Enabling topical and long-term anti-radical properties for percutaneous coronary intervention-related complications by incorporating TEMPOL into electrospun nanofibers

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ABSTRACT Scavenging reactive oxygen species (ROS) by antioxidants has been demonstrated as the most effective strategy for preventing percutaneous coronary intervention (PCI)-related complications. However, topical and long-term delivery of ROS antioxidants to a specific vascular tissue is proven to be a great challenge. Herein, an ROS scavenger of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL) is incorporated into electrospun nanofibers with a tunable loading amount to achieve its topical applicability and long-term anti-radical capability. Biological functions of such TEMPOL-loaded electrospun membranes are evaluated by cell proliferation, ROS-scavenging capability, monocyte adhesion, cell migration, inflammatory molecule secretion and mRNA expression *in vitro*. After optimizing the loading amount of TEMPOL, such an electrospun membrane presents a superior ROS-scavenging and anti-inflammation performance for both endothelial cells and macrophages. The expression of endothelial prothrombotic molecules and the migration of vascular smooth muscle cells (VSMCs) are also effectively inhibited. Thus, it is bravely predicted that the topical use of such a TEMPOL-loaded electrospun system will be a promising pathway for the anti-restenosis therapy, especially when used as a novel coating on stent for long-term and topical delivery of antioxidant drugs.

Keywords: percutaneous coronary interventions, reactive oxygen species, TEMPOL, electrospinning, topical release

INTRODUCTION

Cardiovascular diseases (CVDs) remain the primary

cause of morbidity and mortality globally [1], resulting in approximately 18 million CVD deaths annually [2]. Until now, percutaneous coronary interventions (PCI), including percutaneous transluminal coronary angioplasty (PTCA) and coronary stent implantation, have been widely applied for coronary artery revascularization as a non-surgical approach [3–5]. However, PCI-related complications, such as restenosis and stent thrombosis still occur at a fairly high rate and continue to limit the long-term success of the procedure. The excessive reactive oxygen species (ROS) production induced by PCI operations is recognized as a key role on the pathophysiological changes of vascular cells [6,7]. A high level of ROS would break the balance of the antioxidant defense system in human body, trigger the proliferation and migration of vascular smooth muscle cells (VSMCs) and promote monocyte/macrophage inflammation followed by endothelial dysfunction [8], thus resulting in the in-stent restenosis and/or thrombosis towards the stent failure [9]. Therefore, referring to such a mechanism, reducing the ROS level by using antioxidants should be the most feasible pathway in preventing PCI-related complications.

As one of the most common antioxidants, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL) features a “superoxide dismutase (SOD) mimetic” action [10], which can effectively scavenge and react with the O₂^{•−} to alleviate the harm of ROS. Specifically, as shown in Fig. S1, owing to its unique molecular structure, TEM-

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POL with a relatively small nitroxide molecule can transform O_2^- to H_2O_2 by shuttling the nitroxide radical between the reduced hydroxylamine and the oxidized oxoammonium cation [11,12]. Such an encouraging catalytic mechanism is attributed to its “boat and chair” conformational changes [11,13], showing a more effective performance than the counterpart of SOD. Moreover, benefiting from its function on reducing vascular matrix metalloproteases (MMPs), TEMPOL can diminish VSMC migration and vascular remodeling in injured arteries.

Despite above merits, the systemic administration of TEMPOL should be with a serious caution since some negatively inotropic effects might be caused, including the negative inotropic action, and inhibition of myocardial contractility [6]. Intuitively, topical application of TEMPOL through drug loading is deemed as an ideal approach, with the drug concentration released in an appropriate range and hopefully preserving a long-term anti-radical performance. To this end, we turn to prefer the electrospinning technologies based on following concerns: (i) with a mimicked natural extracellular matrix morphology, electrospun nanofibers have demonstrated as the attractive candidates for topical delivery of therapeutics [14–16]; (ii) encapsulating biologically active molecules (e.g., drugs [17–21], growth factors [22–26], RNAs [14,27]) into electrospun nanofibers is also pretty flexible and controllable in modulating cellular functions; (iii) in comparison with other encapsulating approaches such as hydrogel [28,29], electrospun nanofibers have much higher specific surface area, and would facilitate cell proliferation as well as nutrient transport; (iv) more importantly, electrospinning technology can be perfectly combined with PCI therapy by mounting electrospun drug-loaded nanofibers onto bare metal stents, achieving the topical drug delivery [18,30,31]. Unfortunately, current efforts are mainly focusing on investigating the therapeutic mechanisms and clinical applications of TEMPOL [32,33], whereas few attempts have been reported regarding their drug delivery materials. Until now, to the best of our knowledge, no reports concerning TEMPOL-loaded electrospun nanofibers have yet been proposed or demonstrated.

