Title: Functionalization of CoCr surfaces with cell adhesive peptides to promote HUVECs adhesion, proliferation and migration.

Authors: Maria Isabel Castellanos^{1,2}, Carlos Mas-Moruno^{1,2}, Anna Grau^{1,2}, Xavier Serra-Picamal^{4,5,6}, Xavier Trepats^{4,5,6}, Fernando Albericio⁷, Michael Joner^{8,9}, Francisco Javier Gil^{1,2}, Jose Maria Manero^{1,2}, Marta Pegueroles^{1,2}.*

Affiliations: ¹ Biomaterials, Biomechanics and Tissue Engineering Group, Department of Materials Science and Metallurgical Engineering, Technical University of Catalonia (UPC), ETSEIB, 08028 Barcelona, Spain
² Centre for Research in NanoEngineering (CRNE), UPC, 08028 Barcelona, Spain
⁴ Institute for Bioengineering of Catalonia (IBEC), 08028 Barcelona, Spain
⁵ University of Barcelona and CIBER-BBN, 08036 Barcelona, Spain.
⁶ Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Spain
⁷ Department of Chemistry, University of Barcelona, CIBER-BBN, 08028 Barcelona, Spain.
⁸ Department of Cardiology, Deutsches Herzzentrum München, 80636 Munich, Germany.
⁹ CVPath Institute, Gaithersburg, MD 20878, USA

Corr. Author: Marta Pegueroles, PhD
marta.pegueroles@upc.edu
Department of Materials Science and Metallurgical Engineering
Av. Diagonal, 647
Barcelona, 08028
Spain.

Maria Isabel Castellanos: maria.isabel.castellanos@upc.edu
Carlos Mas-Moruno: carles.mas.moruno@upc.edu
Anna Grau: agraugar@gmail.com
Xavier Serra-Picamal: xserrapicamal@gmail.com
Xavier Trepats: xtrepats@ub.edu
Fernando Albericio: fernando.albericio@irbbarcelona.org
Michael Joner: michaeljoner@me.com
Francisco Javier Gil: francesc.xavier.gil@upc.edu
Jose Maria Manero: jose.maria.manero@upc.edu
Marta Pegueroles: marta.pegueroles@upc.edu

Abstract.

Biomimetic surface modification with peptides that have specific cell-binding moieties is a promising approach to improve endothelialization of metal-based stents. In this study, we functionalized CoCr surfaces with RGDS, REDV, YIGSR peptides and their combinations to promote endothelial cells (ECs) adhesion, proliferation and migration. An extensive characterization of the functionalized surfaces was performed by XPS analysis, surface charge and quartz crystal microbalance with dissipation monitoring (QCM-D), which demonstrated the successful immobilization of the peptides to the surface. Cell studies demonstrated that the covalent functionalization of CoCr surfaces with an equimolar combination of RGDS and YIGSR represents the most powerful strategy to enhance the early stages of ECs adhesion and proliferation, indicating a positive synergistic effect between the two-peptide motifs. Moreover, the YIGSR motif improved EC migration compared to control samples. Although these peptide sequences slightly increased smooth muscle cells (SMCs) adhesion, these values were ten times lower than those observed for ECs. The combination of RGDS with the REDV sequence did not show synergistic effects in promoting the adhesion or proliferation of ECs. The strategy presented in this study holds great potential to overcome clinical limitations of current metal stents by enhancing their capacity to support surface endothelialization.

Keywords

CoCr alloy, surface functionalization, cell adhesive peptides, HUVEC migration, endothelialization, SMCs adhesion

Functionalization of CoCr surfaces with cell adhesive peptides to promote HUVECs adhesion, proliferation and migration.

1. Introduction

Intracoronary stenting is a common practice in interventional cardiology to treat blood vessels with reduced flow due to atherosclerosis. The stent is a metallic mesh tube, which is expanded in the narrowed artery recovering blood flow. Restenosis due to excessive proliferation of smooth muscle cells (SMCs), neointimal hyperplasia, has been a relatively common complication associated with bare metal stents (BMS) after implantation [1–3]. Currently, drug-eluting stents (DES) are polymer-coated stents, which release anti-proliferative drugs that successfully minimize restenosis. However, the drugs not only limit SMCs proliferation but also delay endothelialization of the device after implantation [4]. This may result in higher risk of late in-stent thrombosis, requiring long-term anti-platelet aggregation therapies after implantation. Thus, new surface functionalization should combine the benefit of decreasing acute restenosis, while keeping low levels of thrombogenicity and recovering artery's function.

Biomimetic surface modification with proteins or peptides that have specific cell-binding moieties is a promising approach to improve endothelialization [5,6]. In this regard, stent functionalization with proteins [7], biopolymers [8,9], or with biologically relevant peptide sequences[4,10,11] has been shown to promote endothelial cells (ECs) adhesion. Proteins purified from the extracellular matrix (ECM) have a strong biological efficiency because their native structure and synergistic sequences are preserved. However, this strategy may present problems of immunogenicity, denaturation after sterilization and poor control of functionalization. An alternative to such shortcomings is the use of short synthetic peptides, which are non-immunogenic, easy to purify and can be immobilized on surfaces in a controlled manner [12].

Surfaces modified with bioactive cell-adhesive peptides have shown to mediate anchorage-dependent cell functions, including adhesion, migration and proliferation[13–16]. A prominent example is illustrated by the well-known adhesive sequence RGDS (Arg-Gly-Asp-Ser), present in fibronectin and other ECM proteins, which is recognized as the minimal amino acid sequence necessary to promote cell adhesion [16]. This sequence has been applied to different surfaces including poly(ethylene glycol), polyethylene terephthalate and polytetrafluoroethylene [17,18], poly(L-lactic acid) scaffold [19] and titanium surfaces and its alloys [20,21], in order to improve cell attachment and the bioactivity of the surfaces. Besides the RGDS motif, other adhesive ligands such as the laminin derived YIGSR (Tyr-Ile-Gly-Ser-Arg) sequence[22,23] have been shown to promote EC adhesion and migration without enhancing platelet adhesion [24]. For instance, this peptide has enhanced the adhesion of ECs in hydrogels [13,25], PET [26], polyurethane [27,28], poly(2-hydroxyethyl methacrylate) [29] and decellularized scaffolds [30]. Finally, another cell adhesive sequence found in fibronectin, the REDV (Arg-Glu-Asp-Val) peptide, which targets ECs via the integrin $\alpha_4\beta_1$, has been reported to selectively promote EC adhesion and spreading over SMCs and platelets [22,31]. The REDV peptide has been immobilized onto several polymers such as poly(ethylene glycol) diacrylate hidrogels [32], PET surfaces [33], zwitterionic polycarboxybetaine copolymers [34], and polysaccharide hydrogels [35], aiming at improving the capacity of these surfaces to support endothelialization.

Thus, combining the **RGDS** sequence with either REDV or YIGSR motifs could potentially lead to improved values of EC adhesion. In this regard, the ability of the peptides YIGSR, PHSRN and **RGDS**, and their combinations, to selectively affect the adhesion and migration of ECs and SMCs onto polyethylene glycol (PEG) had been evaluated by Fittkau *et al* [13]. However, the effect of immobilizing mixtures of **RGDS**, YIGSR and REDV peptides onto CoCr alloys, which are widely used as cardiovascular stents, has not been yet explored. The extent and quality of endothelialization strongly depends on the interactions established between functionalized surfaces and ECs. Such process should enhance ECs adhesion and migration [14], but ideally also reduce SMCs migration and proliferation, and prevent platelet adhesion and thrombogenicity [4].

In this work we evaluate the use of equimolar combinations of specific cell adhesive peptides to improve the

endothelialization of CoCr surfaces for cardiovascular applications. To this end, CoCr surfaces were functionalized with the different oligopeptides, and after a thorough characterization of the physicochemical properties of the surfaces, the adhesion, proliferation and migration of ECs, as well as the adhesion of SMCs, were examined at the *in vitro* level.

2. Materials and Methods

2.1 Materials

2.1.1 Metallic surfaces

CoCr alloy (ASTM F90: Co–20Cr–14.6W–10.8Ni–2.5Fe–1.5Mn) (Technalloy, Barcelona, Spain) disks of 8,5 mm diameter and 2 mm thick were subsequently abraded with silicon carbide papers of decreasing grit size (P240, P400, P600, P800 and P1200) and finally polished with suspensions of 1 µm and 0,05 µm alumina powder in distilled water. Prior to the surface treatments, all samples were ultrasonically cleaned with ethanol, distilled water and acetone for 5 min each.

2.1.2 Solid-phase peptide synthesis

The linear peptides **RGDS**, REDV and YIGSR (Fig. 1(a) and Supplementary Material Table S1) were manually synthesized in solid-phase following the Fmoc/tBu strategy and using 2-chlorotrityl-chloride resin (200 mg, loading of 1.0 mmol/g) as previously reported [36]. Briefly, Fmoc-L-amino acids (4 equiv) were sequentially coupled with either ethyl 2-cyano-2-(hydroxyimino)acetate (OxymaPure) (4 equiv), and N,N'-diisopropylcarbodiimide (DIC) (4 equiv); or N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridino-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate (HATU) (4 equiv) and N,N-diisopropylethylamine (DIEA) (8 equiv) as coupling systems. The efficiency of each reaction was monitored using the Kaiser test and/or by HPLC analysis. Once the peptide sequences were completed, cleavage from the resin was accomplished upon treatment with trifluoroacetic acid (TFA)/water/triisopropylsilane (TIS) (85:10:5, v/v/v) for 1-2 h in the presence of small amounts of dithiothreitol (DTT). The peptides were purified by semi-preparative HPLC and characterized by analytical HPLC and MALDI-TOF (Supplementary Material Table S1). All chemicals required for the synthesis, including resins, Fmoc-L-amino acids and coupling reagents, were obtained from Iris Biotech GmbH (Germany) and Sigma-Aldrich (USA).

2.2 Surface functionalization

The peptides covalent immobilization onto CoCr surfaces was achieved through a three-step strategy consisting of (1) activation, (2) silanization and (3) peptide immobilization. Alternatively, peptides were deposited by simple physical adsorption on the CoCr surfaces. The process of CoCr surfaces functionalization is summarized in Fig. 1(b).

2.2.1 Surface activation

The surface of CoCr samples was activated by basic etching with 5 M NaOH solution during 2 h at room temperature (RT) (samples **NA**). Samples treated with the alkaline solution were cleaned twice in distilled water during 30 min. Non-activated CoCr samples were used as controls (**CT**).

2.2.2 Silanization

Activated samples were silanized by immersing the substrates in a 10 ml solution of 0,5 M 3-chloropropyltriethoxysilane (CPTES) (Sigma-Aldrich) and 0.05 M N,N-diisopropylethylamine (DIEA) in anhydrous toluene under nitrogen atmosphere for 1 h at 90°C under vigorous stirring. After silanization was completed, the discs were ultrasonically washed with cyclohexane, isopropanol, distilled water, and acetone, for 15 min each, and finally dried with nitrogen. The CPTES-modified substrates were stored under vacuum. Silanized samples were coded as **NA-CP**.

2.2.3 Peptide attachment

Finally, 100 µl of peptide solutions (RGDS, REDV and YIGSR) at 100 µM in PBS at pH 13.0 (adjusted with Na₂CO₃) and their combinations (1:1) were deposited on the CPTES-grafted surfaces overnight at RT. The

immobilization of the peptides by physical adsorption was done under the same conditions but using PBS at pH 7.0 instead. After the immobilization protocol, samples were washed three times with distilled water. Prior to cell adhesion assays, functionalized samples were blocked for 1 h at 37 °C with 5% (w/v) bovine serum albumin (BSA) in PBS in order to reduce non-specific interactions of the cells with the surface [8,36,41,42]. Functionalized samples were coded as follows: a) Peptide physisorbed surfaces (**CT-peptide**: CT-RGDS, CT-REDV, CT-YIGSR, CT-RGDS+REDV and CT-RGDS+YIGSR); b) Peptide covalently bound surfaces (**NA-CP-peptide**: NA-CP-RGDS, NA-CP-REDV, NA-CP-YIGSR, NA-CP-RGDS+REDV, NA-CP-RGDS+YIGSR) Fig. 1(b).

2.3 Surface characterization

2.3.1 Chemical composition

The chemical analysis of the surface through the process of functionalization was investigated by X-ray photoelectron spectroscopy (XPS) using an XPS equipment (SPECS Surface Nano Analysis GmbH, Germany), equipped with an Al anode XR50 source operating at 150 W and a Phoibos 150 MCD-9 detector XP.

