X. & Leng, X. Physicochemical and functional properties of chitosan-stabilized selenium nanoparticles under different processing treatments. *Food chemistry* **331**, 127378 (2020).
- León, Y., Cárdenas, G. & Arias, M. Synthesis and characterizations of metallic nanoparticles in chitosan by chemical reduction. *Journal of the Chilean Chemical Society* **62**, 3760-3764 (2017).
- Chen, B.-K., Su, C.-T., Tseng, M.-C. & Tsay, S.-Y. Preparation of polyetherimide nanocomposites with improved thermal, mechanical and dielectric properties. *Polymer Bulletin* **57**, 671-681 (2006).
- Jones, E. M., Cochrane, C. A. & Percival, S. L. The effect of pH on the extracellular matrix and biofilms. *Advances in wound care* **4**, 431-439 (2015).
- Lee, D. W., Lim, C., Israelachvili, J. N. & Hwang, D. S. Strong adhesion and cohesion of chitosan in aqueous solutions. *Langmuir* **29**, 14222-14229 (2013).
- Suh, J., Paik, H.-j. & Hwang, B. K. Ionization of Poly (ethylenimine) and Poly (allylamine) at Various pH' s. *Bioorganic Chemistry* **22**, 318-327 (1994).
- Vermeulen, L. M. P. *et al.* Endosomal Size and Membrane Leakiness Influence Proton Sponge-Based Rupture of Endosomal Vesicles. *ACS Nano* **12**, 2332-2345, doi:10.1021/acsnano.7b07583 (2018).
- Chen, H. *et al.* Grafting chitosan with polyethylenimine in an ionic liquid for efficient gene delivery. *PLoS One* **10**, e0121817, doi:10.1371/journal.pone.0121817 (2015).
- Richard, I., Thibault, M., De Crescenzo, G., Buschmann, M. D. & Lavertu, M. Ionization behavior of chitosan and chitosan-DNA polyplexes indicate that chitosan has a similar capability to induce a proton-sponge effect as PEI. *Biomacromolecules* **14**, 1732-1740 (2013).
- Garcia-Fuentes, M. & Alonso, M. J. Chitosan-based drug nanocarriers: where do we stand? *Journal of Controlled Release* **161**, 496-504 (2012).
- Moreira, C. *et al.* Improving chitosan-mediated gene transfer by the introduction of intracellular buffering moieties into the chitosan backbone. *Acta biomaterialia* **5**, 2995-3006 (2009).
- Udalova, I. A. *et al.* IRF5 promotes inflammatory macrophage polarization and Th1/Th17 response. *Nature Immunology* (2011).
- Kim, K.-S. *et al.* The inhibition of JNK MAPK and NF- κ B signaling by tenuifoliside A isolated from *Polygala tenuifolia* in lipopolysaccharide-induced macrophages is associated with its anti-inflammatory effect. *European Journal of Pharmacology* **721**, 267-276 (2013).
- Weiss, G. & Schaible, U. E. Macrophage defense mechanisms against intracellular bacteria. *Immunol Rev* **264**, 182-203, doi:10.1111/imr.12266 (2015).
- Caldwell, R. W., Rodriguez, P. C., Toque, H. A., Narayanan, S. P. & Caldwell, R. B. Arginase: A Multifaceted Enzyme Important in Health and Disease. *Physiol Rev* **98**, 641-665, doi:10.1152/physrev.00037.2016 (2018).
- Page, M. J., Bester, J. & Pretorius, E. The inflammatory effects of TNF-alpha and complement component 3 on coagulation. *Sci Rep* **8**, 1812, doi:10.1038/s41598-018-20220-8 (2018).
- Chen, L. & DiPietro, L. A. Toll-like receptor function in acute wounds. *Advances in wound care* **6**, 344-355 (2017).
- Komi, D. E. A., Khomtchouk, K. & Santa Maria, P. L. A review of the contribution of mast cells in wound healing: involved molecular and cellular mechanisms. *Clinical reviews in allergy & immunology* **58**, 298-312 (2020).
- Wolfs, I. M., Donners, M. M. & de Winther, M. P. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. *Thrombosis and haemostasis* **106**, 763-771 (2011).
- Vannella, K. M. & Wynn, T. A. Mechanisms of organ injury and repair by macrophages. *Annual review of physiology* **79**, 593-617 (2017).