Herein, a first attempt was devoted to scavenging ROS by employing a TEMPOL-loaded poly(*D,L*-lactic acid) (PDLLA) electrospun nanofibrous membranes as the topical release system. Through a coaxial electrospinning method, TEMPOL could be encapsulated into the PDLLA nanofibers and utilized to regulate the response of endothelial cells, smooth muscle cells and macrophage by adjusting its loading amount; and the shell layer of

PDLLA can regulate the release behavior of TEMPOL for a long-term performance. The proliferation of Human umbilical vein endothelial cells (HUVECs) on such membranes demonstrated that excessive TEMPOL loading has a certain cytotoxicity. With an optimized TEMPOL loading amount, such an electrospun membrane presented an effective anti-inflammatory performance by incubating macrophages and HUVECs under inflammatory stimulation, which were also demonstrated by the cell proliferation, corresponding gene expression with a real-time PCR, and enzyme-linked immunosorbent assay (ELISA). Moreover, cell migration stimulation of Human aortic smooth muscle cells (HASMCs) under inflammatory was also evaluated, showing an obvious inhibition by the released TEMPOL. And, its potential as a delivery vehicle for inhibiting intimal hyperplasia in PCI was further demonstrated.

EXPERIMENTAL SECTION

Materials

PDLLA (molecular weight = 88 kDa, polydispersity index = 1.76) was purchased from Jinan Daigang Biomaterial Co. (China). TEMPOL and acetonitrile (high performance liquid chromatography, HPLC) were purchased from Aladdin Reagent Co. (China). Dichloromethane and methanol were purchased from Sinopharm Chemical Reagent Co. (China). Hexafluoroisopropanol (HFIP) was purchased from Shanghai Darui Finechem Co. (China). HUVECs and HASMCs were obtained from ScienCell Research Laboratories (San Diego, California, USA). Human THP-1 monocytes were kindly provided by Dr. Ruoshui Li from the Institute of Cardiovascular Disease, Shanghai, China. Endothelial cell medium (ECM) and smooth muscle cell medium (SMCM) with supplemented materials were purchased from ScienCell Research Laboratories (San Diego, California, USA). RPMI-1640 culture medium was obtained from Gibco Company (USA). Cell counting kit-8 (CCK-8) kit was purchased from Dojindo Laboratories (Japan).

Preparation of TEMPOL-loaded PDLLA electrospun membranes

The shell solution was prepared by dissolving 1.25 g PDLLA in 10 mL HFIP under stirring at room temperature for 12 h; a series of core solutions were prepared by dissolving TEMPOL in deionized water and stirring at room temperature for over 0.5 h with their concentrations listed in Table 1. A coaxial electrospinning setup was used to prepare the TEMPOL-loaded PDLLA elec-

Table 1 Compositions of TEMPOL-loaded PDLLA electrospun membranes with different loading amounts

Samples	PDLLA (mg mL ⁻¹)	TEMPOL (mg mL ⁻¹)	Diameter (nm)	Theoretical loading amount (ng mg ⁻¹)	Actual loading amount (ng mg ⁻¹)	Encapsulation efficiency (%)
P-T-0	125	–	284.4±117.4	–	–	–
P-T-0.1	125	1	415.2±228.1	0.799	0.339±0.002	42.5±0.3%
P-T-0.2	125	2	421.1±245.7	1.597	0.543±0.004	33.9±0.3%
P-T-0.5	125	5	371.8±222.2	3.984	1.489±0.062	37.4±1.6%
P-T-1	125	10	369.6±192.1	7.937	3.091±0.223	38.9±2.8%
P-T-2	125	20	362.8±236.5	15.748	5.582±0.450	35.6±2.9%
P-T-5	125	50	239.0±155.3	38.462	7.017±0.050	18.2±0.1%

trospun membranes, as shown in Fig. 1. For the subsequent coaxial electrospinning, a coaxial spinneret with its internal diameters of 0.5 and 1.5 mm is used in this study. The shell and core solutions were pushed by syringe pump at a speed of 1 and 0.1 mL h⁻¹, respectively, applied with a high voltage of 19.5 kV; and the collection distance was fixed to 15 cm. As a control, the same shell and core solutions without TEMPOL were also applied to prepare the nanofibrous membranes. In this work, all the electrospun membranes were collected for 1 h on a rotating drum followed by a vacuum dry at 40°C for 12 h to remove residual solvent for further usage. And the samples prepared by varying concentrations of the TEMPOL solutions (0, 1, 2, 5, 10, 20 and 50 mg mL⁻¹) were named as P-T-0, P-T-0.1, P-T-0.2, P-T-0.5, P-T-1, P-T-2 and P-T-5, respectively.

