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# *In Vitro* Evaluation of Cenderitide-Eluting Stent I —An Antirestenosis and Proendothelization Approach

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**ABSTRACT:** Despite the success that drug-eluting stents (DESs) have achieved for minimizing in-stent restenosis (ISR), the antirestenotic agents used in DES have been implicated in delayed endothelial healing and impairment of endothelial functions. Cenderitide (CD-NP) is a novel antiproliferation chimeric peptide of semiendothelial origin; thus, this paper aims to demonstrate the selectivity aspect of this new peptide via *in vitro* evaluation on key players in ISR—smooth muscle cells (SMCs) and endothelial cells. The microbicinchoninic acid protein assay was used to investigate the CD-NP release from films and stents. Cenderitide-containing films blended with poly(ethylene glycol) and its copolymer exhibited higher release kinetics compared with neat poly(ε-caprolactone) (PCL) formulation. Cenderitide-eluting stents (CES) was produced by coating bare metallic stents with CD-NP entrapped PCL using an ultrasonic spray coater. The investigation of CD-NP on *in vitro* cells revealed that CD-NP inhibits human coronary smooth muscle cells (HCaSMCs) proliferation but exhibits no effects on human umbilical vein endothelial cells (HUVECs) proliferation. Moreover, CD-NP released up to 7 days displayed inhibitory effects on SMCs proliferation. The CES produced in this work shows that the released CD-NP inhibits HCaSMCs proliferation but did not hamper HUVECs proliferation *in vitro*, suggesting that it has potential to reduce ISR without retarding the endothelialization healing *in vivo*. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3631–3640, 2014
**Keywords:** biodegradable polymer; natriuretic peptide; CD-NP; peptide delivery; controlled release; excipients; formulation; biomaterials

## INTRODUCTION

Cardiovascular disease is the number one cause of death in the world[1]; in fact, 70% of hospitalizations are a resultant of artery occlusion.[2] To date, the use of immunosuppressive agents such as paclitaxel and sirolimus in first-generation drug-eluting stent (DES) successfully overcame stenosis and significantly reduced the incidence of in-stent restenosis (ISR) in stented segments by inhibiting the proliferation and migration of smooth muscle cells (SMCs) proliferation.[3–5] However, amid the successful suppression of neointimal proliferation, the presence of these antiproliferative drugs in the tissue may cause delay in the endothelial coverage and healing. In one recent study, paclitaxel or rapamycin were found to upregulate antifrinolytic factor in human endothelial gene expression, resulting in the prothrombotic effects in DES.[6] For the last decades, DES-related work had been largely dedicated to addressing ISR reduction and relatively less consideration had been given to the initial damage of the endothelial lining from the mechanical deployment of the DES and long-term delay in endothelial healing as a resultant of antiproliferative agents eluted from the DES. From reports, it is now evident that DES delayed healing, particularly from first-generation DESs have significantly increased the risk of late-stage stent thrombosis (LST) that is often fatal.[7–10] The damaged endothelial lining exposes the subintimal layer and this stimulates the activation of platelets where a plethora of chemotactic and mitogenic growth factors are being released. There is an urgent need to address the issue of delayed endothelial healing and yet maintain a delicate balance between inhibition of SMCs and enabling reendothelization of damaged endothelial lining.

In the last 10 years, there have been escalating research effort on the use of natriuretic peptide (NP) as therapeutic agents[11–14] in place of synthetic drugs. In particular, the c-type NP (CNP) is a potent vasodilator and anti-inflammatory agent.[15–18] In rabbits study,[19] continuous infusion of CNP post-balloon catheter injury resulted in suppressed neointima formation. However, the short half-life of CNP and lack of cardiorenal functions limits its utility in disease states requiring chronic administration, such as coronary artery disease (CAD). CD-NP, developed by the Mayo Clinic,[20] is a hybrid of native CNP from human and a C-terminus isolated from the dendroapis NP found in the venom of the green mamba. The addition of the C-terminus tail enhanced the resistance to proteolysis, resulting in increased half-life and prolonged biological actions.[21] Furthermore, the C-terminus tail equips CD-NP with cardio-protective abilities to regulate natriuresis and diuresis.[12,14,21] CD-NP originates from endothelial origin CNP; hence, we believe it is partial endothelial in nature. We postulate that its semiendothelial nature and antiproliferative properties of CD-NP makes it a potential cardio agent for the inhibition of SMCs proliferation without compromising endothelial healing.

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Currently, CD-NP is undergoing initial evaluation in humans by Mayo Clinic to address ventricular remodeling. Our previous work have also demonstrated that controlled release of CD-NP from films and patches sustained the suppression of human cardiac fibroblast proliferation for cardiac remodeling indication.[22] However, its utility as an antirestenotic compound have not yet been tested. Hence, in this work, we set out to elucidate CD-NP's selectivity in cell inhibition. We believe that if CD-NP could selectively inhibit SMC proliferation and yet not compromise endothelial healing, it could potentially replace synthetic immunosuppressant and be used for treatment in occluded vessels. In the treatment of occluded vessels, continuous supply of antiproliferative agents is crucial for effective suppression of neointima proliferation over time; hence, to maximize the potential of CD-NP for this indication, we aim to develop a cenderitide-eluting stent (CES). CES could serve as a local delivery system, providing cenderitide in a sustained and direct manner to the site of injury. Additionally, encapsulating CD-NP within the polymer matrix overcomes the issue of CD-NP clearance, which also reduces unnecessary wastage. In exploring the feasibility of developing such systems, thin films were first used as screening tools to determine the release kinetics and subsequently translated into the development of CES. Biocompatible polymers poly(ε-caprolactone) (PCL) was selected as matrix for entrapment in this work because of its biodegradability and biocompatibility.[23,24] In this paper, we also focus on elucidating the retention of bioactivity of CD-NP released from formulations. Finally, we developed CD-NP-coated stents with suitable release profiles and characteristics for further studies.

