Full length article

Nitric oxide-releasing vascular grafts: A therapeutic strategy to promote angiogenic activity and endothelium regeneration

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ABSTRACT

Small-diameter vascular grafts (SDVGs) are associated with a high incidence of failure due to infection and obstruction. Although several vascular grafts are commercially available, specific anatomical differences of defect sites require patient-based design and fabrication. Design and fabrication of such custom-tailored grafts are possible with 3d-printing technology. The aim of this study is to develop 3d-printed SDVGs with a nitric oxide (NO)-releasing coating to improve the success rate of implantation. The SDVGs were printed from polylactic acid and coated with blending of 10 wt% S-nitroso-N-acetyl-D-penicillamine into the polymeric substrate consisting of poly (ethylene glycol) and polycaprolactone. Our results show that NO is released in the physiological range ($0.5\text{--}4 \times 10^{-10} \text{ mol}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$) for 14 days and NO-releasing coating showed significant antibacterial potential against Gram-positive and Gram-negative strains. It was shown that both NO-releasing and control grafts are biocompatible *in-vitro* and *in-vivo*. Interestingly, the NO-releasing SDVGs dramatically enhanced ECs proliferation and significantly enhanced ECs migration *in-vitro* compared to control grafts. In addition, the NO-releasing SDVGs showed angiogenic potential *in-vivo* which can further prove the results of our *in-vitro* study. These findings are expected to facilitate tissue regeneration and integration of custom-made vascular implants with enhanced clinical success.

Statement of significance

A series of 3d-printed small-diameter vascular grafts (SDVGs, <6 mm) with controlled release of nitric oxide (NO) were prepared to combine the advantages of 3D printing technology and NO-releasing systems. The resulting NO-releasing grafts were promisingly showing sustained NO release in the physiological range over a two weeks period. In addition to the evaluation of endothelial cell migration *in-vitro*, we implanted for the first time the NO-releasing vascular grafts in a chick chorioallantoic membrane (CAM) to investigate the effect of the prepared grafts on the angiogenesis *in-vivo*. The fabricated grafts also exhibited bactericidal properties which prevent the formation of a biofilm layer and can thereby enhance the chance of endothelialization on the surface. Taken together, the innovative combination of rapid and highly accurate 3d-printing technology as a patient-specific fabrication method with NO-releasing coating represents a promising approach to develop bactericidal SDVGs with improved endothelialization.

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1. Introduction

The blood-contacting surface of implanted biomaterial is a competitive site for adhesion and colonization of bacteria or

attachment of host cells and tissue integration, especially during the first 6 h of post-implantation known as “decisive period”. This competitive process is critical to the success of implants to be integrated into surrounding tissue or failure and loss of implants by the formation of bacterial biofilm which prevents tissue integration [1–3]. Therefore, the development of biomaterials with an engineered surface to promote tissue integration and simultaneously kill bacteria is an emerging field in cardiovascular tissue engineering.

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Among blood-contacting biomaterials, small-diameter (<6 mm) vascular grafts (SDVGs) are predisposed to a higher failure rate due to stenosis, infection and poor endothelialization [4–8]. Currently, controlled release of antibiotic molecules and silver nanoparticles are the common approaches to create antibacterial surfaces. Despite the significant bactericidal effect of these releasing systems, the consequent undesirable side effects such as development of antibiotic resistance and cytotoxicity are inevitable, respectively [9–12]. Moreover, this type of antibacterial grafts could not promote endothelialization of newly implanted surface. An important post-operative complication is delayed wound healing and tissue regeneration which affects a considerable population of patients such as individuals suffering from vascular disorders or diabetes and elderly patients worldwide [13]. Therefore, it is an important demand to develop biomaterials with an improved endothelialization and accelerated healing process while combating pathogenic bacteria.

The internal layer of blood vessels is covered with a monolayer of endothelial cells (ECs), which are in direct contact with blood flow and continuously releasing nitric oxide (NO) to regulate the vascular homeostasis [14]. NO is a crucial signaling molecule to promote EC migration and proliferation and acts as an antibacterial agent which can circumvent antibiotic resistance [10,15,16].