- 84 Peter, J. & Thomas, A. Protective and pathogenic function of macrophage subsets. *Nature Reviews Immunology* **11**, 723-737 (2011).
- 85 Ferrante, C. J. & Leibovich, S. J. Regulation of macrophage polarization and wound healing. *Advances in wound care* **1**, 10-16 (2012).
- 86 Wynn, T. A. & Vannella, K. M. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity* **44**, 450-462 (2016).
- 87 Li, Z., Guo, S., Yao, F., Zhang, Y. & Li, T. Increased ratio of serum matrix metalloproteinase-9 against TIMP-1 predicts poor wound healing in diabetic foot ulcers. *Journal of Diabetes and its Complications* **27**, 380-382 (2013).
- 88 Barnes, B. J., Moore, P. A. & Pitha, P. M. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon α genes. *Journal of Biological Chemistry* **276**, 23382-23390 (2001).
- 89 Takaoka, A. *et al.* Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* **434**, 243-249 (2005).
- 90 Zhang, X. & Mosser, D. Macrophage activation by endogenous danger signals. *The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland* **214**, 161-178 (2008).
- 91 Chistiakov, D. A., Myasoedova, V. A., Revin, V. V., Orekhov, A. N. & Bobryshev, Y. V. The impact of interferon-regulatory factors to macrophage differentiation and polarization into M1 and M2. *Immunobiology* **223**, 101-111 (2018).
- 92 Al-Rashed, F. *et al.* Repetitive Intermittent Hyperglycemia Drives the M1 Polarization and Inflammatory Responses in THP-1 Macrophages Through the Mechanism Involving the TLR4-IRF5 Pathway. *Cells* **9**, 1892 (2020).
- 93 Sarett, S. M., Nelson, C. E. & Duvall, C. L. Technologies for controlled, local delivery of siRNA. *J Control Release* **218**, 94-113, doi:10.1016/j.jconrel.2015.09.066 (2015).
- 94 Whitehead, K. A. *et al.* Degradable lipid nanoparticles with predictable in vivo siRNA delivery activity. *Nat Commun* **5**, 4277, doi:10.1038/ncomms5277 (2014).
- 95 Whitehead, K. A. *et al.* In vitro-in vivo translation of lipid nanoparticles for hepatocellular siRNA delivery. *ACS Nano* **6**, 6922-6929, doi:10.1021/nn301922x (2012).
- 96 Juliano, R. L. The delivery of therapeutic oligonucleotides. *Nucleic Acids Res* **44**, 6518-6548, doi:10.1093/nar/gkw236 (2016).
- 97 Kafshgari, M. H., Voelcker, N. H. & Harding, F. J. Applications of zero-valent silicon nanostructures in biomedicine. *Nanomedicine (Lond)* **10**, 2553-2571, doi:10.2217/nnm.15.91 (2015).
- 98 Nel, A., Mädler, L., Velegol, D., Xia, T. & Hoek, E. V.; Somasundaran P.; Klaessig F.; Castranova V.; Thompson M. *Understanding Biophysicochemical Interactions at the Nano-Bio Interface*. *Nat. Mater* **8**, 543-557 (2009).
- 99 Krausgruber, T. *et al.* IRF5 promotes inflammatory macrophage polarization and TH 1-TH 17 responses. *Nature immunology* **12**, 231-238 (2011).
- 100 Dalmás, E. *et al.* Irf5 deficiency in macrophages promotes beneficial adipose tissue expansion and insulin sensitivity during obesity. *Nat Med* **21**, 610-618, doi:10.1038/nm.3829 (2015).
- 101 Seneviratne, A. N. *et al.* Interferon Regulatory Factor 5 Controls Necrotic Core Formation in Atherosclerotic Lesions by Impairing Efferocytosis. *Circulation* **136**, 1140-1154, doi:10.1161/CIRCULATIONAHA.117.027844 (2017).
- 102 Sakaguchi, S. *et al.* Implication of anti-inflammatory macrophages in regenerative moto-neuritogenesis: promotion of myoblast migration and neural chemorepellent semaphorin 3A expression in injured muscle. *Int J Biochem Cell Biol* **54**, 272-285, doi:10.1016/j.biocel.2014.05.032 (2014).
- 103 Jetten, N. *et al.* Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo. *Angiogenesis* **17**, 109-118 (2014).
- 104 Mokarram, N. & Bellamkonda, R. V. A perspective on immunomodulation and tissue repair. *Ann Biomed Eng* **42**, 338-351, doi:10.1007/s10439-013-0941-0 (2014).
- 105 Fan, Y. *et al.* Nanogel encapsulated hydrogels as advanced wound dressings for the controlled delivery of antibiotics. *Advanced Functional Materials* **31**, 2006453 (2021).
- 106 Layzer, J. M. *et al.* In vivo activity of nuclease-resistant siRNAs. *RNA* **10**, 766-771, doi:10.1261/rna.5239604 (2004).

Figure S1. Cell viability of PEI-LBL-NCs and Se@CS NPs in RAW 264.7 cells. Cell viability, measured by the metabolic CellTitre Glo assay, after 4h incubation with PEI-LBL-NCs and Se@CS NPs as a function of Se concentration (ppm).

Figure S2. Cell viability of Se@CS core in RAW 264.7, NIH-3T3, and H1299 cells. Cell viability, measured by the metabolic CellTitre Glo assay, after 4h incubation with Se@CS as a function of Se concentration (ppm). Bars represent mean $\pm$ standard deviation, (ns = not significant, n = 3, *: p < 0.05; **: p < 0.01; ***: p < 0.001).

Figure S3. FFS decomplexation assay. Representative fluorescence fluctuations as measured with FFS for naked siRNA (red) Water Background (BG, Black), NCs (PEI-LBL-NCs, CS-LBL-NCs, jetPRIME-NCs, and RNAiMAX-NCs) 4h incubated in (A) HEPES, (B) CCM, (C) SWF, and (D) NCs 15 min incubated in SDS/DS (PEI-LBL-NCs, CS-LBL-NCs, and jetPRIME-NCs were incubated in sodium dodecyl sulfate (SDS) 2%, and RNAiMAX-NCs incubated with dextran sulfate (DS) 0.1%).