## MATERIALS AND METHODS

### Materials

CD-NP was obtained from American Peptide Company, CA, USA. PCL (Mn: 80,000 g/mol) (Sigma–Aldrich, MO, USA), PEG (Mw: 2000 g/mol) (Merck-Schuchardt, Hohenbrunn, Germany), and copolymer PCL (1.5k)–PEG (5k) (Advanced Polymer Materials Inc., Québec, Canada) were used as received. Phosphate buffer solution (PBS, pH 7.4) was obtained from OHME Scientific, Singapore.

### Film Preparation

The polymer solution was prepared by dissolving PCL pellets in DCM (0.18 g/mL). The peptide solution was prepared by dissolving CD-NP in ethanol. The two solutions were mixed and solvent cast. For formulations containing excipients, weight percentage of PEG and copolymer were added to the solution prior to casting. The cast film was left in room temperature ambience overnight and transferred to the 37°C vacuum oven for 7 days. The thermogravimetric analyzer (TA Instruments Q500; TA Instruments, Research Instruments Pte Ltd, Singapore) was used to verify residual solvent in films (<0.5, wt %). The thicknesses of fabricated films were 0.045 ± 0.005 mm (Elcometer 456, Elcometer (Asia) Pte Ltd, Singapore).

### *In Vitro* Films Release Study

Samples prepared in triplicate were immersed in PBS and replenished at predetermined time points. The amount of peptide released was detected using the microbicinchoninic acid protein assay (Pierce, Thermo Fisher Scientific Inc., MA, USA) using the UV–Vis spectrophotometer UV-2501 (Shimadzu Corp, Kyoto, Japan).

### *In Vitro* Degradation Study

The films underwent similar process described in *In Vitro* Films Release Study. At predetermined time points, samples were rinsed with deionized water and dried in 37°C vacuum oven prior to characterization. The molecular mass was measured using the gel permeability chromatograph Agilent Series 1100 (Agilent Technologies, CA, USA ), and the mass loss was calculated by taking the difference of the initial mass and the final mass of the films over the initial mass.

### Morphological Examination

Samples were gold sputtered under argon atmosphere for 20 s. Surface morphology was observed with the scanning electron microscopy (SEM) AS Jeol 6360 (JEOL Ltd, Tokyo, Japan) at 3 kV.

### *In Vitro* Cell Culture

Human coronary smooth muscle cells and human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Group, Basel, Switzerland. HCaSMCs were grown and maintained using the SmGM-2® medium and 5% of fetal bovine serum (FBS). Only cells from passages 3 to 6 were used for this study. HUVECs were maintained using EGM-2® medium (Lonza) and 2% FBS. Only cells from passage 6 were used for this study. All cells were grown in atmosphere 5% $CO_2$ at 37°C incubator.

### Cell Proliferation

The proliferation rate of HCaSMCs and HUVECs was investigated using the bromo-2′-deoxyuridine (BrdU) assay (Roche). Eight-thousand cells were seeded in 96-well plate for 24 h. Cells underwent serum deprivation for 24 h to induce quiescent. Quiescent cells were incubated with CD-NP and CD-NP was released from formulation for 4 h along with controls. Next, cells were BrdU labeled for 24 h and tested in accordance with the manufacturer's protocol. The relative proliferation rates of investigated groups were compared with positive controls of SmGM-2® or EGM-2® and negative controls were their respective mediums without the addition of FBS. Relative proliferative value of control groups were set as 1. All treatment groups were normalized to positive control group.

### Detection of 3′5′ Cyclic Guanosine Monophospate

Eight-thousand cells were seeded in 48-well plate for 24 h. Cells were incubated with CD-NP in PBS (without magnesium and calcium) containing 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.1% FBS, and 0.5 mM 1-methyl-3-isobutyl xanthine at 37°C for 30 min. Cells were tested using the 3′5′ cyclic guanosine monophosphate (cGMP) assay (GE Health) in accordance with the manufacturer's protocol.

### Coated Stent Preparation

The stents used in this study are closed cell design cobalt–chromium stents with strut dimensions 0.075 × 0.080 $mm^2$ (W × T) (Fortimedix B.V., Nuth, Netherlands). Bare metallic Co–Cr stents were cleaned by immersing in acetone, ethanol, and distilled water for ultrasonic cleaning for 20 min each and

dried in 60°C vacuum oven overnight. The Medicoat™ (Sono-Tek Corporation, NY, USA) was used to spray the coat stents used in this study. Table 1A and 1B give a summary of the film and stents formulations and CD-NP loading, respectively.

### *In Vitro* Stents Release Study

Coated stents were prepared in triplicate. The amount of peptide released was detected using the method described in section *In Vitro Films Release Study*.

### Statistical Analysis

Results are presented in mean ± SD, unless otherwise stated. For cell culture studies, each experiment was performed in triplicate in three separate assays. Statistical analysis was carried out with the Origin 8.0 and GraphPad Prism 5.0. Values were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test; $p < 0.05$ is denoted as statistically significant.

## RESULTS

### *In Vitro* Release from Films

Figure 1 shows the release profiles of CD-NP from PCL and PCL with excipient-addition films. The results show that the release of CD-NP from neat PCL had the lowest initial release of 20.4% and final release of 61.1% by 30 days. An increase in initial release of 26.4% and 66.6% from formulations with addition of 5% and 10% PEG, respectively, was observed. The formulations with addition of copolymer resulted in more pronounced initial release. In 5% and 10% copolymer-addition formulation, peptide from films was released completely within 18 days and 6 h, respectively.