Characterizations

Scanning electron microscopy (SEM) images were obtained with a Hitachi SU8010 (Japan). Transmission electron microscopy (TEM) images were obtained with a JEM-2100 (Japan). The electron paramagnetic resonance (EPR) spectra were recorded at room temperature in a Bruker A300 (Germany) spectrometer operating at 9.85 GHz with 100 kHz magnetic field modulation. The spectra were collected with the following parameters: sweep width 200 G; microwave power 19.23 mW; receiver gain 2.00×10¹; time constant 10.24 ms; conversion time 49.00 ms. High performance liquid chromatography (HPLC, LC 2050, China) analyses were carried out with a mobile phase comprised of acetonitrile and water (95/5, v/v) to determine the actual loading amount of TEMPOL in the as-electrospun membranes. For the TEMPOL release experiment, the mobile phase was changed and comprised of acetonitrile and water (40/60, v/v). More detailed information and statement can be found in the Supplementary information. All samples were run in

triplicate.

In vitro release behaviors of TEMPOL-loaded PDLLA nanofibers

To evaluate the release behavior of TEMPOL from the nanofibers, membranes loaded with different amounts of TEMPOL were immersed with 4 mL phosphate-buffered saline (PBS, pH 7.4) at 37°C for TEMPOL release; at certain time intervals, 1 mL of the above solution was then taken and used for the following HPLC analysis with a mobile phase consisting of acetonitrile and water (40/60, v/v). And, an equal volume of fresh PBS was added into the stock solution for following release incubation. The release percentage was calculated with Equations (1) and (2) as follows:

$$M_t = M_1 + M_2 + \dots + 4M_x, \quad (1)$$

$$Q_t = \frac{M_t}{M_\infty} \times 100\%, \quad (2)$$

where M_t is the cumulative amount of drug released from the electrospun membrane at predetermined time t , M_x ($x = 1, 2, 3, \dots$) is the amount of drug in 1.0 mL PBS at predetermined time, and M_∞ refers to the total amount of drug released from the electrospun membrane which was set as the cumulative amount of drug at day 31, Q_t is the release percentage at predetermined time t .

In vitro performance

Cell culture

Each sample was cut into a complete piece and folded to match the size of culture plate well with the same density of 0.6316–0.7895 mg cm⁻². All the samples with the culture plate were sterilized under ultraviolet light for 2 h. Stainless steel rings were used to prevent the membranes from floating. For cell seeding, electrospun membranes were placed in 24-well or 12-well plates, and cells were

Figure 1 The coaxial electrospinning apparatus and the corresponding electrospinning process. (a) Schematic illustration for the preparation of TEMPOL-loaded PDLLA electrospun membranes; (b) the coaxial spinneret; (c) a Taylor cone observed at 10 kV; (d) a digital photo of the obtained TEMPOL-loaded PDLLA membrane; (e) a representative TEM image of one as-electrospun nanofiber (P-T-1); (f) EPR spectrum of the P-T-1. (g–l) SEM images of the as-fabricated electrospun membranes by varying the concentration of core spinning TEMPOL solutions: (g) P-T-0.1, (h) P-T-0.2, (i) P-T-0.5, (j) P-T-1, (k) P-T-2 and (l) P-T-5, respectively.

seeded upon membranes with a certain concentration. Cells were cultured at 37°C/5% CO₂ to allow for cell adhesion and equilibrium of cell spreading.

HUVECs were cultured in ECM with 5% fetal bovine serum (FBS), 1% endothelial cell growth supplement (ECGS) and 1% penicillin-streptomycin as supplemented materials. HASMCs were maintained in SMCM supplemented with 2% FBS, 1% smooth muscle cell growth supplement (SMCGS). THP-1 monocytes were cultured in RPMI-1640 medium containing 10% FBS and 1% pe-

nicillin-streptomycin. Then the cells were seeded in 12-well or 24-well culture plates and incubated with specific medium at 37°C.

Cell proliferation assay

For the cell viability study, 500 μL of 2×10⁵ HUVECs was incubated on the electrospun nanofibrous membranes in 24-well culture plates, and quantified on day 1, 3 and 5 by CCK-8 assay. The measurement was performed over four biological replicates.

Cell spreading morphology

The spreading of HUVECs on the electrospun membranes was also examined by using SEM, live cells staining and immunofluorescence staining through fluorescence microscope (Olympus, Japan). For SEM analysis, samples were fixed in 2.5% (v/v) glutaraldehyde for 2 h, dehydrated using graded ethanol, and then critical-point-dried before observation. Cell Tracker Green CMFDA (Yeason, China) was used for live cell staining. Stained cells were imaged by fluorescence microscope. Image J was then used to count cell numbers of 4 independent views per well. Von Willebrand factor antibody (vWF, 1:100, Abcam, UK) was used as the endothelial marker, and 4,6-diamidino-2-phenylindole (DAPI, Weiao Biotech; China) was used for cell nuclei staining. The marked cells were imaged by fluorescence microscope.

