

ORIGINAL ARTICLES

Tailored surface design of biodegradable endovascular implants by functionalization of poly (L-lactide) with elastin-like proteins

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Received: July 28, 2015

Accepted: September 13, 2015

Online Published: October 8, 2015

DOI: 10.5430/jbei.v2n1p52

URL: <http://dx.doi.org/10.5430/jbei.v2n1p52>

Abstract

Endovascular implants currently used after cardiovascular events have proven their efficacy. However, strategies are in quest to optimize clinical outcomes. One possibility is the development of polymer surfaces imitating extracellular matrix in order to promote vascular integration of an implanted device. The aim of this study was to develop and investigate methods for covalent immobilization of a synthesized elastin-like protein (ELP) additionally modified with functional domains (RGD, CS5 and P15) promoting endothelial cell proliferation on biodegradable poly (L-lactide) (PLLA) as model endovascular implant surface. Evaluation of the impact of different ELP immobilization methods on PLLA regarding the achievable surface load evidences that the amino activation of PLLA does not have considerable influence, while the reaction sequence as well as the used crosslinker presents determining factors in ELP immobilization. Biocompatibility regarding selective promotion of endothelial cell (EC) adherence and proliferation especially in contrast to smooth muscle cells (SMC) was improved on covalently immobilized but not on physically adsorbed ELP. In summary, we could underline the applicability of a modified ELP-coating for endovascular implant surfaces *in vitro* and provide information on applicable immobilization procedures. Moreover, the latter builds the basis for a wide variety of implant applications, because the developed immobilization strategy should be easily transferable to any ELP with tailored biological functionality by exchange of the integrated active sequences.

Key words

Surface functionalization, Elastin-like protein, Biodegradable polymer, Endothelialization

1 Introduction

With the purpose of saving lives and improving the quality of life, various medical applications are inserted in humans each year. However, reduction of the associated foreign body reaction, involving nonspecific protein adsorption, interaction with immune cells such as neutrophils and macrophages, and giant cell formation and cytokine release possibly leading to encapsulation of the device, still provides substantial room for improvement of the applied biomaterial^[1,2]. This

is especially true for endovascular implants, which are in close and steady contact with blood. Here, the implant material might trigger activation of different cascades of homeostasis possibly resulting in a stent thrombosis^[3]. In order to reduce this surface-induced reactions of the organism the blood contacting artificial devices are more and more coated with inherently better hemocompatible substances such as polysaccharides, enzymes, and anti-inflammatory agents^[4]. Moreover, development and establishment of implant surfaces mimicking the natural local surrounding of cells, the extracellular matrix (ECM), which significantly controls cell adhesion, proliferation and differentiation *in vivo*, is one research focus today^[5]. From the plethora of insoluble and soluble macromolecules contained within ECM, the fibrillar protein collagen is probably the most commonly used coating of for example commercially available polyester vascular grafts^[6]. Furthermore, besides the use of integer ECM components, various ECM peptide sequences including functional domains of proteins, glycoproteins, and proteoglycans have been isolated and grafted on biomaterials to control cell behavior, for example, RGD, and REDV within the CS5 (EEIQIGHIPREDVDYHLYPHG) sequence of fibronectin^[7], laminin-derived recognition sequences IKLLI, IKVAV, LRE, PDSGR, and YIGSR^[8], and the collagen type I derived sequences DGEA^[9] and TPGPQGIAGQRGVV (P15)^[10]. However, these peptides, nicely reviewed by de Mel *et al.*^[5], are not cell-selective as they bind to integrins, present on many epithelial cells. Nevertheless, Blindt *et al.* demonstrated that endothelial progenitor cells (EPC), reported to induce endothelialization resembling the natural innermost layer of blood vessels^[11], could be successfully recruited to polymer-based stent coatings modified with cyclic RGD^[12]. Cell selectivity could be moreover augmented by the coupling of peptides, which have been previously isolated by a phage display technique^[13]. The application of a coating combining both, an integer ECM component with functional peptide domains, might hence be very promising with regard to the creation of an optimal environment for endothelialization, thereby suppressing the surface induced foreign body reaction. Envisioning a coating for vascular grafts mimicking the natural surroundings of endothelial cells (EC), which provides itself a tight non-thrombogenic barrier between the lumen of the vessel and the rest of the vessel wall, one hence has to study the composition of the EC's environment. Stegemann *et al.*^[14] described this region being enriched in collagen IV and laminin encircled by a fenestrated but acellular layer of elastin called the internal elastic lamina. Elastin-like proteins (ELP), artificial repetitive polypeptides consisting of peptide sequences of ECM proteins with previously reported functional cell recognition motifs^[15-17], have already found widespread medical applications in vascular graft tissue engineering^[18]. Instead of directly processing those ELPs into gels^[19], films^[20], foams^[21], or fibers^[22], impregnation and coating of vascular graft surfaces with various ELP via adsorption has been recently demonstrated to yield materials with low incidence of platelet deposition^[23,24]. Especially, an ELP sequence consisting of hydrophobic and hydrophilic blocks was demonstrated to reduce thrombogenicity of the coated material. Although the latter allows the use of the well-established vascular graft materials as polyesters and poly(tetrafluoroethylene), it was shown in previous work that the physical adsorption of ELP to substrates represented an inherently limiting characteristic due to coating instability, resulting in a loss of the anti-thrombotic effect^[25].