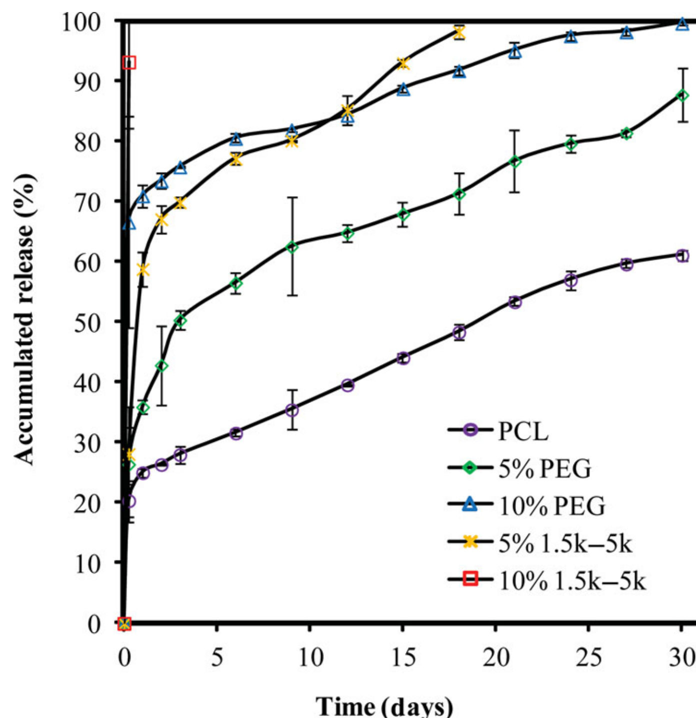


**Figure 1.** Accumulated release profiles of 1% CD-NP-loaded films according to the addition of poly(ethylene glycol) PEG and copolymer PCL (1.5k)–PEG (5k). All release sustained for 30 days except for films with copolymer addition, where early complete release by 18 days and 6 h time point was observed in 5% and 10% copolymer-addition formulations, respectively.

### *In Vitro* Degradation Study

In Figure 2a, the molecular mass study carried out over 30 days showed no significant change for both the PCL and excipient-added formulations. In Figure 2b, the percentage mass loss recorded for PCL was 2.44 ± 0.05% by 30 days. The films with addition of PEG displayed higher mass loss of 4.68 ± 0.43% and 10.10 ± 0.30% for 5% and 10% PEG addition, respectively. Similar mass loss trends were observed in films with copolymers addition, where 5% and 10% copolymer addition experienced 3.97 ± 0.49% and 7.91 ± 0.41% mass loss, respectively. It was observed that increase in mass loss corresponded to the amounts of PEG and copolymer added.

Figure 3 shows the surface morphology of films at 0- and 30-day time point. Figures 3a–3c show films with addition of PEG and copolymer appearing slightly more porous compared with the PCL formulation. After 30 days of immersion, films maintained their integrity (Figs. 3d–3f). The absence of degradation in films corresponded to the molecular mass data

**Table 1.** Summary of the film and stents formulations

A. Films

| Sample Name | Materials Composition | CD-NP Loading (%) |
|---|---|---|
| F-PCL | 100% PCL | 1 |
| F-5% PEG | 95% PCL + 5% PEG | 1 |
| F-10% PEG | 90% PCL + 10% PEG | 1 |
| F-5% 1.5k–5k | 95% PCL + 5% PCL(1.5k)–PEG(5k) copolymer | 1 |
| F-10% 1.5k–5k | 90% PCL + 10% PCL(1.5k)–PEG(5k) copolymer | 1 |

B. Stents

| Sample Name | Materials Composition | CD-NP Loading (%) | Weight of Coating (μg) | Weight of CD-NP (μg)/Stent |
|---|---|---|---|---|
| BMS | Cobalt–Chromium Stent | | | |
| S-PCL | BMS + 100% PCL | 10 | 378 ± 15 | 39 |
| S-5% PEG | BMS + 95% PCL + 5% PEG | 10 | 379 ± 24 | 42 |
| S-10% PEG | BMS + 90% PCL + 10% PEG | 10 | 361 ± 11 | 38 |
| S-5% 1.5k–5k | BMS + 95% PCL + 5% PCL(1.5k)–PEG(5k) copolymer | 10 | 355 ± 7 | 37 |
| S-10% 1.5k–5k | BMS + 90% PCL + 10% PCL(1.5k)–PEG(5k) copolymer | 10 | 375 ± 3 | 38 |

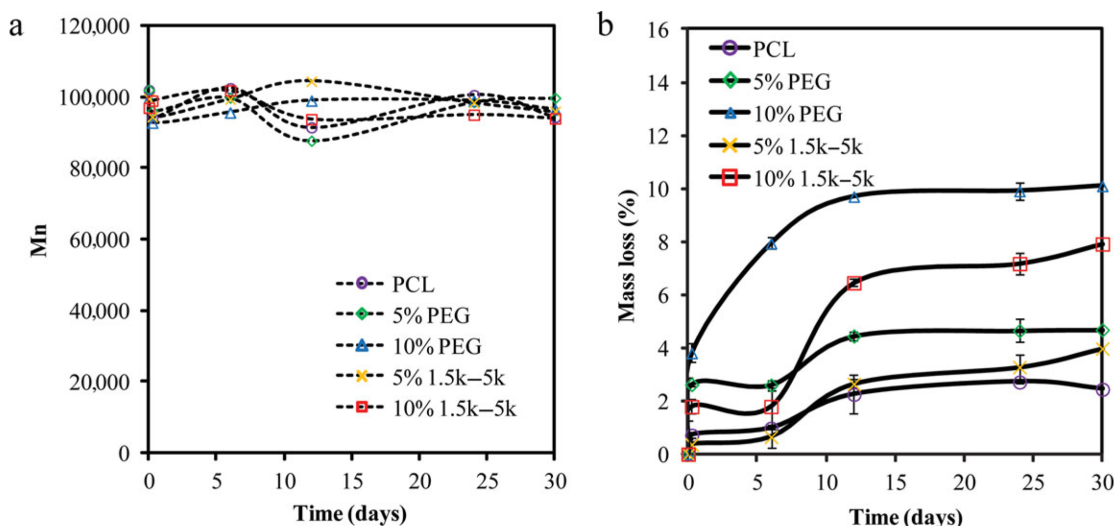


**Figure 2.** Molecular mass and mass loss of CD-NP-loaded films. (a) Molecular mass and (b) mass loss of CD-NP-loaded films according to addition of PEG and copolymer PCL (1.5k)–PEG (5k) over 30 days.

