Direct functionalization of metals by low-pressure plasma improves the clinical performances of coronary stents: From surface modification to in vitro and in vivo validation

S. Diaz-Rodriguez[1], P. Chevallier[1], L. Houssiau[2], G. Caligiuri[3] and D. Mantovani[1]

[1] *Laboratory for Biomaterials and Bioengineering, CRC-I, Dept of Min-Met-Materials Eng., & University Hospital Research Center, Regenerative Medicine, Laval University, Quebec City, G1V 0A6, Canada*
[2] *Laboratoire Interdisciplinaire de Spectroscopie Electronique, Namur Institute of Structured Matter, University of Namur, 61 Rue de Bruxelles, 5000 Namur, Belgium*
[3] *Institut National de la Santé et de la Recherche Médicale U1148, Department of Cardiology, Bichat Hospital, Paris, France*

Abstract: Despite the advances on cardiovascular stents, the need for new strategies that can specifically target complications post-implantation exists. A plasma-based strategy that allows the direct grafting of biomolecules onto CoCr stents to create biomimetic surfaces was developed. A combination of *in vitro* and *in vivo* biological tests confirmed that these devices presented a better re-endothelialisation and lower in-stent restenosis rate than commercial stents. These initial trials demonstrated a promising panorama for PEG-Pept stents.

Keywords: Biomimetic coating, stent, direct plasma amination, preclinical evaluation, L605

1. Introduction

L605 CoCr alloy represents one of the leading alloys used in the manufacture of cardiovascular stents due to its superior mechanical properties that allow the fabrication of thinner devices, when compared to other alloys such as SS316L [1,2]. However, the biocompatibility of this material needs to be modified to assure the reduction of potential complications present due to the implantation of a foreign device in the patient. Thus, properties such as fast endothelialisation, low thrombus formation and anti-inflammatory response are desired by clinics [3]. Strategies to confer these properties involve the deposition of polymeric-based coatings that can be further functionalized with bioactive molecules. Nevertheless, coating homogeneity, resistance to deformation when implanting the device are not achieved due to a lack of coating cohesion and adhesion on bare metallic devices. This is related to the coating deposition technique that are used due to the complex geometry of the stent: dip coating, thin layer deposition, solvent evaporation and sputtering [4].

A different approach that foregoes the deposition of this polymeric intermediate layer, allowing the direct functionalization of the metallic surface with reactive amine groups has been developed [5]. These amine groups are used as anchor points to immobilize biomolecules of interest. This multi-step plasma-based strategy, as shown in Figure 1, is divided in: a) surface preparation, to modulate the properties of the oxide layer b) plasma functionalization, to create the reactive groups using a MW reactor and c) the biomolecule grafting, to improve the biological performance of the device.

This work focuses on the study of the biological performance of the L605 CoCr alloy after its modification with this multi-step plasma-based strategy. A combination of *in vitro* tests on flat samples were proposed in order to predict the *in vivo* performance of the cardiovascular devices. *In vitro* tests involved the incubation of human coronary artery endothelial cells (HCAEC) onto flat surfaces to assess their adhesion, distribution and their phenotype by quantifying the soluble factors release in the supernatant. For that purpose, three specific molecules were selected: a) Vascular cell adhesion molecule-1 (VCAM-1), a protein that mediates the adhesion and interaction of leukocytes onto the endothelium [6], b) interleukin-6 (IL-6) an inflammatory cytokine released during the first steps of endothelial inflammation [7], and c) tissue factor pathway inhibitor (TFPI), a primary inhibitor of the blood coagulation cascade, produced by a healthy endothelium [8]. Regarding *in vivo* studies, the implantation of BMS, drug eluting stents (DES) and the functionalized stent with the bioactive peptide (PEG-Pept) were implanted in porcine coronary arteries, due to their similarities to human arteries [9]. Short term studies, 7 days, were performed to evaluate the re-endothelialization. Furthermore in-stent restenosis was assessed after 28 days of implantation. Thus, by combining both *in vitro* and *in vivo* assays an insight about how these devices, functionalized by this original multi-step plasma-based approach, can perform in a human trial.

