NANOFIBER DRESSING CONSISTING OF ANTISENSE RNA-FUNCTIONALIZED NANODIAMONDS FOR THERAPY OF NON-HEALING WOUNDS IN DIABETIC INDIVIDUALS

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Abstract

Non-healing wounds are serious complication in diabetic patients and represent an attractive challenge for development of suitable carrier system possessing constant and localized release of therapeutic biomolecule into the wound without any undesired side effects. Given the fact that these non-healing wounds are result of impaired balance in metalloproteinases synthesized by immune cells residing the wounds, gene therapy offering knock down of such enzymes is of great interest.

Here we challenged a development of functional and biocompatible wound dressing enabling controlled release of trackable carrier loaded with therapeutic siRNA. Our dressing consists of scaffold from degradable polymer nanofibers enriched with fluorescent nanodiamond particles (FND). We have previously shown the nanodiamond particles are great carriers for antisense RNAs. Their advantages represent high biocompatibility, stable luminescence giving us the possibility to track the carrier system in the wound, and effective release of antisense RNA in the wound. Embedding of nanodiamond-siRNA systems into nanofiber scaffold enables continuous release of siRNA and maintaining the stable siRNA concentration in the wound site resulting in a promotion of wound healing.

We developed FND-siRNA complexes specific to MMP-9 that efficiently inhibit the expression of target MMP-9 mRNA. The complexes were embedded into core/shell nanofibers from PVA and PCL, visualized by confocal microscopy, and characterized by electron microscopy. Real-time PCR was used to assess the silencing effect of siRNA that has been delivered to target murine fibroblasts by FND released from nanofiber dressing. Nanofiber system with embedded FNDs was applied on wounds in diabetic animal models to evaluate its suitability regarding short and long term toxicity, efficacy, and handling *in vivo*.

Our results suggest that using nanodiamond-siRNA complexes with nanofiber-controlled and localized release is a promising approach in gene therapy of non-healing wounds.

Keywords: Electrospun nanofibers, fluorescent nanodiamonds, RNA interference, gene therapy, non-healing wounds

1. INTRODUCTION

According to the annual WHO statistics, the number of diabetic patients almost quadrupled since 1980 and its prevalence is rapidly rising among low- and middle-income countries [1]. There are many efforts being done to prevent, educate and treat the disease as such, but at the same time, the emphasis is put on finding ways to help patients cope with the consequences of the disease. Diabetes is a major cause of a lower limb amputation, resulting from non-healing ulcers, common complications of diabetes [1].

1.1. Non-healing wounds and RNA interference

The wound healing process is characterized by a high complexity and it is usually described as consisting of four main parts which include initiation, inflammation, proliferation, and maturation. Matrix metalloproteinases called gelatinases are enzymes known for their capability to cleave collagen, elastin, basement membranes,

and denatured collagen [2]. During the inflammatory phase of wound healing, the amount of active gelatinases increases in order to clean the wound from old cells and damaged extracellular parts. Once the wound is free of such old tissue, the activity of the metalloproteinases reduces to prevent any removal of newly formed tissue [3]. Therefore, gelatinases play an important role in wound healing, and their dysregulation caused by prolonged inflammatory phase in diabetic individuals results in impaired wound healing. In diabetic animal models, levels of both MMP-2 and MMP-9 active metalloproteinases are significantly elevated [4]. The level of active gelatinase can be regulated by using customized siRNAs that affect the protein expression by RNA interference (RNAi) [5].

1.2. FND carriers for local gene therapy

Fluorescence nanodiamond nanoparticles (FNDs) are suitable carriers that can deliver the siRNA to the cells in a wound site. In addition to their nano-size, the most important properties that make them so attractive include their non-toxicity, high biocompatibility, easily functionalized surface, and non-bleaching/blinking fluorescence [6][7]. The fluorescence of FNDs is given by the nitrogen-vacancy centers (NVCs), useful lattice defects created by irradiation of NDs with high energy particles followed by vacuum annealing [6][8]. Their stable fluorescence can be than well detected by confocal microscopy.