Since the pre-seeding of artificial grafts with autologous ECs is a time-consuming process and the storage and transportation of the final product would be limited, the new approach of tissue engineering is build up on attraction and adhesion of host cells to the surface of the biomaterial *in-vivo* [17]. Therefore, accelerated migration and proliferation of cells towards the luminal site of the implanted graft is highly desirable for a successful integration of the implant into surrounding native tissue. The exogenous NO supplementation such as using NO donors is a promising candidate coating to biomimetically modify the surface of blood-contacting biomaterials such as vascular grafts and stents. There are considerable studies that support the benefits and non-cytotoxic effects of NO-releasing surfaces for mammalian cells. For instance, Goudie et al. fabricated Elast-eon E2As films with 10 wt% S-nitroso-N-acetyl-D-penicillamine (SNAP) and reported zero toxicity of extracts for L-929 mouse fibroblast cells [18,19]. The non-cytotoxic nature of NO was also proved for mouse fibroblast cells from silicone rubber tubings releasing liquid-infused NO [20] and NO-releasing polydimethylsiloxane (PDMS) [21].

Furthermore, ECs were shown to be attracted towards biomaterials with NO release. Recently, the NO-catalytic bioactive coating was developed and the results demonstrated an enhancement of HUVEC adhesion, migration and proliferation [16]. In another study, the NO-generating coating for vascular stents was developed and the results showed the enhancement of EC migration and proliferation and promotion of endothelialization [22].

The next generation of implants is needed to be designed and fabricated to fit with various, individual patients vessel geometry obtained by medical imaging such as MRI and CT scan. To achieve this goal, employing the 3d-printing technology could be extremely helpful. This layer by layer technique is able to fabricate reproducible, cost-effective, accurate and complex anatomical geometries for new grafts [23]. Among different techniques of 3d-printing, *fused deposition modeling* (FDM) is the most popular nozzle based technique which produces objects by extruding thermoplastic filaments like polylactic acid (PLA) [24]. PLA and poly (ϵ -caprolactone) (PCL) are widely used polymers for the fabrication of biodegradable vascular grafts [25], providing appropriate mechanical support and replacing newly regenerated tissue by long-term degradation [26,27].

This study investigated the innovative principle of incorporating 3d-printing technology and a controlled NO release system to develop the next generation of regenerative SDVGs which is characterized by a simple, inexpensive and reproducible method

to fabricate patient-based biodegradable and biocompatible SDVGs. We aimed to accelerate the endothelialization and protect the surface from bacterial infection by controlled release of NO. Therefore, the biodegradable polymers were used in this study and the effect of NO-release in physiologic range on ECs proliferation, viability, morphology and migration were investigated. Moreover, the chorioallantoic membrane (CAM) assay was used as an *in-vivo* model to investigate the biocompatibility and angiogenesis of fabricated grafts and the bactericidal potential of NO-releasing SDVGs was also evaluated.

2. Materials and methods

2.1. Materials

N-Acetyl-D-penicillamine (NAP), sodium nitrate, vanadium (III) chloride (VCl₃), sulfanilamide, N-(1-naphthyl) ethylene diamine dihydrochloride (NEDD), PCL (Mn = 80,000, Mw/Mn < 2), BioUltra grade PEG (Mn = 4000), dimethyl sulfoxide (DMSO) and thiazolyl blue tetrazolium were purchased from Sigma-Aldrich (St. Louis, MO). PLA filament (Mn $\approx$ 100,000, white, diameter 1.75 mm) was supplied by Shenzhen eSUN Industrial Company (Shenzhen, China). Sulfuric acid (H₂SO₄), hydrochloric acid (HCl), orthophosphoric acid (H₃PO₄), methanol, tetrahydrofuran (THF), tryptic soy broth (TSB), Luria broth (LB) and sodium nitrite (NaNO₂) were obtained from Merck (Darmstadt, Germany). Human umbilical vein endothelial cells (HUVECs, NCBI Code: C554) were obtained from the Cell Bank of the Pasteur Institute (Tehran, Iran). Calcium and magnesium free Dulbecco's phosphate buffered saline (PBS, pH 7.4), Dulbecco's modified Eagle's medium (DMED), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco (Scotland, UK).