ROS-scavenging capability

ROS production was evaluated using dihydroethidium (DHE, Beyotime Biotechnology, China) stain. HUVECs and THP-1 cells were seeded under electrospun membranes for three days in complete medium before stimulating with 0.1 mmol L⁻¹ H₂O₂ (Weiao Biotech, China) for 6 h. Electrospun membranes and medium were removed, 10 μmol L⁻¹ DHE was added and cells were incubated for 1 h. DHE was discarded and cells were washed three times with cold PBS. Stained cells were imaged by fluorescence microscope. Image J was then used to count cell numbers of 4 independent views per well.

Monocyte adhesion

THP-1 cells were incubated with 20 mmol L⁻¹ Calcein AM (KeyGEN BioTech, China) for 20 min, then washed twice with PBS. HUVECs were cultured on the membranes for three days to reach over 80% confluence before cells were stimulated by 10 ng mL⁻¹ tumor necrosis factor-α (TNFα, PeproTech, USA) for 2 h. Thereafter, 4×10⁴ Calcein AM-labelled THP-1 cells were incubated with HUVECs at 37°C for 1 h on an orbital incubator. Non-adherent cells were removed by washing for three times with PBS. Fluorescence microscope was used for photograph of cells. Image J was then used to count cell numbers of 5 independent views per well.

Cell migration

HASMCs were cultured on 12-well plates to 90%–95% confluence in SMCM, and serum deprived for 12 h. A straight scratch-wound was made on the cellular layer

using a sterilized 200-μL pipette tip. Cell Tracker Green CMFDA was used to label HASMCs. Electrospun membranes (i.e., P-T-0 and P-T-1) were placed on top of HASMCs. Platelet-derived growth factor subunit B (PDGF-BB, PeproTech, USA, 20 ng mL⁻¹) was added in culture medium and maintained for 24 h. Cell migration was followed by fluorescence microscopy at indicated time points (i.e., 0, 16 and 24 h, respectively) up to wound healing closure. Image J was used to count cell numbers of 5 independent views per well.

ELISA

THP-1 cells were seeded on electrospun nanofibrous membranes (0.6316–0.7895 mg cm⁻²) at a density of 8×10⁴ cells/well in 12-well plates. Phorbol 12-myristate 13-acetate (PMA, 100 ng mL⁻¹; Sigma-Aldrich, USA) was added in the culture medium and maintained for three days for the differentiation of THP-1 cells to M₀ type macrophages. Then cells were stimulated by lipopolysaccharide (LPS, 200 ng mL⁻¹, Sigma-Aldrich, USA) for 2 h. The cell-free culture supernatants were collected for ELISA tests. Cells were harvested for protein concentration measurement using bicinchoninic acid assay kit (BCA; Weiao Biotech, China). Cytokine secretions (interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-18 (IL-18), TNFα) in supernatants of LPS-stimulated cells were measured using high sensitivity ELISA kit (Multi-Sciences, China). Procedures were accomplished according to manufacturer's instructions. The measurement was performed twice from three biological replicates.

Gene expression by real-time PCR

The total RNA was isolated using an adsorption column-based extraction kit (Tiangen Biotech, China) according to the manufacture's protocol. Complementary DNA (cDNA) was synthesized from isolated RNA with PrimeScript RT Master Mix (Takara, Japan). qRT-PCR was performed in triplicates using TB Green Premix Ex Taq II (Takara, Japan) with a CFX96 real-time PCR system and CFX Manager Software (Bio-Rad Laboratories GmbH, Munich, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene. The relative messenger RNA expressions were calculated using comparative cycle threshold (CT) value method ($\Delta C(t)$ and $\Delta\Delta C(t)$). The measurement was performed twice from three biological replicates. qRT-PCR primers were as follows:

IL-1β: Forward-5'-CCACAGACCTTCCAGGAGAATG-3' and Reverse-5'-GTGCAGTTCAGTGATCGTACAGG-3';
IL-6: Forward-5'-AGACAGCCACTCACCTCTTCAG-3'