1.3. Nanofibers mesh loaded with FND carriers

The length of the wound healing process requires constitutive release of FND-siRNA complexes to the wound site. Coaxial electrospun nanofibers have been previously shown to be suitable as a wound dressing and they can be produced from different polymers or their combinations enabling the development of dressing with optimal release kinetics. For the FND-siRNA incorporation, we selected poly-ε-caprolactone (PCL) and polyvinyl alcohol (PVA) [9]. These polymers were built-up to the PVA-core/PCL-shell structure, according to their affinity to water.

2. METHODS

FNDs used in this project are kindly provided by Dr. Vlaďka Petrakova from the Faculty of Biomedical Engineering, Czech Technical University. Surface modification of FND with polymer linker and siRNA is carried out by Ing. Marek Kindermann at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences. FND preparation process including coating of their surface has been described earlier [6][10].

Zeta-potential (z-potential) method was used to characterize nanodiamond complexes as they were functionalized, i. e. pure FNDs, PEI800 coated FNDs and short RNAs linking with the FND-PEI surface. Nanoparticles were kept in phosphate-buffered saline solution while measured by Zetasizer Nano (Malvern).

NIH/3T3 mouse adherent fibroblast cell line was obtained from the ATCC (CRL-1658™) and maintained in Gibco™ DMEM medium (Thermo Fisher), supplemented with 10% Gibco™ FBS (Thermo Fisher) and 44 mg/L Gentamicin (Sandoz) in a humid atmosphere with 5% of CO_2. 24 hours before an experiment, cells were seeded in 70-80% confluency in starvation medium (without FBS). Final concentration of FNDs was 25 µg/ml. Control transfection of siRNA was carried out using X-tremeGENE™ HP DNA Transfection Reagent (Roche) according to manufacturer's protocol.

To visualize nanodiamonds inside NIH/3T3 fibroblasts by confocal microscopy, cells were seeded in density of 500.000 cells/ml on the plates with glass bottom and they were incubated with nanodiamonds. Cell nuclei were stained with Hoechst 33342 (1 µg/ml, Invitrogen) at the end of the incubation period, and the images were recorded with an Olympus FluoView™ FV 1000 confocal microscope (objective 40X, NA 0.95) and they were analysed with Olympus FluoView 2.0 software. Excitation/emission settings on confocal microscope were following: Hoechst (Nuclei) 405 nm/461 nm; FNDs 559 nm/655-755 nm.

Total RNA has been purified using a High Pure RNA isolation kit (Roche), according to manufacturer's instructions. 1ug of total RNA was transcribed using the High Capacity cDNA Archive Kit (Thermo Fisher Scientific). Real time PCR was performed with gene-specific TaqMan® Assay and the TaqMan® Universal PCR Master Mix. Here, β2 microglobulin (B2M) mRNA was used as an internal control. All samples were measured in triplicates using a CFX96 Touch™ Real-Time PCR Detection System (BioRad) and data were analysed using CFX Manager™ Software (BioRad).

Nanofibers were characterized visually by confocal microscopy to capture the fluorescence of incorporated FNDs (559 nm/655-755 nm) and FITC labelled fibers (488 nm/525 nm). Fluorescence-lifetime imaging (FLIM) was used to distinguish the different decay of FND fluorescence lifetime inside fibers. The effect of nanofiber wound dressing on the NIH/3T3 fibroblasts metabolic activity was evaluated using Cell Proliferation Reagent WST-1 (Roche). The cells were seeded in 12 well plates (250.000 cells/well) and nanofibers with and without incorporated FNDs were added in corresponding wells. WST-1 assay was performed according to manufacturer's instructions and the absorbance was measured with Tecan Infinite® 200 PRO microplate reader (450 nm, reference at 630 nm). Nanofibers were visualized using confocal microscopy.

