Materials Science & Engineering C 92 (2018) 875–891

Contents lists available at ScienceDirect

Materials Science & Engineering C

journal homepage: www.elsevier.com/locate/msec

Harnessing the pleiotropic effects of atorvastatin-fenofibrate combination for cardiovascular stents

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ARTICLE INFO

Keywords:
Drug eluting stents
Atorvastatin
Fenofibrate
Anti-thrombotic
Restenosis

ABSTRACT

Atorvastatin and fenofibrate have been conventionally employed as lipid-lowering agents. They also exhibit beneficial effects in the treatment of endothelial dysfunction, oxidative stress and vascular inflammation due to their pleiotropic effects that include vasodilatory and anti-inflammatory effects. These pleiotropic effects may serve to overcome the drawbacks of late stent thrombosis and delayed endothelialization that plague conventional drug eluting stents. However, the combination has not been explored yet as therapeutic coatings in drug eluting stents. The present study aims to investigate the potential of atorvastatin-fenofibrate combination loaded in a biodegradable poly(L-lactide-*co*-caprolactone) polymer film to inhibit thrombus formation and macrophage activation apart from exploring their effect on the proliferation of smooth muscle cells and endothelial cells. The dual drug-loaded polymer films were characterized by spectroscopy and calorimetry. In vitro studies revealed that the combination effectively retarded the proliferation of only smooth muscle cells but not the endothelial cells which augers well for stent applications where rapid re-endothelialization is preferred. Further, the dual drug-loaded films exhibited a marked decrease in the adhesion and activation of platelets and macrophages revealing the potent anti-thrombogenic and anti-inflammatory effects of the combination. The pleiotropic effects of the combination may be attributed to their ability to activate nitric oxide synthase in endothelial cells while mTOR levels remained unaltered by the combination.

1. Introduction

Drug eluting stents (DES) are the major treatment options for cardiovascular disorders [1]. The demand for these interventions is constantly on the rise due to the increased incidence of cardiac disorders in the modern era. Conventional drug eluting stents employ a biodegradable polymer coating incorporating an anti-proliferative drug such as sirolimus, everolimus, tacrolimus, paclitaxel etc., in a bid to curtail smooth muscle cell proliferation [2]. This avoids blocking of the stented artery by the over-proliferating smooth muscle cells – a condition referred to as neointimal hyperplasia or restenosis. The biodegradable polymer serves as a carrier that favors localized and sustained delivery of the anti-proliferative agent [3]. It has been demonstrated that drug eluting stents reduce the incidence of stent restenosis by 50% when compared with their bare metal counterparts [4]. But regardless of their advantage over bare metal stents, DES exhibit delayed healing, lack of complete endothelialization and stent thrombosis arising due to restricted endothelial proliferation at the stented site [5]. Clinical trial data have shown that the cell growth inhibitors such as sirolimus and paclitaxel used in the first and second generation of DES show incomplete endothelialization over the stent struts and eventually lead to thrombosis [6]. Consequently, the patients are required to be on an anti-platelet regimen for the rest of their life. The long-term use of these drugs leads to additional complications that drastically compromise on the quality of life. Hence, innovations in the drug combinations employed in the stent focusing on retarding smooth muscle cell proliferation, inflammatory response and platelet activation but promoting endothelialization are required. Many drugs such as curcumin [7], 6-mercaptopurine [8], gallic acid [9], heparin [10], etc., have been investigated to overcome the shortcomings of DES, but reports reveal that they exhibit less favorable outcomes. Novel therapeutic options such gene [11] and antibody therapy [12] has also been explored to overcome the stent-associated complications. But these approaches are expensive and may face bottlenecks in large-scale commercial use. As a single drug may not be effective in controlling the diverse response of vasculature, multi-drug combinations with anti-proliferative and pro-healing effect have been explored for harnessing the synergistic and multi-targeted action. Tuning a multi-drug formulation for its synergistic, pleiotropic and additive beneficial effects could expand the therapeutic efficacy of drug eluting stents to overcome the current

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https://doi.org/10.1016/j.msec.2018.07.048
Received 10 October 2017; Received in revised form 23 June 2018; Accepted 18 July 2018
Available online 20 July 2018
0928-4931/

conundrum. Unfortunately, only few combinations of drugs have been explored for DES applications. Resveratrol-quercetin, sirolimus-triflusal, paclitaxel-sirolimus and curcumin-paclitaxel are a few combinations that have been tested for stent applications [13–16]. These combinations have displayed promising in vitro and in vivo performance indicating the importance of multi-drug combinations in DES. However, these combinations are yet to successfully complete clinical trials. Concurrently, newer drug combinations need to be investigated to improve the safety and performance of drug eluting stents.

The present study aims to evaluate for the first time a combination of statin and fibrate to address stent-related complications. Atorvastatin and fenofibrate are lipid-lowering drugs prescribed to cardiac patients for maintenance of their lipid profile [17]. Though the major target of statins is HMG CoA reductase, a key enzyme in the cholesterol biosynthesis [18] and fibrates act on peroxisome proliferator activated receptor (PPAR) involved in lipid and glucose metabolic pathways [19], both drugs are known to display pleiotropic effects on other molecular targets and hence their combination have been explored for a broad spectrum of disorders [20]. The beneficial effects of this combination include reduction of atherosclerotic oxidative stress [21] and endothelial dysfunction [22], improve insulin sensitivity in diabetic patients [23], modulate vascular inflammation [24], and hemostatic system [25, 26]. A scan of literature reveals that fenofibrate and atorvastatin independently as well as in combination up-regulate nitric oxide synthase levels [27]. This may augur well for stent-associated applications as nitric oxide synthases play a key role in endothelialization in vascular space [28]. Though several studies have explored this combination for its therapeutic applications in dyslipidemic conditions [29], it has not been explored for coronary stent applications and this forms the crux of the present work.

