Acta Biomaterialia 12 (2015) 146–155

Contents lists available at ScienceDirect

Acta Biomaterialia

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

# Elastin-like recombinamer-covered stents: Towards a fully biocompatible and non-thrombogenic device for cardiovascular diseases

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ARTICLE INFO

*Article history:*
Received 24 April 2014
Received in revised form 29 September 2014
Accepted 22 October 2014
Available online 29 October 2014

*Keywords:*
Elastin-like recombinamers
Gel
Stent
Restenosis
Endothelialization

ABSTRACT

We explored the use of recently developed gels obtained by the catalyst free click reaction of elastin-like recombinamers (ELRs) to fabricate a new class of covered stents. The approach consists in embedding bare metal stents in the ELR gels by injection molding, followed by endothelialization under dynamic pressure and flow conditions in a bioreactor. The mechanical properties of the gels could be easily tuned by choosing the adequate concentration of the ELR components and their biofunctionality could be tailored by inserting specific sequences (RGD and REDV). The ELR-covered stents exhibited mechanical stability under high flow conditions and could undergo crimping and deployment without damage. The presence of RGD in the ELR used to cover the stent supported full endothelialization in less than 2 weeks in vitro. Minimal platelet adhesion and fibrin adsorption were detected after exposure to blood, as shown by immunostaining and scanning electron microscopy. These results prove the potential of this approach towards a new and more effective generation of covered stents which exclude the atherosclerotic plaque from the blood stream and have high biocompatibility, physiological hemocompatibility and reduced response of the immune system.



## 1. Introduction

Vascular stenting is a widely adopted intervention to treat narrowed or weakened (e.g. presenting non-obstructive lesions, aneurysms and dissections) coronary and peripheral vessels by stabilizing them and maintaining them patent. Despite the simplicity of the concept and the knowledge gained in the last decades on the interactions between stent, vascular tissue and blood, the re-occlusion of the vessels after stenting with bare metal stents (BMSs) remains a major problem [1–3]. To avoid this complication, drug eluting stents (DESs) have been developed with the aim of suppressing the proliferation of smooth muscle cells. This concept proved to be successful in preventing cellular ingrowth; however, late stent thrombosis has been reported [4,5], likely occurring because the same drugs hinder the healing of the endothelial layer and the presence of the polymeric coating results in wall inflammation. Recently published clinical studies reported the improved performance of the new generations of drug eluting coronary stents with respect to the occurrence of late thrombosis [6,7].

Recognizing the importance of a rapid and complete endothelial regeneration, stents with the capability of attracting endothelial progenitor cells (EPCs) have been proposed [8]. The mimicry of EPC homing holds great promise for in vivo self-endothelialization of medical devices; however, it depends heavily on the use of not yet identified, highly selective capture molecules able to bind exclusively EPCs [9,10]. Furthermore, the role of EPCs in re-endothelialization following vascular injury and the effect on intimal hyperplasia are still controversial [11,12].

Covered stents represent an alternative family of devices consisting of BMSs in combination with a layer made of synthetic polymers [13,14] or based on naturally occurring materials [15–18]. Autologous native [19] and engineered [20,21] tissue was also used for covering or embedding BMSs. The layer functions as a barrier to exclude the atherosclerotic plaque from the blood flow in the lumen of the vessel, potentially preventing neointima hyperplasia and therefore re-occlusion of the vessel [22]. Ideally, the membrane should also support endothelialization to achieve a physiological hemocompatibility. Furthermore, covered stents

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http://dx.doi.org/10.1016/j.actbio.2014.10.029
1742-7061/

are indicated to seal degenerated vein grafts, cover vascular perforations and exclude symptomatic aneurysms [23,24].

Despite the possible advantages that covered stents might have with respect to bare and coated ones, especially in the peripheral vascular system, their full potential has not been fully explored. The main challenge is to identify the optimal material able to cover the stent in a mechanically stable way under physiological pressure and flow conditions, possessing elasticity to undergo crimping and deployment without damage, biocompatible, hemocompatible and supporting endothelial cell adhesion/proliferation to full coverage.

Here we propose the use of elastin-like recombinamers (ELRs) to fabricate endothelialized covered stents. ELRs are bioengineered polymers based on the natural elastin, produced by recombinant techniques. They possess an extremely low polydispersity, thermosensitive behavior and the possibility to include different bioactive sequences for cell adhesion and proliferation or sequences sensitive to enzymes [25]. These polymers exhibit high biocompatibility [26] and hemocompatibility [27,28]. Notably, because ELRs are formed by the repetition of amino acid sequences present in the natural elastin, they are ignored by the host immune system as well as their degradation products [29,30]. ELRs have been exploited for applications as nanovaccines [31], protein [32] and drug delivery [33,34] and tissue engineering [35]. ELRs gels have demonstrated to be an optimal artificial extracellular matrix for different cell lines, such as fibroblasts and neuroblasts [36], chondrocytes [37,38], epithelial cells from the ocular surface [39] and endothelial cells (human umbilical vein endothelial cells, HUVECs) [40]. In general terms, ELRs are positioned midway between natural products and synthetic polymers. Interestingly, they show the advantages of an engineered material because of the exhaustive control over their composition, functionality and mechanical properties, while still keeping the biological origin and nature. That is why ELRs could be an ideal candidate for creating bioactive and more efficient stents similarly to other biomedical applications where ELRs have the potential to outperform more conventional materials [31–35].

