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**Nanoparticles eluting stents for coronary intervention: Formulation, characterization and
in vitro evaluation**

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ABSTRACT

Coronary artery disease (CAD) is currently a leading cause of death worldwide. In the history of percutaneous coronary intervention for the treatment of CAD, a drug-eluting stent (DES) is recognized as a revolutionary technology that has the unique ability to significantly reduce restenosis and provide both mechanical and biological solutions simultaneously to the target lesion. The aim of the research work was to design and fabricate DES coated with a nanoparticulate drug formulation. Sirolimus, an inhibitor of the smooth muscle cell (SMC) proliferation and migration, was encapsulated in polymeric nanoparticles. The nanoparticle formulation was characterized for various physicochemical parameters. Cell viability and cell uptake studies were performed using human coronary artery smooth muscle cells (HCASMCs). The developed nanoparticle formulation showed enhanced efficacy compared to plain drug solution and exhibited time-dependent uptake into the HCASMCs.

The developed nanoparticle formulation was coated on the Flexinnium™ ultra-thin cobalt-chromium alloy coronary stent platform. The nanoparticle coated stents were characterized for morphology and residual solvent analysis. *In vitro* drug release was also evaluated. *Ex vivo* arterial permeation was carried out to evaluate the nanoparticle uptake from the surface of the stents. The characterization studies together corroborated that the developed nanoparticle coated stent can be a promising replacement of the current drug-eluting stents.

Keywords: Coronary artery disease, Sirolimus, Restenosis, Drug-eluting stents, Nanoparticles, Human coronary artery smooth muscle cells

1. Introduction

Coronary artery disease (CAD), also called Coronary artery Atherosclerosis, Coronary Heart Disease (CHD), or Ischemic Heart Disease (IHD), remains the major cause of morbidity and mortality throughout the world despite the greater understanding of the pathology of the disease, advanced diagnostic imaging and continued improvements in medical treatment options. As per the World Health Organization (WHO) report of 2021, cardiovascular diseases (CVD) caused 17.9 million deaths worldwide in 2019. ([https://www.who.int/news-room/factsheets/detail/cardiovascular-diseases-\(cvds\)](https://www.who.int/news-room/factsheets/detail/cardiovascular-diseases-(cvds))). Among all cardiovascular diseases, CHD ranks as the most prevalent (Roth et al., 2017).

CHD is a multifactorial disease involving inflammatory processes arising from the encounter of certain bacterial products to diverse risk factors such as dyslipidemia, hypertension, hyperglycemia, or adipose tissue-derived cytokines. This leads to the sticking of blood leukocytes to the inner surface of the arterial wall where they ultimately communicate with the endothelial and smooth muscle cells (SMCs) of the endothelium. The migration and proliferation of SMCs from tunica media into intima initiates complex lesion progression which may involve calcification and plaque formation (Wessely et al., 2006a).

Percutaneous coronary intervention (PCI) with stents is a common treatment modality for CHD with coronary artery stenosis of > 70%. Drug-eluting stents (DES) are commonly used in PCI as compared to bare metal stents (Ni et al., 2020). DES carries anti-proliferation drugs mainly sirolimus or other drug molecules from the limus family and paclitaxel. These drugs inhibit the proliferation of SMCs and prevent a further cascade of events that lead to restenosis. Polymers are employed as a carrier for the drug to facilitate drug release.

The challenge of in-stent restenosis (ISR) persists even after 43 years of the first angioplasty performed in 1977 (Meier and Kaltenbach, 2018) despite all the scientific and technological innovations in DES.

To overcome these complications, the current stent research is focused on the enhanced safety and efficacy by optimally combining stent platform, drug, and alteration of drug release kinetics by the development of novel formulations. Nanotechnology offers a new avenue for the improvement of current stent technology. In 1997, Surface-modified cationic poly (lactide-co-glycolide) (PLGA) polymeric nanoparticles were developed for the treatment of restenosis using an *ex vivo* arterial model. The arterial uptake of surface coated nanoparticles was higher (10-time) than the non-coated ones. That study provided, for the first time, a basis to integrate nanocarriers on stent surfaces for the treatment of restenosis (Labhasetwar et al., 1998).

Encapsulation of drugs in nanoparticles may allow higher arterial wall concentrations and residence times than traditional drugs, two important factors for the prevention of restenosis (Caves and Chaikof, 2006). Nanoparticles of various drug molecules have been investigated in the literature and such studies have given promising insights into the nanoparticle interaction *in vivo*. In 1996, Guzman et. al investigated the performance of dexamethasone-loaded nanoparticles in a dog model. They showed that the nanoparticles were present in all three layers of the femoral artery wall after 7 days and in the adventitial layer after 14 days. Furthermore, the plasma concentrations of dexamethasone were not detectable after 3 days, which suggested that the systemic exposure and resulting toxicity effects may be prevented with localized, drug-laden nanoparticles (Guzman et al., 1996). Xiaowei Zhu et al. demonstrated the use of bilayered poly(lactide-co-glycolide) nanoparticles (NPs) containing VEGF (Vascular endothelial growth factor) plasmid and paclitaxel in the rabbit model. They showed that paclitaxel exhibited delayed

