Layer-by-layer biofabrication of coronary covered stents with clickable elastin-like recombinamers

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Abstract

Coronary artery disease is the leading cause of death around the world. Endovascular stenting is the preferred treatment option to restore blood flow in the coronary arteries due to the lower perioperative morbidity when compared with more invasive treatment options. However, stent failure is still a major clinical problem, and further technological solutions are required to improve the performance of current stents. Here, we developed coronary stents covered with elastin-like recombinamers (ELRs) by exploiting a layer-by-layer technique combined with catalyst free click-chemistry. The resulting ELR-covered stents were intact after an in vitro simulated implantation procedure by balloon dilatation, which evidence the elastic performance of the membrane. Additionally, the stents were mechanically stable under high flow conditions,

which is in agreement with the covalent and stable nature of the click-chemistry crosslinking strategy exploited during the ELR-membrane manufacturing and the successful embedding of the stent. Minimal platelet adhesion was detected after blood exposure in a Chandler loop as shown by scanning electron microscopy. The seeding of human endothelial progenitor cells (EPCs) on the ELR-membranes resulted in a confluent endothelial layer. These results prove the potential of this strategy to develop an advanced generation of coronary stents, with a stable and bioactive elastin-like membrane to exclude the atherosclerotic plaque from the blood stream or to seal coronary perforations and aneurysms, while providing a non-thrombogenic luminal surface and favouring the endothelialization.

Keywords: coronary stents, layer-by-layer, elastin-like recombinamers, biobased covered stents, click-chemistry, hemocompatibility.

1. INTRODUCTION

Coronary artery disease is responsible for approx. 20% of all deaths in Europe [1]. Endovascular stenting is a minimally invasive procedure to restore blood flow in the coronary arteries, which offers lower perioperative morbidity when compared with more invasive treatment options. Despite the great progress made with coronary stenting [2], restenosis and thrombosis after stent implantation are still major clinical complications that lead to stent failure and often to the need for reintervention [3]. Coated stents (e.g. drug eluting polymer coated stents and biofunctionalized endothelial progenitor cell-capturing stents) [4] and covered stents [5] have been proposed as a solution for these complications. Coronary covered stents feature a membrane that covers the metallic struts and functions as a physical barrier that can i) exclude the underlying atherosclerotic plaque from the blood flow, potentially preventing the ingrowth of smooth muscle cells and therefore the reocclusion of the stented vessel [6] and ii) seal

coronary artery perforations [7]. The success of such an approach relies mainly on the properties of the membrane's material. Ideally, it should be hemocompatible, mechanically stable and elastic enough to allow the stent's configurational changes upon implantation [5].

Unfortunately, the synthetic materials originally used for covering small-caliber stents (Table 1) display limited hemocompatibility, and their use has resulted in restenosis, thrombotic [8, 9] and inflammatory events [10, 11].

Table 1: Materials employed for the fabrication of covered coronary stents and clinical outcomes.

Covering material	**Illustrative devices**	**Limitations/ outcomes**	**Refs**
Polytetrafluoroethylene (PTFE)	- JOSTENT® GraftMaster Stent Graft (Abbot, USA) - Symbiot ™ (Boston Scientific, USA)	-No improvement in clinical outcomes when compared to bare metal stents and in many cases, association with a higher incidence of restenosis and thrombosis	[12-16]
Polyethylene terephthalate (PET)	- MGuard Coronary Stent System (InspireMD Ltd, Israel)	- High incidence of major adverse cardiac events	[17]
Polyurethane (PU)	PK Papyrus stent (BIOTRONIK, Germany)	- No differences in major adverse cardiac events in patients treated with PU-CS or PTFE-CS after 1-year follow-up	[18]
Equine pericardium	AneugraftDx (Amnis Therapuetics Ltd, Israel)	- Potential risk of disease transmission	[19, 20]
Autologous vein	Any balloon expandable metal stent	- Time consuming fabrication - Limited availability - Patient morbidity	[21]

The need to address these issues has motivated the search for new membranes with non-thrombogenic properties. In this regard, the use of bio-based materials (i.e. materials that have either a biological origin or a bio-inspired chemical composition) has been proposed as

alternative to the synthetic ones with the rationale of eliciting a more favourable host response [22]. Equine pericardium coronary covered stents are currently available on the market (Table 1), but the xenogenic origin may represent a limitation. Autologous veins are also used in the operating room to cover bare metal coronary stents, but their use is hampered by their limited availability and the invasiveness of the harvesting procedure. Therefore, advanced materials, able to provide a non-thrombogenic surface while offering off-the-shelf availability, are required to address the unmet clinical need of coronary covered stents.