Recently, we showed the successful modification of ELRs by the Huisgen 1,3-dipolar cycloaddition of azides and alkynes [41] according to the concept of click chemistry [42,43]. In this way, we could produce gels under physiological conditions, in a completely cell-friendly process without the need of any catalyst. The biocompatibility of the click reactions has been tested in the presence of cells [44–46] and inside living organisms [47]. Click reactions have been largely employed in drug discovery [48], bioconjugation with proteins [46] and DNA [49], cell surface labeling [50] and surface modifications with ELRs [51] among other molecules.

Here we used the newly developed ELR-catalyst free click gels (ELR-CFCGs) [41] to cover self-expandable stents by embedding in the gels by the injection molding technique. The ELR-covered stents were endothelializated dynamically in a bioreactor with the goal of obtaining an endovascular device with (1) a physical barrier for the atherosclerotic plaque; (2) physiological hemocompatibility; (3) mechanical stability; and (4) no inflammatory reaction.

The quality of the embedding process was evaluated by scanning electron microscopy (SEM); the mechanical stability of the ELR layer was tested under high shear stress conditions. The ability of ELR-CFCGs with different biofunctional sequences to support adhesion and proliferation of HUVECs was tested by immunocytology. The catheter-based delivery of the ELR stents was simulated by crimping the devices and keeping them in the crimped position for 20 min, representing the maximum estimated delivery time. After deployment the stents were exposed to blood contact in a Chandler loop [52,53] for 1 h and the surface performance was evaluated in terms of platelet adhesion by immunochemistry and fibrin deposition by SEM.

## 2. Materials and methods

### 2.1. ELR biosynthesis, modification and characterization

The ELRs used in this work were obtained by using standard genetic engineering techniques. Their purification was performed with several cycles of temperature-dependent reversible precipitations, by centrifugation below and above their transition temperature ($T_t$), making use of the intrinsic thermal behavior of these compounds [54]. The ELRs were subsequently dialyzed against purified water and freeze-dried. Three different ELRs were obtained: VKVx24, a structural recombinamer without any bioactive sequence; HRGD6, a recombinamer with the universal cell-adhesion epitope (RGD) and REDVx10, bearing a cell-adhesion domain (REDV) which is more specific for endothelial cells than RGD. The amino-acid sequences of these polymers are: MESLLP VG VPGVG [VPGKG(VPGVG)$_5$]$_{23}$ VPGKG VPGVG VPGVG VPGVG VPGV for VKVx24; MGSSHHHHHHSSGLVPRGSHMESLLP [(VPGIG)$_2$ (VPGKG)(VPGIG)$_2$]$_2$AVTGRGDSPASS[(VPGIG)$_2$(VPGKG)(VPGIG)$_2$]$_2$ for HRGD6 and MESLLP[(VPGIG)$_2$(VPGKG)(VPGIG)$_2$EEIQIGHIPRED VDYHLYP(VPGIG)$_2$(VPGKG)(VPGIG)$_2$(VGVAPG)$_3$]$_{10}$V for REDVx10. The purity and chemical characterization of ELRs were verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) amino acid composition analysis, differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) [54,55]. ELRs were chemically modified by transformation of the ε-amine group in the lateral lysine chain to bear cyclooctine and azide groups, as we recently reported [46]. In addition to VKVx24-cyclooctine and HRGD6-$N_3$, novel VKVx24-$N_3$, REDV-$N_3$, REDVx10-cyclooctine, HRGD6-cyclooctine were prepared and characterized by NMR, Fourier transform infrared spectroscopy (FTIR), and DSC (see Figs. S.1–S.6 in Supporting information).

### 2.2. Gel formation (ELRs-CFCGs)

Gels were formed by catalyst free click reactions between an azide group and an activated cyclooctine group. Solutions of ELRs-cyclooctine and ELRs-azide were prepared in purified water at the desired concentration and kept at 4 °C for at least 24 h. ELRs-CFCGs were obtained by mixing the solutions (ELR-cyclooctine, ELR-azide) at 4 °C inside the appropriate mold (e.g. well plates and cylindrical and tubular molds). After 15 min at 4 °C, the gels were completely formed. Specifically VKVx24-VKVx24 (VKV-CFCG), HRGD6-HRGD6 (RGD-CFCG) and REDVx10-REDVx10 (REDV-CFCG) were obtained and characterized by DSC and FTIR (see Figs. S.7 and S.8 in Supporting information).

### 2.3. Rheological measurements

Rheological experiments were performed on a strain-controlled AR-2000ex rheometer (TA Instruments USA). Cylinder-shaped gels, cast in a custom-made mold, were placed between the nonporous parallel stainless steel plates (12 mm diameter) of the rheometer. The gap between the plates was adjusted using a normal force of ~0.2 N in order to prevent slippage. A gap higher than 1000 μm was always reached after the sample relaxed until equilibrium. Measurements were carried out at two temperatures: 4 °C (formation temperature), and 37 °C. Sample temperature was controlled and maintained by using a Peltier device. For each kind of ELRs-CFCG, nine samples were tested. Two different measurements