93 release from the core of the nanoparticles and inhibited the restenosis phenomena *in vivo* (Zhu et
94 al., 2017). Nakano et. al have reported that nanoparticles are taken up rapidly by endothelial cells
95 and are retained in the intracellular space. They reported that stents coated with nanoparticles using
96 electrodeposition were also able to deliver fluorescein-isothiocyanate (FITC) within the vessel
97 wall up to four weeks (Nakano et al., 2009). Chorny et al. developed magnetic nanoparticles of
98 paclitaxel which in the presence of the magnetic field, facilitated the localization of nanoparticles
99 and resulted in the reduction of ISR (Chorny et al., 2010). Luderer et al. demonstrated that
100 sirolimus-loaded poly (D, L-lactide) nanoparticles of the size 250 nm were more effective than the
101 free drug to prevent the proliferation of smooth endothelial cells (Luderer et al., 2011). Prabha, S.,
102 et al evaluated the size dependency on the uptake of nanoparticles. The smaller-sized nanoparticles
103 (70 nm) had a higher uptake (27-fold) than 200 nm diameter nanoparticles in the COS-7 cell line
104 (Prabha et al., 2002). Increasing evidence supports the idea that a coating of drug encapsulated
105 nanoparticles (NPs) onto an angioplasty balloon or stent surface is a promising approach to
106 significantly enhance and prolong drug delivery to the arterial wall.

107 Sirolimus is an inhibitor of the proliferation and migration (Poon et al., 1996) of coronary artery
108 smooth muscle cells. Sirolimus binds to the immunophilin, FK binding protein-12 (FKBP-12), to
109 generate an immunosuppressive complex. The sirolimus: FKBP-12 complex binds to and inhibits
110 the activation of the mammalian target of rapamycin (mTOR), a key regulatory kinase. (M Vilella-
111 Bach et al., 1999) This inhibits interleukin (IL)-2-mediated signal transduction suppressing T-cell
112 proliferation and the progression from the G1 to the S phase of the cell cycle (Hirenkumar K.
113 Makadia and Siegel, 2011). Selection of the drug for the present study was done based on proven
114 scientific evidence which shows the superiority of sirolimus over paclitaxel in improving critical
115 parameters associated with restenosis and adverse vascular events such as inhibition of smooth

muscle cell migration and favorable pattern of restenosis, i.e., focal (<10 mm) for sirolimus vs 50% Proliferative lesion (>10 mm) extending outside stent (Wessely et al., 2006b). Another meta-analysis study (Kastrati et al., 2005) compared the performance of Cypher (sirolimus-eluting) and Taxus (Paclitaxel eluting) stents in clinical trials. The superior performance of the Cypher stent was observed regarding angiographic restenosis and target lesion revascularization (TLR). In a randomized, controlled, single-blind trial comparing sirolimus-eluting stents with paclitaxel-eluting stents, the rate of major adverse cardiac events (MACE) at nine months was 6.2 percent in the sirolimus-stent group and 10.8 percent in the paclitaxel-stent group (Windecker et al., 2005). In the comparative RCT trial (The REALITY Trial), Quantitative coronary angiography (QCA) measurements indicated a more profound inhibition of neointimal hyperplasia by sirolimus-eluting stent than paclitaxel-eluting stents (Morice et al., 2006). In another study, the sirolimus-eluting stent showed significantly higher inhibition of neointimal hyperplasia compared with the paclitaxel-eluting stent in complex lesions. A significant reduction in peri-stent plaque was observed only with sirolimus-eluting stent (Petronio et al., 2007). Another five clinical trials which compared sirolimus vs paclitaxel for TLR and LLL (Late Lumen Loss), repeatedly demonstrated the superiority of sirolimus (Moliterno, 2005).

Recent pooled analyses suggest a significant reduction of thrombosis risk at 4 years post stent implantation (Byrne et al., 2012) and a significant reduction in Target Vessel Revascularization (TVR) (Lupi et al., 2014) in patients treated with biodegradable polymer DES. Among the various available biodegradable polymers, poly lactic-co-glycolic acid (PLGA) is most popular because of its long clinical history, tunable physical characteristics, sustained drug delivery, and most importantly it is FDA approved (Hirenkumar K Makadia and Siegel, 2011).

Some of the limitations of sirolimus and other limus drugs in currently marketed polymer matrix coated stents such as intrinsic molecular instability once in solution and inability to achieve long-term tissue retention despite local drug delivery (Gray and Granada, 2010) (Kolachalama et al., 2013) can be effectively overcome by encapsulating into polymer nanoparticles. We hypothesize that the stent coated with drug-eluting nanoparticles could have a more profound anti-restenotic effect than the current, clinically employed matrix drug-coated stent because of the peculiar characteristics of nanoparticles such as size-dependent uptake and localization, prolonged drug release, prevention of systemic drug exposure as demonstrated in the literature. Interestingly, nanoparticles have potential to penetrate through atherosclerotic and calcified lesions which can lead to bioavailability enhancement of the antirestenotic drug (Montserrat Vilella-Bach et al., 1999). These characteristics of the nanoparticles can possibly aid in dose reduction and improved clinical outcomes. Hence, we anticipate that the devised nanoparticle eluting stent would prove a promising tool increase the functional duration and efficacy of the stent to tackle the lingering challenges such as ISR and TLR.