In this context, we established a covalent surface binding of an ELP sequence, additionally modified with functional domains (RGD, CS5 and P15) assumed to promote endothelial cell proliferation, via an ϵ -amino group of lysine (K) incorporated as fourth residue within the pentapeptide sequence of ELP (VPGXG) to amino-activated poly (L-lactide) (PLLA). Therefore, we synthesized the ELP sequence X_2 -RGD- X_2 , with $X_2 = [\text{ELP-CS5-(ELP)}_2\text{-P15-(ELP)}_2\text{-CS5-ELP}]_2$ containing repeats of the classical ELP $[(\text{VPGIG})_2\text{-VPGKG-(VPGIG)}_2]$, the CS5 sequence from alternative spliced fibronectin including the binding domain REDV, and the P15 sequence from collagen type I. PLLA has been chosen as model material since it is nowadays often applied as biocompatible and biodegradable platform^[26, 27] or coating with possible drug incorporation^[28, 29] for endovascular implants.

The herein reported study focuses on evaluating the impact of different ELP immobilization methods on PLLA regarding the achievable surface load and biocompatibility in terms of selective promotion of EC adherence and proliferation especially in contrast to smooth muscle cells (SMC), identified as one main principal cause for vascular graft occlusion.

2 Methods

2.1 ELP preparation

2.1.1 ELP cloning

Afforded DNA sequences encoding for the different peptide sequences contained in X₂-RGD-X₂ (see Table 1) were purchased from Eurofins Genomics (Ebersberg, Germany). As previously reported by McDaniel *et al.*, these DNA fragments were subcloned into pET24a (+), amplified and combined to ELP-CS5-ELP and ELP-P15-ELP by recursive directional ligation for generation of 2 repeats of the sequence [ELP-CS5-(ELP)₂-P15-(ELP)₂-CS5-ELP]₂ called X₂^[30]. This method of recursive directional ligation was used again to encompass the RGD sequence between two X₂ sequences. The resulting plasmid pET24a+-X₂-RGD-X₂ was subsequently transformed into commercially available *E. coli* BL21 (DE3) (Life Technologies GmbH, Darmstadt, Germany) for protein expression with subsequent ELP purification.

Table 1. Abbreviations of peptide sequences contained in the synthesized ELP sequence X₂-RGD-X₂

Abbreviation	Sequence
ELP-RGD-ELP	(VPGIG) ₂ -VPGKG-(VPGIG) ₂ -YAVTGRGDSPASS-(VPGIG) ₂ -(VPGKG)-(VPGIG) ₂
ELP-CS5-ELP	(VPGIG) ₂ -VPGKG-(VPGIG) ₂ -EEIQIGHIPREDVDYHLYPHG-(VPGIG) ₂ -(VPGKG)-(VPGIG) ₂
ELP-P15-ELP	(VPGIG) ₂ -VPGKG-(VPGIG) ₂ -GTPGPQGIAGQRGVVG-(VPGIG) ₂ -(VPGKG)-(VPGIG) ₂

2.1.2 ELP expression and purification

Based on a modified protocol established by McPherson *et al.* the synthetic X₂-RGD-X₂ protein was extracted from cultured bacteria^[31]. Briefly, an overnight pre-culture of *E. coli* BL21(DE3)-pET24a+-X₂-RGD-X₂ was used to inoculate a main culture with an optical density of OD₆₀₀ = 0.04 containing 100 µg/ml kanamycin. After reaching an OD₆₀₀ of 0.6 the ELP expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.5 mM). Following 3 hours of continued growth, monitored via OD₆₀₀, the bacteria were harvested by centrifugation at 4,400 × g for 15 min at room temperature. After resuspension in a small volume of distilled water the bacteria were lysed in an ultrasonic water bath for 10 min in cold water. After centrifugation for 20 minutes at 4°C, and 10,000 × g the supernatant containing soluble ELPs was transferred in new reaction tubes. After adding the same volume of 2 × TN-buffer (100 mM Tris-HCl, pH 8.0, 1 N NaCl) the reaction tubes were incubated in a water bath (45°C, 15 min) to induce polymerization of soluble ELPs. Resultant polymers were isolated by centrifugation (1,500 × g for 30 min at 40°C). The supernatant was removed and the ELPs were dissolved in cold distilled water by pipetting up and down. The procedure of cold centrifugation followed by addition of 2 × TN-buffer and warm centrifugation was repeated three-times to purify the recombinant ELPs. The protein was stored at -20°C in distilled water.

2.1.3 ELP analysis

To analyze the enriched ELP from *E. coli* culture, identical samples volumes were separated by 10% SDS-PAGE, then fixed in 40% ethanol with 10% acetic acid and staining was performed using a commercial available Roti-Blue Colloidal Coomassie solution (Roth AG, Arlesheim, Switzerland). After an incubation of 5 h the gels were washed four times with 25% methanol and the staining was digitalized using the ChemiDocMP imaging system (Bio-Rad Laboratories, Cressier, Switzerland). The protein content was determined using a BCA-assay (Thermo Scientific) according to the manufacturer's protocol.

2.2 Polymer film fabrication

PLLA films (Resomer[®] L210, Mw = 280,000 g/mol, Boehringer Ingelheim, Ingelheim, Germany) were prepared via a pouring procedure as described previously^[32].

2.3 PLLA surface activation

PLLA surface activation was carried out via plasma etching in an ammonia radio frequency plasma generator, aminolysis with hexamethylenediamine (HMDA), and silanization with 3-aminopropyltriethoxysilane (APTES) after oxygen plasma treatment. All activation procedures are described in detail in our previous study^[32].