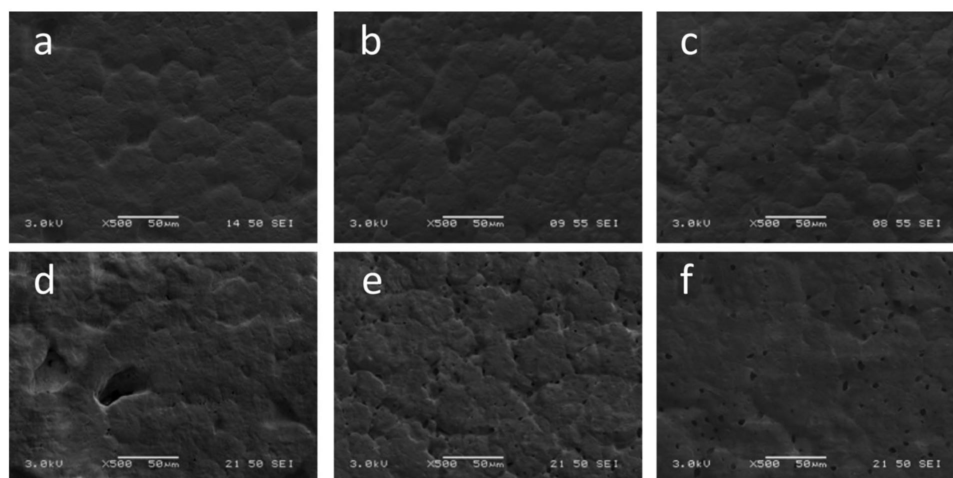


**Figure 3.** Surface morphology of films loaded with CD-NP. SEM micrograph of 1% CD-NP-loaded PCL film, 1% CD-NP 10% PEG-loaded PCL film, and 1% CD-NP 10% copolymer PCL (1.5k)–PEG (5k)-loaded PCL film on day 0 (a, b, and c) and after day 30 release (d, e, and f) in respective order.

(Fig. 2a). In addition, the PCL with PEG addition formulations were selected for the rest of the studies, because of the reasonable initial burst and sustained release profile.

### *In Vitro* Effects of CD-NP on HCaSMCs and HUVECs

As SMCs inhibition is the main factor in mitigating ISR, we carried out *in vitro* studies to evaluate the inhibitory effects of CD-NP on HCaSMCs. From literature, effective NP concentrations have been reported in molar concentrations between $10^{-9}$ M and $10^{-5}$ M, which is 0.00037 ($10^{-9}$ M)–37 μg/mL ($10^{-5}$ M). Figure 4a shows the proliferation rate of HCaSMCs upon addition of CD-NP in concentrations of 37, 0.37, and 0.0037 μg/mL compared with the control. The DNA synthesis in HCaSMC was suppressed by CD-NP, where inhibition occurred at doses between 37 and 0.0037 μg/mL but beyond 0.0000037 μg/mL inhibition was ineffective (not shown). The relative proliferation rates in CD-NP treatment groups were 0.82–0.86. Using ANOVA, there was no significant dose dependency among concentrations of 37, 0.37, and 0.0037 μg/mL. Figure 4b shows the relative proliferation rates of HUVEC after the addition of CD-NP solution in concentrations of 37, 0.37, and 0.0037 μg/mL compared with the control. The relative proliferation rates of CD-NP treatment groups fell in the range of 0.98–0.99. CD-NP exhibited no proliferative or inhibitory effects on HUVECs.

Figures 4c and 4d present the effect of CD-NP on the production of cGMP in HCaSMCs and HUVECs, respectively. For both cell types, the addition of CD-NP resulted in elevated cGMP compared with control, implying that CD-NP elicits particulate guanylyl cyclase (GC) receptor activation. The addition of CD-NP at concentrations of 37 μg/mL, the highest concentration studied, produced more than two times cGMP amounts compared with the control in HCaSMCs and approximately five times more cGMP in HUVECs. There appears to be a dose-dependent relationship between CD-NP concentration and cGMP levels ($p < 0.05$) for both HCaSMCs and HUVECs.

### Bioactivity Study of Encapsulated and Released CD-NP

The cGMP assay was carried out to verify whether encapsulated CD-NP retained the ability to elevate cGMP alike