PLGA-Sirolimus nanoparticles have been investigated previously by many research groups for various applications. However, the reported formulations did not show all the required characteristics, i.e., high drug loading, high entrapment efficiency (EE), and low particle size. High drug loading is essential to reduce coating duration and ease of commercial manufacturing and technology transfer while low particle size and high entrapment efficiency are important for improving uptake and sustaining the release of the drug, respectively.

Narrow particle size distribution is often achieved by membrane filtration. Lyophilization is often employed to convert the nanoparticle into powder form. In many instances, the high entrapment efficiency and high drug loading represented are calculated from the lyophilized

nanoparticles from which the free drug had been removed by ultracentrifugation. Although these techniques yield desired formulation properties, they add extra steps in formulation development and hamper industrial scalability.

PLGA nanoparticles stabilized by PVA and PVA-Tween 80 were developed by emulsification diffusion method for drug delivery to glioma cells. The nanoparticles were collected by centrifugation and lyophilized. They exhibited the particle size of 232 and 247 nm having 43% and 28% EE, respectively. The drug loading was not defined in this study (Escalona-Rayo et al., 2019). PLGA-Carbopol NPs were developed for restenosis. The nanoparticles were separated by ultracentrifuge and resuspended and filtered through a 0.45 μm size membrane. The EE of 74-79% with a particle size of 157-256 nm was obtained. The nanoparticles were lyophilized which resulted in high drug loading of 2-6% (Zou et al., 2009). PLGA NPs were prepared using single emulsion method having particle size of 265 nm, 69% EE, and drug loading of 11.6% (Shi et al., 2014). Katiyar *et al* reported PLGA NPs having particle size of 132-152 nm having 70% EE with 0.01% w/v sirolimus loading (Katiyar et al., 2016). PLGA NPs were prepared using the oil-in-water (O/W) single emulsion solvent evaporation method. The lyophilized nanoparticles exhibited 274 nm particle size, 80% EE, and loading of 160 μg drug per mg of NPs.(Acharya et al., 2009).

In another study, freeze-dried PLGA NPs were prepared using the oil/water solvent evaporation technique. The nanoparticles showed the 307 nm size having 81% EE with a drug loading of 502 ng/mg of polymer. (Das et al., 2008)(Haddadi et al., 2008).

In this investigation, from the best of our knowledge, for the first time, we report sirolimus-loaded PLGA nanoparticles having all the required characteristics for effective arterial permeation and industrial-scale adaptability to achieve product realization. The developed nanoparticles have a low particle size (~ 100 nm) high entrapment efficiency ($\sim 82\%$) and high drug loading (1mg/mL).

The sirolimus-loaded nanoparticles were coated onto ultrathin (60 μm) Co-Cr stent using ultrasonic spray coating technique. Further, the nanoparticles coated stents were evaluated for *in vitro* release and tissue drug permeation the *ex vivo* artery model.

2. Materials

Poly (lactic-co-glycolic acid) (PLGA) (50:50) was procured from Evonic Industries (Essen, Germany). According to the manufacturer's specification, the inherent viscosity of this polymer was 0.45 dL/g (0.5 w/v % in chloroform, 30 $^{\circ}\text{C}$) and the residual monomer was 1.0 weight%. Sirolimus was obtained from Fujian Kerui Pharmaceutical Co., Ltd, China. Tocopheryl polyethylene glycol succinate (TPGS) 1000 and acetone were obtained from Sigma Aldrich, USA. Water was purified using a MilliQ system (Millipore).

3. Methods

3.1 Preparation of sirolimus loaded nanoparticles

The PLGA nanoparticles were synthesized by a modified nanoprecipitation technique as reported earlier (Fessi et al., 1989). Briefly, The organic solution was prepared by dissolving PLGA in acetone (3 mg/mL). In the case of drug-loaded nanoparticles, sirolimus was dissolved in this polymer solution, while Rhodamine was also dissolved along with sirolimus to prepare dye-loaded PLGA nanoparticles. Sirolimus was loaded at the concentration of 0.1 % w/v in the nanoparticle formulation.

The organic solution was added dropwise (25G syringe needle) to an aqueous surfactant solution (0.1% w/v TPGS 1000) at a constant flow rate while continuously stirred by a magnetic stirrer. After the addition of the organic phase, the mixture was kept on stirring on a magnetic stirrer overnight to allow complete evaporation of the organic solvent.

3.2. Characterization of sirolimus loaded nanoparticles

3.2.1. Particle size and Zeta potential

The average particle size, size distribution, and zeta potential of the PLGA nanoparticle formulation were determined by means of dynamic light scattering (DLS) (Zetasizer Nano-ZS 90, Malvern Instruments GmbH, Herrenberg, Germany). The intensity of scattered light was detected at 90° to the incident beam. All the data analysis was performed in automatic mode. The measured size was presented as the average value of 20 runs, with triplicate measurements within each run.