2.4 ELP immobilization

The immobilization of ELP to the differently amino-activated PLLA surfaces was performed via three reaction strategies (see Figure 1, a-c), described in the following, applying the two different crosslinker disuccinimidylsuberate (DSS) and tris(succinimidyl) aminotri-acetate (TSAT), both from Thermo Scientific. Prior to immobilization experiments, the crosslinker were either dissolved in phosphate buffered saline (Dulbecco's PBS, pH 7.2 [DPBS]) or dimethyl sulfoxide (DMSO) at a concentration of 80 μ M and ELP in DPBS at a concentration of 1 μ M. Reaction strategy a involves ELP crosslinking prior to surface attachment by incubating 40 μ l of ELP solution with 10 μ l of crosslinker solution in DPBS at 17°C in a cryostate (MultiTemp III, Pharmacia BioTech, Freiburg, Germany) in order to avoid precipitation of the ELP. After 24 h one PLLA sample (\varnothing = 6 mm) and 10 μ l of crosslinker solution in DPBS were added and the reaction proceeded for another 24 h at 17°C. Reaction strategy b in contrast foresees simultaneous ELP crosslinking and surface attachment by adding 40 μ l of ELP solution and 10 μ l of crosslinker solution in DPBS directly to one PLLA sample (\varnothing = 6 mm). After 24 h incubation at 17°C another 10 μ l of crosslinker solution in DPBS were added and the reaction proceeded for 24 h at 17°C according to reaction strategy a. Reaction strategy c starts with attachment of the crosslinker to the amino-activated surface by adding 40 μ l DMSO and 10 μ l crosslinker solution in DMSO, in order to avoid hydrolysis in aqueous media, to one PLLA sample (\varnothing = 6 mm). After 24 h incubation at 17°C, the solution was withdrawn and 40 μ l ELP solution and, 10 μ l crosslinker in DPBS and 10 μ l DBPS were added and the reaction proceeded for 24 h at 17°C according to reaction strategy a and b. For ELP modification via simple physical adsorption without crosslinking, the first step was omitted and the second step was performed with 40 μ l ELP solution, and 20 μ l DBPS. Prior to characterization, all samples were dried in a vacuum chamber.

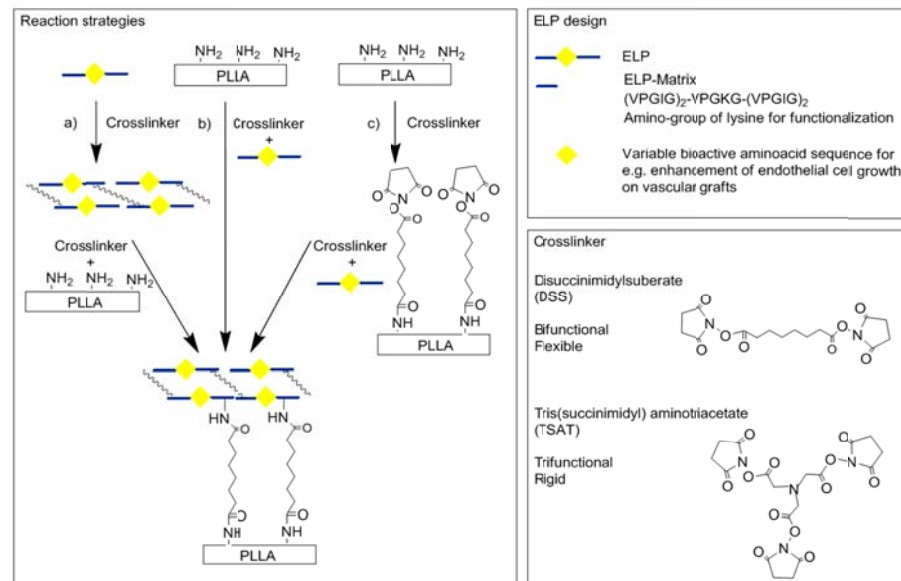


Figure 1. Left: Reaction strategies for ELP immobilization to amino-activated PLLA surfaces. a) ELP crosslinking prior to surface attachment, b) simultaneous ELP crosslinking and surface attachment, c) surface attachment of the crosslinker prior to ELP binding and crosslinking. Right: ELP design (top) and applied crosslinker DSS and TSAT (bottom).

2.5 Characterization of PLLA surface modifications

2.5.1 Contact angle measurements

Contact angles were measured by the sessile drop method (Contact Angle System, OCA 20, Dataphysics Instruments GmbH, Filderstadt, Germany).

2.5.2 Scanning electron microscopy

Examination of the modified PLLA samples was carried out in a Philips XL 30 ESEM (Philips Electron Optics, Eindhoven, Netherlands) operating in the ESEM mode. Representative micrographs are shown.

2.5.3 BCA protein assay for quantification of immobilized ELP

A BCA protein assay (Thermo Scientific) was employed to quantify the amount of ELP on the model implant surfaces. Four identically modified PLLA foils were placed into 1.5 ml vials and incubated for 2 h at 17°C with 300 µl of working reagent containing the following in a 50:1 ratio: a 0.1 M NaOH solution containing sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate and 4.0% cupric sulfate pentahydrate. Standards (25 ng to 5,000 ng BSA were prepared in 25 µl DPBS, pipetted into separate wells of a 96-well plate (in triplicate) and after lyophilization incubated for 2 h at 17°C with 300 µl working reagent. Both standards and samples were assayed at 562 nm in a spectrophotometer (BIOMATE 3S, Thermo Scientific).