The biodegradable polymer employed for the stent coating needs to be sufficiently elastic to withstand the crimping pressure and expansion on deployment in the blood vessel. In addition to being biocompatible, the polymer should also possess slow degradation rates to ensure sustained release of the drug(s) over an extended period. Current generation of DES employ poly(L-lactide-*co*-glycolide) (PLGA) for coating stents [30]. Poly(L-lactide) (PLL) and poly(caprolactone) (PCL) have also been explored for stent coating [31] due to their biocompatibility and slow degrading properties. However, PLGA and PLL are more brittle and hence have the risk of cracking during deployment of the stent. In the present work, we have employed biodegradable films of the copolymer poly(L-lactide-*co*-caprolactone) (PLCL 70:30) as it possesses a viscoelastic nature and a tunable degradation rate apart from being biocompatible [32]. The higher lactide content in the copolymer controls the degradation rate while the caprolactone content imparts sufficient elastic nature and mechanical strength thereby making it a promising stent coating material. In spite of the promising properties of PLCL, there are very few attempts to explore this polymer for stent coating. The present work explores the feasibility of entrapping atorvastatin and fenofibrate in PLCL films for suppressing restenosis and thrombosis and promoting endothelialization for possible coronary stent applications that has hitherto never been explored before with this combination.

2. Materials and methods

2.1. Materials

Poly(L-lactide-*co*-ε-caprolactone) (7030LCL) M_w = 230 kDa (Evonik, Germany), atorvastatin calcium (Swapnroop Drugs & Pharmaceuticals, India), fenofibrate (Sigma-Aldrich, USA) and acetone (Merck, India) were procured for the study. Fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (DPBS) and antibiotics (penicillin-streptomycin (P/S)) were purchased from Gibco, USA. CellTiter96-AQueous one solution was purchased from Promega, USA. Live-Dead cell viability kit was procured from Molecular Probes, USA. Human umbilical vein endothelial cells (HUVEC) were purchased from Hi-Media laboratories and macrophages, IC-21, was obtained from NCCS, Pune. Human vascular smooth muscle cells (hVSMC) were a kind gift from Madras Diabetic Research Foundation (MDRF), Chennai.

2.2. Fabrication of dual drug loaded and pristine PLCL films

Atorvastatin and fenofibrate loaded PLCL films were prepared by solvent casting method. PLCL was dissolved in acetone and stirred for 2 h at room temperature. Pre-determined concentrations of the drugs were added to the polymer solution and stirred for proper mixing. The solution was drop cast on a glass slide and allowed to air dry for 24 h. Dried films were then removed and stored in vacuum desiccator for three days. Pristine PLCL films were obtained by drop-casting PLCL solution followed by air-drying and then stored in a vacuum desiccator. Surface morphology of the films was evaluated using field emission scanning electron microscopy (FE-SEM, JSM 6701F, JEOL, Japan). Dried films were sputter coated with gold before imaging.

2.3. Fourier transform infrared spectroscopy (FTIR)

Atorvastatin, fenofibrate, PLCL film and dual drug loaded PLCL film samples were characterized using Fourier transform infrared (FT-IR) spectrometry (Spectrum 100, Perkin Elmer, USA). The samples were pelletized using KBr (IR grade, Merck, Germany) and the IR spectra of the films and free drugs were recorded between 4000 and 400 cm^{-1} for 50 scans.

2.4. Differential scanning calorimetry (DSC)

The thermal profiles of atorvastatin, fenofibrate, pristine polymer and dual drug-loaded PLCL films were recorded using differential scanning calorimetry (DSC, TA Instruments, DSC Q20, USA). Thermograms were recorded in an inert environment between −100 to 200 °C with a nitrogen flux of 50 mL/min and a cooling rate of 10 °C/min.

2.5. Tensile strength

Tensile property of pristine and dual drug-loaded PLCL films was determined in a uniaxial tensile testing machine (Instron 3345, UK). Films were cut to the dimension of 50 mm × 10 mm and mounted on the gripping unit for measurements. A load of 500 N was applied with an extension rate of 1 mm/min (n = 10).

2.6. In vitro release study

Drug release profiles of single and dual drug-loaded PLCL films containing 1 mM of each drug were recorded in 0.1 M phosphate buffered saline (PBS) at a pH 7.4, 80 rpm and 37 °C for 60 days. The release medium was collected at pre-determined time points and replaced with same amount of fresh medium. Samples were analyzed using UV–Visible spectrophotometry (Lambda 25, Perkin Elmer, USA) at 246 and 286 nm for atorvastatin and fenofibrate respectively. The release profiles of atorvastatin and fenofibrate from PLCL film were analyzed using different kinetic equations tabulated in Table 1.

2.7. In vitro degradation study

Pristine, single and dual drug-loaded PLCL films were incubated in PBS (pH 7.4) at 37 °C under constant stirring at 80 rpm. The medium was replaced every alternate day. At pre-determined time points, the films were taken out, rinsed with distilled water and air-dried followed by vacuum drying. Morphology of the films was imaged using FE-SEM. The percentage weight loss was calculated by weighing the films before (W_O) and after the degradation study (W_f) and using the following