The elastin-like recombinamers (ELRs) are a family of artificial polymers bioinspired by the pentapeptide VPGVG present in the natural elastin [18, 19]. The ELRs show the advantages of an engineered material because of the exhaustive control over their composition thanks to their recombinant nature, while maintaining inherent properties of the natural elastin (i.e. elastic mechanical behaviour, hemocompatibility and bioactivity) [20]. ELRs are therefore excellent candidates for the fabrication of devices intended to be in contact with blood [23, 24]. Besides the membrane material, the fabrication strategy represents a paramount aspect to be considered, as it must be suitable for covering a device with a very small diameter (e.g. 2-4 mm).

Here, we show the fabrication of covered coronary stents by combining the efficiency, stability and selectivity of catalyst-free-click chemistry [25, 26], with a layer-by-layer dip-coating technique, and the hemocompatibility and elasticity of ELRs. We tested the resulting click-ELR-coronary covered stents for their ability to withstand deployment by balloon catheter and high shear stress flow. We also evaluated the hemocompatibility of the stents and their capability to support endothelialization.

2. MATERIALS AND METHODS

2.1. Layer-by-layer fabrication of ELR-covered coronary stents

Two ELRs modified with either cyclooctyne or azide groups (Table 2) [27] were dissolved at 100 mg/mL in PBS at 4 °C. ELR-c is a structural recombinamer while ELR-a contains the RGD adhesion sequence. For the dip coating process, ELR solutions were kept at 4 °C in separate cylindrical containers and the coronary stents (custom-made by R.T.M. Rainer Trapp Medizintechnik GmbH (Germany), L 605 Co-Cr-alloy, polished, with a length of 14.9 mm, an outer diameter of 1.8 mm and a strut thickness of 90 µm) were sequentially immersed in the ELR solutions and washed in PBS to remove the excess of polymer. The dipping procedure was repeated 5 times. The covered stents were stored in ethanol 70%.

Table 2: Amino acid sequences of each ELR and the corresponding reactive groups used for catalyst-free-click chemistry (specifically Huisgen 1,3-dipolar cycloaddition of azides and alkynes). The reactive groups were incorporated by chemical modification of the lysine residues.

ELR	Amino acid sequence	Reactive group
ELR-c	MESLLPVGVPGVG[VPGKG(VPGVG)$_5$]$_{23}$VPGKGVPGVGV PGVGVPGVGVPGV	Cyclooctyne
ELR-a	MGSSHHHHHHSSGLVPRGSHMESLLP[(VPGIG)$_2$(VPGK G)(VPGIG)$_2$]$_2$AVTG**RGD**SPASS [(VPGIG)$_2$(VPGKG)(VPGIG)$_2$]$_2$	Azide

2.2. Mechanical stability of ELR-covered coronary stents

The ELR-coronary covered stents (n=3) were exposed for 24 h to high shear stress flow in a closed-loop flow system to evaluate the robustness of the polymer covering. The system consisted of two peristaltic pumps (Ismatec, MCP Process) connected in parallel to a fluid reservoir through silicone tubes (inner diameter 6.4 mm; Ismatec). The stents were fixed in the expanded state in a tube with a diameter of 3 mm, and then subjected to the pulsatile arterial pressure of 80-120 mmHg. The resulting flow rate was set to 300 mL/min resulting in a mean shear stress of 1.5 Pa, according to the equation [28].

$$P = (4*\eta*Q)/(\pi*r^3) \qquad \text{Eq. 1}$$

where P is the shear stress (in Pa), η the dynamic viscosity (in Pa*s), r is the inner radius (in m) of the covered stent and Q the volumetric flow rate (in m^3*s^{-1}).