2. Materials and methods

Sample preparation: L605 CoCr round flat samples of 12.7 mm, for in vitro tests, were successively cleaned for 10 min in ultrasonic baths, with acetone, water and methanol, before air dried. Then, they were electropolished to homogenize the interfacial oxide layer as described in [5,10]. The amination of the electropolished (EP) alloy was performed in post-discharge by a MW plasma reactor (Plasmionique Inc., Varennes, Canada) using a two-step process with N_2 and H_2 (grade 4.8 and 5.0, respectively, Linde, Qc, Canada) as described elsewhere [5]. Finally, the covalent grafting of the biomolecule was performed using polyethylene glycol bis carboxylic (PEG) as linking arm. First, PEG was activated in MES buffer (pH 4.75) with EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) for 30 min, then grafted on the aminated

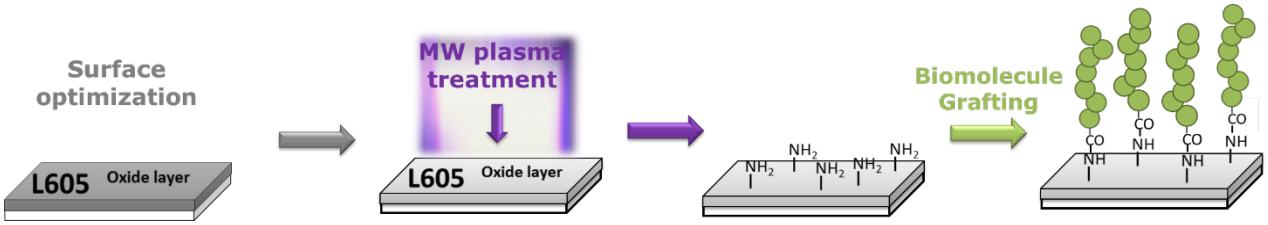


Figure 1 Multi-step procedure for the covalent grafting of biomolecules on bare metallic L605 Co-Cr alloys. This procedure is divided in: 1) surface optimization, 2) plasma functionalization and 3) biomolecule grafting.

surface for 1 hour. After several washings, the activation of the free carboxylic group of the linking arm was then performed using EDC to react with the peptide, $2.5x10^{-5}$ M in phosphate buffer saline (PBS) for 3 hours, leading to a stable covalent binding. Thereafter, the grafted samples were washed five times with water, and air dried [5].

As regards samples for *in vivo* tests, Multilink® (Abbott) commercial stents were deployed and unmounted from their catheter. Before amination step, they were cleaned by ultra-sonic bath. Samples were mounted vertically on a tungsten support. Similar to flat samples, the plasma amination was carried out in a two-step process using a mixture of N_2 and H_2 in after glow. The plasma amination of stents was performed twice on the device, flipping the device after the first treatment to create a homogeneous functionalization. After plasma amination, the grafting of PEG as linking arm and the grafting of the peptide were performed as previously detailed. After the peptide grafting, stents were cramped back onto the catheter using tweezers and sterilized by beta radiations at 25 kGγ (Ionisos, Chaumesnil, France). Surface modifications were assessed by XPS and ToF-SIMS analyses for surface chemical composition for flat and plasma treated stents, respectively.

In vitro tests: HCAECs were incubated in Endothelial cells growth medium MV2 (Promocell) supplemented with nutrients and growth factors needed by ECs. Flat samples (EP and PEG-Pept) were placed in 24-well plates and sterilized in ethanol. After sterilization, 200,000 cells suspended in their growth medium were seeded on the surface for 48 h. After incubation, supernatants were collected to study the ECs phenotype by multiplex assay analysis whilst the cells attached on the surface were stained for the nuclei and CD31 intensity count.

Immunostaining of CD31 and the nuclei was performed as follows: samples were washed with PBS, then incubated during night in a solution containing 1 % of bovine serum albumin (BSA) and 0.02 % of fish gelatin in PBS with mouse anti-human CD31 (clone JC70/A, 10 µl/mL, Dako M0823). After PBS rinsing, a second incubation for 1h with the secondary antibody for CD31 (goat anti-mouse IgG, A488, 5 µl/mL) was performed. After this, the nuclei staining was performed by the addition of Hoechst (10 µg/mL) for 5 minutes and the samples were fixed. Images were obtained under an Axio Observer inverted fluorescence microscope (Zeiss), equipped with the Zen software (Zeiss). Analysis of the intensity and nuclei counting was performed using the LEICA QWIN® software and custom macro written in QUIPS language.