and Reverse-5'-TTCTGCCAGTGCCTCTTTGCTG-3';
 IL-18: Forward-5'-GATAGCCAGCCTAGAGGTATGG-3' and Reverse-5'-CCTTGATGTTATCAGGAGGATTCA-3';
 TNF α : Forward-5'-CTCTTCTGCCTGCTGCACTTTG-3' and Reverse-5'-CCTTGATGTTATCAGGAGGATTCA-3';
 VCAM-1: forward-5'-GATTCTGTGCCACAGTAAGG C-3' and Reverse-5'-TGGTCACAGAGCCACCTTCTTG-3';
 Tissue factor (TF): Forward-5'-CAGAGTTCACACCTT ACCTGGAG-3' and Reverse-5'-GTTGTTCCCTTCTGAC TAAAGTCCG-3';
 E-selectin: Forward-5'-GGACACCACAAATCCCAGTC TG-3' and Reverse-5'-TCGCAGGAGAACTCACAACCTG G-3';
 Plasminogen activator inhibitor-1(PAI-1): Forward-5'-GGACACCACAAATCCCAGTCTG-3' and Reverse-5'-TCGCAGGAGAACTCACAACCTGG-3';
 GAPDH: Forward-5'-GTCTCCTCTGACTTCAACAGC G-3' and Reverse-5'-ACCACCCTGTTGCTGTAGCCA A-3'.

Statistical analysis

All quantitative data are presented as mean $\pm$ standard deviation (SD). Statistical difference between groups was analyzed by unpaired t-test and one-way ANOVA test. p value 0.05 was considered to be statistically significant between groups.

RESULTS AND DISCUSSION

Characteristics of the TEMPOL-loaded PDLLA electrospun membranes

In this study, a coaxial electrospinning process was used to incorporate TEMPOL into nanofibers (Fig. 1a–d). PDLLA was used here to form the shell layer of the core-shell nanofibers due to its good biocompatibility and electro-spinnability (Fig. 1a). Fabricated as such, TEMPOL can be effectively incorporated into the electrospun PDLLA nanofibers (Fig. 1d). Here, it should be noted that the as-electrospun nanofibers in such a coaxial electrospinning system presented a fuzzy core-shell structure with no clear boundary, which is mainly due to the good compatibility between the solutions of the shell layer and the core layer (Fig. 1e and Fig. S2). As a powerful technique for studying the motion of nitroxyl radicals, EPR spectra were further utilized to study the EPR signals of the as-obtained membranes. As shown in Fig. 1f, a characteristic triplet signal of the nitroxyl radical was observed clearly, confirming the presence of TEMPOL

radicals in the electrospun membranes [34,35].

Moreover, the loading amount of TEMPOL can be adjusted by simply varying the concentration of TEMPOL solution (i.e., the core layer spinning solution). The surface morphology of the as-electrospun membranes with different loading amounts of TEMPOL was demonstrated by SEM (Fig. S3 and Fig. 1g–l). As shown in Fig. 1g–l, all fibers are smooth and randomly oriented with no visible beads-on-a-string or spindles-on-a-string phenomena, yet with their diameters uneven. Such a size unevenness might be due to the complexity and compatibility of dual spinning solutions in such a coaxial electrospinning system, thus affecting the uniform formation of the nanofibers. As the inner spinning TEMPOL concentration increased from 1 to 50 mg mL⁻¹, the electrospun nanofibers only displayed a slight change in their diameters, i.e., (415.2 $\pm$ 228.1) nm (P-T-0.1), (421.1 $\pm$ 245.7) nm (P-T-0.2), (371.8 $\pm$ 222.3) nm (P-T-0.5), (369.6 $\pm$ 192.1) nm (P-T-1), (362.8 $\pm$ 236.5) nm (P-T-2) and (239.0 $\pm$ 155.3) nm (P-T-5), respectively (Table 1).

In vitro release of TEMPOL

The actual loading amounts of TEMPOL in such PDLLA nanofibers were further determined by HPLC with their calculated results recorded in Table 1. Obviously, the actual loading amount of TEMPOL is increased with increasing spinning solution concentration, while their encapsulation efficiency presents a significant decrease from ~42.5% for P-T-0.1 to ~18.2% for P-T-5. Such a declined encapsulation efficiency can be attributed to that higher concentrations of TEMPOL have more tendency to migrate to the surface or near the surface of nanofibers during the electrospinning process, thus leading to a certain loss during the subsequent processing procedures, which is similar to some previous reports [36,37].

To study the releasing behaviors, *in vitro* release measurements were conducted on such TEMPOL-loaded PDLLA electrospun membranes which were monitored by HPLC for 19 days (Fig. 2). As shown in Fig. 2a, it was found that the release behavior of TEMPOL for all the samples experienced two stages: (i) the initial burst release in the first day with a release percentage of 40% to 70%, and (ii) the continuous slow release to a platform for the following 18 days. The initial burst release is mainly attributed to the diffusion of TEMPOL on the surface or under very near the surface of fibers [38]. Comparably, the releasing rates of P-T-0.5 and P-T-1 in 24 h were obvious slower than others; it is assumed that appropriate TEMPOL loading in such electrospun membranes can also preserve a low TEMPOL release rate that are pre-