The elements present on the surface were qualitatively evaluated by low-resolution survey spectra, whereas high-resolution spectra of determined elements (C 1s, N 1s, O 1s, Cr 2p, Co 2p, Si 2s, Cl 2p) were recorded with pass energy of 35 eV at 0.1 eV steps at a pressure below 5×10^{-9} mbar. Binding energies of the peaks were referenced to the C 1s peak maximum at 284.8 eV. CasaXPS spectrum software (Casa Software Ltd. UK) was used to analyse and deconvolute XPS spectra.

2.3.2 Surface charge

A streaming potential instrument (Surpass Electrokinetic Analyzer, Anton Paar, Austria) with an adjustable gap cell was used to determine the zeta-potential (ζ) of the surfaces. Automatic titrations were performed using a 1 mM KCl solution as electrolyte and the pressure ramp run up to a maximum pressure of 500-mbar. The electrolyte solution was initially adjusted to pH = 9.0 using 0.1 M KOH and titrated by adding 0.1 M HCl down to pH 3.0. The isoelectric point (IEP) and the surface charge at pH 7.4 were determined using VisioLab software (Anton Paar, Austria).

2.3.3 Quartz crystal microbalance with monitoring dissipation (QCM-D)

The quantification and characterization of the peptide layer attached to the surfaces was performed using a QCM-D (D-300, Q-Sense, Sweden). Co-20Cr-15W-10Ni-1.5Mn (QSX999) sensors were purchased at Q-Sense. The fundamental mode of the sensors was at 4.95 MHz. Prior to use, the sensors were cleaned as follows: (1) 10 min sonication with ethanol; (2) 10 min sonication with acetone; (3) 10 min sonication with MiliQ ultrapure water; and (4) a 10 min-treatment in a UV/ozon chamber (BioForce Nanosciences, USA). Measurements were performed at 25°C by monitoring changes in frequency, Δf (Hz), and dissipation, ΔD ($\times 10^{-6}$), in real-time using Qsoft software (Q-Sense). Raw data was analyzed using QTools software (Q-Sense). Frequency and dissipation curves were fitted to a Voigt viscoelastic model to yield the adsorbed mass and thickness of the peptide layer, as well as kinetic information. The description of the Voigt model and details on its implementation using a QCM-D are reported elsewhere [43].

To monitor the adsorption of the peptides, first the baseline was completely stabilized with PBS for 30-60 min, and then the peptides (**RGDS**, **REDV**, **YIGSR**) were introduced at 100 μ M concentrations and maintained in the sensor chamber for 120 min. Finally, the surfaces were rinsed with PBS for 10 min.

2.4 In vitro cell studies

2.4.1 Cells

Human umbilical vein endothelial cells (HUVECs) (Lonza Group Ltd., Switzerland) were grown in EC basal medium (EBM®) supplemented with 5% (v/v) fetal bovine serum (FBS), 0.1% (v/v) gentamicin sulphate amphotericin (GA-1000), 0.4% (v/v) recombinant human fibroblast growth factor (rhFGF), 0.1% (v/v) recombinant human epidermal growth factor (rhEGF), 0.1% (v/v) ascorbic acid, 0.1% (v/v) vascular endothelial growth factor

(VEGF), 0.1% (v/v) recombinant Long R insulin (R3-IGF-1) and 0.04% (v/v) hydrocortisone (EBM and all supplements were obtained from Lonza). Human vascular coronary artery smooth muscle cells (CASMCs) (Lonza) were grown in SMC basal medium (SmBm®) supplemented with 5% FBS, 0.1% GA-1000, 0.1% rhEGF, 0.2% rhFGF and 0.1% Insulin (Lonza). Both cell types were incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO₂. The culture medium was changed every 2 days. Cell culture was performed in Nunc cell flasks (Thermo Scientific, Denmark) pre-coated with 1 µg/ml of fibronectin (FN) in phosphate buffered saline (PBS). Upon reaching 70-90% confluence, cells were harvested by trypsin/EDTA (Sigma-Aldrich), centrifuged and subcultured into a new flask or re-suspended in a serum-free medium and used in cell assays. All experiments were conducted using HUVECs at passages 4 to 8 [37,38] and CASMCs at passages 4 to 6 [39,40].

2.4.2 Fluorescein diacetate (FDA)-staining

FDA-staining (Sigma-Aldrich) was used to conduct a preliminary screening and determine the best spacer system in the cell adhesive peptides (Ahx₃ vs. Gly₃). To this end, HUVECs were seeded on the functionalized surfaces at a density of 2 x 10⁴ cells/disk and incubated in serum-free medium. After 4 h of cell culture, FDA was directly added to the medium to reach a final concentration of 1 µg/ml, and left to react for 3 min. Living cells, the only ones able to convert the dye into a fluorescent analogue, were analyzed with an inverted fluorescent microscope (Upright Microscope Nikon E1000, Japan).

2.4.3 LDH metabolic activity

The number of HUVECs on the biofunctionalized surfaces was quantified by measuring the released active lactate dehydrogenase (LDH) enzyme after 4 h of incubation using the cytotoxicity detection kit LDH (Roche Applied Science, Germany). HUVECs were seeded on CoCr samples at a density of 2 x 10⁴ cells/disk and incubated with serum-free medium. Cells were lysed with 300 µl of mammalian protein extraction reaction (M-PER) (Pierce, USA) per well and the release of LDH was measured with a spectrophotometer (Infinite M200 Pro, Tecan, Switzerland) at 490 nm. The quantity of the LDH was determined by comparison to a standard curve prepared with known cell concentrations (0, 5000, 10000, 15000, 20000, 25000 and 30000 cells/well) under identical conditions.

2.4.4 Cellular adhesion analysis by immunofluorescence

The adhesion and spreading of HUVECs and CASMCs on the biofunctionalized surfaces was analyzed by means of immunofluorescence staining of actin fibers and nuclei. Cells were cultured in serum-free media at a concentration of 2 x 10⁴ cells/disk during 4 hours. After this time, cells attached to the metal surfaces were fixed for 10 min with 3,7 % (w/v) paraformaldehyde (PFA) (Sigma-Aldrich) in PBS, and permeabilized with 100 µL/disk of 0,05% (w/v) triton X-100 (Sigma-Aldrich) in PBS for 15 min. Washings between steps were all performed with PBS for 3 x 5 min. Next, samples were incubated with 100 µl of phalloidin (1:300) (AlexaFluor 546, ThermoFisher) in PBS for 30 min, and in a final step, nuclei of cells were stained with 50 µl of DAPI (1:1000) in PBS for 10 min in the dark. Finally the samples were mounted with aqueous mounting medium DAKO (Agilent Technologies, USA) on microscope slides and were viewed and photographed by fluorescent microscopy Olympus B2-2 (Olympus, Japan).

The microscope settings were kept constant allowing comparative measurements at several points on the surface. Different cell morphology parameters were studied by ImageJ-FIJI software (NIH, USA): total area occupied by cells and cell number normalized by the image area (cells/cm²).

2.4.5 Cell proliferation

To corroborate the biological effect of the peptides in cell growth, cell proliferation and inhibitory cell rate with soluble peptides assays were also conducted for HUVECs.

Cell proliferation onto modified CoCr surfaces, was analyzed by live cell imaging with an upright fluorescent microscope Olympus B2-2 (Olympus). HUVECs were labelled with the cell membrane fluorescent linker PKH67 (PKH67-GL, Sigma-Aldrich) 24 h prior to cell proliferation assays. Afterwards, 8·10³ labelled cells/well were seeded and incubated in serum free-medium for 4 h. For longer incubation times (24, 48 and 72 h) cells were cultured with complete medium.

For the inhibitory cell proliferation assay with soluble peptides, 8×10^3 cells/well were seeded onto tissue culture polystyrene (TCPS) with serum free-medium during 4 h. Then, the medium was changed to complete medium. Finally, after 20 h, soluble peptides were added at 100 μ M in fresh complete medium (timepoint 0). Cell inhibition rate was calculated after 24, 48 and 72 h of incubation with the soluble peptides, by counting the adhered cells onto TCPS by optical microscopy. Inhibition rates of proliferation were calculated in comparison to the cell proliferation values obtained for TCPS cultured in completed medium without peptides [44].

2.4.6 Wound healing migration studies

To investigate the process of wound healing and cell migration, HUVECs were first stained with the cell membrane labeling PKH67 reagent as previously described, and then seeded on the functionalized CoCr samples. The wound healing processes were reproduced by using an Ibidi culture-insert (Ibidi, Germany). Thus, a suspension of 2×10^4 labeled HUVECs were placed in both sides of the insert, and incubated for 4 h in serum free medium. After that, the insert was removed creating a cell-free gap of $500 \mu\text{m} \pm 50 \mu\text{m}$ and the well refreshed with complete medium. HUVEC cell migration was then monitored using up-right motorized microscope and NIS elements software (Nikon Eclipse Ni-E, Japan). Time-series recording started approximately 15 min after removing the insert-culture. The image acquisitions (fluorescence images) were manually acquired across all the generated-gaps. The experiment lasted 2 days, and acquisitions were taken every 4 h. To capture the complete picture of the closing gap, four images were acquired at 10X, approximately overlapping by 5%. The four images were manually stitched using ImageJ-FIJI software (NIH, USA). The contour of the monolayer was determined and the area occupied by the monolayer was measured for representative time points at 4 h, 24 h, and 48 h. The quantification of the recovery percentage was calculated at 4 h, 24 h and 48 h normalized to initial point at $t = 0$ h for each surface.

2.5 Statistical analysis

All data are represented as mean values $\pm$ standard deviations (SD). Experiments were done using triplicates of each condition and cellular studies were repeated at least in three independent assays to ensure reproducibility except wound healing migration studies that was repeated twice. ANOVA with multiple comparisons Fisher's, Tukey's and non-parametric Mann-Whitney U-test were used to determine statistically significant differences (p -value < 0.05 between the different groups). Statistical analysis was performed using Minitab software (Minitab Inc., USA) and SPSS Statistics 20 (IBM, USA).

3. Results and discussion

3.1 Design of the coating peptides

The design of the coating molecule is crucial for a successful surface functionalization and it should consider not only a bioactive cell-binding motif, but also an appropriate spacer and anchoring groups. Whereas the active sequence of the peptide determines its biofunctionality, the anchoring group should provide a strong and chemo selective binding of the molecule to the material. Finally, the spacer ensures an optimal presentation and accessibility of the cell-binding motif to cell-expressed receptors.

In our work, the selected bioactive sequences RGDS, REDV and YIGSR were synthesized containing either three units of aminohexanoic acid (Ahx_3) or three glycine residues (Gly_3), in order to determine the best spacer system to support EC adhesion (Fig.1(a) and Supplementary Material Table S1). The anchoring group was a thiol for all synthesized biomolecules.

The analysis of HUVEC adhesion after 4 h of incubation showed improved values of cell number and spreading onto CoCr surfaces functionalized with peptides bearing the Ahx_3 spacer compared to the Gly_3 (Supplementary Material Fig. S1). Although the differences were not statistically significant, the trend was evident for all surfaces, regardless of the method of immobilization. Therefore, an increased spacer length ($\text{Ahx}_3 > \text{Gly}_3$) was translated in improved cell responses, in agreement with previous studies [45,46]. Thus, Ahx_3 was selected as optimal spacer to conduct the rest

of experimental studies of this work.

3.2 CoCr biofunctionalization and surface characterization

To immobilize peptides onto CoCr surfaces, two approaches were considered: physical adsorption and covalent binding *via* silanization using CPTES as coupling agent (Fig. 1(b)). Physical adsorption commonly involves less stable bonding compared to silanization procedures where covalent attachment between surfaces and peptides is expected [42,47]. To facilitate silanization, CoCr surfaces were activated with NaOH, a treatment that generates accessible hydroxyl groups on the surface required for a successful silane coupling [8]. Moreover, NaOH etching on CoCr surfaces did not significantly alter surface morphology or mean-roughness values compared to non-treated surfaces [8]. Finally, the covalent binding of the peptides to the silanized surfaces was conducted in Na₂CO₃ buffer solution at pH=13.0 to enhance the direct nucleophilic substitution between the free thiol group of the peptides and the organosilanes.