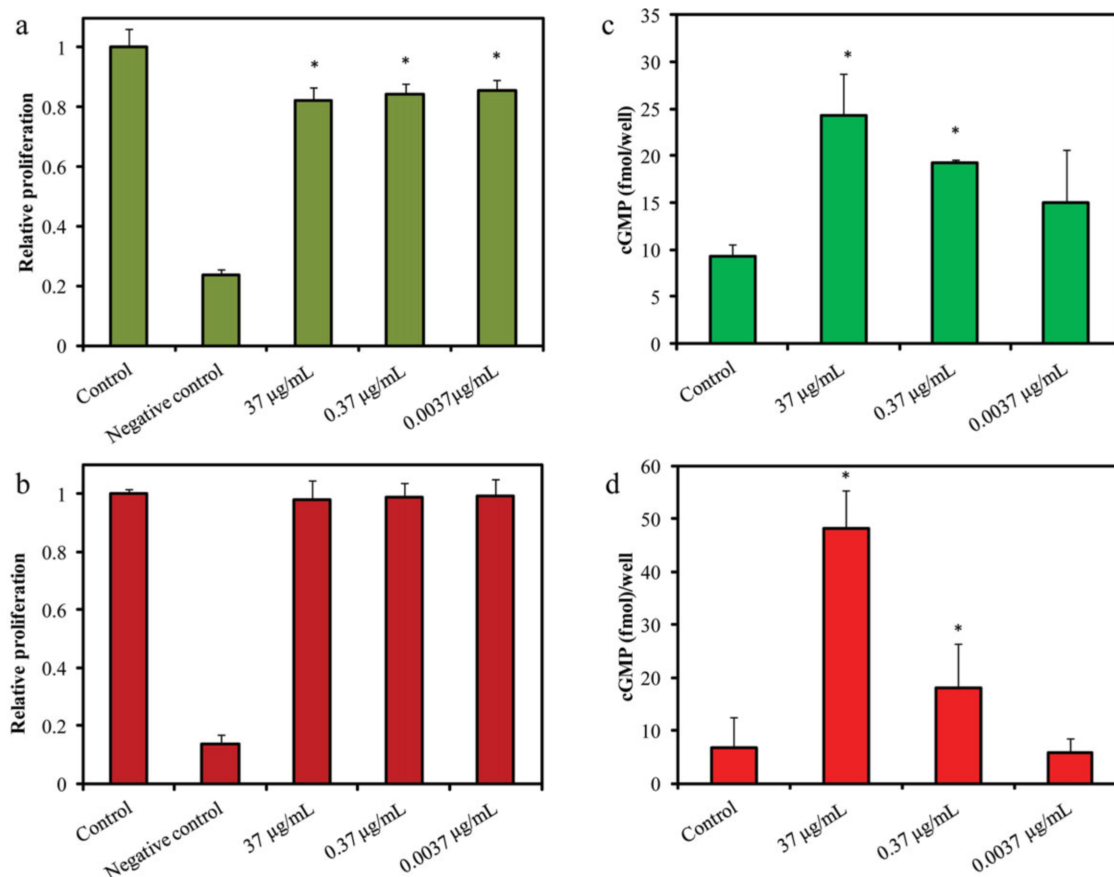


**Figure 4.** CD-NP's effect on HCaSMCs and HUVECs. Effects of addition of CD-NP on the relative proliferation in (a) HCaSMCs and (b) HUVECs measured via colorimetric BrdU uptake normalized to control and (cGMP generation in (c) HCaSMCs and (d) HUVECs induced by different CD-NP concentration; $^*p < 0.05$.

nonencapsulated CD-NP. Figure 5a shows the cGMP levels produced by the CD-NP released from the different polymer formulations compared with the control group. Both the PCL formulation and PEG addition formulations showed statistically significant production of cGMP compared with the control. Although the degree of bioactivity could not be quantified, the elevated production of cGMP implied that the released peptide retained bioactivity.

To investigate the effects of released CD-NP on HCaSMCs, CD-NP released at 1st, 3rd, and 7th day time points were tested. The relative proliferation of HCaSMCs is shown in Figure 5b. The encapsulated peptide (1st, 3rd, and 7th day)

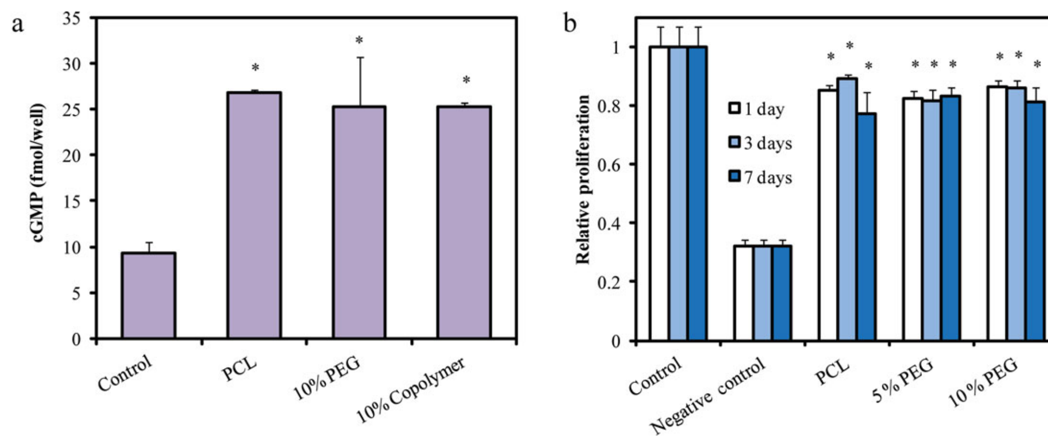


**Figure 5.** Effects of released CD-NP on HCaSMCs. (a) cGMP generation in HCaSMCs induced by CD-NP released from different CD-NP-loaded formulation, $^*p < 0.05$ compared with control and (b) relative proliferation of CD-NP released from different CD-NP-loaded formulation (released in 1 day, 3 days, and 7days) in HCaSMCs via BrdU; $^*p < 0.05$.

suppressed DNA synthesis compared with the control in a statistically significant manner ($p < 0.05$), indicating that the encapsulated peptide retained inhibitory capability. The inhibitory effect between different formulations was compared and there was no statistical significance between the groups, suggesting that PEG addition in this work neither enhanced nor decreased the inhibitory effects. Also, the inhibitory effect of peptide released at different time points was compared, and the absences of statistical significance suggest that CD-NP inhibitory effect did not deteriorate over time.