Figure 2 *In vitro* release curves. Raw release data are given for (a) the entire 19-day experiment with the inset showing the burst release in the 1st day; (b) Korsmeyer-Peppas plots for the TEMPOL release from the TEMPOL-loaded PDLLA electrospun membranes.

ferable for a long-term scavenge of ROS. To further study the releasing mechanism, the data were further fitted according to the zero-order model, one-order model, Higuchi model and Korsmeyer-Peppas model, respectively [39,40], and analyzed with the Equations (3–6) as follows:

zero-order model:

$$Q = kt, \quad (3)$$

one-order model:

$$\ln(1 - Q) = -kt, \quad (4)$$

Higuchi model:

$$Q = kt^{1/2}, \quad (5)$$

Korsmeyer-Peppas model:

$$Q = kt^n, \quad (6)$$

where Q is the release percentage, k is the rate constant, and n in Korsmeyer-Peppas model is an exponent which sheds some insights on the drug release mechanism. As calculated and shown in Fig. 2b, Figs S4 and S5, and Table S1, the fitting of TEMPOL release kinetics presented a preferable match to the Korsmeyer-Peppas model (Table S1); rather than the other ones (i.e., Higuchi model, zero-order model and one-order model). Additionally, all samples have their n values lower than 0.45, corresponding to a Fickian diffusion mechanism [39,41,42], which is also in consistent with above releasing behaviors.

Adhesion and proliferation behavior of HUVEC on TEMPOL-loaded PDLLA electrospun membranes

To investigate the cytotoxicity of excessive TEMPOL on HUVECs, we investigated the cell proliferation behavior

Figure 3 CCK-8 results of HUVECs cultured on the TEMPOL-loaded PDLLA electrospun membranes with different loading amounts for 1, 3 and 5 days, respectively. Data are presented as means ± SD ($n = 4$, ** $p < 0.01$, *** $p < 0.001$).

on the TEMPOL-loaded electrospun membranes with different loading amounts by CCK-8 assay. As shown in Fig. 3 and Fig. S6, it can be found that the sample with a higher TEMPOL loading (i.e., P-T-2 and P-T-5) presented a certain cytotoxicity; the cell proliferation was therefore inhibited in all the incubation experiments from 1 to 5 days.

Furthermore, living HUVECs on the electrospun membranes were stained and detected by Cell Tracker Green CMFDA; and the results was shown in Fig. 4. After an incubation for three days, all the samples present a good biocompatibility, and an obvious cell proliferation can be observed on P-T-1 (Fig. 4b) with an equivalent cell counts to the control sample of P-T-0 (Fig. 4a). In comparison, the cell counts on the sample of P-T-5 was much lower than the other two counterparts (Fig. 4c, d), exhibiting a significant inhibition effect caused by the

Figure 4 Live cell staining of HUVECs by Cell tracker green CMFDA. The HUVECs were incubated for three days on the electrospun membranes of (a) P-T-0, (b) P-T-1, and (c) P-T-5, respectively; and (d) their cell counts. Data are presented as means $\pm$ SD ($n = 4$, $p < 0.05$).

excessive TEMPOL; such a result is also in consistence with the result of CCK-8 assay (Fig. 3).

Additionally, SEM and fluorescence microscope were further used to study the cell morphology of HUVECs after a 3-day incubation on the P-T-0, P-T-1 and P-T-5 samples. As observed by SEM (Fig. 5a–c), the HUVECs on the three samples spread over the entire region of the membranes with cobblestone-like morphologies. Besides, the P-T-0 and P-T-1 membranes exhibited excellent cellular biocompatibility for HUVECs growth in comparison with the P-T-5 membrane. The endothelial expression of vWF marker was confirmed in cells on day 3. From the fluorescence micrographs (Fig. 5a1–c3), it can be seen that HUVECs on the P-T-1 and P-T-5 samples exhibited positive vWF expression, which is in consistence with their typical endothelial phenotype [27]. Furthermore, the results displayed that the electrospun membranes loaded with appropriate TEMPOL (i.e., P-T-1

Figure 5 SEM and fluorescence microscope images of the HUVECs incubated for three days on the electrospun membranes of (a) P-T-0, (b) P-T-1, and (c) P-T-5, respectively. Green: vWF, blue: DAPI.

sample) could well maintain the shape and phenotype of HUVECs, suggesting an excellent performance in keeping the cell activities.

ROS-scavenging and anti-inflammation performance

Previous reports have well demonstrated that ROS produced by macrophages has multiple adverse impact on vascular cells (e.g., the dysfunction and activation of endothelial cells and VSMCs) [43,44], and eventually result in the restenosis as mentioned in the introduction section. Consequently, it is highly desired to know whether these TEMPOL-loaded PDLLA membranes can effectively inhibit the ROS production. To this end, THP-1-derived macrophages pre-incubated with the electrospun membranes for three days were utilized, and H_2O_2 (0.1 mmol L^{-1}) was used as a stimulator to trigger the ROS production in THP-1 cells which can be probed by a fluorescent dye of DHE. ROS level was significantly elevated after H_2O_2 stimulation of THP-1-derived macrophages with P-T-0 (Fig. 6a, b). In comparison, DHE fluorescent signals of the macrophages with P-T-1 sample presented an obvious attenuation of ROS production (Fig. 6c), exhibiting an effective ROS scavenging performance (Fig. 6d).