Water contact angle measurements on the progressively modified CoCr surfaces indicated a successful peptide attachment (Fig. 2(a)). NaOH etching and further attachment of CPTES did not significantly change water contact angle compared to CT, as observed in previous studies [8]. In contrast, the immobilization of all peptides increased surface hydrophilicity compared to silanized (NA-CP) and plain (CT), surfaces. According to the Hopp and Woods scale for amino acid hydrophilicity [48], RGDS, REDV, and YIGSR peptides have hydrophilicity values of 6.3, 7.5 and -0.8, respectively. Therefore, it should not come as surprise that YIGSR-immobilized CoCr surfaces had a notably higher hydrophobicity than RGDS- and REDV-coated samples, which displayed similar wettability. Moreover, variances in the quantity of immobilized peptide onto CoCr surfaces, could also explain wettability differences.

Zeta-potential at pH 7.4 and isoelectric point (IEP) values for the physisorbed and silanized RGDS, REDV and YIGSR CoCr surfaces are presented in Fig. 2(a). The IEP is around pH ~ 3.5 for all series except for NA and CT-RGDS [49]. Fig. 2(b) shows representative zeta-potential curves as a function of the electrolyte pH. As expected, the obtained zeta-potential curves showed a steadily more negative zeta potential, as the solution was more alkaline. The surface charge is due to the preferential adsorption of negatively charged electrolyte anions, such as OH⁻ and Cl⁻. The zeta-potential at physiological pH (7.4) was found to be significantly more negative after surface activation with NaOH and subsequent silanization with CPTES (CT: -26.4 mV, NA: -31.1 mV, NA-CP: -38.7 mV) (Fig. 2(a)). These values were further modified upon peptide binding. After surface functionalization, differences in surface charge at pH 7.4 were detected depending on the nature of the immobilized peptide. Immobilization of RGDS peptide led to surfaces with lower electronegativity compared to REDV and YIGSR, being this effect more pronounced for silanized surfaces (NA-CP: -38.7 mV vs. NA-CP-RGDS: -18.0 mV). In contrast, REDV and YIGSR functionalized surfaces highly increased surface electronegativity for physisorbed CT surfaces (CT: -26.4 mV vs. CT- REDV: -38.1 mV; CT-YIGSR: -31.3 mV) and maintained electronegativity values for silanized surfaces (NA-CP: -38.7 mV vs. NA-CP-REDV: -34.6 mV and NA-CP-YIGSR: -32.9 mV) at physiological pH.

XPS results further confirmed the immobilization of the peptide coatings on the silanized surfaces. Table 1 displays the quantitative elemental composition of the XPS survey spectra of treated surfaces. Untreated CoCr surfaces showed characteristic C 1s (285 eV), Co 2p, Cr 2p and O 1s (530 eV) peaks. The activation process by NaOH etching did not alter the atomic concentration of C 1s, in correlation with wettability results and confirming that this activation treatment did not eliminate the carbonaceous species adsorbed from the environment. Nevertheless, a significant increase in the level of O 1s signal was found, probably indicating the successful formation of -OH groups at the surface level. The emergence of Cl 2p (NA-CP: 0.2%) and Si 2s (NA-CP: 0.8%) peaks on NA-CP samples confirmed the presence of CPTES and, thus, the presence of the silane on CoCr surfaces. Upon peptide immobilization, XPS analysis showed a strong increase of N 1s (1.2-15.5%) and C 1s (35.6-63.4%) signals, as well as a significant decrease in Co 2p, Cr 2p, and O 1s peaks. Noteworthy, peptide binding to CoCr surfaces resulted in a

reduction to almost undetectable levels of Si and Cl signals. This observation was mainly attributed to the release of chlorine ions during the nucleophilic attack of the thiol groups of the peptides and, also, to the full coverage of the underlying layer of organosilanes.

To further characterize the biofunctionalization of CoCr surfaces, high resolution C 1s (Supplementary Material Fig. S2(a)) and O 1s (Supplementary Material Fig. S2(b)) XPS spectra were recorded for CT, NA, CT-YIGSR and NA-CP-YIGSR surfaces. The C1s peak of untreated CoCr surfaces (CT) was deconvoluted in four different peaks with binding energies at 284.7, 282.7, 285.7 and 288.5 eV, and attributed to C-H/C-C, Cr_3C_2 , C-OH and C=O groups, respectively [36,37,39,40]. Functionalized surfaces (CT-YIGSR, NA-CP-YIGSR) exhibited the presence of the peptide/protein characteristic amide group, N-C=O, 288.1 eV. The higher proportion of amide signal was found for silanized samples, indicating a greater extent of peptide attachment for the silanization process. These results correlate also with the percentages of N 1s previously observed for these samples (Table 1). The analysis of the O 1s peak of CT surfaces revealed three contributions at 529.9, 531.5 and 532.8 eV assigned to O^{2-} , -OH and H_2O groups [40,43,50,51]. NaOH etching successfully activated CoCr surfaces as it increased the relative intensity of the hydroxyl group signal from 49.89%, for CT, up to 57.22%, for NA, while decreasing the signal of oxides and H_2O . Concerning the efficiency of peptide biofunctionalization, the deconvolution of O 1s peak showed that the intensity of O=C-N/OH signal, 530.9 eV, increased from CT (49.89%) to CT-YIGSR (66.41%) and from NA (57.22%) to NA-CP-YIGSR (87.51%). Thus, high resolution O1s analysis also confirms that silanization process immobilizes a highest amount of peptide than physical adsorption.

Taken together, all these results clearly indicate a successful immobilization of the cell adhesive peptides on CoCr surfaces by both physical adsorption and silanization.

3.3 Physisorbed peptide layer characteristics

In the present study, the initial real-time adsorption behavior of RGDS, REDV and YIGSR peptides on CoCr sensors was monitored by QCM-D. Table 2 indicates the characteristics of the adsorbed peptide layer in terms of thickness (nm), peptide density (ng/cm^2), viscosity (kg/ms) and shear elastic modulus (MPa). Surprisingly, peptide physisorption on CoCr sensors yielded a higher peptide thickness and density compared to silanized sensors. This trend was observed for all peptides of the study; indicating lower peptide immobilization efficiency through silanization. This observation seems to be in disagreement with our previous characterization by XPS. However, the fact that, in this case, QCM sensors were not activated before silanization, could explain such non-optimal peptide immobilization. Thus, as a proof of concept, CoCr sensors were activated by oxygen plasma prior to silanization with CPTES and finally functionalized with YIGSR (PL-CP-YIGSR). NaOH etching could not be used as an activation method since it damaged the surface of CoCr sensors. Interestingly, the activation of the sensors with oxygen plasma remarkably increased the efficiency of peptide immobilization. This result confirms that surface activation is an essential step prior to silanization to obtain a high efficiency of peptide attachment.

Moreover, the extent of thickness and surface mass density of immobilized peptide onto CoCr sensors was as follows: RGDS ($153,42 \text{ ng}/\text{cm}^2$) > REDV ($92,33 \text{ ng}/\text{cm}^2$) > YIGSR ($50,43 \text{ ng}/\text{cm}^2$) (Table 2). Such differences may be expected on the basis of distinct electrostatic interactions between the biomolecules and the CoCr sensors surface [52,53]. At physiological pH, REDV and RGDS peptides are negatively charged, but YIGSR total net charge is zero. This is in agreement with the higher amounts of adsorbed REDV and RGDS biomolecules onto CoCr sensors in comparison to YIGSR.

3.4 Adhesion of HUVECs and CASMCS

The adhesion of HUVECs and CASMCS onto the different substrates was investigated to understand the effect of surface biofunctionalization on the preferential cell behavior. Fluorescent micrographs and the amount of cells that adhered on modified CoCr surfaces after 4 h are shown in Fig. 3 for HUVECs and in Fig. 4 for CASMCS.

The activation process by NaOH etching did not enhance HUVECs adhesion compared to CT, while it considerably increased the amount of adhered CASMCs after 4 h of cell culture. Actually, the ratios of HUVEC to CASMC (number of HUVEC adhered/ number of CASMC adhered) on CT and NA were of 24.5 and 3.2, respectively. Thus, untreated CoCr surfaces seem to reduce the nonspecific adhesion of SMCs, while changes in the chemical composition of the surfaces after NaOH activation (i.e. the presence of a higher amount of hydroxyl groups on NA) show a positive effect on CASMCs adhesion.

Biofunctionalized surfaces clearly increased the number of adhered HUVECs independently of the type of immobilized peptide. Moreover, cell area was also enhanced on these surfaces, as visualized by immunofluorescence (Fig. 3(a) and Supplementary Material Table S2). Therefore, these results confirm that biofunctionalization with cell adhesive peptides is a good strategy to enhance endothelialization of CoCr surfaces. In detail, significant differences in HUVECs adhesion were detected depending on the peptide and immobilization strategy used. Physisorbed RGDS and YIGSR peptides significantly enhanced cell adhesion compared to REDV or the peptide combinations. In contrast, the equimolar combination of RGDS with YIGSR significantly improved cell adhesion on the silanized series. In fact, the highest level of cell adhesion on CoCr surfaces was obtained for NA-CP-RGDS+YIGSR samples, indicating a positive synergistic effect between these two peptide motifs. This behavior, however, was not detected on physisorbed CT-RGDS+YIGSR surfaces, probably related to a lower quantity of immobilized peptide onto the surface. As determined in XPS studies, the amount of immobilized peptide was higher for NA-CP-RGDS+YIGSR (N 1s: 7.6%) compared to CT-RGDS+YIGSR (N 1s: 6.3%).

The adhesion and morphology of CASMCs was also influenced by the immobilized peptide onto the surface. In particular, combinations of peptides on the silanized surfaces (NA-CP-RGDS+YIGSR and NA-CP-RGDS+REDV) and physisorption of YIGSR (CT-YIGSR) enhanced CASMCs adhesion compared to CT samples. Nevertheless, values of adhered SMCs were much lower than the number of adhered ECs.

As a whole, it should be highlighted that the amount of HUVECs attached onto all the modified surfaces after 4 h of incubation was one order of magnitude higher compared to the attachment of CASMCs. Such preferential binding of HUVECs is highly relevant because previous studies have demonstrated that greater numbers of SMCs compared to ECs in the vascular lumen leads to intimal hyperplasia [4,38,54]. The fact that the immobilization of RGDS (either by physisorption or silanization) on CoCr surfaces also exhibited selective cell adhesion capacity towards ECs came as a surprise. However, it is well known that both ECs and SMCs need an optimal **RGDS** concentration for effective adhesion [13,55] and it is plausible that in our surfaces the concentration required for satisfactory supporting SMC adhesion was not achieved. Moreover, the physicochemical properties of the CoCr alloy may also play an important role on such behavior.

3.5 Proliferation of HUVECs

Fig. 5(a) shows HUVECs proliferation on modified CoCr surfaces after 4, 24, 48 and 72 h of incubation. RGDS+YIGSR-coated surfaces, both physisorbed and silanized, significantly enhanced ECs proliferation in comparison to plain and NaOH etched CoCr samples, displaying higher numbers of cells at each time point. RGDS, YIGSR and RGDS+REDV-coated surfaces showed similar proliferation rates, which in general were higher than CT or NA. No clear trend was found when comparing physisorbed vs. covalently bonded samples. Moreover, to confirm the specificity of the peptides in promoting cell growth, the peptides were also incubated as soluble antagonists in the media and their inhibitory effect on cell proliferation studied (Fig. 5(b)). As shown in Fig. 5(b), the highest inhibitory rate corresponds to the RGDS+YIGSR mixture, which correlates with the most effective proliferation of HUVECs.

In summary, the results of adhesion and proliferation of HUVECs indicate that the immobilization of the equimolar mixture of RGDS+YIGSR peptides by means of silanization (NA-CP-RGDS+YIGSR) exhibits beneficial properties in terms of endothelialization capacity.

3.6 Cell migration studies

ECs have restricted capability for vessel and arterial regeneration, and therefore their migration from the surrounding tissue plays an important role in the endothelialization process [4,14]. For this reason, the migration of HUVECs onto CT, NA-CP-RGDS, NA-CP-REDV and NA-CP-YIGSR was monitored in serum-containing culture media by fluorescent microscopy (Fig. 6). Serum was necessary for maintaining cell viability and normal migration rates during the 48 h experiments. To reduce the effect of adsorbed serum-protein that could cover and, thus, attenuate the bioactivity of peptides immobilized on surfaces, cell attachment during the first 4 h was performed under serum-free conditions. The fluorescent microscopy images showed the total area occupied by HUVECs at each time-point of the study.