2.2. Design of CAD model and 3d-printing of SDVGs

The tubular SDVG was designed using Rhinoceros® CAD software (version 5 SR8, Robert McNeel & Associates). Height, internal diameter and wall thickness of objects were kept at 10 mm, 5 mm and 0.2 mm, respectively. The CAD model data were saved in STL format and sliced layer by layer with CURA slicing software (Ultimaker BV, Geldermalsen, Netherlands). The G-code was created in CURA and imported into the home-made FDM 3d-printer (optimized in our lab) to create the grafts. The SDVGs were deposited layer by layer on a heated platform by extrusion of PLA filament through a 220 °C nozzle with 200 μ m internal diameter. It has been shown that parallel struts represent a more homogeneous structure compared to perpendicular printed struts [28]. Therefore, the struts were printed parallel to each other.

2.3. Synthesis of SNAP

A modified protocol for SNAP synthesis was used in this study [29]. Briefly, 1000 mg of NAP was dissolved in 25 ml of methanol. Next, 15 ml of 1 M HCl, 500 μ l of H₂SO₄ (98%) and 724.5 mg of NaNO₂ were added and mixed, respectively. After 15 min of stirring and 45 min of ice-cooling, the liquid portion was evaporated under air flow at room temperature. The dark green crystals were rinsed and filtered by ice-cooled deionized water and dried by the freeze-drying procedure. The final green crystals were kept in the darkness at –20 °C.

2.4. Characterization of SNAP

Ten mM grounded SNAP was dispersed in deionized water and the ultraviolet–visible (UV–Vis) spectra were recorded in the

wavelength range of 200–700 nm using a UV–Vis spectrophotometer (Lambda 25, Perkin Elmer, Singapore). The SNAP crystal morphology was inspected under polarized optical microscopy (Olympus, Japan).

2.5. Preparation of NO-releasing and control coatings

By doping the SNAP molecules into polymeric matrices, the crystal form of SNAP with strong intermolecular interactions will be formed which result in slow dissolution and NO release [30,31]. In a previous study, we compared PEG, PCL and PEG-PCL as SNAP reservoirs and concentrations of 5, 10, 15 and 20 wt% SNAP. The results displayed that PEG-PCL (1:1) with 10% SNAP covered with a PCL topcoat (tc) layer is the optimum polymeric matrix to release NO for 14 days in the physiological range [32]. According to our previous results, the coating solution to create a SNAP reservoir layer was prepared by dissolving 50 mg PEG and 50 mg PCL in 1 ml THF. This solution was used for coating of control samples. For NO-releasing grafts, 10 wt% SNAP was also added to the coating solution. Two hundred μ l of donor-storing solution was injected inside each rotating graft in 2 steps in darkness. After solvent evaporation at room temperature, the SNAP containing coating layer was dip-coated in PCL solution prepared by dissolving 100 mg PCL in 1 ml THF to create the topcoat layer. This top-coat layer was created to cover the main coating and to control the release kinetics. The final grafts (Fig. 1c) were freeze-dried and kept at -20°C .

2.6. Morphological evaluation of SDVGs

The coated grafts were loaded on supporting stubs covered with adhesive carbon stickers and sputter coated with a thin layer of gold (Au) (Fisons Instruments, UK). The morphology of specimens was observed using a scanning electron microscope (Streoscan S360, Leica Cambridge, UK) at the acceleration voltage of 20 KV.

2.7. NO release measurement

The prepared UV-sterilized grafts with 1 cm length were immersed in 10 ml PBS as releasing medium and incubated at 37°C in darkness ($n = 6$). At pre-determined time-points, 150 μ l samples were collected for NO-measurement. The amount of NO was measured using a modified Griess assay [14,33,34]. Briefly, 50 μ l sample and 50 μ l of 1:1 mixture of 2 g sulfanilamide in 100 ml deionized water with 3.44 ml H_3PO_4 (85%) and 0.2 g NEDD in 100 ml deionized water were loaded in 96-well ELISA plate and incubated at room temperature for 15 min under gentle shaking. Next, due to the inability of the Griess assay to detect the nitrate amount [35], nitrate content was reduced to nitrite by transferring 50 μ l of the VCl_3 solution, prepared by dissolving 400 mg VCl_3 in 50 ml 1 M HCl and 60 min incubation at 60°C to be detectable by Griess reaction. Subsequently, the plate was cooled down at ambient temperature and the absorbance was measured at 550 nm. The NO concentration was calculated using standard curves generated from serially diluted NaNO_2 solutions.