3.2.2 Encapsulation efficiency and Drug loading

The nanoparticle formulation was centrifuged at 70,000 rpm by means of ultracentrifuge (Beckman Coulter) for 30 min to ensure the precipitation and separation of polymeric nanoparticles. The supernatant was analyzed for the free drug by HPLC. To determine the total drug content, the nanoparticle formulation (0.5 mL) was completely dissolved in 2 mL acetonitrile by vortex mixing for 1 min followed by bath sonication for 10 min. The resulted transparent drug solution was analyzed by HPLC for the determination of the sirolimus concentration.

In order to determine the sirolimus concentration, an isocratic HPLC method was developed and validated. The column used was Waters Atlantis® C18, 4.6× 250, 5 µm. The chromatographic conditions were: 50 °C column temperature, methanol/water (80/20 v/v) eluent, 1.0 mL/min flow rate, 278 nm detection UV, 1 to 100.0 µg/mL calibrated measurement range, and a detection limit of 0.054 µg/mL.

The encapsulation efficiency was calculated by the following equation:

$$\% \text{ Drug Encapsulation} = \frac{(D_t - D_s) \times 100}{D_t}$$

Where, D_t =Total drug content, D_s =Drug content of supernatant after centrifugation

228 3.2.3. Surface morphology of nanoparticles

229 A drop of the nanoparticle formulation was dried on a piece of black electrical carbon conductive
230 tape. The nanoparticle-coated stent and carbon tape were sputtered with Platinum for about 80
231 seconds. The resulting sample was analyzed by Scanning Electron Microscopy (SEM).

232 3.2.4. *In vitro* degradation of nanoparticles

233 *In vitro* degradation study was carried out to study the time-dependent degradation behavior of
234 sirolimus loaded polymeric nanoparticles. Briefly, nanoparticles were prepared and separated by
235 centrifugation at 70,000 rpm to remove the free drug and the surfactant. The separated
236 nanoparticles were redispersed in PBS pH7.4 containing 0.02% sodium azide as a preservative in
237 glass vials.

238 The samples were kept at 37°C for a period of 3 months. Sampling was taken at weekly intervals
239 to determine the pH of the sample and molecular weight of the polymer. To determine the
240 molecular weight of the polymer, the nanoparticles were separated by ultracentrifugation at 70,000
241 rpm. The resulting pellet was collected and stored at -70°C until analyzed for molecular weight.
242 The solid pellet was dissolved in chloroform. The resulting solution was subjected to Gel
243 Permeation Chromatography (GPC) for the determination of the molecular weight of the polymer.

244 3.2.5. Cell culture study

245 HCASMCs were purchased from Thermo Fischer Scientific (USA). HCASMCs were cultured in
246 the smooth muscle cell growth medium (M231, Gibco™, Thermo Fischer Scientific, USA) mixed
247 with Smooth Muscle Growth Supplement (SMGS, Gibco™, Thermo Fischer Scientific, USA),
248 which contains required growth regulators, namely 4.9% v/v fetal bovine serum (FBS), 2 ng/mL
249 human basic fibroblast growth factor, 0.5 ng/mL human epidermal growth factor, 5 ng/mL

Heparin, 2 µg/mL recombinant human insulin-like growth factor-I, 0.2 µg/mL BSA. Antibiotic-Antimycotic (Gibco, ThermoFisher, USA) solution containing amphotericin B, penicillin and streptomycin was added to the supplemented medium at the concentration of 250 ng/mL, 100 units/mL and 100 µg/mL, respectively to prevent bacterial and fungal contamination.

HCASMCs were grown in 25 cm² cell culture flasks up to 80% confluence in their respective cell culture medium at 37 °C under 5% CO₂. The cells were harvested by trypsinization (0.025% trypsin; 0.025% EDTA in PBS), collected by centrifugation (7 min at 1200 rpm), resuspended and counted in a Neubauer chamber.

3.2.5.1 Cell viability assay

The cells were seeded at a density of 2000 cells per well in a 96-well microtiter plate (tissue culture grade; flat, clear bottom) and incubated for 24 h at 37 °C under 5% CO₂ before conducting a cell viability test. Four groups of formulations were studied, i.e., group 1, drug nanoparticles (separated by ultracentrifugation); group 2, nanoparticle formulation (containing free drug); group 3, drug solution and group 4, blank nanoparticles. The supernatant was removed from the wells and replaced by formulation of the test groups prepared in cell culture medium and incubated for 24 h at 37 °C under 5% CO₂. Then the old media containing test formulation was replaced by fresh media after giving two washes to each well with fresh media followed by the addition of 20 µL of resazurin solution (60 µg/mL) to each well. The cells were incubated for 2 h, during which resazurin was reduced to resorufin. Resorufin fluorescence (excitation wavelength at 544 nm and emission wavelength at 590 nm) was measured with a fluorimeter (Fluostar Optima, BMG, Germany). All samples were run in triplicate. The relative cell viability was calculated as the percentage of untreated cells.