Cell migration was evaluated through the injury coverage ratio, which was defined as the percentage of injury recovered by cells (Fig. 6). After 4 h and 24 h, migration of HUVECs was visible on all surfaces, with the highest area of recovery found onto YIGSR silanized surfaces. Finally, after 48 h the injury model on NA-CP-YIGSR surfaces was totally covered by HUVECs indicating a complete healing. The REDV sequence showed a slightly lower capacity to support the migration of ECs but higher than the RGDS motif. The capacity of REDV to interact with specific integrin's expressed by HUVECs (e.g. $\alpha_4\beta_1$) could explain such observation. These results support the capacity of the sequence YIGSR in enhancing ECs adhesion and migration.

Overall, cell studies determined that the functionalization of CoCr surfaces with cell adhesive peptides represents a good strategy to enhance HUVECs adhesion, proliferation and migration. In particular, the combination of RGDS with YIGSR seems an optimal solution to enhance surface endothelialization. Although such combination of peptides also increased SMCs adhesion, which can lead to non-desired enhancement of intimal hyperplasia, these values of adhesion were ten times lower than those of EC adhesion. Therefore, the strategy presented in this study holds great potential to overcome clinical limitations of current stents by enhancing surface endothelialization.

Conclusions

The aim of the present work was to investigate and characterize the biofunctionalization of CoCr surfaces with cell adhesive peptides in order to enhance the adhesion, proliferation and migration of ECs onto these materials. The present study demonstrates the effectiveness of immobilizing RGDS, REDV and YIGSR peptides, and especially the equimolar combination of RGDS and YIGSR on CoCr surfaces to enhance ECs adhesion spreading without significantly enhancing SMCs. These results could be beneficial in the development of new strategies to increase the endothelialization of coronary stents, while preventing restenosis and thrombosis.

Acknowledgments

Authors acknowledge the Spanish Government for financial support through project [MAT 2012-30706], co-funded by the EU through European Regional Development Funds, and the Agency for Administration of University and Research Grants of the Government of Catalonia [2014 SGR 1333]. M.I.C. would like to thank the Generalitat de Catalunya for funding through a FI Scholarship. C. Mas-Moruno thanks the support of the Secretary for Universities and Research of the Ministry of Economy and Knowledge of the Government of Catalonia [2011-BP-B-00042] and the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme [FP7-PEOPLE-2012-CIG, REA Grant Agreement 321985].

References

- [1] P. Wenaweser, C. Rey, F.R. Eberli, M. Togni, D. Tüller, S. Locher, et al., Stent thrombosis following bare-metal stent implantation: success of emergency percutaneous coronary intervention and predictors of adverse outcome. *Eur. Heart J.* 26 (2005) 1180–7.
- [2] T. Simard, B. Hibbert, F.D. Ramirez, M. Froeschl, Y.-X. Chen, E.R. O'Brien, The evolution of coronary stents: a brief review., *Can. J. Cardiol.* 30 (2014) 35–45.
- [3] F. Nazneen, G. Herzog, D.W.M. Arrigan, N. Caplice, P. Benvenuto, P. Galvin, et al., Surface chemical and physical modification in stent technology for the treatment of coronary artery disease., *J. Biomed. Mater. Res. B. Appl. Biomater.* 100 (2012) 1989–2014.
- [4] Y. Wei, Y. Ji, L.-L. Xiao, Q. Lin, J. Xu, K. Ren, et al., Surface engineering of cardiovascular stent with endothelial cell selectivity for in vivo re-endothelialisation., *Biomaterials.* 34 (2013) 2588–99.
- [5] W. Zheng, Z. Wang, L. Song, Q. Zhao, J. Zhang, D. Li, et al., Endothelialization and patency of RGD-functionalized vascular grafts in a rabbit carotid artery model, *Biomaterials.* 33 (2012) 2880–2891. doi:10.1016/j.biomaterials.2011.12.047.
- [6] A. de Mel, G. Jell, M. Stevens, A. Seifalian, Biofunctionalization of biomaterials for accelerated in situ endothelialization: a review, *Biomacromolecules.* 9 (2008) 2969–79.
- [7] G. Li, P. Yang, W. Qin, M.F. Maitz, S. Zhou, N. Huang, The effect of coimmobilizing heparin and fibronectin on titanium on hemocompatibility and endothelialization, *Biomaterials.* 32 (2011) 4691–4703.
- [8] M.I. Castellanos, F.J. Gil, J.M. Manero, M. Pegueroles, Biofunctionalization of REDV elastin-like recombinamers on a CoCr surface selective improves endothelialization for cardiovascular applications, *Colloids Surfaces B Biointerfaces.* 127 (2015) 22–32.
- [9] J.C. Rodríguez-Cabello, S. Prieto, J. Reguera, F.J. Arias, A. Ribeiro, Biofunctional design of elastin-like polymers for advanced applications in nanobiotechnology., *J. Biomater. Sci. Polym. Ed.* 18 (2007) 269–286.
- [10] H. Shin, Biomimetic materials for tissue engineering, *Biomaterials.* 24 (2003) 4353–4364.
- [11] M. Joner, Q. Cheng, S. Schönhofer-Merl, M. Lopez, S. Neubauer, C. Mas-Moruno, et al., Polymer-free immobilization of a cyclic RGD peptide on a nitinol stent promotes integrin-dependent endothelial coverage of strut surfaces, *J. Biomed. Mater. Res. - Part B Appl. Biomater.* 100 B (2012) 637–645.
- [12] J.H. Collier, T. Segura, Evolving the use of peptides as components of biomaterials., *Biomaterials.* 32 (2011) 4198–204.
- [13] M.H. Fittkau, P. Zilla, D. Bezuidenhout, M.P. Lutolf, P. Human, J. a Hubbell, et al., The selective modulation of endothelial cell mobility on RGD peptide containing surfaces by YIGSR peptides., *Biomaterials.* 26 (2005) 167–74.
- [14] S. Kouvroukoglou, K.C. Dee, R. Bizios, L. V McIntire, K. Zygourakis, Endothelial cell migration on surfaces modified with immobilized adhesive peptides, *Biomaterials.* 21 (2000) 1725–1733.
- [15] U. Hersel, C. Dahmen, H. Kessler, RGD modified polymers: biomaterials for stimulated cell adhesion and beyond, *Biomaterials.* 24 (2003) 4385–4415.
- [16] M.D. Pierschbacher, E. Ruoslahti, Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature.* (1984) 30–33.
- [17] S. VandeVondele, J. Voros, J.A. Hubbell, RGD-grafted poly-L-lysine-graft-(polyethylene glycol) copolymers block non-specific protein adsorption while promoting cell adhesion, *Biotechnol. Bioeng.* 82 (2003) 784–790.
- [18] S.P. Massia, J.A. Hubbell, Human endothelial cell interactions with surface- coupled adhesion peptides on a nonadhesive glass substrate and two polymeric biomaterials, *J. Biomed. Mater. Res. -Part A*, 25 (1991) 223–242.
- [19] J.C. Stendahl, L. Li, R.C. Claussen, S.I. Stupp, Modification of fibrous poly(l-lactic acid) scaffolds with self-assembling triblock molecules, *Biomaterials.* 25 (2004) 5847–5856.
- [20] T.D. Sargeant, M.S. Rao, C.Y. Koh, S.I. Stupp, Covalent functionalization of NiTi surfaces with bioactive peptide amphiphile nanofibers, *Biomaterials.* 29 (2008) 1085–1098.
- [21] M. Schuler, G.R. Owen, D.W. Hamilton, M. de Wild, M. Textor, D.M. Brunette, et al., Biomimetic modification of titanium dental implant model surfaces using the RGDSP-peptide sequence: A cell morphology study, *Biomaterials.* 27 (2006) 4003–4015.
- [22] J.A. Hubbell, S.P. Massia, Endothelial cell-selective materials for tissue engineering in the vascular graft

- via a new receptor, *Nat. Biotechnol.* 9 (1991) 568.
- [23] S.P. Massia, J. a Hubbell, Covalent surface immobilization of Arg-Gly-Asp- and Tyr-Ile-Gly-Ser-Arg-containing peptides to obtain well-defined cell-adhesive substrates., *Anal. Biochem.* 187 (1990) 292–301.
- [24] H.-W. Jun, J.L. West, Modification of polyurethaneurea with PEG and YIGSR peptide to enhance endothelialization without platelet adhesion., *J. Biomed. Mater. Res. B. Appl. Biomater.* 72 (2005) 131–9.
- [25] W. Wang, L. Guo, Y. Yu, Z. Chen, R. Zhou, Z. Yuan, Peptide REDV-modified polysaccharide hydrogel with endothelial cell selectivity for the promotion of angiogenesis, *J. Biomed. Mater. Res. Part A.* 103 (2015) 1703–1712.
- [26] Y. Lei, M. Rémy, C. Labrugère, M.-C. Durrieu, Peptide immobilization on polyethylene terephthalate surfaces to study specific endothelial cell adhesion, spreading and migration, *J. Mater. Sci. Mater. Med.* 23 (2012) 2761–2772.
- [27] J.H. Oh, J.S. Lee, K.M. Park, H.T. Moon, K.D. Park, Tyrosinase-mediated surface grafting of cell adhesion peptide onto micro-fibrous polyurethane for improved endothelialization, *Macromol. Res.* 20 (2012) 1150–1155.
- [28] W.S. Choi, J.W. Bae, Y.K. Joung, K.D. Park, M.H. Lee, J.-C. Park, et al., Fabrication of endothelial cell-specific polyurethane surfaces co-immobilized with GRGDS and YIGSR peptides, *Macromol. Res.* 17 (2009) 458–463.
- [29] T. Ren, S. Yu, Z. Mao, S.E. Moya, L. Han, C. Gao, Complementary Density Gradient of Poly(hydroxyethyl methacrylate) and YIGSR Selectively Guides Migration of Endothelial cells, *Biomacromolecules.* 15 (2014) 2256–2264.
- [30] J.S. Lee, K. Lee, S.-H. Moon, H.-M. Chung, J.H. Lee, S.H. Um, et al., Mussel-Inspired Cell-Adhesion Peptide Modification for Enhanced Endothelialization of Decellularized Blood Vessels, *Macromol. Biosci.* 14 (2014) 1181–1189.
- [31] M. Veis, O. Veis, M.C. Martin, F. Asphahani, M. Zhang, Short Peptides Enhance Single Cell Adhesion and Viability on Microarrays Short Peptides Enhance Single Cell Adhesion and Viability on Microarrays, *Langmuir.* 23 (2007) 4472–4479.
- [32] W.J. Seeto, Y. Tian, E.A. Lipke, Peptide-grafted poly(ethylene glycol) hydrogels support dynamic adhesion of endothelial progenitor cells, *Acta Biomater.* 9 (2013) 8279–8289.
- [33] Y. Wei, Y. Ji, L. Xiao, Q. Lin, J. Ji, Different complex surfaces of polyethyleneglycol (PEG) and REDV ligand to enhance the endothelial cells selectivity over smooth muscle cells, *Colloids Surfaces B Biointerfaces.* 84 (2011) 369–378.
- [34] Y. Ji, Y. Wei, X. Liu, J. Wang, K. Ren, J. Ji, Zwitterionic polycarboxybetaine coating functionalized with REDV peptide to improve selectivity for endothelial cells, *J. Biomed. Mater. Res. Part A.* 100A (2012) 1387–1397.
- [35] Q.K. Lin, Y. Hou, K.F. Ren, J. Ji, Selective endothelial cells adhesion to Arg-Glu-Asp-Val peptide functionalized polysaccharide multilayer, *Thin Solid Films.* 520 (2012) 4971–4978.
- [36] C. Mas-Moruno, R. Fraioli, F. Albericio, J.M. Manero, F.J. Gil, A novel peptide-based platform for the dual presentation of biologically-active peptide motifs on biomaterials, *ACS Appl. Mater. Interfaces.* 6 (2014) 6525–6536.
- [37] B.C. Heng, P.P. Bezerra, Q.R. Meng, D.W.-L. Chin, L.B. Koh, H. Li, et al., Adhesion, proliferation, and gene expression profile of human umbilical vein endothelial cells cultured on bilayered polyelectrolyte coatings composed of glycosaminoglycans., *Biointerphases.* 5 (2010) 53–62.
- [38] A. Andukuri, W.P. Minor, M. Kushwaha, J.M. Anderson, H.-W. Jun, Effect of endothelium mimicking self-assembled nanomaterials on cell adhesion and spreading of human endothelial cells and smooth muscle cells., *Nanomedicine.* 6 (2010) 289–97.
- [39] S. Habibzadeh, L. Li, S. Omanovic, D. Shum-Tim, E.C. Davis, Biocompatibility of Ir/Ti-oxide coatings: Interaction with platelets, endothelial and smooth muscle cells, *Appl. Surf. Sci.* 301 (2014) 530–538.
- [40] Y. Ding, Z. Yang, C.W.C. Bi, M. Yang, J. Zhang, S.L. Xu, et al., Modulation of protein adsorption, vascular cell selectivity and platelet adhesion by mussel-inspired surface functionalization, *J. Mater. Chem. B.* 2 (2014) 3819.
- [41] P. Rocas, M. Hoyos- Nogués, J. Rocas, J.M. Manero, J. Gil, F. Albericio, et al., Installing Multifunctionality on Titanium with RGD-Decorated Polyurethane-Polyurea Roxithromycin Loaded Nanoparticles: Toward New Osseointegrative Therapies, *Adv. Healthc. Mater.* 4 (2015) 1956–1960.
- [42] R. Fraioli, F. Rechenmacher, S. Neubauer, J.M. Manero, J. Gil, H. Kessler, et al., Mimicking bone