The completeness of the ELR-layer was evaluated by measuring the difference of the dry weight of the ELR-covered stents before and after exposure to the flow. The dry weight was determined after storing the ELR-covered stent in a 70 % ethanol solution overnight and subsequently placing it in an oven (Binder GmbH, Germany) at 60 °C for 90 min. Values are expressed as mean ± standard deviation (SD). Wilcoxon signed rank test was used for the statistical analysis.

2.3. Balloon expansion of the ELR-covered coronary stents

The stents were positioned on the balloons (TREK coronary dilation catheter, Abbot), and expanded up to a pressure of 8 atm, corresponding to a stent diameter of 3.4 mm (supplementary video S1) as advised by the manufacturer.

2.4. Thrombogenicity assay

Human blood was drawn from healthy volunteers and mixed with 3.2% sodium citrate using an S-Monovette CPDA1 device (Sarstedt, Germany) at a volumetric ratio of 1:9 to prevent coagulation. PVC tubes (CODAN pvd Medical GmbH) were filled with 1.5 mL of human blood. The blood and the ELR-covered stent were placed into a Chandler-loop system, formed by a closed PVC tube mounted on the rotating head of a roller pump (company) [29, 30]. The rotational speed of the Chandler loop resulted in a shear rate of 429 s^{-1} and a shear stress of ~ 1.5 Pa (considering a blood dynamic viscosity of 3.5 $x10^{-3}$ Pa*s), within the range of physiological values [31]. After 1 h of exposure to the blood flow, the stents were washed with PBS and cut into pieces for SEM visualization. The same procedure was done with the commercially available ePTFE grafts (GORE-TEX®) as controls. The platelet covered-area was assessed by image analysis with ImageJ software [32] by analyzing three different regions per sample, each with an area of 461 μm^2. SEM images were coloured with MountainsMap7 SEM software courtesy of Digital Surf, France.

2.5. Scanning electron microscopy

Samples for SEM investigation were fixed in 3% glutaraldehyde in 0.1 M Sorenson's buffer (pH 7.4) at room temperature for 1h. They were rinsed with sodium phosphate buffer (0.2 M, pH 7.39, Merck) and dehydrated consecutively in 30%, 50%, 70% and 90% acetone and then three times in 100% acetone for 10 minutes. After critical-point-drying in CO_2, they were sputter-coated (Leica EM SC D500) with a 20 nm gold-palladium layer. Images were obtained with an ESEM XL 30 FEG microscope (FEI, Philips, Eindhoven, the Netherlands) with accelerating voltage of 10 kV.

2.6. Cell isolation and culture

Endothelial progenitor cells (EPCs) were isolated from peripheral blood of human adult volunteers. Anticoagulated blood of healthy donors was carefully added to the separating solution Histo-Paque-1077 (Sigma-Aldrich, St Louis, Missouri), and centrifuged at 400 g at room temperature for 30 min. The layer containing mononuclear cells was gently washed twice with PBS. The cell pellet was resuspended with 15 mL of endothelial cell growth medium (Endothelial Cell Growth Medium MV2; PromoCell, Heidelberg, Germany) containing epidermal growth factor (5 ng/mL), basic fibroblast growth factor (10 ng/mL), insulin-like growth factor (20 ng/mL), vascular endothelial growth factor 165 (0.5 ng/mL), ascorbic acid (1 µg/mL) and hydrocoecisone (0.2 µg/mL), and transferred into T-75 culture flask precoated with human fibronectin (1 mg/cm^2, Sigma-Aldrich, St. Louis, MO). The cells were cultured in 5% CO_2 and 95% humidity at 37 °C. EPCs colonies were trypsinized (0.25% trypsin/0.02% ethylenediaminetetraacetic acid solution (Gibco, Karlsruhe, Germany)) and transferred to T-25 culture flask precoated with type I rat-tail collagen (5 mg/cm^2, BD Biosciences, San Jose, CA). Cells with 70-80% of confluence were trypsinized and transferred into T-75 culture flasks. Cells up to passage 9 were used for all experiments.

To study the ability to support endothelialization, the ELR membranes were incubated with a suspension of EPCs (5x10^4 cells/mL) for 6 h, after which the medium was exchanged. The scaffolds were then cultured for additional 18 h and subsequently investigated by immunohistochemistry followed by confocal microscopy.