3.2.5.2 Cell uptake study by Confocal laser scanning microscopy

In order to investigate the uptake of sirolimus-loaded PLGA nanoparticles into cells, sirolimus nanoparticles containing Rhodamine B were used. In the wells of a 12-well plate, ethanol-treated and flame-dried round coverslips were placed. 1 mL of the cell suspension was seeded at a cell density of 35000 cells/well. The plate was incubated at 37 °C and 5% CO₂ for 24 h. Further, the cells were exposed to 1 mL of Rhodamine B loaded nanoparticles and plain Rhodamine B solution for different periods-30 min, 1 h, 2 h, 4 h, 8 h, 16 h and 24 h. A negative control containing only complete media was maintained to standardize the evaluation. After each time point, the cells were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS) and 1 mL of DPBS containing 5 ng/mL of DAPI (4, 6-diamidino-2-phenylindole) was added to all wells and incubated for 15 minutes in order to stain the cell nuclei. The coverslips were then taken out from the wells and a drop of VectaShield® mounting medium (Vector Laboratories, USA) was added to all. Clean slides were placed on these coverslips making sure no air bubble was trapped in between the coverslips and the slides. The borders of the coverslips were sealed and then the imaging was performed under CSLM (Confocal Laser Scanning Microscopy) (Carl Zeiss 510 LSM meta, Germany) using excitation and Emission filter for Rhodamine B. (Ex: 540 nm, Em: 625 nm)

3.2.5.3 Cell uptake study by Flow cytometry

In a 12 well plate, 1 mL of the cell suspension was seeded at a cell density of 35000 cells/well. The plate was incubated at 37 °C and 5% CO₂ for 24 h. Further, the cells were exposed to 1 mL of Rhodamine B loaded nanoparticles and plain Rhodamine B solution for different periods-30 min, 1 h, 2 h, 4 h, 8 h, 16 h and 24 h. A negative control containing only complete media was also maintained to standardize the evaluation. After each time point, the cells were washed twice with DPBS and the cells were collected by trypsinization. Centrifugation (7 min at 1200 rpm) was

performed to get cell pellet, which was resuspended in fresh media and analyzed by flow cytometry.

3.3. Stent coating of sirolimus loaded nanoparticles

Coating of sirolimus nanoparticles (NP) was done on Flexinnium®, which is a stent having ultrathin strut (60 µm) design with a cobalt-chromium platform (12 mm length). In-house-developed coating equipment containing an ultrasonic spraying gun was used for the coating of nanoparticles on the stent surface. The ultrasonic spray coating technique is currently perceived as the best method for achieving the desired results. Sirolimus was coated at a standard concentration of 1 µg/mm² of the stent.

3.4. Characterization of nanoparticle coated stents

3.4.1. Residual solvent content

In order to determine the residual solvent (acetone) in the prepared nanoparticles, headspace gas chromatography was performed. Briefly, five nanoparticle coated stents were sealed in a glass vial containing Dimethylacetamide (DMA) (1 mL). The vial was sonicated for 10 min to dissolve the nanoparticles completely and the vial was then subjected to analysis to detect the presence of acetone. The sample was held at 50 °C for 10 min and then the temperature was increased at 20 °C/min up to 150 °C.

3.4.2. Surface morphology of the nanoparticle coated stent

The nanoparticle-coated stent was mounted on a black electrical carbon conductive tape and sputter-coated with Platinum for about 80 seconds. The resulting sample was analyzed by Scanning Electron Microscopy.

3.4.3. Drug solubility and stability

In preparation for the drug release studies, the sirolimus solubility and stability were analyzed in different media at 37 °C. For this purpose, 5 µg/mL sirolimus was dissolved in phosphate buffer pH 7.4 containing 0.05 and 0.5% polyoxyethylene alkyl ether non-ionic surfactant (POE surfactant) or phosphate buffer pH 4 containing POE surfactant. The resulting solutions were continuously stirred at 37 °C in glass cells of Franz diffusion cell apparatus. The drug content in these solutions was measured over a time period of up to 24 h by means of HPLC. All samples were run in triplicate.

3.4.4. Accelerated *in vitro* release study

The time course of sirolimus release from sirolimus-loaded PLGA nanoparticle coated stents into the release medium was determined at 37 °C. Two nanoparticle coated stents were placed in 2 mL buffer media (PBS pH 4 containing 0.5% POE surfactant). The elution medium was continuously shaken by a magnetic stirrer and renewed at each sampling time point in order to avoid saturation and sirolimus degradation in the medium. The medium exchange was conducted by centrifugation (30 min at 70,000 rpm) and by the suction of the supernatant. The resulting pellet was redispersed by means of sonication in a fresh elution medium. The supernatant was analyzed by HPLC. The study was performed in triplicate for each time interval, i.e., 2 h, 4 h, 6 h, 8 h, 12 h, 24 h and 36 h.

3.4.5. *Ex vivo* permeation

The nanoparticle coated stents were crimped on the balloon catheter and sterilized by ethylene oxide before using in the study. The schematic diagram of the *ex vivo* permeation model is presented in Figure 1. The required size of the goat aortic tissue was mounted inside a piece of the peristaltic pump tubing and secured by thread knots. The stent was expanded inside the prepared

arterial lumen with the help of an inflation device for 30 sec at a nominal pressure of 7 bar. This simulated artery containing stent was mounted securely between the peristaltic pump tubing, as shown in Figure 1. Care was taken to prevent the leakage of the media from the tubing during the circulation. The simulated biological fluid containing 1% sodium azide was used as a circulating media. The media was kept in a water bath to maintain the temperature of 37 °C. The flow rate of the media was kept at 35 mL/min, which is equivalent to the average blood flow in coronaries (Di Mario et al., 1993, Bandomir et al., 2015). The experiment was performed in triplicate (n=3) for each time point, i.e., day 1, day 2 and day 3.