2.8. Leachate collection from SDVGs

To obtain the extract from the grafts, 10 mg of NO-releasing or control grafts were incubated in 10 ml of DMEM medium at 37°C according to ISO 10993-5:2009 test [19,20,36]. After 24 h, the grafts were removed from the solution and kept in the fridge. Ten % FBS and 1% penicillin–streptomycin solution were freshly added to the leachates before experiments [19,20,36].

2.9. Cell culture

HUVECs were cultured in DMEM supplemented with 10% FBS with 1% penicillin–streptomycin antibiotic solution in humidified air containing 5% CO_2 at 37°C .

2.10. Morphological study of HUVECs

A confluence monolayer of HUVECs was detached from T-75 flask using trypsin and viable cells were counted by trypan blue staining. To assess the effects of leachates from NO-releasing and control grafts on HUVECs morphology and proliferation, cells were diluted in leachates and 10^4 cells/well were seeded in 96-well plate ($n = 3$) and incubated at 37°C . After 24 h, the morphology of cells was inspected by an inverted microscope (Olympus, Japan) and images were captured for further analysis. Carbon dot staining was also performed to further analyze the morphology of cells using a fluorescent microscope (Olympus, Japan).

2.11. Cell migration assay

The migration rate of ECs from adjacent healthy endothelium is crucial for successful endothelialization of vascular implant. The scratch assay was used to investigate the effect of NO-releasing and control grafts on EC migration rate. HUVECs were seeded in 96-well plates ($n = 3$) at a density of 10^4 cells/well and incubated at 37°C to obtain a confluent monolayer. A vertical scratch wound was created with a 100- μ l pipette tip and the culture media was aspirated and cells were rinsed with PBS to remove detached cells. Since the migration of cells from the adjacent vessels is critical for endothelialization, the scratch line is a cell-free zone which can mimic the graft's edges after implantation. The wounded monolayers were incubated with leachate of NO-releasing grafts, control grafts, or fresh culture media (positive control) for 24 h at 37°C . Inverted microscope (Olympus, Japan) was used to monitor the cell migration at 0 h and 24 h after scarification.

2.12. Cytocompatibility assay

To assess cell viability in presence of leachates, MTT colorimetric assay was carried out on day 1 and 3 after cell seeding. Hundred μ l of HUVEC suspension (5×10^3 cells/well) in leachates supplemented with 10% FBS were seeded in 96-well plates and incubated at 37°C and 5% CO_2 . At each time point, the medium was aspirated and 110 μ l of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in PBS was added to each well ($n = 6$). After 4 h incubation at 37°C and 5% CO_2 in darkness, the supernatant was replaced with 110 μ l of DMSO and shaken for 15 min to dissolve the formazan crystals produced by viable cells. Hundred μ l of the supernatant was transferred to a new 96-well plate and the absorbance was measured at 570 nm by using a microplate reader (Bio-Tek, USA) [37,38].

2.13. Angiogenesis and biocompatibility assessment in-vivo

In order to determine the effect of produced SDVGs on angiogenesis and cytotoxicity *in-vivo*, the CAM assay has been carried out. Briefly, fertilized eggs at embryonic day 1 (ED1) were purchased from a local company and incubated at 37°C in a 60% humidified atmosphere and rotated twice a day. On ED3, 1.5 ml of albumen was aspirated with a syringe to enhance an empty space under the eggshell and the shell's hole created by the needle was covered with tape and incubated at the same environment. On ED8, an approximate 1 cm^2 square shape window was created with a sharp blade under sterile conditions and the shell fragment was removed to access the CAM beneath. The UV-sterilized grafts with

2.5 mm length were randomly distributed ($n = 3$) and gently placed on the CAM. The eggshell fragment was put back on the window and sealed with a sterile tape. The eggs were further incubated horizontally in the same environment without rotation. After 72 h of incubation, the window was opened and the CAM was inspected and the images and videos were captured by a camera [38,39]. The images were quantitatively analyzed using WimCAM online software and the vascular junctions were counted in rectangular equally sized frames.