Finally, the measure of pro-inflammatory and anti-coagulant endothelial cell biomarkers in the supernatants was achieved using a custom multiplexed cytometric bead assay (Luminex technology). EC culture supernatants were centrifuged, transferred to Eppendorf tubes, and diluted 1:2 in order to avoid over saturation. Analyses were performed in 96-well plate with a Bio-Plex 200 (Bio-rad inc.) [11].

In vivo tests: Three groups of stents were implanted in the coronary arteries of 8 female farm pigs: BMS unmodified, everolimus-eluting stents (Xience, Abbott) and PEG-Pept. Animals were sedated and anesthesia was maintained throughout all the chirurgical procedure. Stents were deployed on the three epicardial coronary arteries (interventricular, left circumflex and right coronary arteries) with the help of coronary angiography. The balloon was inflated to a maximal pressure of 8 atm for 30 s, deflated and withdrawn deploying the stent in the artery.

As regards the endothelialisation studies, after 7 days of implantations, the animals were euthanized, and the stented arteries were explanted and fixed in formaldehyde for 48h. Arteries were cut longitudinally in halves to expose the luminal side. Further dehydrations were performed and coated in gold in order to be suitable for SEM imaging. Strut endothelialisation and platelet adherence were evaluated by imaging mode. Further analyses were performed by staining transversal cuts of the arteries embedded in poly(methylmethacrylate) with Masson's trichrome. Finally, for in-stent restenosis, angiographies of the stented coronary arteries were taken to observe the potential reduction in the lumen.

3. Results and discussion

Surface characterization: Chemical composition changes were followed up by XPS survey analyses (Table 1). First, the results evidenced that the amination step was effective through nitrogen incorporation on the surface. Then, the decrease of the percentage of metals allowed to conclude that the peptide covalent grafting was reached.

Table 1. XPS survey analyses of samples.

Sample	O	C	N	Metals
Clean	46.0 ± 1.4	39.9 ± 2.0	2.0 ± 0.7	10.1 ± 1.4
EP	51.9 ± 1.4	42.9 ± 1.1	-	4.8 ± 0.2
-NH_2	49.9 ± 0.8	27.5 ± 1.8	11.0 ± 0.1	11.8 ± 1
PEG-Pept	45.0 ± 2.2	34.9 ± 1.7	4.5 ± 0.7	10.9 ± 1.8

As regards stent functionalization, ToF-SIMS analyses in imaging positive mode confirmed the presence of a specific fragments related to the chemical composition of the peptide ($C_2H_6N^+$, alanine). Further, as observed in Figure 2, it was distributed homogeneously on the surface, thus, confirming the peptide immobilization.

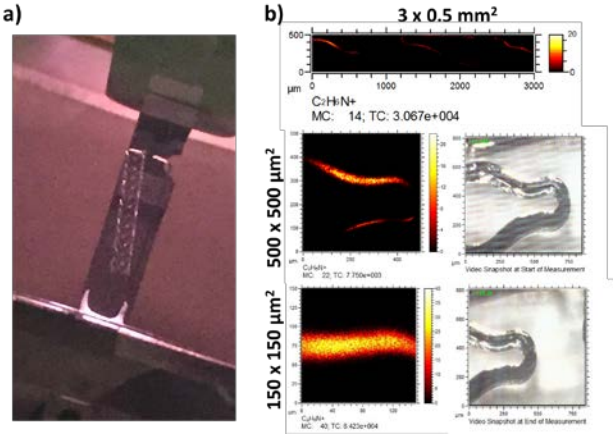
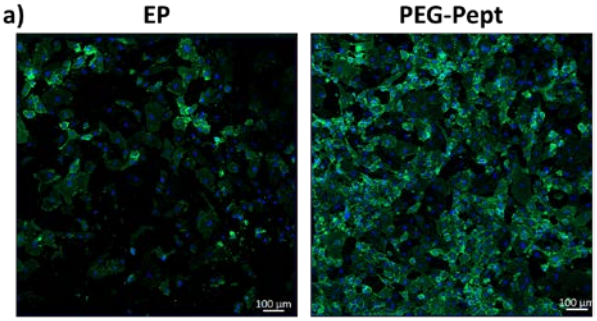


Figure 2. a) Image of stent mounted on a tungsten filament for plasma treatment in MW reactor. b) ToF-SIMS images of the peptide grafted stent assessed from a specific fragment of the peptide ($C_2H_6N^+$, alanine) in positive mode at different scales.