2.7. Immunohistochemistry

ELR membranes seeded with EPCs were fixed with methanol-free formaldehyde (Roth, Karlsruhe, Germany) at 4% for 1h at room temperature. Nonspecific sites were blocked and the cells were permeabilized by incubation in 5% normal goat serum (NGS, Dako) in 0.1% Triton-PBS. The samples were incubated overnight at 4°C with a 1:100 dilution of the primary mouse anti-CD31 antibody (P8590, Sigma). After washing three times with PBS, the samples were incubated for 1 h at room temperature with a 1:400 dilution of AlexaFluor 594 goat anti-mouse antibody (A11005, Invitrogen). The samples were washed three times with PBS, and then incubated with Triton X-100 at 0.1 % in PBS for 5 min. The membranes were then incubated for 45 min at room temperature with Acti-stain^{TM} 488 fluorescent phalloidin (7:1000 in PBS). Samples were counterstained with 4',6- diamidino- 2-phenylindole (DAPI) nucleic acid stain (Molecular Probes). Images were acquired using a Zeiss LSM 710 confocal laser scanning microscope.

3. RESULTS

3.1. Layer-by-layer fabrication of ELR-covered coronary stents

The layer-by-layer fabrication approach (Figure 1 a) resulted in a complete click-ELR membrane which uniformly covered the whole stent with no defects or voids detected by macroscopic and microscopic inspection (Figure 1 c).