Diabetic model was established using the C57/BL6 mouse male and an intraperitoneal administration of streptozotocin (STZ). Animals were starved for 4 hours prior the injection, they received the dose of 40 mg/kg/day for 5 consecutive days. Blood glucose was measured two weeks after last STZ injection and values over 8.3 mmol/l were considered as statistically higher than control animal values [11].

3. RESULTS

Measuring of z-potential confirmed the proper formation of the FND-PEI-RNA complex. It is clear from results in **Figure 1** that the FNDs exhibit negative z-potential, after modification with PEI800 the value moves to positive values, enabling the RNA to link electrostatically, shifting the value back to negative.

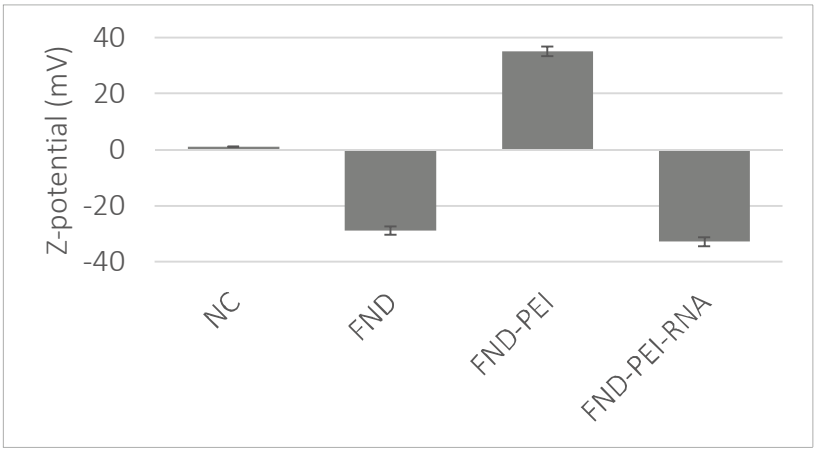

Figure 3 Z-potential results showing the FND, FND-PEI and FND-PEI-RNA values (in mV), control sample (NC) contained only phosphate-buffered saline

3T3 fibroblasts growing on glass visualized by confocal microscopy showed internalized nanodiamonds in cytoplasm (red signal, **Figure 2b**) after focusing on nuclei (blue signal, **Figure 2b**). SiRNA designed for murine MMP-9 activity reduction was verified *in vitro*, there was a significant decrease of normalized expression (**Figure 2a**) after 24 hours of stimulation with siRNA. 3T3 fibroblasts growing in presence of nanofibers on 12 well plates were measured in terms of their metabolic activity and the results are shown in **Figure 2c**. There were multiple control samples including wells without cells and wells with 3T3 cells that had promoted metabolic activity (FBS 10%).