Moreover, the inflammatory responses of THP-1 cells on such membranes were further investigated. As shown in Fig. 7, the mRNA expression and secretion of proinflammatory cytokines on the P-T-1 samples revealed a remarkable anti-inflammatory effect in comparison with

the P-T-0 counterpart, including the $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6 and IL-18 . Notably, P-T-5 sample with much higher TEMPOL loading did not present a better anti-inflammation performance than P-T-1 sample, with the main reason that overwhelming scavenging of ROS would be detrimental because the existence of oxidants also have some positive functions, e.g., homeostatic and well-calibrated signaling [45]. Based on these results, it has demonstrated that such electrospun membranes with an appropriate TEMPOL loading can effectively counteract induced oxidative stress and reduce secretion of inflammatory cytokines in macrophages; which is consistent with previous reports that conducted by direct administration of TEMPOL [46].

Anti-inflammation and anti-monocytes adhesion of HUVECs on TEMPOL-loaded PDLLA membranes

It has well demonstrated that the vascular endothelium is vulnerable to the tissue damage by oxidants during intravascular interventions [9], thus leading to dysfunctional endothelium followed by the activation of the endothelium cells and increasing of monocyte adhesion from blood circulation. Such a pathological evolution is assumed as a key inducement in pathogenesis of in-stent restenosis [47,48]. Furthermore, vascular injury would trigger the expression of prothrombogenic molecules in dysfunctional endothelium, which is a crucial factor of early in-stent thrombosis resulting in stent failure [49]. To study the effects of these TEMPOL-loaded PDLLA membranes on the above negative consequences caused by dysfunction of endothelium cells, $\text{TNF}\alpha$ was used as a stimulator to trigger the dysfunction of HUVECs pre-incubated with the electrospun membranes.

As shown in Fig. 8, the results have demonstrated that $\text{TNF}\alpha$ stimulation would enhance mRNA expression of cell adhesion molecules on the P-T-0 sample (i.e., E-Selectin and VCAM-1 in Fig. 8a, b). Such an mRNA expression presented a much lower level on TEMPOL-loaded PDLLA samples, especially for the P-T-1 and P-T-2 samples (Fig. 8a, b). Monocytes adhesion tests were also performed to further demonstrate their anti-inflammatory effect in HUVECs (Fig. 9). Similarly, TEMPOL-loaded PDLLA membranes have significantly attenuated the adhesion of THP-1 monocytes to the $\text{TNF}\alpha$ -stimulated HUVECs by using the P-T-1 sample (Fig. 9).

Moreover, the inhibition effect on expression of prothrombogenic factors of endothelial cells was further studied. As shown in Fig. 8c and d, an increased mRNA expression of prothrombogenic factors (TF, Plasminogen

Figure 6 DHE staining of ROS in THP-1 cells. THP-1 cells were seeded on samples for three days before a H_2O_2 (0.1 mmol L^{-1}) stimulation for 6 h. (a) DHE-labelled THP-1 cells cultured on the (a, b) P-T-0 sample (a) without H_2O_2 and (b) with H_2O_2 stimulation, and on the (c) P-T-1 sample with H_2O_2 stimulation. (d) Quantitative analysis of integrated optical density. Data are presented as means $\pm$ SD ($n = 4$, ** $p < 0.01$).

Figure 7 Inhibition of inflammation in THP-1 cells on the TEMPOL-loaded PDLLA electrospun membranes. THP-1 cells were incubated with P-T-1 samples for three days followed by a stimulation of LPS for 2 h. Expression of (a) IL-6, (b) TNFα, (c) IL-1β and (d) IL-18 was detected by qRT-PCR, and the protein level of (e) IL-6, (f) TNFα, (g) IL-1β and (h) IL-18 was detected by ELISA of THP-1 cells cultured on the different samples. Data are presented as means ± SD ($n = 3$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Activator Inhibitor-1 (PAI-1)) was observed in HUVECs after a stimulation by TNFα for 2 h. Only P-T-1 and P-T-2 displayed a significant inhibition on mRNA expression of PAI-1. All samples except the P-T-0.5 exhibited significant inhibition on mRNA expression of TF. We have also performed a detailed analysis of biological response of HUVECs to these electrospun membranes under inflammatory conditions, hopefully to provide more information about potential application in preventing or treating endothelial dysfunction after PCI procedure.