Figure 1. Schematic diagram of *ex vivo* permeation model

4. Results and Discussion

4.1 Characterization of sirolimus loaded nanoparticles

4.1.1 Particle size and Zeta potential

The average particle size of the sirolimus-loaded polymeric nanoparticles was 100.5 ± 5.2 nm with a polydispersity index of 0.072 ± 0.002 . The nanoparticles with such lower size between 1-100 nm have unique properties to permeate through biological membranes, cells, and tissues (Khan et al., 2019). The zeta potential is an index of the stability of nanoparticles. The higher the magnitude, the higher the stability and monodispersity expected (Bala et al., 2005). The zeta potential of sirolimus loaded nanoparticle formulation was found to be -13.9 ± 3.79 mV. The negative surface charge is attributed to the presence of terminal carboxylic groups of the polymer as reported previously (Stolnik et al., 1995). The slightly negative charge might facilitate the penetration of the nanoparticles through the cell membrane (Wilhelm et al., 2003).

4.1.2 Encapsulation efficiency and Drug loading

Encapsulation of sirolimus in prepared nanoparticles was found to be $82 \pm 3\%$. The high entrapment assured drug stability and sustained release. The sirolimus loading was found to be 0.1% w/v of the formulation. The high drug loading ensured the use of less spray volume needed to achieve desired drug loading on the stent during the stent coating process.

4.1.3 Surface morphology of nanoparticles

The size and shape of nanoparticles were determined by means of SEM. SEM images revealed that the nanoparticles are a spherical in shape with a narrow particle size distribution, ranging from

about 70 to 150 nm, as illustrated in Figure 2. The result obtained was consistent with the particle size obtained by DLS.

Figure 2: SEM image of sirolimus nanoparticles

4.1.4 In-vitro degradation of nanoparticles

The pH of the nanoparticle dispersion media decreases gradually with the increase in time, suggesting the accumulation of acidic polymer degradation products viz. lactic acid and glycolic acid as illustrated in Figure 3.

Figure 3: The pH change profile of the nanoparticle dispersion media

The molecular weight of the polymer decreased with time, as shown Figure 4. The polymer was not detected by GPC after 9 weeks, indicating complete polymer degradation. The results are indicative of the time duration of polymeric nanoparticle degradation at physiological conditions (pH 7.4, 37 °C). The polymer chains were degraded in the buffered solution by biphasic kinetics. Within the first 7 weeks, approximately 56% of the polymer molecular weight was reduced. This initial slow degradation process is followed by acceleration, resulting in a molecular weight reduction of 84 % after 8 weeks. The acceleration might be caused by the reduction in the size of nanoparticles facilitating bulk erosion at a higher incubation period. Also, the increased comparative availability and the interaction of the media with the nanoparticles may have resulted in a higher degradation rate at higher incubation intervals.

Figure 4: Molecular weight vs. time profile of sirolimus nanoparticles

4.1.5. Cell culture study

4.1.5.1 Cell Viability Assay

The nanoparticle formulation (containing free drug and nanoparticles) exhibited the lowest cell viability, as shown in Figure 5. This can be due to the combined effect of free drug availability and the sustained release of the drug from nanoparticles.

The 50% cytotoxic concentration (CC50) value of the sirolimus was calculated after plotting the log of drug concentration vs. normalized response using Graphpad Prism 6 software. The CC50 values with a 95% confidence interval are presented in Table 1. It was found that the nanoparticle formulation had the lowest CC50 value, hence the highest antiproliferative activity. Statistical analysis was performed between group 2 and other studied groups to find out the significant difference at concentrations close to CC50 (20 μ M and 10 μ M). The results are presented in Table 2. It was found that the sirolimus loaded nanoparticles had significantly higher activity compared to drug solution and blank nanoparticles.

Figure 5: Cell viability study of sirolimus nanoparticles

Table 1: CC₅₀ values of different groups containing sirolimus studied in the cell viability assay

Group	Formulation	CC ₅₀ Value	95% Confidence Interval
1	Drug nanoparticles	22.7 µM	20.16 to 25.56 µM
2	Nanoparticle Formulation (Drug nanoparticles + Free Drug)	16.07 µM	13.93 to 18.53 µM
3	Drug solution (Free Sirolimus)	20.28 µM	18.15 to 22.65 µM

Table 2: Statistical analysis (2 way ANOVA, Tukey's multiple comparisons test) between the nanoparticle formulation and other groups studied in the cell viability assay

Groups	Drug Concentration (μM)	Adjusted P-Value	Significant	Summary
Group 2 vs Group 1	20	0.0639	No	Not Significant
	10	0.0216	Yes	*
Group 2 vs. Group 3	20	0.0292	Yes	*
	10	0.0125	Yes	*
Group 2 vs. Group 4	20	< 0.0001	Yes	****
	10	0.0002	Yes	***

4.1.5.2 Cell uptake study by Flow cytometry

In the quantitative analysis of the nanoparticle uptake by the cells using flow cytometry, it was found that there was an appreciably quick uptake of the sirolimus nanoparticles. A scatter plot and histogram of unlabeled cells are shown in Figure 6 a-b.