In vitro performance: After the composition evaluation of the surface, *in vitro* biological tests were performed to study the endothelial cell behaviours when in contact with the functionalized material. As it can be observed in Figure 3, the adhesion and distribution of the HCAECs were evaluated. It can be noticed that the direct functionalization and the biomolecule grafting significantly improves the presence of HCAECs on the L605 surface, from 133 ± 35 to 292 ± 29 cells. Furthermore, the adhesion of the peptide onto the surface also increased the intensity and the area of the marked CD31 which could be correlated to the potential formation of monolayer of endothelial cells, crucial for the wound healing after the stent implantation.



b)

Sample	Nuclei	CD31 Area (µm²)	CD31 intensity
EP	133 ± 35	6731.5 ± 3974	40108 ± 21164
PEG-Pept	292 ± 29	27873 ± 5909	179000 ± 46189

Figure 3. a) Fluorescent images after cell viability studies of HCAEC incubated for 48h onto an electropolished surface and PEG-Pept. b) Quantitative data of the staining, functionalizing the bare metallic surface with the linking arm and the peptide increases quantity (number of nuclei) and viability (intensity of CD31 staining) of cells.

Then, the EC phenotype was assessed to predict how these functionalized surfaces would behave in an *in vivo* environment. As it can be observed in Figure 4, the direct immobilization of the peptide onto the bare metallic surface decreased the presence of IL-6 and VCAM-1, molecules related to an inflammation reaction whilst increased the presence of TFPI, related to an anti-thrombotic behaviour. Thus, *in vitro* results confirmed that the covalent immobilization of the peptide increased the endothelialisation and the anti-thrombotic behaviour whilst decreased the probability to present an inflammation with this material.

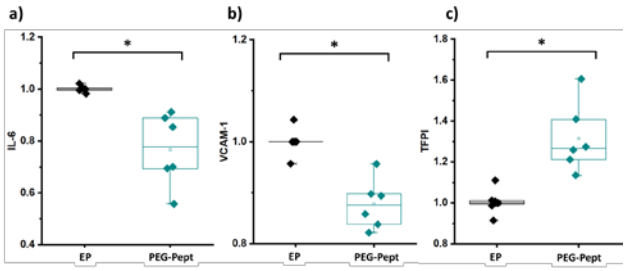


Figure 4 Quantification of the soluble factors of HCAECs after 48h of incubation with the surfaces, normalized to EP. It can be observed that both inflammation markers, VCAM-1 and IL-6, decreased with the presence of the peptide on the surface. Furthermore, the grafted surface with the proposed strategy increased its anti-thrombotic activity when compared to EP, as evidenced by TFPI.

In vivo performance: After 7 days of implantation, stented arteries were explanted from the euthanized animals to study the degree of stent strut endothelialisation, evaluated by SEM images and histology. As observed in Figure 5a, both BMS and PEG-Pept presented a complete strut covering by the endothelium, which was not the case of DES that shown a weakly coverage. This could be related to the mechanism of action of the eluted drug that avoided the proliferation of cells, thus compromising the wound healing at short term. Furthermore, as observed in Figure 5b, after studying the arterial wall, only PEG-Pept demonstrated a low adhesion of leukocytes and platelets, which further appeared in a non-activated state, which could be related to the anti-thrombotic activity of the functionalized device.

Finally, the in-stent restenosis was evaluated after 28 days of implantation. Angiographies were performed in order to observe the reduction in the lumen where the stent was implanted. As it can be observed in Figure 6, both DES and PEG-Pept did not present a significant difference among them in terms of in-stent restenosis, but a significant difference when compared to BMS, which presented a stenotic artery.

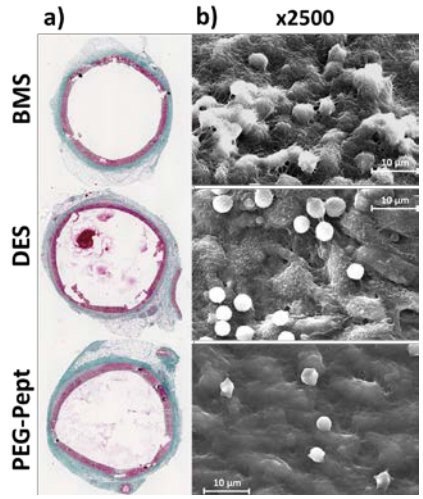


Figure 5. a) Histological analyses with, trichrome staining, of the transversal section of a porcine coronary artery after 7 days of implantation. It can be observed that BMS and PEG-Pept presented a better re-endothelialisation after 7 days. b) SEM images of extracted porcine coronary. PEG-Pept presented the less quantity of leukocytes and platelets adhered to the surface when compared to the other devices.