2.14. In-vitro antimicrobial evaluation

The antibacterial activity of NO-releasing and control grafts was tested against both Gram-positive (*S. aureus*, ATCC 8325-4) and Gram-negative (*E. coli*, ATCC 25922) bacteria using a disc diffusion assay. The 3d-printed discs with 200 μm thickness and 5 mm diameter were prepared and coated with NO-releasing or control coating. *E. coli* and *S. aureus* solutions were prepared at a concentration of 10^5 CFU·ml $^{-1}$ and were homogeneously streaked on LB and TSB agar plates ($n = 3$), respectively [40–42]. After inoculation, the UV-sterilized discs were loaded on the plates and incubated at 37 °C. After 24 h, the inhibition zones were inspected and recorded with a camera.

Moreover, the viability of strains after incubation with the NO-releasing and control specimens was also investigated by the CFU counting method. Briefly, 10^7 CFU·ml $^{-1}$ of *S. aureus* and *E. coli* inoculum were prepared in PBS + 10% culture medium [32] (TSB and LB, respectively). The NO-releasing and control specimens were incubated in 2 ml of bacterial solutions with 200 rpm shaking at 37 °C. After 24 h, 100 μl of the bacterial solution were collected from each tube and plated on blood agar and LB agar for CFU counting.

2.15. Statistical analysis

All data were expressed as mean $\pm$ standard deviation (SD). One-way or two-way analysis of variance (ANOVA) was performed for statistical analysis and followed by Tukey or Bonferroni post-tests, respectively. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. 3d-printed and coated SDVGs

Considering the advantages of 3d-printing technology for preparation of patient-specific implants, the FDM 3d-printer was used in the current study to fabricate SDVGs. In an earlier research, we investigated the effect of printing parameters on creating the vascular grafts with homogeneous morphology from PLA filament using a FDM printer [25]. In the current work, the luminal surface of 3d-printed SDVGs (Fig. 1a) was modified with NO-releasing coating (Fig. 1c) to improve the bactericidal properties and the healing process of implants *in-vivo*. The PEG, as a hydrophilic polymer to absorb water and facilitate the SNAP dissolution [43,44], and PCL, as a hydrophobic polymer with slow degradation [45,46], were blended to prepare the donor-storing coating. It has been shown that the addition of PEG to the PCL solution can decrease the interfacial tension of the solution which consequently enhances the shrinkage of the coating after the solvent evaporation and subsequently a porous microstructure will be formed [47,32]. The obtained porous structure is beneficial to enhance the water uptake and improve the NO flux compared to NO release by PCL erosion from pure PCL matrix. Based on the result of our previous study that indicated the PEG-PCL-SNAP with PCL topcoat (tc) as an optimum SNAP reservoir to release NO in the physiological range

for 14 days [32], the PEG-PCL-SNAP-tc was chosen in this study for further evaluation.

Representative morphological features of 3d-printed grafts and same grafts with NO-releasing layer covered by PCL topcoat are given in Fig. 1. It is evident from the figure that 3d-printed grafts were created from smooth circular struts with uniform orientation and morphology (Fig. 1b). After top-coating, the morphology was changed to be rougher but the location of struts is still visible (Fig. 1d).

3.2. SNAP characterization

Fig. 2a shows the UV-Vis spectra of 10 mM synthesized SNAP in deionized water. The appearance of characteristic absorbance maxima at 340 and 590 nm illustrated the presence of S-NO bond in synthesized SNAP [48,18,48]. The representative absorbance of SNAP crystals indicated the purity of the synthesized donor without any extra peaks related to impurities. Fig. 2b represents the morphology of SNAP crystals on a glass slide evaluated by polarized optical microscopy.