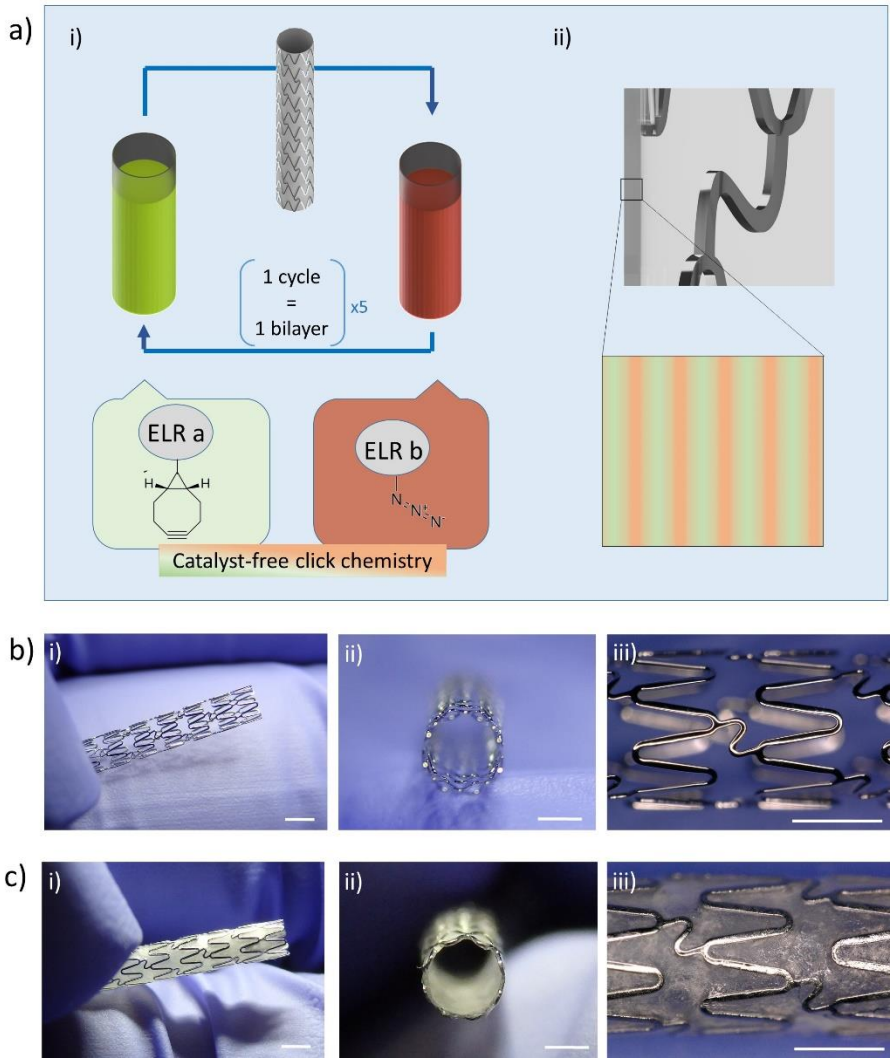

Figure 1: Fabrication of the click-ELR coronary covered stents. a) i) Schematic of the layer-by-layer technique, in which the stent is sequentially immersed in the solution of ELR-azide and ELR-cyclooctyne to create a crosslinked ELR-covering membrane by means of catalyst-free click-chemistry; ii) Cross-section scheme of the click-ELR coronary covered stent; b) Bare metal coronary stent. i) lateral and ii) frontal view; iii) detailed view of the struts; c) ELR-covered coronary stent i) lateral and ii) frontal view; iii) detailed view of the struts covered with the click-ELR-membrane. Scale bars: 1 mm.

3.2. Simulated delivery procedure of ELR-covered stent and mechanical stability

The ELR-membrane was able to undergo the increase in diameter from 1.8 mm to 3.4 mm during balloon expansion without rupture (Figure 2 a and Supplementary video S1). SEM analysis revealed a thickness of the ELR-layer of approximately 30 µm after expansion (Figure 2 c). The dry weight of the ELR-covered stent after exposure to arterial shear stress (1.5 Pa)

was not significantly different from that before the exposure (Figure 2 d), indicating a stable covering membrane.