2.5.4 CBQCA assay for quantification of ELP crosslinking

As an estimation of the degree of internal ELP crosslinking, dissolved ELP prior crosslinking and after crosslinking, following experimental conditions of the second step of reaction strategy c described above omitting the addition of PLLA foils, was determined by labelling the primary amino groups with ATTO-TAG CBQCA (3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde). For this assay, 15 µl of the sample were mixed with 120 µl DPBS, 5 µl potassium cyanide (20 mM in dH₂O) and 10 µl ATTO-TAG CBQCA (5 mM in DPBS) and incubated for 1 h at 17°C. Fluorescence measurement was performed with a plate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Germany) applying an excitation wavelength (λ_{exc}) of 485 nm and a detection wavelength (λ_{em}) of 520 nm.

2.6 Cell assays

2.6.1 Culture of human primary cells

Primary human coronary artery smooth muscle cells (HCASMC) and endothelial cells (HCAEC) of different donors were obtained from PromoCell GmbH (Heidelberg, Germany) and cultured in a humidified atmosphere at 37°C with 5% CO₂ using optimized culture media as recommended by the manufacturer. Prior to seeding of the cells for determination of viability and proliferation and fluorescence microscopy the different PLLA surfaces were placed into the wells of 96-well plates, weighted with Teflon rings and disinfected with 70% ethanol (incubated for 10 minutes). After three washing steps with PBS, HCAEC or HCASMC were seeded at a density of 5×10^3 cell/well and incubated in respective growth medium for 48 h, respectively.

2.6.2 Cell viability assay

Cell viability was determined using the commercially available Fluometric Cell Viability Kit I (PromoKine GmbH, Heidelberg, Germany). Three hours prior to the final measurement the culture medium was changed and 10% resazurin solution was added. After 3 hours incubation the supernatant was transferred in a new 96-well plate and fluorescence of resorufin was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm using the Infinite_M200 microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Presented data were normalized to those obtained from cells cultured on untreated PLLA surfaces. The cells were subsequently used for determination of cell proliferation.

2.6.3 Cell proliferation assay

Cellular proliferation was assessed using the commercially available BrdU Cell proliferation Kit (Roche, Basel, Switzerland). After 24 h adding the BrdU labelling solution to the medium and after incubation at 37°C the incorporation of BrdU was determined by ELISA. Fluorescence values detected by a microplate reader (Tecan) were normalized to those observed in cells cultured on untreated PLLA surfaces.

2.6.4 Cell count by fluorescence microscopy

For detection of cell numbers the cells were seeded and washed twice with DPBS after 48 h of culture. After that the cells were incubated 10 minutes with DAPI diluted 1: 2,000 in PBS for nucleic staining. After washing of the cells with PBS detection of the fluorescence was conducted by fluorescence scan of each microtiter plates using the PALM MicroBeam (Carl Zeiss, Jena, Germany; SP5-II-MATRIX, Leica, Heerbrugg, Switzerland). Nuclei were counted using the FIJI image software (ImageJ, freeware, supported by several institutions). The observed nuclei numbers were normalized to the counts observed on untreated PLLA surfaces.

2.7 Statistics

Statistics was performed using IBM SPSS software 20.0 GraphPad PRISM 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Indicated statistical differences were analyzed using the non-directional Mann-Whitney U-test, assuming $P < .05$ as significantly different and $P < .01$ as highly significantly different.

3 Results

3.1 Protein analysis

The *E. coli* protein synthesis was monitored via measurement of the OD_{600} , indicating the bacterial concentration. Results show that bacteria, which were treated with IPTG for induction of ELP expression, reduce their growth and change their protein expression machinery to the synthesis of the ELP compared to cells which did not receive IPTG (see Figure 2b). After purification of proteins from bacteria by repetitive cold and warm centrifugation, run SDS page gels reveal a clear band at 86 kDa, corresponding to the X_2 -RGD- X_2 construct, for *E. coli* treated with IPTG and not for *E. coli*, which omitted the IPTG addition (see Figure 2c).

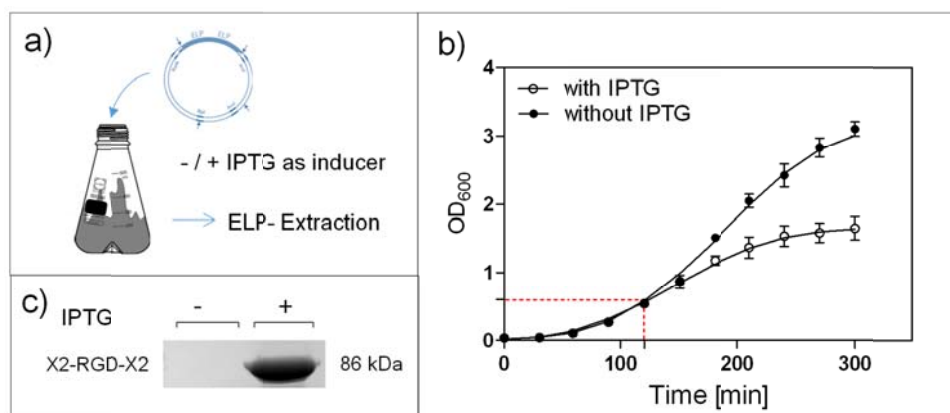


Figure 2. Expression and synthesis of ELP. a) The plasmid containing the genetic information for the ELP was transformed into BL21(D3)pLysS *E. coli*. The bacterial production of the synthetic protein was induced by supplementation of IPTG (final conc. 0.5 mM). b) Monitoring of the growth of *E. coli* with and without IPTG (dashed red lines mark the time of IPTG addition) via OD_{600} measurements, mean \pm SD, $n = 3$. c) After purification a Coomassie stained 10% SDS page gel shows a protein band for bacteria with and no band for bacteria without IPTG.