- extracellular matrix: Integrin-binding peptidomimetics enhance osteoblast-like cells adhesion, proliferation, and differentiation on titanium, *Colloids Surfaces B Biointerfaces*. 128 (2015) 191–200.
- [43] F. Höök, B. Kasemo, T. Nylander, C. Fant, K. Sott, H. Elwing, Variations in coupled water, viscoelastic properties, and film thickness of a Mefp-1 protein film during adsorption and cross-linking: a quartz crystal microbalance with dissipation monitoring, ellipsometry, and surface plasmon resonance study., *Anal. Chem.* 73 (2001) 5796–804.
- [44] F. Rechenmacher, K. Steigerwald, B. Laufer, S. Neubauer, T.G. Kapp, L. Li, et al., The Integrin Ligand c(RGDf(NMe)Nal) Reduces Neointimal Hyperplasia in a Polymer-Free Drug-Eluting Stent System., *ChemMedChem*. 9 (2014) 1413.
- [45] F. Rechenmacher, S. Neubauer, C. Mas-moruno, P.M. Dorfner, J. Polleux, J. Guasch, et al., A Molecular Toolkit for the Functionalization of Titanium-Based Biomaterials That Selectively Control Integrin-Mediated Cell Adhesion, *Chemistry (Easton)*. (2013) 9218–9223.
- [46] C. Mas-Moruno, P.M. Dorfner, F. Manzenrieder, S. Neubauer, U. Reuning, R. Burgkart, et al., Behavior of primary human osteoblasts on trimmed and sandblasted Ti6Al4V surfaces functionalized with integrin α v β 3-selective cyclic RGD peptides, (2012) 87–97.
- [47] E. Salvagni, G. Berguig, E. Engel, J.C. Rodriguez-Cabello, G. Coullerez, M. Textor, et al., A bioactive elastin-like recombinamer reduces unspecific protein adsorption and enhances cell response on titanium surfaces., *Colloids Surf. B. Biointerfaces*. 114 (2014) 225–33.
- [48] T.P. Hopp, K.R. Woods, Prediction of protein antigenic determinants from amino acid sequences., *Proc. Natl. Acad. Sci. U. S. A.* 78 (1981) 3824–3828.
- [49] M. Kosmulski, Compilation of PZC and IEP of sparingly soluble metal oxides and hydroxides from literature, *Adv. Colloid Interface Sci.* 152 (2009) 14–25.
- [50] J.T. Jr, K. Muirhead, D. Pan, Tracking immune cell proliferation and cytotoxic potential using flow cytometry, *Flow Cytom.* 699 (2011) 119–164.
- [51] M. Nagyova, L. Slovinska, J. Blasko, I. Grulova, M. Kuricova, V. Cigankova, et al., A comparative study of PKH67, DiI, and BrdU labeling techniques for tracing rat mesenchymal stem cells., *In Vitro Cell. Dev. Biol. Anim.* 50 (2014) 656–63.
- [52] M. Rabe, D. Verdes, S. Seeger, Understanding protein adsorption phenomena at solid surfaces., *Adv. Colloid Interface Sci.* 162 (2011) 87–106.
- [53] Y.R. Corrales Ureña, L. Wittig, M. Vieira Nascimento, J.L. Faccioni, P.N. Lisboa Filho, K. Rischka, Influences of the pH on the adsorption properties of an antimicrobial peptide on titanium surfaces, *Appl. Adhes. Sci.* 3 (2015).
- [54] H. Ceylan, A.B. Tekinay, M.O. Guler, Selective adhesion and growth of vascular endothelial cells on bioactive peptide nanofiber functionalized stainless steel surface., *Biomaterials*. 32 (2011) 8797–805.
- [55] B.K. Mann, J.L. West, Cell adhesion peptides alter smooth muscle cell adhesion, proliferation, migration, and matrix protein synthesis on modified surfaces and in polymer scaffolds, *J Biomed Mater Res.* 60 (2002) 86–93.

Figures legends:

Fig. 1. (a) Chemical structure of the cell adhesive peptides. The linear peptides contained a bioactive sequence (highlighted in red), a spacer unit (green) and an anchoring group (blue). In this study two spacer systems were used: three units of aminohexanoic acid (Ahx₃) or three glycine residues (Gly₃). (b) Scheme of the biofunctionalization of CoCr surfaces with cell adhesive peptides process showing the process of activation and covalent binding or physical adsorption.

Fig. 2. (a) Isoelectric point (IEP) and apparent zeta-potential (ZP), at pH 7.4, of modified CoCr surfaces. (b) Zeta potential vs. pH curves of CT physisorbed (white) and NA chemisorbed (black) CoCr surfaces.

Fig. 3. (a) Fluorescence images of the HUVEC cells and; (b) quantification of the cell number for the HUVECs cultured for 4 h on CT physisorbed and NA chemisorbed modified CoCr surfaces. Bar: 1 mm. Groups identified with the same letters are not statistically different ($p>0.05$).

Fig. 4. (a) Fluorescence microscopy images of the CAsMC cells and. (b) Quantification of the cell number for the CAsMCs cultured for 4 h on CT physisorbed and NA chemisorbed modified CoCr surfaces. Bar: 1 mm. Groups identified with the same letters are not statistically different ($p>0.05$).

Fig. 5. (a) Proliferation of HUVEC cells on modified CoCr surfaces after 4, 24, 48 and 72 hours of culture. Cells were previously stained with fluorescent cell linker PKH67. Quantification of cells was done by fluorescent microscopy. (b) HUVECs inhibition rate onto TCPS in the presence of soluble peptides (100 μ M) was measured at the time the peptide was added into the culture medium (day 0) and after 24, 48 and 72 hours.

Fig. 6. Migration of HUVECs on plain (CT) and silanized (NA-CP-RGDS, NA-CP-REDV, NA-CP-YIGSR) CoCr surfaces at beginning, 4, 24 and 48 hours by fluorescent microscopy. Percentage values correspond to total percentage of recovery by cells.

1 Title: **Functionalization of CoCr surfaces with cell adhesive peptides to promote HUVECs**
2 **adhesion and proliferation.**
3
4 Authors: Maria Isabel Castellanos^{1,2}, Carlos Mas-Moruno^{1,2}, Anna Grau^{1,2}, Xavier Serra-Picamal^{4,5,6},
5 Xavier Trepas^{4,5,6}, Fernando Albericio⁷, Michael Jone^{8,9}, , Francisco Javier Gil^{1,2}, **Maria Pau**
6 **Ginebra**^{1,2,10}, Jose María Manero^{1,2}, Marta Pegueroles^{1,2} *.
7
8 Affiliations: ¹ Biomaterials, Biomechanics and Tissue Engineering Group, Department of Materials Science
9 and Metallurgical Engineering, Technical University of Catalonia (UPC), ETSEIB, 08028
10 Barcelona, Spain
11 ² Centre for Research in NanoEngineering (CRNE), UPC, 08028 Barcelona, Spain
12 ⁴ Institute for Bioengineering of Catalonia (IBEC), 08028 Barcelona, Spain
13 ⁵ University of Barcelona and CIBER-BBN, 08036 Barcelona, Spain.
14 ⁶ Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Spain
15 ⁷ Department of Chemistry, University of Barcelona, CIBER-BBN, 08028 Barcelona, Spain.
16 ⁸ Department of Cardiology, Deutsches Herzzentrum München, 80636 Munich, Germany.
17 ⁹ CVPath Institute, Gaithersburg, MD 20878, USA
18 ¹⁰ **Institute for Bioengineering of Catalonia (IBEC), 08028 Barcelona, Spain**
19
20
21
22 Corr. Author: Marta Pegueroles, PhD
23 marta.pegueroles@upc.edu
24 Department of Materials Science and Metallurgical Engineering
25 Av. Diagonal, 647
26 Barcelona, 08028
27 Spain.
28
29
30
31 Maria Isabel Castellanos: maria.isabel.castellanos@upc.edu
32 Carlos Mas-Moruno: carles.mas.moruno@upc.edu
33 Anna Grau: agraugar@gmail.com
34 Xavier Serra-Picamal: xserrapicamal@gmail.com
35 Xavier Trepas: xtrepas@ub.edu
36 Fernando Albericio: fernando.albericio@irbbarcelona.org
37 Michael Jone: michaeljone@me.com
38 **Maria Pau Ginebra**: maria.pau.ginebra@upc.edu
39 Francisco Javier Gil: francesc.xavier.gil@upc.edu
40 Jose María Manero: jose.maria.manero@upc.edu
41 Marta Pegueroles: marta.pegueroles@upc.edu
42
43
44
45
46
47
48

Abstract.

Biomimetic surface modification with peptides that have specific cell-binding moieties is a promising approach to improve endothelialization of metal-based stents. In this study, we functionalized CoCr surfaces with RGDS, REDV, YIGSR peptides and their combinations to promote endothelial cells (ECs) adhesion and proliferation. An extensive characterization of the functionalized surfaces was performed by XPS analysis, surface charge and quartz crystal microbalance with dissipation monitoring (QCM-D), which demonstrated the successful immobilization of the peptides to the surface. Cell studies demonstrated that the covalent functionalization of CoCr surfaces with an equimolar combination of RGDS and YIGSR represents the most powerful strategy to enhance the early stages of ECs adhesion and proliferation, indicating a positive synergistic effect between the two-peptide motifs. Although these peptide sequences slightly increased smooth muscle cells (SMCs) adhesion, these values were ten times lower than those observed for ECs. The combination of RGDS with the REDV sequence did not show synergistic effects in promoting the adhesion or proliferation of ECs. The strategy presented in this study holds great potential to overcome clinical limitations of current metal stents by enhancing their capacity to support surface endothelialization.

Keywords

CoCr alloy, surface functionalization, cell adhesive peptides, HUVEC proliferation, endothelialization, SMCs adhesion

Functionalization of CoCr surfaces with cell adhesive peptides to promote HUVECs adhesion and proliferation.

1. Introduction

Intracoronary stenting is a common practice in interventional cardiology to treat blood vessels with reduced flow due to atherosclerosis. The stent is a metallic mesh tube, which is expanded in the narrowed artery recovering blood flow. Restenosis due to excessive proliferation of smooth muscle cells (SMCs), neointimal hyperplasia, has been a relatively common complication associated with bare metal stents (BMS) after implantation [1–3]. Currently, drug-eluting stents (DES) are polymer-coated stents, which release anti-proliferative drugs that successfully minimize restenosis. However, the drugs not only limit SMCs proliferation but also delay endothelialization of the device after implantation [4]. This may result in higher risk of late in-stent thrombosis, requiring long-term anti-platelet aggregation therapies after implantation. Thus, new surface functionalization should combine the benefit of decreasing acute restenosis, while keeping low levels of thrombogenicity and recovering artery's function.

Biomimetic surface modification with proteins or peptides that have specific cell-binding moieties is a promising approach to improve endothelialization [5,6]. In this regard, stent functionalization with proteins [7], biopolymers [8,9], or with biologically relevant peptide sequences[4,10,11] has been shown to promote endothelial cells (ECs) adhesion. Proteins purified from the extracellular matrix (ECM) have a strong biological efficiency because their native structure and synergistic sequences are preserved. However, this strategy may present problems of immunogenicity, denaturation after sterilization and poor control of functionalization. An alternative to such shortcomings is the use of short synthetic peptides, which are non-immunogenic, easy to purify and can be immobilized on surfaces in a controlled manner [12].