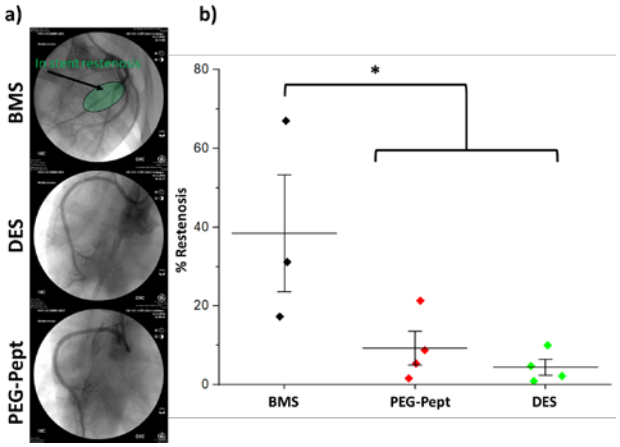


Figure 6. a) Coronary angiographies of BMS, DES and PEG-Pept obtained before the euthanasia of the animals at day 28 to study. b) Estimation of the in-stent restenosis based on angiographies. Both DES and PEG-Pept decreased the *in vivo* in-stent restenosis compared to BMS.

4. Conclusion

A multi-step plasma-based strategy allowed the direct functionalization of commercial cardiovascular devices with a bioactive peptide having anti-thrombotic, anti-inflammatory and pro-endothelialisation activity using PEG as a linking arm. The immobilization of this molecule was confirmed by XPS survey analyses on the flat surfaces and by ToF-SIMS imaging mode on functionalized stents. Furthermore, the *in vitro* biological assays demonstrated that the grafting of PEG-Pept onto the metallic surface of L605 significantly improved the biological properties of the metallic L605 surface. Indeed, the grafted surface promoted the presence of endothelial cells with an anti-thrombotic and anti-inflammatory phenotype in comparison with the crude metallic surface. These promising i*n vitro* results were used to predict functionalized stent behaviour *in vivo* in porcine model.

Regarding *in vivo* tests, it was found that stents functionalized by this original strategy presented a better re-endothelialisation after 7 days when compared to commercial DES, with a low adhesion of leukocytes and platelets when compared to BMS. Moreover, after 28 days of implantation, PEG-Pept did not present a significant decrease on the lumen diameter, which was not the case for BMS where in-stent restenosis was found after this period.

Overall, these initial trials gave a promising panorama for PEG-Pept stents. Further studies with long term trials, an increased number of specimens, must be performed in order to understand if these devices are suitable for the next step: human trials.

5. Acknowledgements

Sergio Diaz-Rodriguez was a recipient of a doctoral scholarship from the NCPRM (www.ncprm.ulaval.ca). Our thanks to Prof. Frederic Chaubet (U. Paris XIII) for scientific help and guidance. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), the CHU de Québec Research Center, The Ministry of Economy, Science and Innovation of Quebec, and the Linkage Grants Québec/Wallonie-Brussels of the Ministry of International Relations and "*La Francophonie*" of Quebec Province.

6. References

[1] J. Pache et al. J. Am. Coll. Cardiol., **41**, 8, 1283–1288 (**2003**).
[2] G. Mani, et al. Biomaterials, **28**, 1689-1710, (2007).
[3] B. Ratner, et al. Annu. Rev. Biomed. Eng., **6** (1), 41–75 (2004).
[4] John, A. A. et al. RSC Adv., **5** (49), 39232–39244 (2015).
[5] Diaz-Rodriguez, S. et al. Plasma Process. Polym., **15**, e1700214 (2018).
[6] J. Cook-Mills et al. Antioxid. Redox Signal., **15** (6), 1607–1638 (2011).
[7] T. Tanaka, et al. Cold Spring Perspect. Biol., **6** (10), a016295–a016295 (2014).
[8] J. P. Wood et al. Blood, **123** (19), 2934–2943 (2014).
[9] M. J. Byrom et al. J. Vasc. Surg., **52** (1), 176–195 (2010).
[10] S. Diaz-Rodriguez et al. RSC Adv., **9**, 2292–2301 (2019).
[11] Bioplex instruction Manual. Accessed on January 29th of 2019. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10044282.pdf