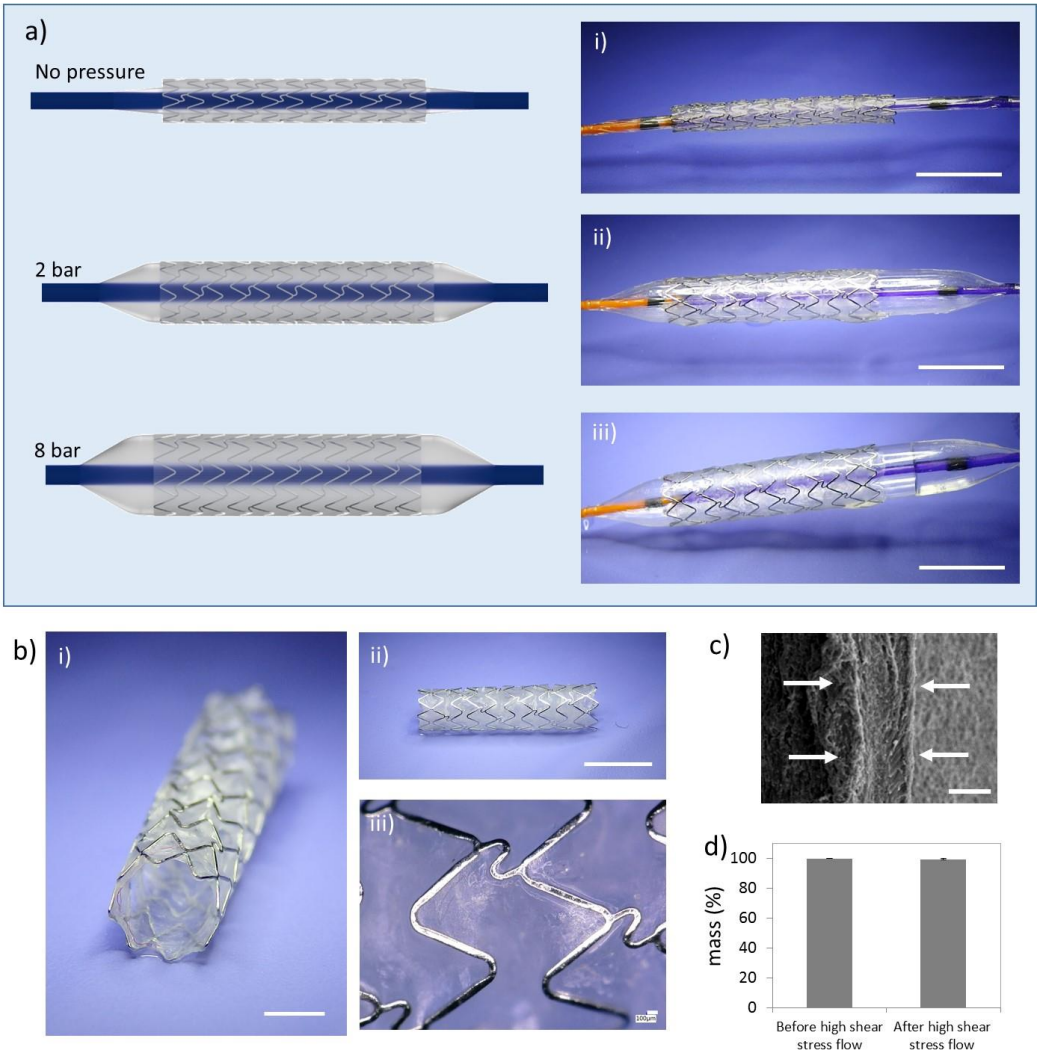

Figure 2: Simulated in vitro ELR-stent delivery procedure and mechanical stability. a) Balloon-expansion of the ELR-covered stent; i) initial state before starting the expansion (stent diameter: 1.8 mm); ii) intermediate state (balloon pressure 2 bar); iii) final state (balloon pressure: 8 bar; stent diameter: 3.4 mm); b) Deployed ELR-covered stent; i) semi-frontal image; ii) lateral image; iii) detailed view of the ELR membrane; c) SEM image showing the thickness of the layer (white arrows, approx. 30 µm); d) Assessment of the dry-weight of the covered stent before and after high shear stress flow. Scale bars: a) i-iii) 5 mm; b) i) 2 mm, ii) 5 mm; iii) 100 µm. c) 20 µm.

3.3. Thrombogenicity evaluation

After stent delivery and exposure to blood under physiological shear stress in the Chandler loop system, no macroscopic blood clots were found on the surface of the covered stents. Further microscopic analysis by SEM revealed a complete and homogeneous ELR-membrane, which completely embedded the struts and bridged the area in between (Figure 3 a-b). The zoom-in views of the luminal side of the stents showed minimal platelet adhesion on the surface of the ELR-covered stents, in contrast with the high number of activated platelets present on the GORE-TEX® surfaces used as control. Further image analysis showed that ELR-stents had a platelet covered area 5 fold-smaller than GORE-TEX® (Figure 3 g).

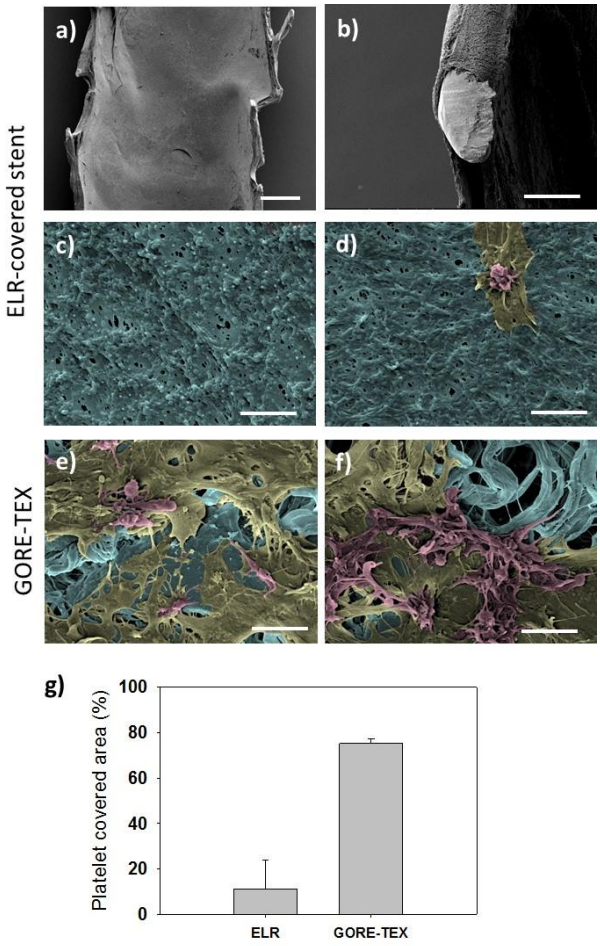

Figure 3: Thrombogenicity assessment. SEM images of the ELR-covered stents and GORE-TEX® after blood exposure. a) Overview of the luminal surface of the ELR-covered stent; b) Detailed view of the stent strut embedded with the ELR-membrane; c)-d) Detailed views of the

lumen of the ELR-covered stent; e)-f) Detailed views of the lumen of GORE-TEX® used as control; g) Percentage of area covered by platelets for ELR-covered stent and for GORE-TEX®. Scale bars: a) 500 µm; b) 100 µm; c)-f) 5 µm.