Surfaces modified with bioactive cell-adhesive peptides have shown to mediate anchorage-dependent cell functions, including adhesion, migration and proliferation [13–16]. A prominent example is illustrated by the well-known adhesive sequence RGDS (Arg-Gly-Asp-Ser), present in fibronectin and other ECM proteins, which is recognized as the minimal amino acid sequence necessary to promote cell adhesion [16]. This sequence has been applied to different surfaces including poly(ethylene glycol), polyethylene terephthalate and polytetrafluoroethylene [17,18], poly(L-lactic acid) scaffold [19] and titanium surfaces and its alloys [20,21], in order to improve cell attachment and the bioactivity of the surfaces. Besides the RGDS motif, other adhesive ligands such as the laminin derived YIGSR (Tyr-Ile-Gly-Ser-Arg) sequence[22,23] have been shown to promote EC adhesion and migration without enhancing platelet adhesion [24]. For instance, this peptide has enhanced the adhesion of ECs in hydrogels [13,25], PET [26], polyurethane [27,28], poly(2-hydroxyethyl methacrylate) [29] and decellularized scaffolds [30]. Finally, another cell adhesive sequence found in fibronectin, the REDV (Arg-Glu-Asp-Val) peptide, which targets ECs via the integrin $\alpha_4\beta_1$, has been reported to selectively promote EC adhesion and spreading over SMCs and platelets [22,31]. The REDV peptide has been immobilized onto several polymers such as poly(ethylene glycol) diacrylate hidrogels [32], PET surfaces [33], zwitterionic polycarboxybetaine copolymers [34], and polysaccharide hydrogels [35], aiming at improving the capacity of these surfaces to support endothelialization.

Thus, combining the RGDS sequence with either REDV or YIGSR motifs could potentially lead to improved values of EC adhesion. In this regard, the ability of the peptides YIGSR, PHSRN and RGDS, and their combinations, to selectively affect the adhesion of ECs and SMCs onto polyethylene glycol (PEG) had been evaluated by Fittkau *et al* [13]. However, the effect of immobilizing mixtures of RGDS, YIGSR and REDV peptides onto CoCr alloys, which are widely used as cardiovascular stents, has not been yet explored. The extent and quality of endothelialization strongly depends on the interactions established between functionalized surfaces and ECs. Such process should enhance ECs adhesion and migration [14], but ideally also reduce SMCs migration and proliferation, and prevent platelet adhesion and thrombogenicity [4].

In this work we evaluate the use of equimolar combinations of specific cell adhesive peptides to improve the

endothelialization of CoCr surfaces for cardiovascular applications. To this end, CoCr surfaces were functionalized with the different oligopeptides, and after a thorough characterization of the physicochemical properties of the surfaces, the adhesion and proliferation of ECs, as well as the adhesion of SMCs, were examined at the *in vitro* level.

2. Materials and Methods

2.1 Materials

2.1.1 Metallic surfaces

CoCr alloy (ASTM F90: Co–20Cr–14.6W–10.8Ni–2.5Fe–1.5Mn) (Technalloy, Barcelona, Spain) disks of 8,5 mm diameter and 2 mm thick were subsequently abraded with silicon carbide papers of decreasing grit size (P240, P400, P600, P800 and P1200) and finally polished with suspensions of 1 µm and 0,05 µm alumina powder in distilled water. Prior to the surface treatments, all samples were ultrasonically cleaned with ethanol, distilled water and acetone for 5 min each.

2.1.2 Solid-phase peptide synthesis

The linear peptides RGDS, REDV and YIGSR (Fig. 1(a) and Supplementary Material Table S1) were manually synthesized in solid-phase following the Fmoc/tBu strategy and using 2-chlorotrityl-chloride resin (200 mg, loading of 1.0 mmol/g) as previously reported [36]. Briefly, Fmoc-L-amino acids (4 equiv) were sequentially coupled with either ethyl 2-cyano-2-(hydroxyimino)acetate (OxymaPure) (4 equiv), and N,N'-diisopropylcarbodiimide (DIC) (4 equiv); or N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridino-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate (HATU) (4 equiv) and N,N-diisopropylethylamine (DIEA) (8 equiv) as coupling systems. The efficiency of each reaction was monitored using the Kaiser test and/or by HPLC analysis. Once the peptide sequences were completed, cleavage from the resin was accomplished upon treatment with trifluoroacetic acid (TFA)/water/triisopropylsilane (TIS) (85:10:5, v/v/v) for 1-2 h in the presence of small amounts of dithiothreitol (DTT). The peptides were purified by semi-preparative HPLC and characterized by analytical HPLC and MALDI-TOF (Supplementary Material Table S1). All chemicals required for the synthesis, including resins, Fmoc-L-amino acids and coupling reagents, were obtained from Iris Biotech GmbH (Germany) and Sigma-Aldrich (USA).

2.2 Surface functionalization

The peptides covalent immobilization onto CoCr surfaces was achieved through a three-step strategy consisting of (1) activation, (2) silanization and (3) peptide immobilization. Alternatively, peptides were deposited by simple physical adsorption on the CoCr surfaces. The process of CoCr surfaces functionalization is summarized in Fig. 1(b).

2.2.1 Surface activation

The surface of CoCr samples was activated by basic etching with 5 M NaOH solution during 2 h at room temperature (RT) (samples **NA**). Samples treated with the alkaline solution were cleaned twice in distilled water during 30 min. Non-activated CoCr samples were used as controls (**CT**).

2.2.2 Silanization

Activated samples were silanized by immersing the substrates in a 10 ml solution of 0,5 M 3-chloropropyltriethoxysilane (CPTES) (Sigma-Aldrich) and 0.05 M N,N-diisopropylethylamine (DIEA) in anhydrous toluene under nitrogen atmosphere for 1 h at 90°C under vigorous stirring. After silanization was completed, the discs were ultrasonically washed with cyclohexane, isopropanol, distilled water, and acetone, for 15 min each, and finally dried with nitrogen. The CPTES-modified substrates were stored under vacuum. Silanized samples were coded as **NA-CP**.

2.2.3 Peptide attachment

Finally, 100 µl of peptide solutions (RGDS, REDV and YIGSR) at 100 µM in PBS at pH 13.0 (adjusted with Na₂CO₃) and their combinations (1:1) were deposited on the CPTES-grafted surfaces overnight at RT. The immobilization of the peptides by physical adsorption was done under the same conditions but using PBS at pH 7.0

instead. After the immobilization protocol, samples were washed three times with distilled water. Prior to cell adhesion assays, functionalized samples were blocked for 1 h at 37 °C with 5% (w/v) bovine serum albumin (BSA) in PBS in order to reduce non-specific interactions of the cells with the surface [8,36,41,42]. Functionalized samples were coded as follows: a) Peptide physisorbed surfaces (**CT-peptide: CT-RGDS, CT-REDV, CT-YIGSR, CT-RGDS+REDV and CT-RGDS+YIGSR**); b) Peptide covalently bound surfaces (**NA-CP-peptide: NA-CP-RGDS, NA-CP-REDV, NA-CP-YIGSR, NA-CP-RGDS+REDV, NA-CP-RGDS+YIGSR**) Fig. 1(b).

2.3 Surface characterization

2.3.1 Chemical composition

The chemical analysis of the surface through the process of functionalization was investigated by X-ray photoelectron spectroscopy (XPS) using an XPS equipment (SPECS Surface Nano Analysis GmbH, Germany), equipped with an Al anode XR50 source operating at 150 W and a Phoibos 150 MCD-9 detector XP.

The elements present on the surface were qualitatively evaluated by low-resolution survey spectra, whereas high-resolution spectra of determined elements (C 1s, N 1s, O 1s, Cr 2p, Co 2p, Si 2s, Cl 2p) were recorded with pass energy of 35 eV at 0.1 eV steps at a pressure below 5×10^{-9} mbar. Binding energies of the peaks were referenced to the C 1s peak maximum at 284.8 eV. CasaXPS spectrum software (Casa Software Ltd. UK) was used to analyse and deconvolute XPS spectra.

2.3.2 Surface charge

A streaming potential instrument (Surpass Electrokinetic Analyzer, Anton Paar, Austria) with an adjustable gap cell was used to determine the zeta-potential (ζ) of the surfaces. Automatic titrations were performed using a 1 mM KCl solution as electrolyte and the pressure ramp run up to a maximum pressure of 500-mbar. The electrolyte solution was initially adjusted to pH = 9.0 using 0.1 M KOH and titrated by adding 0.1 M HCl down to pH 3.0. The isoelectric point (IEP) and the surface charge at pH 7.4 were determined using VisioLab software (Anton Paar, Austria).

2.3.3 Quartz crystal microbalance with monitoring dissipation (QCM-D)

The quantification and characterization of the peptide layer attached to the surfaces was performed using a QCM-D (D-300, Q-Sense, Sweden). Co-20Cr-15W-10Ni-1.5Mn (QSX999) sensors were purchased at Q-Sense. The fundamental mode of the sensors was at 4.95 MHz. Prior to use, the sensors were cleaned as follows: (1) 10 min sonication with ethanol; (2) 10 min sonication with acetone; (3) 10 min sonication with MiliQ ultrapure water; and (4) a 10 min-treatment in a UV/ozone chamber (BioForce Nanosciences, USA). Measurements were performed at 25°C by monitoring changes in frequency, Δf (Hz), and dissipation, ΔD ($\times 10^{-6}$), in real-time using Qsoft software (Q-Sense). Raw data was analyzed using QTools software (Q-Sense). Frequency and dissipation curves were fitted to a Voigt viscoelastic model to yield the adsorbed mass and thickness of the peptide layer, as well as kinetic information. The description of the Voigt model and details on its implementation using a QCM-D are reported elsewhere [43]. To monitor the adsorption of the peptides, first the baseline was completely stabilized with PBS for 30-60 min, and then the peptides (**RGDS, REDV, YIGSR**) were introduced at 100 μ M concentrations and maintained in the sensor chamber for 120 min. Finally, the surfaces were rinsed with PBS for 10 min.

2.4 In vitro cell studies

2.4.1 Cells

Human umbilical vein endothelial cells (HUVECs) (Lonza Group Ltd., Switzerland) were grown in EC basal medium (EBM®) supplemented with 5% (v/v) fetal bovine serum (FBS), 0.1% (v/v) gentamicin sulphate amphotericin (GA-1000), 0.4% (v/v) recombinant human fibroblast growth factor (rhFGF), 0.1% (v/v) recombinant human epidermal growth factor (rhEGF), 0.1% (v/v) ascorbic acid, 0.1% (v/v) vascular endothelial growth factor (VEGF), 0.1% (v/v) recombinant Long R insulin (R3-IGF-1) and 0.04% (v/v) hydrocortisone (EBM and all

supplements were obtained from Lonza). Human vascular coronary artery smooth muscle cells (CASMCs) (Lonza) were grown in SMC basal medium (SmBm®) supplemented with 5% FBS, 0.1% GA-1000, 0.1% rhEGF, 0.2% rhFGF and 0.1% Insulin (Lonza). Both cell types were incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO₂. The culture medium was changed every 2 days. Cell culture was performed in Nunc cell flasks (Thermo Scientific, Denmark) pre-coated with 1 µg/ml of fibronectin (FN) in phosphate buffered saline (PBS). Upon reaching 70-90% confluence, cells were harvested by trypsin/EDTA (Sigma-Aldrich), centrifuged and subcultured into a new flask or re-suspended in a serum-free medium and used in cell assays. All experiments were conducted using HUVECs at passages 4 to 8 [37,38] and CASMCs at passages 4 to 6 [39,40].

2.4.2 Fluorescein diacetate (FDA)-staining

FDA-staining (Sigma-Aldrich) was used to conduct a preliminary screening and determine the best spacer system in the cell adhesive peptides (Ahx₃ vs. Gly₃). To this end, HUVECs were seeded on the functionalized surfaces at a density of 2 x 10⁴ cells/disk and incubated in serum-free medium. After 4 h of cell culture, FDA was directly added to the medium to reach a final concentration of 1 µg/ml, and left to react for 3 min. Living cells, the only ones able to convert the dye into a fluorescent analogue, were analyzed with an inverted fluorescent microscope (Upright Microscope Nikon E1000, Japan). **Total area occupied by cells was calculated based on five images (1661,52 µm x 1246,14 µm) with an average number of 40-60 cells/image.**

2.4.3 LDH metabolic activity

The number of HUVECs on the biofunctionalized surfaces was quantified by measuring the released active lactate dehydrogenase (LDH) enzyme after 4 h of incubation using the cytotoxicity detection kit LDH (Roche Applied Science, Germany). HUVECs were seeded on CoCr samples at a density of 2 x 10⁴ cells/disk and incubated with serum-free medium. Cells were lysed with 300 µl of mammalian protein extraction reaction (M-PER) (Pierce, USA) per well and the release of LDH was measured with a spectrophotometer (Infinite M200 Pro, Tecan, Switzerland) at 490 nm. The quantity of the LDH was determined by comparison to a standard curve prepared with known cell concentrations (0, 5000, 10000, 15000, 20000, 25000 and 30000 cells/well) under identical conditions.