### Characterization of Stents

Figures 6a–6c show the SEM micrograph of coated stents from PCL and formulations with the addition of 5% and 10% PEG, respectively. Overall, all formulations had uniform and smooth surface coatings. There were no visible cracks between struts or signs of peptide aggregation. The addition of 10% PEG results in slightly dimpled morphology. Figure 6d shows the SEM of the stent before angioplasty balloon expansion, and Figure 6e shows the SEM of stent after angioplasty balloon expansion. Expanding the stents did not result in observable cracks or coating delamination, signifying that the stents produced were able to withstand compressive and tensile strains from stents expansion. To measure the coating thickness, the coating surface was intentionally scratched with a sharp blade. Figure 6f shows the thickness ($15.69 \pm 0.72$ μm) of the coating measured under SEM.

### *In Vitro* Release from Stents

Figure 7a plots the cumulative peptide release from stents formulation with and without the addition of PEG. The PCL formulations observed a 3% initial release (24 h), whereas the addition of 5% and 10% PEG increased the initial release of CD-NP to 18% and 26%, respectively. This indicates that peptide release is associated to the amount of excipient in the PCL. Figure 7b shows the amount of peptide released. The PCL formulation displayed consistent release throughout the 30 days, releasing approximately 0.33 μg daily. PEG formulations displayed higher amount release, particularly on the 1st day. Following the burst release (24 h), 5% PEG and 10% PEG formulations released 0.40 and 0.50 μg daily.

To compare the amount of CD-NP released from the stents and films, the cumulative peptide released from PCL and PCL with PEG-addition films were replotted in Figure 7c, and the daily release was plotted in Figure 7d. The cumulative trend of CD-NP released from the stents is similar to that in the films. In Figure 7d, the PCL formulation released $1.86 \pm 2.04$ μg over 30 days, whereas 5% PEG and 10% PEG formulations observed $2.43 \pm 0.97$ and $2.24 \pm 2.15$ μg between day 2 and day 30 post its burst release.

## DISCUSSION

For patients with CAD, performing a percutaneous coronary intervention procedure accompanied with a DES serves as mechanical means to patent the vessel, whereas its ability to elute antiproliferative drugs concomitantly restrains the neointima proliferation. However, the deployment of DES would inevitably injure the endothelial layer and the presence of immunosuppressive drugs eluted into the tissue halts the gratifying outcomes of DES because of the nonselectivity of the drug for SMCs and endothelial cells (ECs), resulting in incomplete or delayed endothelialization of the vessel.[3,4,9] The lack of adequate healing gives rise to LST and ISR post angioplasty, which prolongs the use of dual antiplatelet therapy in stented patients and in some cases exacerbating to fatal LST.

The current study sought to develop a novel peptide eluting stent incorporating designer peptide, CD-NP, which we postulate would inhibit neointima hyperplasia without compromising the endothelization of the vessel because of its endothelial origin. The study was conducted in three successive stages. In the first stage, we investigated the encapsulation and release of CD-NP from films. In the second stage, we verified our postulation of CD-NP's cell discriminatory property. The outcome of the *in vitro* cell study demonstrated that CD-NP inhibited HCaSMCs but did not affect the proliferation of HUVECs. In the final stage, we verified the bioactivity of CD-NP encapsulated in films and proceed to develop CES.

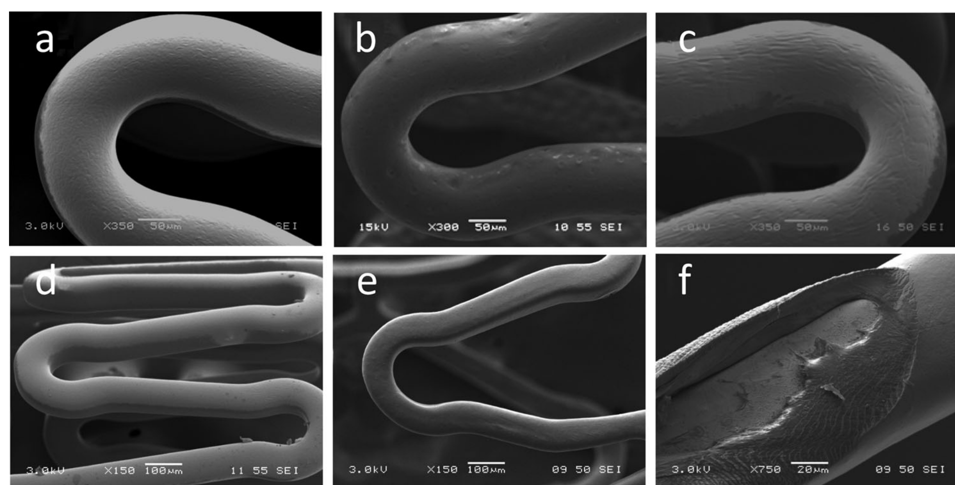


**Figure 6.** Surface morphology of CD-NP-loaded coated stents. SEM micrograph of 10% CD-NP-loaded stent coating of (a) PCL formulation, (b) 10% addition of PEG, (c) 10% addition of copolymer PCL (1.5k)–PEG (5k); intact coating of CD-NP-loaded stent (d) before and (e) after expansion; and (f) sectioned away stents coating.