3.2 Impact of PLLA surface activation on ELP immobilization

PLLA surface activation is necessary for covalent immobilization of ELP in order to generate enough functional amine groups. Here, we investigated three different surface activation treatments: an ammonia plasma treatment, an aminolysis with HMDA and a combination of oxygen plasma treatment and silanization with APTES. While we described the impact of the activation procedure regarding abundance of generated amino groups and penetration depth within PLLA in a previous paper [32], we here focused on obtainable ELP surface loads.

Although no significant difference in ELP surface loads was observed, a tendency for higher surface loads on purely plasma- in comparison to wet-chemically activated surfaces was detected independent of the chosen reaction strategy (see Figure 1a) ELP crosslinking prior to surface attachment, b) simultaneous ELP crosslinking and surface attachment, c) surface attachment of the crosslinker prior to ELP binding and crosslinking.) and the applied crosslinker (DSS, TSAT) (see Figure 3a). Figure 3b shows the results in detail for reaction strategy c.

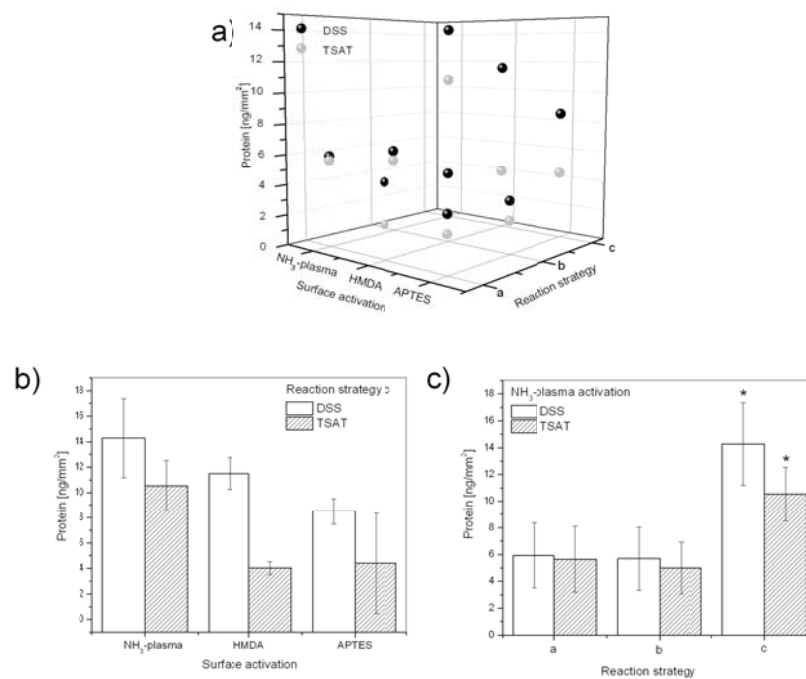


Figure 3. Impact of surface activation procedure (NH₃-plasma, HMDA, APTES), reaction strategy (a) ELP crosslinking prior to surface attachment, b) simultaneous ELP crosslinking and surface attachment, c) surface attachment of the crosslinker prior to ELP binding and crosslinker) and crosslinker (DSS, TSAT)) on ELP surface loads on PLLA. a) Summary of results, b) impact of surface activation and applied crosslinker on ELP surface loads applying reaction strategy c, c) impact of reaction strategy and crosslinker on ELP surface loads for NH₃-plasma activated PLLA. Bars show mean ± SD of at least 5 experiments. **P* < .05 compared to the differently treated surfaces with the same crosslinker.

3.3 Impact of reaction strategy on ELP immobilization

In contrast to the surface activation procedure, the applied reaction strategy revealed a significant impact on obtained ELP surface loads. In this context, surface attachment of the crosslinker prior to ELP binding and crosslinking (reaction strategy c) yielded significantly higher ELP surface loads on PLLA in comparison to the two further evaluated strategies for the three surface activations investigated and both applied crosslinker (see Figure 3a). Figure 3c shows the results in detail for purely plasma-activated PLLA.

3.4 Impact of crosslinker on ELP immobilization

While the chosen crosslinker (DSS or TSAT) showed no influence on ELP surface load obtained by reaction strategy a and b, DSS allowed the immobilization of considerable higher ELP amounts on PLLA independent on the applied surface activation in comparison to TSAT and no crosslinker applying reaction strategy c (Figure 3a, 3b). Estimated ELP crosslinking degrees by a CBQCA-fluorescence assay and contact angle measurements, underline this observation by a higher abundance of unreacted primary amino groups in ELP after crosslinking with TSAT than with DSS (88% vs. 17% with regard to non-crosslinked ELP, see Figure 4a) and a more distinct change in contact angle with regard to PLLA surfaces with nonspecifically adsorbed ELP (see Table 2), respectively. Representative ESEM micrographs of a cross section and a top view of NH₃-plasma-activated PLLA surfaces after ELP immobilization via reaction strategy c without crosslinking and applying DSS as crosslinker are shown in Figure 4b. The deposition of a thin protein layer using DSS as crosslinker can be visualized especially in the cross section (see Figure 4b, a) while hardly any deposit is visible on surfaces, omitting the addition of DSS (Figure 4b, b). Application of TSAT let to a comparable appearance of PLLA surfaces in electron microscopy (picture not shown). White particles, observed in top views of all surfaces (see Figure 4b, b-d), even on untreated PLLA, likely correspond to dust or salt deposition from used buffers.