3.3. NO release profile

It has been reported that a hydrophobic top-coat layer covering the SNAP-reservoir coating can optimize the NO-release kinetics [49,50]. In this study, the PCL top-coat was used to control the NO-release profile. In order to evaluate the NO-release profile, the prepared grafts were soaked in PBS at 37 °C and the SNAP diffusion into PBS was monitored on different time points. On the first day of soaking, an initial burst release was detected which can be helpful for the success of SDVG during the “decisive period” of implantation. After passing the “decisive period”, from the second day of implantation the grafts needed to release NO in the physiological range ($0.5\text{--}4 \times 10^{-10}$ mol·cm $^{-2}$ ·min $^{-1}$) [10,49] of NO which continuously secreted from ECs. Fig. 3 shows the controlled NO-release behavior of SDVGs over 14 days. From day 2 onward, the NO-release was in the physiologic range which is shown in the green zone.

It should be mentioned that the NO flux was measured after UV sterilization. It has been shown that UV irradiation cannot alter the chemical structure of PCL [50], which covered the outer surface of the prepared grafts. In addition, approximately 99% of the SNAP active content was retained in the coating after UV irradiation for 1.5 h [18].

3.4. Proliferation, morphology and migration of ECs

Endothelium regeneration of the luminal surface is a crucial factor in the clinical success of SDVGs especially while the NO flux decreases after a couple of weeks. After implantation of SDVGs, the ECs migrate from the adjacent vessel and grow from the cut edges towards the center of the lumen to form a complete monolayer [51]. In the endothelialization process, the EC motility and migration speed would be critical for tissue healing.

It has been shown that one of the physiological roles of NO is to stimulate EC proliferation [16,22]. As a proof of concept, the proliferation and morphology of HUVECs after 24 h was inspected by optical and fluorescent microscopy. Fig. 4 represents the phase contrast and fluorescent images of HUVECs after 24 h incubation with different leachate media. Cell proliferation was clearly higher in presence of NO which agreed with the results of previous studies [15,16]. Although in the NO-releasing condition, the cells grow more confluent whereas in both conditions, cells represent the spread out (elongated) morphology displaying an appropriate environment for cell growth. This cellular elongation is required for angiogenesis and network formation [52]. These results indi-

Fig. 1. Representative morphological assessment of the SDVGs. Macroscopic top view of 3d-printed PLA SDVG (a) and coated SDVG (c). SEM images of cross-sectional view of pristine SGVG (b) and coated SDVG (d).

Fig. 2. UV-Vis spectroscopy and morphology of synthesized SNAP crystals. (a) UV-vis absorption spectra of synthesized SNAP crystals corresponding to the S-NO bond. (b) Microscopic view of SNAP crystals under polarized light.

Fig. 3. NO release profile from NO-releasing SDVGs over a 14 days period. NO flux was measured in PBS at 37 °C in darkness. The green band represents the physiological range of NO flux. All data were measured by modified Griess assay. Data are expressed as mean $\pm$ SD (n = 6). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Proliferation and morphology of HUVECs after 24 h incubation with leachate of NO-releasing and control grafts. Images obtained by inverted microscopy (a, b, scale bar: 200 µm) and fluorescence microscopy (c, d, scale bar: 100 µm).

cate that NO-releasing 3d-printed SDVGs can promote proliferation of ECs and consequently accelerate endothelial regeneration *in-vivo*.

ECs play a key role in the wound healing process of vascular grafts [54,55]. The migration rate of ECs towards the wounded site of implantation is an important factor which can accelerate the healing process after surgery. As a consequence, an accelerated regeneration of the endothelium monolayer after implantation

could reduce the risk of bacterial infection and thrombosis. Yang et al. reported that the NO release accelerated HUVEC proliferation and migration [16]. It has been shown that exogenous NO donor can up-regulate the expression of cytoskeletal proteins in ECs which are critical for elongation of actin filaments, known as VASP, and consequently improve the migration [13].

Fig. 5a shows the migration of ECs after 24 h incubation in presence of different leachates or culture medium. The obtained images

Fig. 5. Migration of HUVECs after 24 h of incubation with different leachates or culture medium. (a) The monolayer of HUVECs was scratched with a 100 µl pipette tip. The dotted lines indicate the location of initial wound edges at the beginning of the experiment and after 24 h incubation with different leachates or culture medium. Scale bar: 200 µm. (b) The percentage of wound closure was calculated after 24 h compared with the initial gap between the borders of scratches. Data are expressed as mean ± SD (n = 3).

were quantitatively analyzed with the WimScratch online software program. The results demonstrate that NO-releasing significantly enhanced the migration rate of ECs and that after 24 h the wounded area in presence of NO was decreased by 42% compared to control and positive control (culture medium) (Fig. 5b). However, for the control grafts and positive control, cells showed no difference in migration speed. This confirms the accelerating effect of NO on the EC migration rate, which would be promising for faster wound closure after implantation.