3.4. Endothelialization

The culture of human EPCs on the surface of the click-ELR membranes resulted in a confluent endothelial layer after 1 day, as shown by the CD31 immunostaining (Figure 4). This bioactive behaviour is in agreement with the presence of the integrin-mediated adhesion epitope RGD in the ELR (Figure 4).

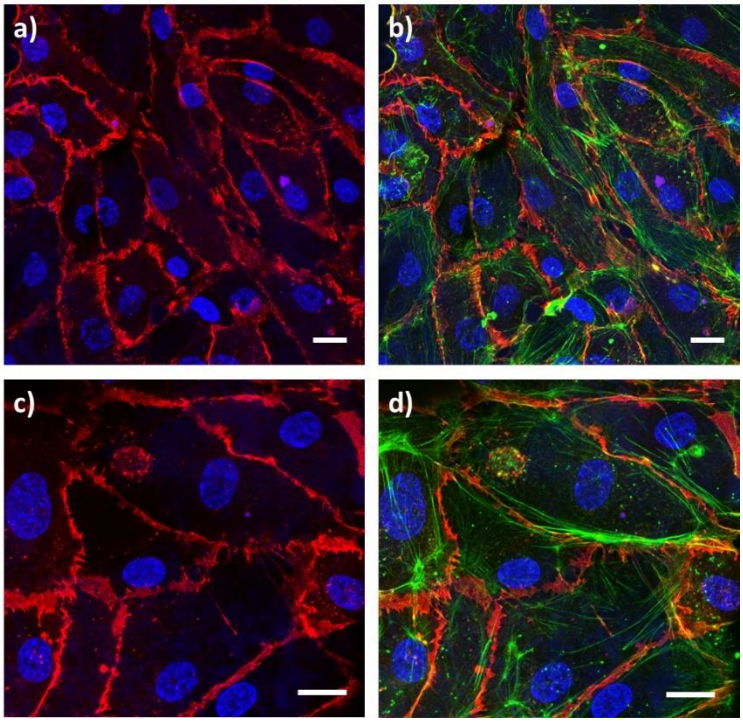

Figure 4. Endothelial progenitor cell layer obtained after 1 day of culture. a) and c) Confocal microscopy images showing CD31 staining (red) with DAPI as nuclear counterstain (blue); b) and d) Confocal microscopy images showing CD31 (red) and phalloidin (green) staining with DAPI as nuclear counterstain (blue). Scale bars: a)-d) 20 µm.

4. DISCUSSION

The concept of the covered coronary stents relies on a membrane that bridges the area between the stent struts. The membrane material should ideally be hemocompatible to avoid thrombosis and be mechanically stable upon implantation to function as physical barrier either to prevent restenosis or to seal coronary perforations and aneurysms [33]. Thrombosis and restenosis are still major clinical complications associated with covered stents. Indeed, stents covered with synthetic traditional polymers (i.e. PET and ePTFE) suffer from the lack of hemocompatibility, and can elicit severe inflammatory reactions and thrombus formation, leading to low patency rates [34]. While drug-eluting stents loaded with anti-proliferative and anti-thrombotic drugs have been suggested as a solution to avoid thrombosis, intimal hyperplasia and subsequent stent occlusion, they have been also associated with delayed endothelialisation and allergic reactions, which hampers the expected benefits of the drug-delivery strategy [35, 36].

Alternative solutions that combine advanced biomaterials with suitable fabrication techniques are, therefore, needed in order to develop covered stents that address the aforementioned current limitations. This represents an extra challenge for coronary stents, whose small diameter (in the order of a few millimeters) makes it difficult to use manufacturing techniques that are applicable to peripheral stents (e.g. injection moulding [24, 37] or suturing of preformed membranes on bare metal stents [22]). Additionally, the covering-membrane must be elastic to withstand the change in the diameter during the implantation.