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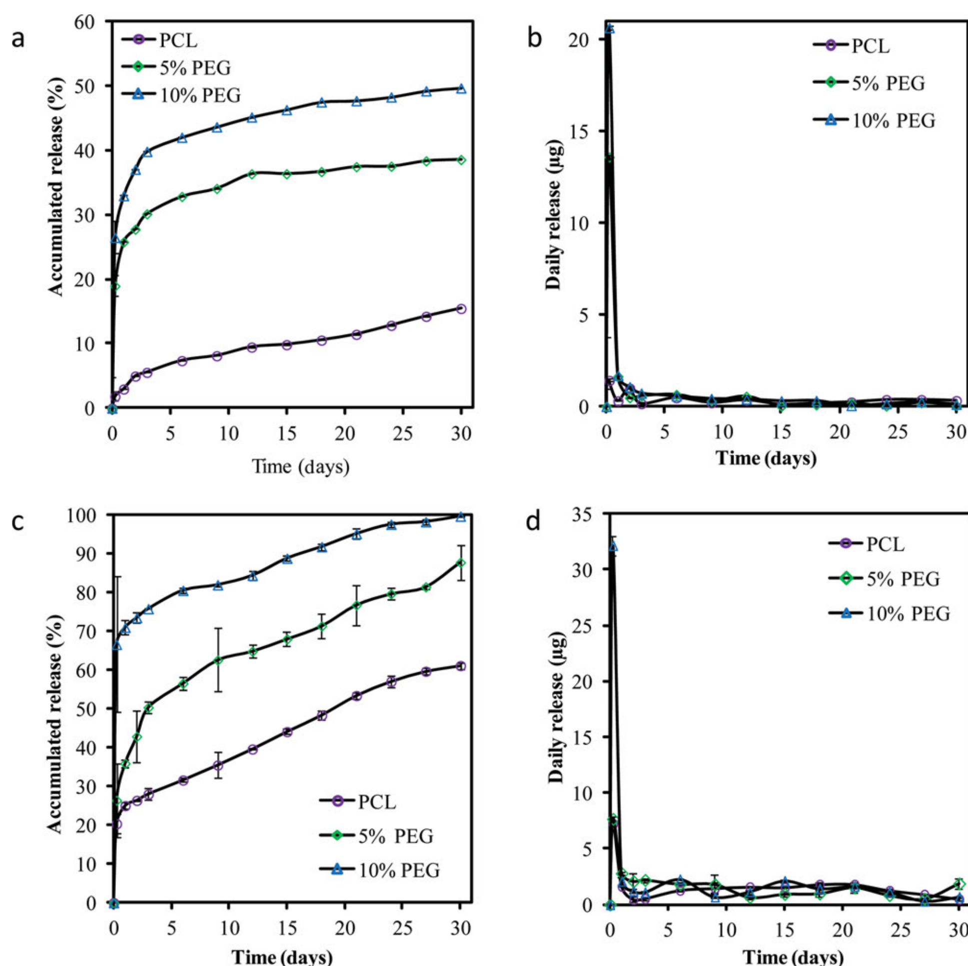


**Figure 7.** The comparison of CD-NP accumulated and daily release from between the stents (a and b) and films (c and d).

The therapeutic dose is critical in determining an efficacious or adverse outcome; hence, it is important to understand the peptide release kinetics and mechanism. In CD-NP-loaded neat PCL films, a two phase release consisting of an initial burst release followed by subsequent release was observed till 30 days. The initial burst release is likely because of the dissolution of peptide near the surface of the films, whereas the subsequent release could be attributed to the diffusion of the peptide from the polymer. Our molecular mass study showed an absence of polymer degradation, which is expected as PCL takes approximately 2–4 years for complete degradation.[25,26] PCL displayed slow release kinetics because of the present of high-crystalline domains that inhibits the water penetration into the segment[25]; hence, the approach of adding excipients was adopted. Our study showed that the addition of hydrophilic excipient PEG resulted in a corresponding increase in the initial and final release. This observation could be attributed to the physical changes in the matrix upon the addition of these excipients.[27–31] The SEM micrograph of films with PEG addition had observable pores on the surface of films, suggesting that the formation of microcavities provided alternative pathways for the peptides to exit. Furthermore, the faster peptide release is likely associated to the peptides leaching out along with the excipients. This postulation was further supported by the correlation between the mass loss and peptide release results. Three-dimensional visual evidence of colocalization of protein and hydrophilic excipients within films was also reported.[29] In this work, we also examined an amphiphilic excipient copolymer PCL–PEG. However, the copolymers addition formulations fell short in achieving the desired sustained release. In 5% and 10% copolymer-addition formulation, peptide from films was released completely within 18 days and 6 h. The rapid CD-NP release from copolymer-addition formulation was not expected and this seemed to suggest that CD-NP in copolymer formulations was distributed near the surface of the films, possibly because of severe phase separation between the hydrophilic and hydrophobic phases in the system.

The implantation of stent induces acute local inflammation, which induces migration and proliferation of SMCs leading to ISR. In our study, CD-NP in the concentration range of 0.0037–37 µg/mL showed nondose-dependent inhibition on SMCs proliferation. The suppressive capability of NPs had been attributed to the elevation of intracellular cGMP,[19,32] which is activated by NPs binding with particulate GC receptors.[12,14] In our study, CD-NP elicited cGMP production in a dose-dependent manner in a similar manner like native NPs.[12,14] However, the lack of correlation between the stimulation of cGMP production and the inhibition of DNA synthesis suggest that CD-NP's inhibitory effects observed in SMCs might not be solely because of the activation of GC receptors.[33] Apart

from inhibiting SMCs proliferation, ensuring unperturbed endothelium regeneration is crucial to avert further progression of ISR and LST. Endothelium is an important vascular component that regulates the synthesis of growth factors, nitric oxide, and protein matrixes.[9] Our proliferation studies showed that CD-NP did not inhibit the growth of ECs, suggesting that CD-NP displayed cell-type discrimination inhibition. From the literature, native CNP has been reported to mediate the inhibition of SMCs but promotes the proliferation of HUVECs.[14,19,32,34] In SMCs, CNP mediates inhibition via NPR-C activation (cGMP production) and ERK 1/2 phosphorylation. ERK 1/2 in SMCs triggers the expression of p21waf1/cip1 and p27kip1 expression, thereby preventing cell cycle progression and proliferation. On the contrary, CNP induces cell proliferation in HUVEC via NPR-C activation and increase in ERK ½ and Akt activation. The activation results in augmenting cyclin D1 expression, which facilitates cell cycle progression. Therefore, CNP-induced cGMP regulates cell cycle protein expression and hence proliferation differently in SMCs and HUVEC. As CD-NP and CNP are similar in structure, we believe that the cell discrimination effects observed in CD-NP may be similar to the mechanisms of CNP. However, further studies need to be carried out to confirm this. Figure 8 illustrates how cell-type discrimination inhibition of CD-NP makes it superior compared with the immunosuppressive drugs typically used in DES.