Figure 6 a-b: Flow cytometry analysis for control or unlabeled cells: (a) A scatter plot of unlabeled cells, showing a gate, P2, used to define the events subsequently analyzed for fluorescence. SSC and FSC are side scattered light and forward scattered light,

respectively. (b) A histogram was created to determine the autofluorescence of unlabeled cells. The gate 'G' is defined as the intrinsic PE fluorescence of unlabeled cells.

In the scatter plot of cells incubated with the Rhodamine-B labeled nanoparticles, the position of the gate P2 was unchanged from the control analysis. A histogram of the cells that have uptaken Rhodamine B labeled nanoparticles that fell within the gate P2 and were analyzed for PE fluorescence. Events that fell within the gate 'G' were considered negative for cell uptake. The position of the gate, G, remained unchanged from the control analysis. The % uptake of the RHB labeled nanoparticles is also indicated in the plot.

Nanoparticle formulation was taken up within 1 h, where 32.8% and 21.4% uptakes were monitored in the CC50 and half CC50 concentration group (Figure 7 a- b). For both the groups, i.e., CC50 and half CC50 concentrations of sirolimus, the level of fluorescence was found to increase for the time points monitored up to 8 h, as shown in Figure 8 a-b , Figure 9 a-b and Figure 10 a-b. The uptake of the nanoparticles in a very short time span gave the assurance that the HCASMCs favor the endothelialization of nanoparticles in a time-dependent manner. Cell death was observed beyond 8 h, which indicates that the effect of the nanoparticles can be observed in a short period of time as the sirolimus is released and exerts its action. A time-dependent increase in uptake of nanoparticles for an extended period within the cell assured its continuous effect for a longer period.

Figure 7 a-b: Flow cytometry analysis for cells after 1 h incubation with RhB labeled nanoparticles (a) At CC50 concentration (b) At half CC50 concentration

Figure 8 a-b: Flow cytometry analysis for cells after 2 h incubation with RhB labeled nanoparticles (a) At CC50 concentration (b) At half CC50 concentration

Figure 9 a-b: Flow cytometry analysis for cells after 4 h incubation with RhB labeled nanoparticles (a) At CC50 concentration (b) At half CC50 concentration

Figure 10 a-b: Flow cytometry analysis for cells after 8 h incubation with RhB labeled nanoparticles(a) At CC50 concentration (b) At half CC50 concentration

4.1.5.3 Cell uptake study by Confocal Laser Scanning Microscopy

In the qualitative analysis of the nanoparticles uptake by the cells using confocal microscopy, the uptake was clearly seen in 1 h incubation itself from the images for both CC50 and half CC50 concentrations of sirolimus, as shown in

Figure Figure 11(a) and Figure 12(a). The uptake is centered mainly in the cytoplasmic region of the cell for 1 h and the nanoparticles were seen to concentrate in the nucleus too at time points

beyond 1 h. The location was determined using a counter dye DAPI which stains the nuclear region of the cells, and the Rhodamine B-contained nanoparticle formulation was found to have traversed to the nuclear region of the cells in time points beyond 1 h. The imaging of the cells at further time points (2 h, 4 h, and 8 h) confirmed the time-dependent increase in uptake of the formulation for both CC50 and half CC50 concentrations of sirolimus as shown in Figure 11 b-d and Figure 12

b-d

(a) 1 h

(b) 2 h

(c) 4 h

(d) 8 h

Figure 11 a-d: Confocal images of HCASMC cell lines after uptake of nanoparticles at different time points at CC50 concentration of sirolimus (A) DAPI (B) Rhodamine B (C) Bright Field (D) Merge

Figure 12 a-d: Confocal images of HCASMC cell lines after uptake of nanoparticles at different time points at half CC50 concentration of sirolimus (A) DAPI (B) Rhodamine B (C) Bright Field (D) Merge

The cell culture study demonstrated that the nanoparticle formulation had the lowest cell viability and hence the highest efficiency to inhibit smooth muscle cell proliferation. The quantitative uptake study showed the time-dependent uptake of the nanoparticles within the HCASMCs. The qualitative uptake study revealed that the nanoparticles are uptaken into the cytoplasm as well as in the nucleus of the cells. The study also showed a time-dependent increase in uptake, supporting the quantitative analysis by flow cytometry.

4.2. Stent coating of sirolimus loaded nanoparticles

Sirolimus loaded nanoparticle formulation was coated successfully using ultrasonic spray gun at optimized coating conditions. The formulation adhered well on the stent surface without the need for a drying step after coating. This could be attributed to requirement of low spray volume of nanoparticle formulation having high drug loading.

4.3. Characterization of nanoparticle coated stent

4.3.1. Residual solvent content

Acetone was not detected in the GC-MS analysis as depicted in the GC-MS chromatogram (Figure 13 a-b). The mass spectrum revealed the peak of $[\text{CO}_2]^+$ molecular ion at m/z of 43.96. No peak corresponding to acetone was detected in either GC or Mass spectra. Since the boiling point of DMA is 165 °C, and the temperature was raised up to 150 °C, no peak of DMA was detected. This confirms that the final product of nanoparticles coated and sterilized stent does not contain any residual solvent.