This finding is in agreement with the previous studies on NO-releasing stents [16,22] that reported significant enhancement of HUVEC's proliferation and migration by NO donor supplementation in the culture medium. Their *in-vivo* results also demonstrated that NO-releasing stents promote re-endothelialization and reduce the chance of re-stenosis [16]. The present study advances and takes our prior work into the next level by examining the EC migration and proliferation to investigate the healing process of SDVGs *in-vitro*.

The endothelial regeneration on the lumen of synthetic vascular grafts without NO-releasing is limited *in-vivo* which consequently increases the thrombogenicity and risk of contamination [56–58]. The results of the current study demonstrate the promotion of endothelialization in response to NO release, which acts as an anti-coagulating layer with bactericidal properties and can promisingly increase the success rate of implantation.

3.5. Cytocompatibility assessment

Treatment of cells with biomaterials and chemical agents can alter cell viability and metabolism and it is necessary to evaluate the cytotoxicity of new products. In order to assess the cytotoxicity of NO-releasing and control grafts, MTT assay was performed. The findings summarized in Fig. 6 illustrate the non-cytotoxic effect of manufactured grafts both in a short-term (24 h) and long-term (72 h) period. This finding is in line with previous study results which also reported zero toxicity of leachate from films with 10 wt% SNAP [18,19,59,20]. Moreover, the results revealed that EC proliferation rate was significantly higher after 24 h incubation with NO-releasing graft's leachate compared to the control group. After 72 h, the propagation of ECs was also significantly higher than after 24 h in which demonstrates the support of cell growth in the presence of leachates, although the difference of NO-releasing and control group was not statistically significant at 72 h. Our results are consistent with earlier reports of biocompat-

ibility and the higher rate of EC proliferation by NO treatment [16,20].

3.6. In-vivo angiogenesis and biocompatibility evaluation

The CAM assay is a cost-effective, minimally invasive, simple and rapid method to evaluate the biocompatibility and angiogenic potential of biomaterials and even used as a bioreactor [39,60]. CAM assay is also a standard method used to assess the activity and toxicity of drug delivery systems. The chemicals in the CAM can absorb through the membrane and reach the systemic circulation and therefore affect the development of the embryo [61].

In this study, CAM assay was used to investigate the effect of grafts with or without NO-release on the viability of chick embryo and angiogenesis *in-vivo*. To the best of our knowledge, this is the first report of 3d-printed vascular graft implantation on the CAM membrane and investigation of the resulting angiogenesis and embryonic viability.

The results (Fig. 7a–b) demonstrate that control grafts were compatible with the CAM model since they did not display any damage to blood vessels or embryo viability. Fig. 7c shows the significant increase in formation of new vascular junctions in the presence of NO-release (97.66 ± 7.50) compared to the control groups (73 ± 9.84) after 72 h. The higher rate of angiogenesis from the existing blood vessels in response to NO can further prove the controlled release of NO in the physiological range and the results of our *in-vitro* EC migration behavior.

Moreover, new capillaries branching were grown on the SDVGs wall, even more concentrated around NO-releasing SDVGs compared to control grafts, which is promising for EC stimulation to migrate to the lumen of the implant.

In the angiogenesis process, ECs locally proliferate in response to an angiogenic stimulus and play a vital role in this process [53]. The angiogenic potential of NO in the CAM assay is in agreement with our results of HUVEC migration rate *in-vitro*. The present study confirms the angiogenic potential of NO which can be concluded that by using NO-releasing systems, the coverage of ECs could be improved on the lumen of SDVGs implants. The results of this study are in agreement with a previous report of angiogenic potential of Spermine NONOate as an exogenous NO donor [62]. Another study also reported that the local NO release enhanced angiogenesis *in-vivo* [63]. The enhancement of angiogenesis in response to NO-releasing, both *in-vitro* and *in-vivo*, can also illustrate long-term antibacterial properties of grafts as previously described [64,65]. Moreover, this angiogenic activity which is essential for tissue healing [66,67] is promising for tissue regeneration and integration after implantation.