Very often, manufacturing process causes proteins and peptides to undergo unintentional alteration, resulting in decline or loss of pharmacological functions and effects. Moreover, there are stringent structural requirements for the NP to be bioactive.[34] Our study shows that CD-NP released from all formulations elicited elevation of cGMP, implying that bioactivity was retained. From the SMCs inhibition studies, CD-NP released up to 7 days from formulations with and without PEG addition displayed inhibitory effects on SMCs. The addition of PEG has been reported to aid in retention of bioactivity by protecting proteins and peptides from exposure of organic solvents.[35,36] We could not verify that PEG addition had enhancing effects to bioactivity retention. But we confirmed that there is no loss of bioactivity because of the addition of PEG, which implies that the addition of PEG could be used as a means to tailor release profiles without compromising bioactivity.

Poststenting rapid proliferation of SMCs occurs until 28 days; hence, a sustained delivery of antiproliferative up to 28 days is required to effectively counter ISR.[7,9,10] From the film release study, we learnt that neat PCL and formulations with 5% and 10% additions of PEG were able to give low, medium, and high peptide release profiles up to 30 days. Thus, these three formulations were selected for stents development. The stent coatings produced by ultrasonic spray coating yielded uniform and smooth surface and the absence of delamination was verified by pre- and postexpansion studies. Overall, the stents formulations observed similar release trends as the film formulations. The addition of PEG observed an increased in peptide release for both films and stents that was expected.[37,38] We believe that the release from the PEG formulations followed the "colocalization model,"[29,39] where peptide colocalized with hydrophilic excipients release along with the efflux of the excipients. The subsequent release of the peptide is dependent on the leaching rates of the excipients and the rate of diffusion of peptide out of the films and stents. Although, a slightly lower peptide cumulative release was observed in stents formulations compared with the films formulations. The difference observed could be attributed to the difference in the peptide distribution in the films and stents, given the faster the drying process in spray-coated stents than film casting, more uniformity in peptide distribution throughout the coating is expected compared with the films. In addition, the dense surface of coated stent versus porous surface of films might have also resulted in the

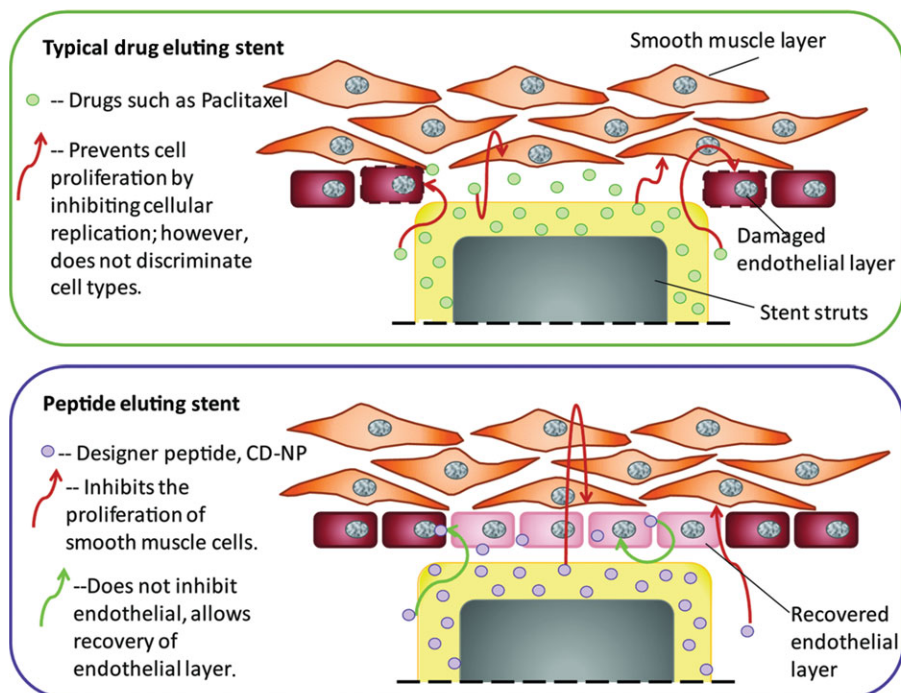


**Figure 8.** Illustration of cellular effects of immunosuppressive drugs from DES and CD-NP from CES.

difference. The daily CD-NP release from 1% loaded films and 10% loaded CES was compared and found to be similar. This implies that a comparable *in vitro* cell effect is expected from the stents and films.

Our study shows that CD-NP is effective in inhibiting SMCs proliferation without hampering ECs proliferation and the achievement of sustained controlled release up to 30 days in coated CES. However, the main limitation of this study is that only *in vitro* evaluation was carried out. For further clinical consideration, it is essential to consider its *in vivo* performance. Hence, an animal study of CES is currently underway to investigate the effect of sustained-release CD-NP on the SMCs and ECs *in vivo*.

## CONCLUSIONS

In this study, we proved that partial endothelial origin CD-NP effectively inhibited SMCs proliferation, but did not hamper HUVECs proliferation. We have also attained sustained release from films and stents and manipulate its rate of release by blending with PEG and copolymer. CD-NP has never been tested for ISR indication; hence, this study is essential before proceeding to *in vivo* evaluation. It is clear that CES has the potential to replace currently used DESs.

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