2.4.4 Cellular adhesion analysis by immunofluorescence

The adhesion and spreading of HUVECs and CASMCs on the biofunctionalized surfaces was analyzed by means of immunofluorescence staining of actin fibers and nuclei. Cells were cultured in serum-free media at a concentration of 2 x 10⁴ cells/disk during 4 hours. After this time, cells attached to the metal surfaces were fixed for 10 min with 3,7 % (w/v) paraformaldehyde (PFA) (Sigma-Aldrich) in PBS, and permeabilized with 100 µL/disk of 0,05% (w/v) triton X-100 (Sigma-Aldrich) in PBS for 15 min. Washings between steps were all performed with PBS for 3 x 5 min. Next, samples were incubated with 100 µl of phalloidin (1:300) (AlexaFluor 546, ThermoFisher) in PBS for 30 min, and in a final step, nuclei of cells were stained with 50 µl of DAPI (1:1000) in PBS for 10 min in the dark. Finally the samples were mounted with aqueous mounting medium DAKO (Agilent Technologies, USA) on microscope slides and were viewed and photographed by fluorescent microscopy Olympus B2-2 (Olympus, Japan).

The microscope settings were kept constant allowing comparative measurements at several points on the surface. Different cell morphology parameters were studied by ImageJ-FIJI software (NIH, USA): total area occupied by cells and cell number normalized by the image area (cells/cm²).

2.4.5 Cell proliferation

To corroborate the biological effect of the peptides in cell growth, cell proliferation and inhibitory cell rate with soluble peptides assays were also conducted for HUVECs.

Cell proliferation onto modified CoCr surfaces, was analyzed by live cell imaging with an upright fluorescent microscope Olympus B2-2 (Olympus). HUVECs were labelled with the cell membrane fluorescent linker PKH67 (PKH67-GL, Sigma-Aldrich) 24 h prior to cell proliferation assays. Afterwards, 8·10³ labelled cells/well were seeded and incubated in serum free-medium for 4 h. For longer incubation times (24, 48 and 72 h) cells were cultured with complete medium.

For the inhibitory cell proliferation assay with soluble peptides, 8×10^3 cells/well were seeded onto tissue culture polystyrene (TCPS) with serum free-medium during 4 h. Then, the medium was changed to complete medium. Finally, after 20 h, soluble peptides were added at 100 μ M in fresh complete medium (timepoint 0). Cell inhibition rate was calculated after 24, 48 and 72 h of incubation with the soluble peptides, by counting the adhered cells onto TCPS by optical microscopy. Inhibition rates of proliferation were calculated in comparison to the cell proliferation values obtained for TCPS cultured in completed medium without peptides [44].

2.5 Statistical analysis

All data are represented as mean values $\pm$ standard deviations (SD). Experiments were done using triplicates of each condition and cellular studies were repeated at least in three independent assays to ensure reproducibility except wound healing migration studies that was repeated twice. ANOVA with multiple comparisons Fisher's, Tukey's and non-parametric Mann-Whitney U-test were used to determine statistically significant differences (p-value < 0.05 between the different groups). Statistical analysis was performed using Minitab software (Minitab Inc., USA) and SPSS Statistics 20 (IBM, USA).

3. Results and discussion

3.1 Design of the coating peptides

The design of the coating molecule is crucial for a successful surface functionalization and it should consider not only a bioactive cell-binding motif, but also an appropriate spacer and anchoring groups. Whereas the active sequence of the peptide determines its biofunctionality, the anchoring group should provide a strong and chemo selective binding of the molecule to the material. Finally, the spacer ensures an optimal presentation and accessibility of the cell-binding motif to cell-expressed receptors.

In our work, the selected bioactive sequences RGDS, REDV and YIGSR were synthesized containing either three units of aminohexanoic acid (Ahx₃) or three glycine residues (Gly₃), in order to determine the best spacer system to support EC adhesion (Fig.1(a) and Supplementary Material Table S1). The anchoring group was a thiol for all synthesized biomolecules.

The analysis of HUVEC adhesion after 4 h of incubation showed improved values of cell number and spreading onto CoCr surfaces functionalized with peptides bearing the Ahx₃ spacer compared to the Gly₃ (Supplementary Material Fig. S1). Although the differences were not statistically significant, the trend was evident for all surfaces, regardless of the method of immobilization. Therefore, an increased spacer length (Ahx₃ > Gly₃) was translated in improved cell responses, in agreement with previous studies [45,46]. Thus, Ahx₃ was selected as optimal spacer to conduct the rest of experimental studies of this work.

3.2 CoCr biofunctionalization and surface characterization

To immobilize peptides onto CoCr surfaces, two approaches were considered: physical adsorption and covalent binding *via* silanization using CPTES as coupling agent (Fig. 1(b)). Physical adsorption commonly involves less stable bonding compared to silanization procedures where covalent attachment between surfaces and peptides is expected [42,47]. To facilitate silanization, CoCr surfaces were activated with NaOH, a treatment that generates accessible hydroxyl groups on the surface required for a successful silane coupling [8]. Moreover, NaOH etching on CoCr surfaces did not significantly alter surface morphology or mean-roughness values compared to non-treated surfaces [8]. Finally, the covalent binding of the peptides to the silanized surfaces was conducted in Na₂CO₃ buffer solution at pH=13.0 to enhance the direct nucleophilic substitution between the free thiol group of the peptides and the organosilanes.

Water contact angle measurements on the progressively modified CoCr surfaces indicated a successful peptide attachment (Fig. 2(a)). NaOH etching and further attachment of CPTES did not significantly change water contact

angle compared to CT, as observed in previous studies [8]. In contrast, the immobilization of all peptides increased surface hydrophilicity compared to silanized (NA-CP) and plain (CT), surfaces. According to the Hopp and Woods scale for amino acid hydrophilicity [48], RGDS, REDV, and YIGSR peptides have hydrophilicity values of 6.3, 7.5 and -0.8, respectively. Therefore, it should not come as surprise that YIGSR-immobilized CoCr surfaces had a notably higher hydrophobicity than RGDS- and REDV-coated samples, which displayed similar wettability. Moreover, variances in the quantity of immobilized peptide onto CoCr surfaces, could also explain wettability differences.

Zeta-potential at pH 7.4 and isoelectric point (IEP) values for the physisorbed and silanized RGDS, REDV and YIGSR CoCr surfaces are presented in Fig. 2(a). The IEP is around pH $\sim$ 3.5 for all series except for NA and CT-RGDS [49]. Fig. 2(b) shows representative zeta-potential curves as a function of the electrolyte pH. As expected, the obtained zeta-potential curves showed a steadily more negative zeta potential, as the solution was more alkaline. The surface charge is due to the preferential adsorption of negatively charged electrolyte anions, such as OH⁻ and Cl⁻. The zeta-potential at physiological pH (7.4) was found to be significantly more negative after surface activation with NaOH and subsequent silanization with CPTES (CT: -26.4 mV, NA: -31.1 mV, NA-CP: -38.7 mV) (Fig. 2(a)). These values were further modified upon peptide binding. After surface functionalization, differences in surface charge at pH 7.4 were detected depending on the nature of the immobilized peptide. Immobilization of RGDS peptide led to surfaces with lower electronegativity compared to REDV and YIGSR, being this effect more pronounced for silanized surfaces (NA-CP: -38.7 mV vs. NA-CP-RGDS: -18.0 mV). In contrast, REDV and YIGSR functionalized surfaces highly increased surface electronegativity for physisorbed CT surfaces (CT: -26.4 mV vs. CT-REDV: -38.1 mV; CT-YIGSR: -31.3 mV) and maintained electronegativity values for silanized surfaces (NA-CP: -38.7 mV vs. NA-CP-REDV: -34.6 mV and NA-CP-YIGSR: -32.9 mV) at physiological pH.

XPS results further confirmed the immobilization of the peptide coatings on the silanized surfaces. Table 1 displays the quantitative elemental composition of the XPS survey spectra of treated surfaces. Untreated CoCr surfaces showed characteristic C 1s (285 eV), Co 2p, Cr 2p and O 1s (530 eV) peaks. The activation process by NaOH etching did not alter the atomic concentration of C 1s, in correlation with wettability results and confirming that this activation treatment did not eliminate the carbonaceous species adsorbed from the environment. Nevertheless, a significant increase in the level of O 1s signal was found, probably indicating the successful formation of -OH groups at the surface level. The emergence of Cl 2p (NA-CP: 0.2%) and Si 2s (NA-CP: 0.8%) peaks on NA-CP samples confirmed the presence of CPTES and, thus, the presence of the silane on CoCr surfaces. Upon peptide immobilization, XPS analysis showed a strong increase of N 1s (1.2-15.5%) and C 1s (35.6-63.4%) signals, as well as a significant decrease in Co 2p, Cr 2p, and O 1s peaks. Noteworthy, peptide binding to CoCr surfaces resulted in a reduction to almost undetectable levels of Si and Cl signals. This observation was mainly attributed to the release of chlorine ions during the nucleophilic attack of the thiol groups of the peptides and, also, to the full coverage of the underlying layer of organosilanes.

To further characterize the biofunctionalization of CoCr surfaces, high resolution C 1s (Supplementary Material Fig. S2(a)) and O 1s (Supplementary Material Fig. S2(b)) XPS spectra were recorded for CT, NA, CT-YIGSR and NA-CP-YIGSR surfaces. The C1s peak of untreated CoCr surfaces (CT) was deconvoluted in four different peaks with binding energies at 284.7, 282.7, 285.7 and 288.5 eV, and attributed to C-H/C-C, Cr₃C₂, C-OH and C=O groups, respectively [36,37,39,40]. Functionalized surfaces (CT-YIGSR, NA-CP-YIGSR) exhibited the presence of the peptide/protein characteristic amide group, N-C=O, 288.1 eV. The higher proportion of amide signal was found for silanized samples, indicating a greater extent of peptide attachment for the silanization process. These results correlate also with the percentages of N 1s previously observed for these samples (Table 1). The analysis of the O 1s peak of CT surfaces revealed three contributions at 529.9, 531.5 and 532.8 eV assigned to O²⁻, -OH and H₂O groups [40,43,50,51]. NaOH etching successfully activated CoCr surfaces as it increased the relative intensity of the hydroxyl group signal from 49.89%, for CT, up to 57.22%, for NA, while decreasing the signal of oxides and H₂O.

Concerning the efficiency of peptide biofunctionalization, the deconvolution of O 1s peak showed that the intensity of O=C-N/OH signal, 530.9 eV, increased from CT (49.89%) to CT-YIGSR (66.41%) and from NA (57.22%) to NA-CP-YIGSR (87.51%). Thus, high resolution O1s analysis also confirms that silanization process immobilizes a highest amount of peptide than physical adsorption. Taken together, all these results clearly indicate a successful immobilization of the cell adhesive peptides on CoCr surfaces by both physical adsorption and silanization.

3.3 Physisorbed peptide layer characteristics

In the present study, the initial real-time adsorption behavior of RGDS, REDV and YIGSR peptides on CoCr sensors was monitored by QCM-D. Table 2 indicates the characteristics of the adsorbed peptide layer in terms of thickness (nm), peptide density (ng/cm²), viscosity (kg/ms) and shear elastic modulus (MPa). Surprisingly, peptide physisorption on CoCr sensors yielded a higher peptide thickness and density compared to silanized sensors. This trend was observed for all peptides of the study; indicating lower peptide immobilization efficiency through silanization. This observation seems to be in disagreement with our previous characterization by XPS. However, the fact that, in this case, QCM sensors were not activated before silanization, could explain such non-optimal peptide immobilization. Thus, as a proof of concept, CoCr sensors were activated by oxygen plasma prior to silanization with CPTES and finally functionalized with YIGSR (PL-CP-YIGSR). NaOH etching could not be used as an activation method since it damaged the surface of CoCr sensors. Interestingly, the activation of the sensors with oxygen plasma remarkably increased the efficiency of peptide immobilization. This result confirms that surface activation is an essential step prior to silanization to obtain a high efficiency of peptide attachment.