Figure 4. a) Evaluation of loss of amino groups of ELP during crosslinking (DSS, TSAT) in solution by CBQCA labelling measured via fluorescence spectroscopy ($\lambda_{exc}=485\text{ nm}$, $\lambda_{em}=520\text{ nm}$) and impact of crosslinker on ELP surface load applying reaction strategy c on NH₃-plasma activated PLLA. Bars show mean \pm SD of at least 5 experiments. b) Representative ESEM micrographs of a cross section (a, b) and a top view (c, d) of NH₃-plasma-activated PLLA surfaces after ELP immobilization via reaction strategy c without crosslinking (a, c) and applying DSS as crosslinker (b, d).

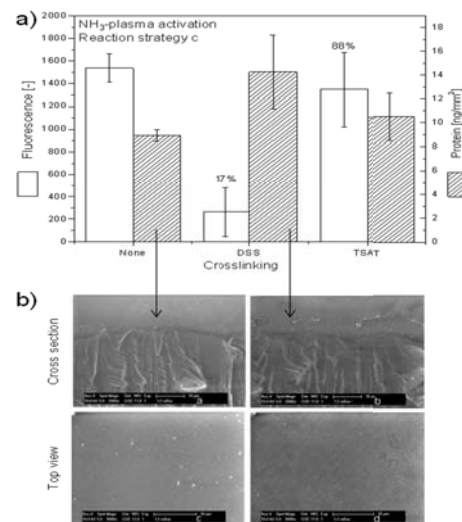


Table 2. Contact angles of the modified PLLA surfaces (n = 5) in preparation for and after nonspecific adsorption and covalent attachment of ELP via DSS and TSAT, Data shown as mean \pm SD

	- ELP	+ ELP
PLLA	75 \pm 5	<i>n.d.</i>
PLLA-NH3	52 \pm 3	54 \pm 3
PLLA-NH3-DSS	54 \pm 4	63 \pm 6
PLLA-NH3-TSAT	51 \pm 5	57 \pm 4

3.5 Assessment of the cellular behavior on PLLA surfaces

In order to assess the impact of ELP modification of biodegradable PLLA surfaces on the surface attachment of HCAEC and HCASMC *in vitro*, cellular viability and proliferation assays were performed on untreated PLLA, NH₃-plasma-activated PLLA (NH₃-Plasma), and ELP covalently coupled to NH₃-plasma-activated PLLA (X₂-RGD-X₂ + DSS) using DSS as crosslinker and reaction strategy c (surface attachment of the crosslinker prior to ELP binding and crosslinking). Further surface activation procedures, reaction strategies and crosslinker described above were not investigated due to lower observed ELP surface loads. Instead, we included NH₃-plasma activated PLLA samples with physically adsorbed ELP (X₂-RGD-X₂) in order to study the influence of the covalent binding.

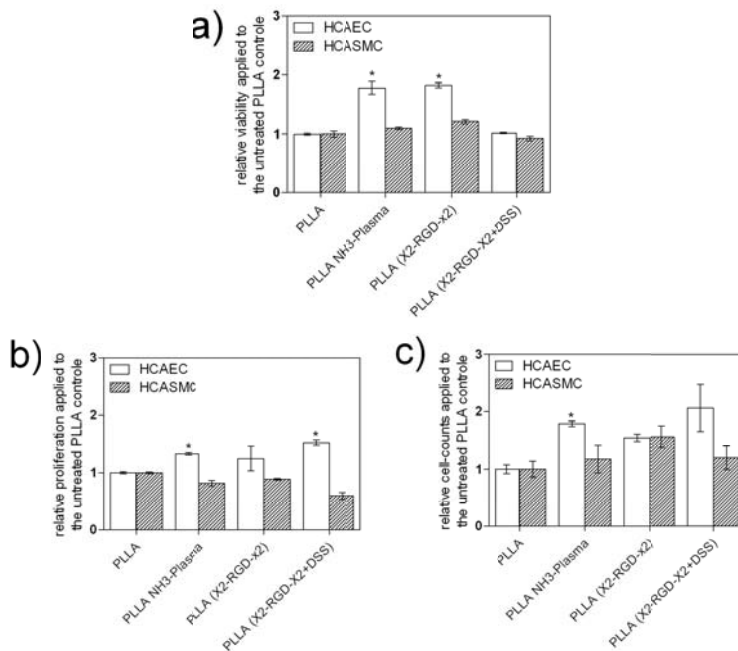


Figure 5. Influence of PLLA untreated, PLLA-NH₃-plasma activated, PLLA-X₂-RGD-X₂ non-crosslinked and PLLA-X₂-RGD-X₂ crosslinked with DSS surfaces on viability, proliferation and cell number of human coronary artery endothelial cells (HCAEC) and smooth muscle cells (HCASMC) cultivated for 48 h, respectively. a) Viability of HCAEC and HCASMC at these surfaces by using resazurin assay. b) Proliferation of HCAEC and HCASMC at PLLA surfaces was measured by BrdU labeling. c) Cell number of HCAEC and HCASMC at these surfaces by DAPI staining and fluorescence microscopy. Data are represented as mean ± SEM, n = 3; normalized to PLLA untreated.