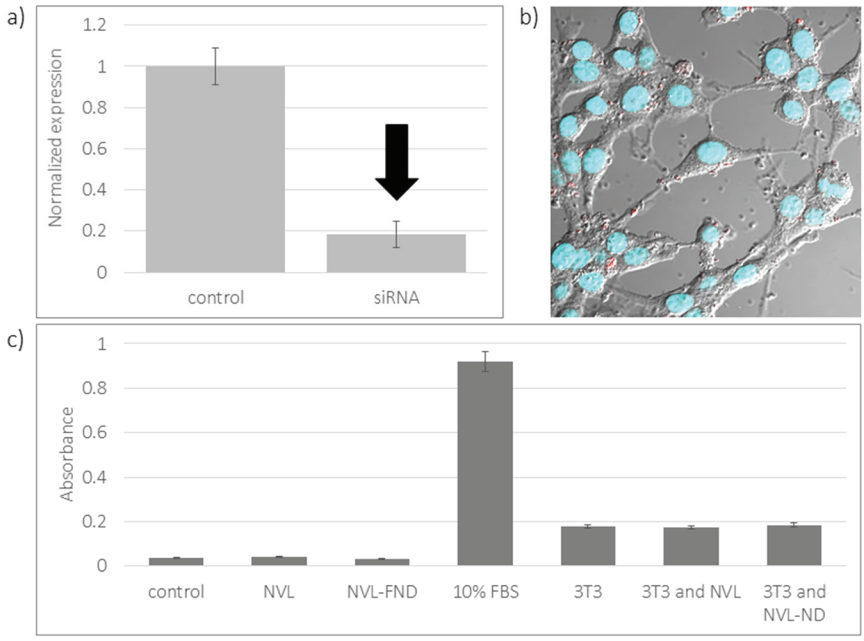

Figure 4 In vitro efficacy of siRNA on 3T3 fibroblasts, a) normalized expression comparison of 3T3 cells before and after siRNA stimulation, b) 3T3 fibroblasts with incorporated FNDs (red signal), c) WST-1 assay with 3T3 cells growing in presence of nanofibers; *Control* (DMEM medium), *NVL* (nanofiber mesh), *NVL-FND* (nanofiber mesh with embedded FNDs), *10% FBS* (3T3 cells stimulated with full media), *3T3* (cells in DMEM), *3T3 and NVL* (3T3 cells and nanofiber mesh), *3T3 and NVL-FND* (3T3 cells and nanofiber mesh with embedded FNDs)

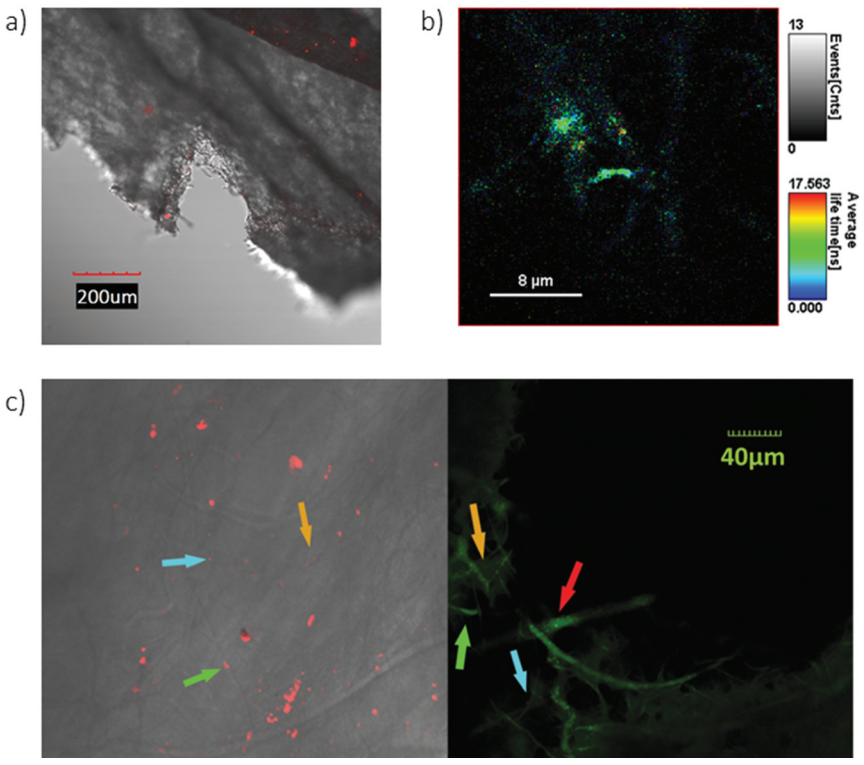

Figure 5 a) confocal microscopy of the thin end part of the nanofiber mesh with FNDs (red signal), b) FLIM of nanofibers with FNDs, c) confocal microscopy of FNDs (red signal, left image) in nanofibers and nanofibers labelled with FITC (green signal, right image)