In the movies, the vascular motion at systole and diastole and the movement of the embryo is observable which confirms the viability of the embryo and the biocompatibility of grafts. From the anatomical aspect, the CAM artery and vein were distinguishable by their color. In contrast to the internal circulation, in the extra-embryonic circulatory system the arterial blood is deoxygenated and therefore the artery looks darker than the vein [68]. In both control (Supplementary video 1) and NO-releasing (Supplementary video 2) groups, as expected, the wall motion of the vein is negligible while the arterial motion was remarkable being in line with earlier reports [68].

3.7. Antibacterial properties of NO-releasing coating

NO is a natural molecule with bactericidal properties by damaging the microbial DNA, proteins and lipids [69]. The prolonged NO-releasing in a controlled manner is expected to inhibit bacterial growth on the biomaterial surface. To investigate the antibacterial activity of the NO-releasing coating, the inhibition zone assay was

Fig. 6. Cytocompatibility evaluation of grafts. EC viability measured by MTT assay after 24 h and 72 h of incubation with leachates of NO-releasing and control grafts. The proliferation rate was significantly higher ($P < 0.05$) in the NO-releasing group ($n = 6$) compared to the control group ($n = 6$) after 24 h. The growth rate was also significantly higher ($P < 0.001$) after 72 h which illustrates the propagation of ECs. Data are expressed as mean $\pm$ SD ($n = 6$).

Fig. 7. Angiogenesis evaluation of NO-releasing and control grafts. Transparent CAM vascular network over the yolk sac implanted with (a) NO-releasing graft and (b) control graft after 72 h. The main artery which is bifurcated and looks darker marked with white arrows, the main vein marked with a black arrow, the embryo showed with a yellow arrow and the graft marked with a star. (c) Quantitative analysis of angiogenesis by counting vascular junctions based on the WimCAM data. Data are expressed as mean $\pm$ SD ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

carried out using Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) strains. In this method, the inhibition of bacterial growth is directly related to antibacterial properties of the biomaterial. Fig. 8 reveals the inhibition halos of NO-releasing and control coatings on agar plates after 24 h of incubation at 37 °C. In both strains,

NO-releasing coating inhibited the growth of bacteria with an approximately 13 mm diameter zone of inhibition. The SNAP-free control grafts did not display any inhibitory zone in both species. The results of the CFU counting method also showed a reduction of 99.99–100% of microorganisms in presence of NO-releasing

Fig. 8. Disc diffusion assay to evaluate the antibacterial potential of coatings. The inhibition halos of NO-releasing (left) and control (right) discs against the growth of *E. coli* and *S. aureus* strains after 24 h incubation at 37 °C. Data are expressed as mean $\pm$ SD ($n = 3$).

grafts after 24 h while the bacteria incubated with control grafts proliferated and displayed a steady-state level [32] which was in agreement with inspected inhibition zone halos.

These findings confirm that fabricated NO-releasing SDVGs can significantly reduce the viability of bacteria after implantation. The inhibition of bacterial growth can promisingly reduce the chance of biofilm formation which should further be investigated. The results are in accordance with results of previous studies [40–42,32].

4. Conclusion

In this study, a composite NO-releasing coating was developed on 3d-printed vascular grafts. This coating can release NO in a controlled manner which mimics the native endothelium properties. The results showed that NO-releasing grafts enhance the migration rate of ECs which consequently accelerate tissue regeneration at the site of implantation. This faster endothelium recovery is favorable while endogenous NO production is disrupted because of ECs damaged during operation and can result in the success of implantation in terms of inhibition of thrombosis and infection. Moreover, the NO-releasing SDVGs exhibited a remarkable angiogenic potential *in-vivo* and antibacterial properties against both Gram-positive and Gram-negative species *in-vitro*. Considering these promising results, NO-releasing 3d-printed SDVGs represent a promising approach to prepare more successful SDVGs with accelerated healing and bactericidal properties.

Declaration of Competing Interest

There is no conflict of interest to declare.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.actbio.2019.05.002>.

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