Here, we propose the elastin-like recombinamers, as the biopolymers of choice for covering coronary stents. The ELRs have shown their potential in a wide range of applications due to their good biocompatibility [38, 39], low thrombogenicity [23, 24] and their elastic properties [40]. Specifically, we have used two ELRs that were chemically modified with cyclooctyne and azide to enable their stable crosslinking via catalyst-free click-chemistry reaction.

Catalyst-free click-chemistry (and specifically Huisgen 1,3-dipolar cycloadition of azides and alkynes) outperforms other cross-linking reactions, as it features i) high chemoselectivity, so that the reactant pairs do not interfere with other biomolecules or cellular reactions [25], ii) high yields under mild, non-toxic conditions; iii) rapidity; iv) no risk of the release of excess crosslinking reagents, as the chemical reactive groups are incorporated into the molecules intended to be crosslinked; v) no need of catalyst, guaranteeing the cytocompatibility of the reaction [26].

By exploiting the click-chemistry as a reliable cross-linking strategy and the layer-by-layer dip-coating as a simple and reproducible fabrication approach, we have created ELR covered coronary stents (Figure 1). The resulting stents were placed on a balloon to undergo a simulated delivery procedure, in which they were expanded from 1.8 to 3.4 mm (Figure 2), to test their suitability for percutaneous implantation into coronary arteries. After stent deployment, neither macroscopic nor microscopic tears were observed when exposed to arterial flow and pressure conditions (Figure 2). The realization of an intact membrane enables also the use of these stents to seal aneurysms or arterial perforations, besides restoring the flow through occluded vessels by excluding the plaque or damaged tissue.

Besides the mechanical elasticity and their potential to act as a physical barrier and to inhibit intimal hyperplasia, the click-elastin membrane might also provide an antithrombogenic surface that avoids platelet adhesion and activation. In order to check this, we deployed our ELR-covered stents in a Chandler loop system and exposed them to whole human blood under dynamic arterial flow. There were no signs of leukocyte adhesion, and only few platelets were observed by SEM, in sharp contrast with the high number of platelets adhered and activated on the GORE-TEX® surface (Figure 3). These results agree with previous studies which proposed elastin and elastin-like polymers as excellent candidates for the fabrication of blood-contacting

devices [23, 24, 41, 42] and support the use of ELRs for the fabrication of covered stents that will be in direct contact with the blood before endothelialization.

In addition to the intrinsic hemocompatibility shown in the blood-contacting test, the anti-thrombogenicity of the ELR-covered stents is further guaranteed by their ability to support the formation of a confluent endothelial layer, as shown by culturing human EPCs (Figure 4). Circulating EPCs are increasingly recognized as important contributors to vascular prosthesis endothelialization, making them important mediators of implant compatibility [43, 44]. Besides its paramount role as anti-thrombogenic surface, the endothelium is involved in several aspects of vascular biology, e.g. blood vessel tone, hemostasis, neutrophil recruitment and hormone trafficking [45, 46]. Therefore, the ability of the covered stent to support endothelialization is of key importance to achieve the success of the implant.

It is important to consider that a stented artery presents a geometry based on the stent strut configuration, which contrasts with the smoothness of the healthy arteries. This inevitably leads to a disturbed local hemodynamics (i.e. turbulent flow) [47], which has been related to the stimulation of vascular smooth muscle cell proliferation [48] and platelet activation [49]. Indeed, the thickness of the stent-struts plays an important role in-stent restenosis, with thinner struts showing considerably lower restenosis rates [50-52]. Importantly, covering the stent with the ELR-membrane bridges the stent-struts, with a concomitant reduction in the height changes in between the stent struts, which may further contribute to the positive outcome of the device.

Overall, we have established a strategy that combines the simplicity of the layer-by-layer technique with the biocompatibility and specificity of the catalyst-free click-chemistry to develop a new class of coronary covered stents. The elastic membrane provided physiological hemocompatibility to the implant, and enabled endothelialization, while representing a physical barrier for the atherosclerotic plaque and smooth muscle cells ingrowth. Additionally, the

recombinant nature of the ELR allows for the introduction of further biological cues (e.g. immunomodulatory cues, antimicrobial peptides) to develop a platform that enables stent fabrication with tailored biofunctionalities.

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Data availability

The data associated with this manuscript is available upon request to the corresponding author.

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