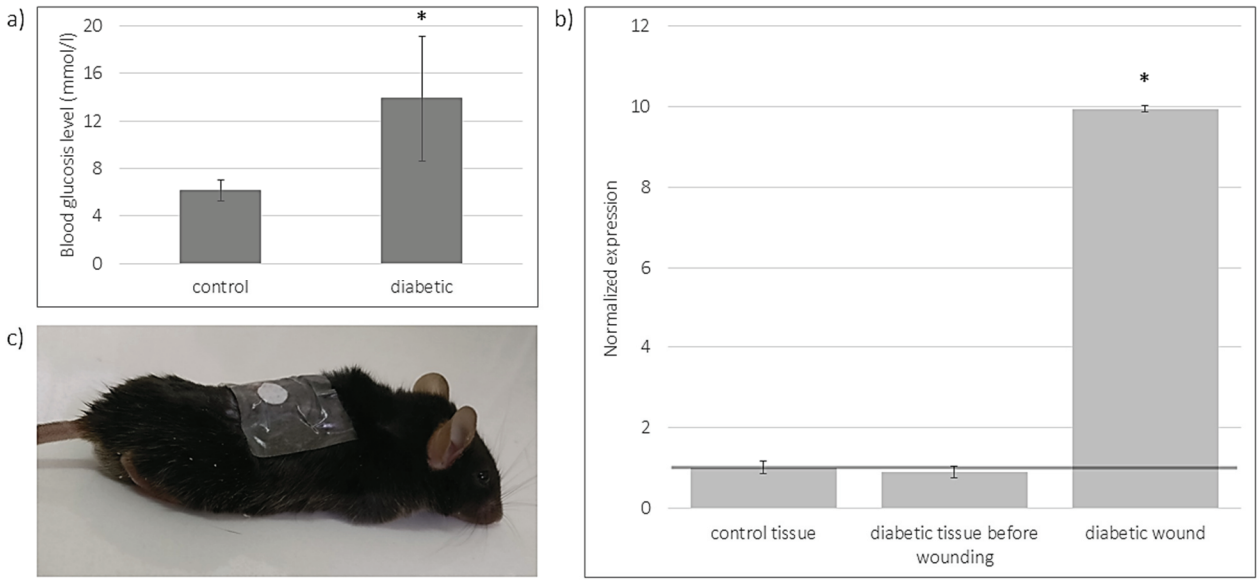

Figure 6 *In vivo* results. a) C57/BL6 animal model blood glucose level 2 weeks after STZ injections, b) level of MMP-9 expression in C57/BL6 tissue after wounding (comparison non-wounded and wounded diabetic tissue), c) C57/BL6 model with wound and nanofiber application

Diabetic animal models developed high blood glucose levels 2 weeks after last STZ injection (**Figure 4a)**. *In vivo* experiments showed high expression of MMP-9 mRNA (**Figure 4b**) in diabetic animal models when compared to unwounded tissue. **Figure 4c** shows C57/BL6 animal model with nanofiber applied on the wound.

3.1. DISCUSSION

The distribution of nanoparticles inside nanofiber system in the confocal microscopy images seems rather homogeneous with occasional clusters. This homogeneity is crucial for the final wound dressing product. FNDs proved the ability to enter the 3T3 fibroblast cytoplasm and thus deliver the siRNA. WST-1 assay confirmed that presence of nanofibers is not influencing the metabolic activity of fibroblasts in vitro and transfection of siRNA by nanodiamonds resulted in knock-down of target mRNA.

4. CONCLUSION

We presented system designed for siRNA delivery in non-healing wounds by combining two different nanomaterials. Thanks to nanofibers, this system is capable of gradual release and thanks to nanodiamonds it is capable to visualize the cargo delivery. We have developed stable hyperglycaemia in C57/BL6 animal models with elevated level of MMP-9 expression in their wound and we have designed nanodiamond-based siRNA carrier enabling decrease of target MMP-9 siRNA expression.

ACKNOWLEDGEMENTS

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