Results revealed no influence of any investigated surface modification on cellular viability of HCASMC (mean viability normalized to PLLA in cells ± SEM: PLLA-NH₃-Plasma: 1.11 ± 0.03; X₂-RGD-X₂: 1.22 ± 0.05; X₂-RGD-X₂ + DSS: 0.93 ± 0.05; see Figure 5a). However, for endothelial cells we observed significant higher enzymatic activity as detected by resazurin metabolism on PLLA after NH₃-plasma activation and ELP deposition via physical adsorption (PLLA-NH₃-Plasma: 1.78 ± 0.15; PLLA-X₂-RGD-X₂: 1.82 ± 0.06; *P* < .05; see Figure 5a). Interestingly, covalent ELP coupling to PLLA via DSS shows no effect (X₂-RGD-X₂ + DSS: 1.02 ± 0.02, see Figure 5a). In addition to the above mentioned assessment of viability, experiments to detect the proliferative behavior of HCASMC and HCAEC cultured on modified PLLA surfaces were performed. Detection of BrdU-fluorescence incorporated within DNA as marker of proliferation revealed no significant change in proliferation of HCASMC on PLLA after NH₃-plasma activation and ELP deposition via physical adsorption (mean proliferation normalized to PLLA: cells ± SEM; NH₃-Plasma 0.82 ± 0.07; X₂-RGD-X₂: 0.89 ± 0.02; see Figure 5b). Moreover, after covalent ELP coupling to PLLA via DSS BrdU fluorescence was even significantly decreased (0.59 ± 0.09, *P* < .05; Figure 5b). In contrast to HCASMC, HCAEC proliferate better on PLLA after all investigated modifications (PLLA-NH₃-Plasma: 1.33 ± 0.03, *P* < .05; X₂-RGD-X₂: 1.25 ± 0.31; X₂-RGD-X₂ + DSS 1.53 ± 0.07, *P* < .05; see Figure 5b).

In order to verify the observed BrdU-based proliferation data, we counted cells after staining with DAPI and visualization by fluorescence microscopy. Quantification of HCASMC cultured on modified PLLA surfaces showed no significant change in cell numbers compared to cells growing on untreated PLLA (mean cell count normalized to PLLA: cells ± SEM; PLLA-NH₃-Plasma 1.17 ± 0.34; X₂-RGD-X₂: 1.55 ± 0.27; X₂-RGD-X₂ + DSS: 1.18 ± 0.29; see Figure 5c). In contrast, culture of HCAEC on PLLA after modification led to higher cell numbers compared to untreated PLLA surfaces (PLLA-NH₃: 1.79 ± 0.07, *P* < .05; X₂-RGD-X₂: 1.55 ± 0.09; X₂-RGD-X₂ + DSS: 2.06 ± 0.59; see Figure 5c). Representative images of the digitalized DAPI staining of HCAEC and HCASMC are shown in Figure 6.

4 Discussions

With the purpose of developing a coating material for biodegradable endovascular implants, which not only mimics the natural surroundings of endothelial cells but also provides additional cellular recognition motifs in order to further enhance endothelialization without inducing SMC proliferation, we synthesized an ELP including the fibronectin- and collagen type I-derived peptides RGD, CS5 and P15. Elastin, which forms the internal elastic lamina in the region of endothelial cells^[14], was thus combined with recognition motifs known to provide cellular adhesion. The CS5 peptides was first introduced by the group of Liu *et al.* demonstrating a significant enhancement of adhesion and spreading of endothelial cells^[7]. Furthermore, it was demonstrated that the combination of CS5 with elastin domains promotes cell proliferation, angiogenesis, tissue repairing and healing^[33]. Additionally incorporating the collagen type I-derived peptide P15 is based on the study of Li *et al.* stating an effective promotion of the adhesion and proliferation of human umbilical endothelial cells with fewer efficiency on smooth muscle cells^[34]. We hence estimated that the combination of RGD, CS5 and P15 sequences with the elastin-derived pentapeptides VPGIG and VPGKG in one protein might be a promising coating material for biodegradable endovascular grafts.