Figure 13 a-b: (a) GC spectrum and (b) MS spectrum for residual solvent determination

4.3.2. Surface morphology of nanoparticle coated stents

Nanoparticles coated stent demonstrated smooth surface without any coating defects by SEM, as shown in Figure 14 (a). After coating on the stent surface, nanoparticles exhibited a spherical shape with a narrow particle size distribution, ranging from about 70 to 120 nm, as illustrated in Figure 14 (b). Also, sirolimus-loaded nanoparticles did not show any aggregation on the stent surface. The results confirmed that the coating process and ethylene oxide sterilization has no adverse effect on particle size and morphology.

Figure 14 a-b: Surface morphology by SEM (a) Nanoparticle coated stent strut (b)

Nanoparticles observed on the stent surface

4.3.3. Drug solubility and stability

Sirolimus was found to be highly unstable in the PBS pH 7.4 due to hydrolytic degradation of the lactone moiety. Also, the drug has very poor solubility of $6 \pm 2 \mu\text{g/mL}$ in PBS pH 7.4 with 0.05% POE surfactant. Sirolimus has good solubility $85 \pm 3 \mu\text{g/mL}$ but poor stability (Figure 15) in PBS pH 7.4 containing 0.5% POE surfactant. Sirolimus was degraded more than 50% after 12 h in this media. Our findings are consistent with previously reported data (Simamora et al., 2001)(Wang et al., 2006). Sirolimus exhibited good stability (>90%) on day 4 (Figure 16) in PBS pH 4 containing 0.5% POE surfactant. Sirolimus has minimum dissociation at this pH range below 6.0. Therefore, buffering the media at pH 4 gave required stability for constructing the release profile (Raval et al., 2012).

Figure 15: Sirolimus stability in PBS pH 7.4

Figure 16: Sirolimus stability in PBS pH 4

4.3.4. Accelerated *in vitro* release study

The *in vitro* drug release profile of nanoparticle coated stents showed sustained drug release for up to 36 h. The *in-vitro* drug release of nanoparticle coated stents showed a biphasic release profile

consisting of an initial burst release phase followed by a slower release phase (Figure 17). Initial burst release for about 2 h could occur due to the free, non-encapsulated drug and surface adsorbed drug on the nanoparticles. This initial burst release is clinically beneficial and desirable in order to achieve high drug concentrations in the targeted tissue immediately after stent angioplasty. The continuous slower drug release for the period of 36 h is controlled by the slow erosion of polymeric nanoparticles and diffusion of the drug from the core of the nanoparticles.

Figure 17: Accelerated *in-vitro* release profile of sirolimus from nanoparticle coated stents

4.3.5. *Ex vivo* permeation

The results demonstrated that the nanoparticles permeated the arterial wall in a sustained fashion (Figure 18 a). The tissue drug retention was observed throughout the length of the study period. This can be attributed to nanoparticle encapsulation of the drug and slow erosion inside the tissue to release to drug. The nanoparticle coated stents also exhibited the initial burst drug release in the arterial tissue that is desirable for the instant therapeutic action to the implantation triggered smooth muscle cell proliferation (Figure 18 a). As illustrated in Figure 18 b, the % drug loss in

the release media was less than 20% which reflects loss of drug in blood in *in vivo* conditions. At the end of day 3, more than 65% of the drug was retained on the stent which ensures that the nanoparticle coating stays intact in the presence of flowing media and the drug remains stable inside the core of the nanoparticle. The *ex vivo* study was performed for three days due to constraints in tissue viability. The *ex vivo* study gave important insights into the tissue uptake of nanoparticles from the coated stents.

Figure 18 a-b: Results of *ex vivo* tissue permeation of sirolimus

5. Conclusions

Sirolimus-loaded polymeric nanoparticles were successfully developed and characterized. The developed formulation exhibited low particle size and polydispersity index, negative zeta potential, spherical surface morphology, high entrapment efficiency, and high drug loading. Further, the formulation exhibited 9 weeks of degradation time. The cell culture study demonstrated that the nanoparticle formulation had the lowest cell viability and hence the highest efficiency to inhibit smooth muscle cell proliferation. The quantitative uptake study demonstrated the time-dependent uptake of the nanoparticles within the HCASMCs. The qualitative uptake study revealed that the

nanoparticles are uptaken into the cytoplasm as well as in the nucleus of the cells. The study also showed a time-dependent increase in uptake, supporting the results of quantitative analysis.

The nanoparticles were successfully coated on the metallic stent platform with smooth surface morphology. The characterization results demonstrated that the particle size on the coated stents is not affected during the coating and sterilization process. The nanoparticle stents exhibited sustained release of the drug from the nanoparticles. The results of the *ex vivo* permeation in the excised artery model demonstrated that the nanoparticles permeated the arterial wall in a sustained fashion with tissue drug retention. Collectively, the developed sirolimus loaded nanoparticle stent exhibited desired properties and can be a promising replacement for the current polymer and sirolimus matrix coated metallic stents.

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