HASMCs migration under TEMPOL-loaded PDLLA electrospun membranes

Migration of activated VSMCs often leads to the pathological changes of vessel including restenosis after PCI [50]. Vascular injury could induce VSMC migration through various cytokines secreted by inflammatory cells, platelets and VSMCs. To study the migration behavior of VSMCs on such electrospun membranes, we firstly utilized the most potent stimulant of PDGF-BB to stimulate the VSMCs followed by an incubation on the membranes

Figure 8 Inhibition of adhesive molecules and prothrombogenic factor expression in HUVECs by TEMPOL-loaded membranes. HUVECs were incubated with the samples for three days before a stimulation of TNFα for 2 h. qRT-PCR analysis of (a) E-Selectin, (b) VCAM-1, (c) PAI-1 and (d) TF mRNA expression of HUVECs cultured on different samples. Data are presented as means ± SD ($n = 3$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

Figure 9 Inhibition of THP-1 monocytes adhesion onto the TEMPOL-loaded membranes pre-incubated with HUVECs. HUVECs were incubated with the samples for three days before stimulated by TNFα for 2 h. Fluorescence images of adhered THP-1 cells: (a) without and (b) with TNFα stimulation on the control P-T-0 samples; and (c) with TNFα stimulation on P-T-1 sample; (d) quantitative analysis of adhered THP-1 cells. Data are presented as means ± SD ($n = 5$, *** $p < 0.001$).

for 24 h [51], and the results are shown in Fig. 10 and Fig. S7. Specifically, it is clearly shown that the migration of PDGF-BB-stimulated HASMCs was effectively inhibited on the P-T-1 membrane (i.e., P-T-1+PDGF-BB) at 16 h when compared with P-T-0 counterpart (i.e., P-T-0+PDGF-BB). Such an inhibition effect was more apparent when observed at 24 h with about a 50% reduction of HASMCs migration (Fig. 10d). These results have well demonstrated that the TEMPOL-loaded electrospun membranes have the capability in counteracting the migration of cytokine-activated VSMCs.

CONCLUSIONS

In summary, we have successfully encapsulated TEMPOL into PDLLA nanofibers *via* a coaxial electrospinning process which provides the possibility for the topical release of antioxidants to scavenge ROS induced after the PCI therapy. Specifically, the actual loading amount of TEMPOL is tunable by simply changing the spinning concentration for the core layer; the PDLLA shell can also

Figure 10 HASMCs migration under cultured different samples ((a) P-T-0 without PDGF-BB stimulation, (b) P-T-0 with PDGF-BB stimulation and (c) P-T-1 with PDGF-BB stimulation) at different times ((1) 0 h, (2) 16 h and (3) 24 h). (d) quantitative analysis of migration index. Data are presented as means $\pm$ SD ($n = 5$, * $p < 0.05$, ** $p < 0.01$).

serve as a protect layer for a long-term release of TEMPOL from the electrospun membranes. The *in vitro* performance indicated that the electrospun membranes with optimized TEMPOL loading amount (P-T-1) presented a good cytocompatibility with HUVECs, ROS-scavenging and anti-inflammation performance in macrophages; it could also significantly attenuated adhesive molecules (e.g., VCAM-1, E-Selectin) and expression of prothrombotic factors (e.g., TF, PAI-1) in HUVECs, the adhesion of THP-1 cells as well as the migration of HASMCs. Therefore, when used as coating on a stent for topically scavenging ROS, such a novel electrospun system has a great potential to address the restenosis and thrombosis issues after PCI therapy in practice.

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Supplementary information Experimental details and supporting data are available in the online version of the paper.

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负载TEMPOL的静电纺丝纳米纤维实现PCI相关并发症治疗的局部长抗自由基性能

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摘要 使用抗氧剂清除活性氧(ROS)被证实是应对经皮冠状动脉介入治疗(PCI)相关并发症的有效方法, 但抗氧剂向特定组织的长期局部输送不易实现. 本文将抗氧剂TEMPOL可控地负载入静电纺丝纳米纤维以实现其局部应用和长期抗自由基能力, 制得TEMPOL负载薄膜的生物功能通过细胞增殖、ROS清除能力、单核细胞黏附、细胞迁移、炎症因子mRNA水平的表达及分泌等体外实验进行了表征. 薄膜在优化TEMPOL负载量后, 对内皮细胞和巨噬细胞表现出优异的清除ROS和抗炎症能力; 同时有效抑制内皮细胞促血栓形成因子的表达和血管平滑肌细胞的迁移. 因此, 我们预测该TEMPOL负载电纺体系的局部使用, 如用作长期局部输送抗氧化药物的血管支架涂层, 可以有效治疗血管再狭窄和抑制血栓形成.