The synthesis of the ELP sequence could be successfully established and verified by electrophoresis (SDS-PAGE) with Coomassie staining (see Figure 2c). The characteristics of elastin being soluble at lower temperatures (< 35°C) and not soluble at higher temperatures including the physiological body temperature of 37°C was used during the isolation procedure of the elastin like protein after *in vitro* production^[31]. After the synthesis of the novel coating material we focused on its immobilization to PLLA as model biodegradable implant surface. Within this context, we investigated several surface activation procedures, reaction strategies and crosslinker with regard to achievable surface loads of ELP. Surface activation of PLLA is necessary in order to allow covalent attachment of ELP. Within this study, we investigated three different surface activation treatments: a non-thermal treatment with ammonia plasma (NH₃-plasma)^[35], aminolysis with HMDA^[36] and a combination of an oxygen plasma (O₂-plasma) treatment and silanization with APTES^[37]. It is well known that plasma treatment is restricted to the surface with a very low penetration depth of only 10 nm^[35], while aminolysis has been reported to occur as deep as 50 µm^[36], and silanization often yields multilayer formation due to condensation of hydrolyzed silanol groups between each other^[38]. Hence, one might assume a considerable higher density of amino groups on aminolyzed and silanized PLLA, which we could indeed evidence in a previous study^[32]. Nevertheless, we here detect no significant impact of the applied surface activation on ELP loading (see Figure 3a, 3b). It hence seems reasonable to suppose that already the amount of generated amino groups on the surfaces largely exceeds the amount of immobilizable ELP. In contrast to the surface activation procedure, the applied reaction strategy revealed a significant impact on obtained ELP surface loads. Efficient surface attachment seems to afford a first coupling of the crosslinker to the surface and a subsequent ELP attachment (see Figure 1, reaction strategy c). ELP crosslinking prior to surface attachment (see Figure 1, reaction strategy a) and simultaneous ELP crosslinking and surface attachment (see Figure 1, reaction strategy b) resulted in significantly lower ELP surface loads (see Figure 3a, 3c), comparable to the amount of ELP immobilized via physical adsorption without the application of crosslinker. It hence seems that the crosslinker preferentially reacts with the free amino groups of the protein in solution instead of the amino groups at the PLLA surface. We dedicate this observation to the higher probability of collision in solution by reduced sterical hindrance. This assumption can be moreover underlined by the fact, that the nature of the applied crosslinker has no impact using reaction strategy a and b, while the application of DSS resulted in higher ELP surface loads compared to TSAT using reaction strategy c (see Figure 4c). This most obviously results from a higher ELP crosslinking, evidenced by a lower abundance of unreacted primary amine groups (see Figure 4a), and the associated deposition of ELP multilayers. The difference in the crosslinking degree might be explained by the structure of the two crosslinker. While TSAT bears three amine-reactive NHS-esters at short spacer arms, possibly poorly accessibly by the terminal amino groups of ELP, DSS is bifunctional with a flexible C6 spacer, favoring ELP crosslinking. Representative electron micrographs, shown in Figure 4b, nicely underline the above stated deposition of ELP multilayer. While a thin deposited layer of protein is visible using DSS, hardly any deposit is visible on surfaces, applying TSAT or omitting the addition of crosslinker (see Figure 4b). For the *in vitro* investigation of ELP-modified PLLA surfaces with regard to attachment, viability and proliferation of

HCAEC and HCASMC, we applied the functionalization strategy with highest achievable ELP surface loads. Thus, DSS was reacted with amino-modified PLLA surfaces prior to ELP binding and crosslinking (X_2 -RGD- X_2 + DSS). Amino activation of PLLA, which did not evidence any impact on ELP surface deposition, was carried out via NH_3 -plasma treatment, which was less time consuming than further investigated wet-chemical treatments. In order to observe the influence of the covalent binding, we included NH_3 -plasma activated PLLA samples with physically adsorbed ELP (X_2 -RGD- X_2) and as reference untreated PLLA and NH_3 -plasma-activated PLLA (NH_3 -Plasma). Stability of the covalent immobilization during the time interval of cell experiments was evidenced via lacking ELP in the supernatant as determined via BCA assay.

Our study revealed enhanced viability, proliferation and cell counts of HCAEC on the NH_3 -plasma activated PLLA-surfaces (Figure 5, a-c). This plasma activation is necessary to bind the X_2 -RGD- X_2 peptide via the lysine residue to the PLLA. However, it has to be noted that the surface modification induced by plasma activation is a temporary state and is not applicable for stable induction of endothelialization^[39]. Physical adsorption of ELP to PLLA resulted in no significantly increased proliferation and attachment of HCAEC (see Figure 5b, 5c). This might be explained by the detachment of the peptides from the coating, or a disadvantageous arrangement of immobilized peptides. In this context, it has been reported in literature before, that the physical adsorption of ELP to substrates represented an inherently limiting characteristic due to coating instability, resulting in a loss of the anti-thrombotic effect^[25]. In contrast, covalently immobilized ELP allowed the generation of PLLA surfaces with improved endothelialization without enhancing smooth muscle cell attachment and proliferation (see Figure 5b, 5c). This underlines on one hand the necessity of the covalent binding and on the other hand the successful EC-selective effect of the adhesion motifs RGD, CS5 and P15. The synthesized ELP seems hence a promising coating material for biodegradable endovascular implants.

5 Conclusions

Taken together, we developed and investigated different strategies for modification of PLLA with modified ELP, combining both an integer ECM component with functional peptide domains, with the purpose of creating an optimal environment for endothelialization and thereby suppressing the surface-induced foreign body reaction. Concerning the functionalization strategy, our findings show that the kind of amino activation of PLLA does not have considerable influence on the amount of deposited ELP, while the reaction sequence and the used crosslinker present a determining factor in ELP immobilization. With regard to biological functionality, we could show that in contrast to both, untreated PLLA and PLLA modified with physically adsorbed ELP, PLLA with covalently crosslinked ELP promoted endothelial cell adherence and proliferation without favoring smooth muscle cell proliferation. Hence, the ELP-modified surfaces are promising for the generation of endovascular implants with rapid endothelialization, associated with a lower risk of occlusion and thrombosis. However, the evaluation of thrombogenicity of the novel surfaces needs further research. In a wider context, we assume that the use of modified ELP-coatings on biodegradable polymers might allow for the establishment of implant surfaces with tailored surface functionality by an adapted design of the ELP construct. Provided information within the present study can be used as basis for the immobilization procedure of such ELP constructs.

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