Moreover, the extent of thickness and surface mass density of immobilized peptide onto CoCr sensors was as follows: RGDS (153,42 ng/cm²) > REDV (92,33 ng/cm²) > YIGSR (50,43 ng/cm²) (Table 2). Such differences may be expected on the basis of distinct electrostatic interactions between the biomolecules and the CoCr sensors surface [52,53]. At physiological pH, REDV and RGDS peptides are negatively charged, but YIGSR total net charge is zero. This is in agreement with the higher amounts of adsorbed REDV and RGDS biomolecules onto CoCr sensors in comparison to YIGSR.

3.4 Adhesion of HUVECs and CASMCs

The adhesion of HUVECs and CASMCs onto the different substrates was investigated to understand the effect of surface biofunctionalization on the preferential cell behavior. Fluorescent micrographs and the amount of cells that adhered on modified CoCr surfaces after 4 h are shown in Fig. 3 for HUVECs and in Fig. 4 for CASMCs.

The activation process by NaOH etching did not enhance HUVECs adhesion compared to CT, while it considerably increased the amount of adhered CASMCs after 4 h of cell culture. Actually, the ratios of HUVEC to CASMC (number of HUVEC adhered/ number of CASMC adhered) on CT and NA were of 24.5 and 3.2, respectively. Thus, untreated CoCr surfaces seem to reduce the nonspecific adhesion of SMCs, while changes in the chemical composition of the surfaces after NaOH activation (i.e. the presence of a higher amount of hydroxyl groups on NA) show a positive effect on CASMCs adhesion.

Biofunctionalized surfaces clearly increased the number of adhered HUVECs independently of the type of immobilized peptide. Moreover, cell area was also enhanced on these surfaces, as visualized by immunofluorescence (Fig. 3(a) and Supplementary Material Table S2). Therefore, these results confirm that biofunctionalization with cell adhesive peptides is a good strategy to enhance endothelialization of CoCr surfaces. In detail, significant differences in HUVECs adhesion were detected depending on the peptide and immobilization strategy used. Physisorbed RGDS and YIGSR peptides significantly enhanced cell adhesion compared to REDV or the peptide combinations. In contrast, the equimolar combination of RGDS with YIGSR significantly improved cell adhesion on the silanized series. In fact, the highest level of cell adhesion on CoCr surfaces was obtained for NA-CP-RGDS+YIGSR samples,

indicating a positive synergistic effect between these two peptide motifs. This behavior, however, was not detected on physisorbed CT-RGDS+YIGSR surfaces, probably related to a lower quantity of immobilized peptide onto the surface. As determined in XPS studies, the amount of immobilized peptide was higher for NA-CP-RGDS+YIGSR (N 1s: 7.6%) compared to CT-RGDS+YIGSR (N 1s: 6.3%).

The adhesion and morphology of CSMCs was also influenced by the immobilized peptide onto the surface. In particular, combinations of peptides on the silanized surfaces (NA-CP-RGDS+YIGSR and NA-CP-RGDS+REDV) and physisorption of YIGSR (CT-YIGSR) enhanced CSMCs adhesion compared to CT samples. Nevertheless, values of adhered SMCs were much lower than the number of adhered ECs.

As a whole, it should be highlighted that the amount of HUVECs attached onto all the modified surfaces after 4 h of incubation was one order of magnitude higher compared to the attachment of CSMCs. Such preferential binding of HUVECs is highly relevant because previous studies have demonstrated that greater numbers of SMCs compared to ECs in the vascular lumen leads to intimal hyperplasia [4,38,54]. The fact that the immobilization of RGDS (either by physisorption or silanization) on CoCr surfaces also exhibited selective cell adhesion capacity towards ECs came as a surprise. However, it is well known that both ECs and SMCs need an optimal RGDS concentration for effective adhesion [13,55] and it is plausible that in our surfaces the concentration required for satisfactory supporting SMC adhesion was not achieved. Moreover, the physicochemical properties of the CoCr alloy may also play an important role on such behavior.

3.5 Proliferation of HUVECs

Fig. 5(a) shows HUVECs proliferation on modified CoCr surfaces after 4, 24, 48 and 72 h of incubation. RGDS+YIGSR-coated surfaces, both physisorbed and silanized, significantly enhanced ECs proliferation in comparison to plain and NaOH etched CoCr samples, displaying higher numbers of cells at each time point. RGDS, YIGSR and RGDS+REDV-coated surfaces showed similar proliferation rates, which in general were higher than CT or NA. No clear trend was found when comparing physisorbed vs. covalently bonded samples. Moreover, to confirm the specificity of the peptides in promoting cell growth, the peptides were also incubated as soluble antagonists in the media and their inhibitory effect on cell proliferation studied (Fig. 5(b)). As shown in Fig. 5(b), the highest inhibitory rate corresponds to the RGDS+YIGSR mixture, which correlates with the most effective proliferation of HUVECs.

In summary, the results of adhesion and proliferation of HUVECs indicate that the immobilization of the equimolar mixture of RGDS+YIGSR peptides by means of silanization (NA-CP-RGDS+YIGSR) exhibits beneficial properties in terms of endothelialization capacity.

Overall, cell studies determined that the functionalization of CoCr surfaces with cell adhesive peptides represents a good strategy to enhance HUVECs adhesion and proliferation. In particular, the combination of RGDS with YIGSR seems an optimal solution to enhance surface endothelialization. Although such combination of peptides also increased SMCs adhesion, which can lead to non-desired enhancement of intimal hyperplasia, these values of adhesion were ten times lower than those of EC adhesion. Therefore, the strategy presented in this study holds great potential to overcome clinical limitations of current stents by enhancing surface endothelialization.

Conclusions

The aim of the present work was to investigate and characterize the biofunctionalization of CoCr surfaces with cell adhesive peptides in order to enhance the adhesion, proliferation and migration of ECs onto these materials. The present study demonstrates the effectiveness of immobilizing RGDS, REDV and YIGSR peptides, and especially the equimolar combination of RGDS and YIGSR on CoCr surfaces to enhance ECs adhesion spreading without significantly enhancing SMCs. These results could be beneficial in the development of new strategies to increase the endothelialization of coronary stents, while preventing restenosis and thrombosis.

1 **Acknowledgments**

2 Authors acknowledge the Spanish Government for financial support through project [MAT 2012-30706], co-funded
3 by the EU through European Regional Development Funds, and the Agency for Administration of University and
4 Research Grants of the Government of Catalonia [2014 SGR 1333]. M.I.C. would like to thank the Generalitat de
5 Catalunya for funding through a FI Scholarship. C. Mas-Moruno thanks the support of the Secretary for Universities
6 and Research of the Ministry of Economy and Knowledge of the Government of Catalonia [2011-BP-B-00042] and
7 the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme [FP7-
8 PEOPLE-2012-CIG, REA Grant Agreement 321985].

References

- [1] P. Wenaweser, C. Rey, F.R. Eberli, M. Togni, D. Tüller, S. Locher, et al., Stent thrombosis following bare-metal stent implantation: success of emergency percutaneous coronary intervention and predictors of adverse outcome. *Eur. Heart J.* 26 (2005) 1180–7.
- [2] T. Simard, B. Hibbert, F.D. Ramirez, M. Froeschl, Y.-X. Chen, E.R. O'Brien, The evolution of coronary stents: a brief review., *Can. J. Cardiol.* 30 (2014) 35–45.
- [3] F. Nazneen, G. Herzog, D.W.M. Arrigan, N. Caplice, P. Benvenuto, P. Galvin, et al., Surface chemical and physical modification in stent technology for the treatment of coronary artery disease., *J. Biomed. Mater. Res. B. Appl. Biomater.* 100 (2012) 1989–2014.
- [4] Y. Wei, Y. Ji, L.-L. Xiao, Q. Lin, J. Xu, K. Ren, et al., Surface engineering of cardiovascular stent with endothelial cell selectivity for in vivo re-endothelialisation., *Biomaterials.* 34 (2013) 2588–99.
- [5] W. Zheng, Z. Wang, L. Song, Q. Zhao, J. Zhang, D. Li, et al., Endothelialization and patency of RGD-functionalized vascular grafts in a rabbit carotid artery model, *Biomaterials.* 33 (2012) 2880–2891. doi:10.1016/j.biomaterials.2011.12.047.
- [6] A. de Mel, G. Jell, M. Stevens, A. Seifalian, Biofunctionalization of biomaterials for accelerated in situ endothelialization: a review, *Biomacromolecules.* 9 (2008) 2969–79.
- [7] G. Li, P. Yang, W. Qin, M.F. Maitz, S. Zhou, N. Huang, The effect of coimmobilizing heparin and fibronectin on titanium on hemocompatibility and endothelialization, *Biomaterials.* 32 (2011) 4691–4703.
- [8] M.I. Castellanos, F.J. Gil, J.M. Manero, M. Pegueroles, Biofunctionalization of REDV elastin-like recombinamers on a CoCr surface selective improves endothelialization for cardiovascular applications, *Colloids Surfaces B Biointerfaces.* 127 (2015) 22–32.
- [9] J.C. Rodríguez-Cabello, S. Prieto, J. Reguera, F.J. Arias, A. Ribeiro, Biofunctional design of elastin-like polymers for advanced applications in nanobiotechnology., *J. Biomater. Sci. Polym. Ed.* 18 (2007) 269–286.
- [10] H. Shin, Biomimetic materials for tissue engineering, *Biomaterials.* 24 (2003) 4353–4364.
- [11] M. Joner, Q. Cheng, S. Schönhofer-Merl, M. Lopez, S. Neubauer, C. Mas-Moruno, et al., Polymer-free immobilization of a cyclic RGD peptide on a nitinol stent promotes integrin-dependent endothelial coverage of strut surfaces, *J. Biomed. Mater. Res. - Part B Appl. Biomater.* 100 B (2012) 637–645.
- [12] J.H. Collier, T. Segura, Evolving the use of peptides as components of biomaterials., *Biomaterials.* 32 (2011) 4198–204.
- [13] M.H. Fittkau, P. Zilla, D. Bezuidenhout, M.P. Lutolf, P. Human, J. a Hubbell, et al., The selective modulation of endothelial cell mobility on RGD peptide containing surfaces by YIGSR peptides., *Biomaterials.* 26 (2005) 167–74.
- [14] S. Kouvroukoglou, K.C. Dee, R. Bizios, L. V McIntire, K. Zygourakis, Endothelial cell migration on surfaces modified with immobilized adhesive peptides, *Biomaterials.* 21 (2000) 1725–1733.
- [15] U. Hersel, C. Dahmen, H. Kessler, RGD modified polymers: biomaterials for stimulated cell adhesion and beyond, *Biomaterials.* 24 (2003) 4385–4415.
- [16] M.D. Pierschbacher, E. Ruoslahti, Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature.* (1984) 30–33.
- [17] S. VandeVondele, J. Voros, J.A. Hubbell, RGD-grafted poly-L-lysine-graft-(polyethylene glycol) copolymers block non-specific protein adsorption while promoting cell adhesion, *Biotechnol. Bioeng.* 82 (2003) 784–790.
- [18] S.P. Massia, J.A. Hubbell, Human endothelial cell interactions with surface- coupled adhesion peptides on a nonadhesive glass substrate and two polymeric biomaterials, *J. Biomed. Mater. Res. -Part A*, 25 (1991) 223–242.
- [19] J.C. Stendahl, L. Li, R.C. Claussen, S.I. Stupp, Modification of fibrous poly(l-lactic acid) scaffolds with self-assembling triblock molecules, *Biomaterials.* 25 (2004) 5847–5856.
- [20] T.D. Sargeant, M.S. Rao, C.Y. Koh, S.I. Stupp, Covalent functionalization of NiTi surfaces with bioactive peptide amphiphile nanofibers, *Biomaterials.* 29 (2008) 1085–1098.
- [21] M. Schuler, G.R. Owen, D.W. Hamilton, M. de Wild, M. Textor, D.M. Brunette, et al., Biomimetic modification of titanium dental implant model surfaces using the RGDSP-peptide sequence: A cell morphology study, *Biomaterials.* 27 (2006) 4003–4015.
- [22] J.A. Hubbell, S.P. Massia, Endothelial cell-selective materials for tissue engineering in the vascular graft

- via a new receptor, *Nat. Biotechnol.* 9 (1991) 568.
- [23] S.P. Massia, J. a Hubbell, Covalent surface immobilization of Arg-Gly-Asp- and Tyr-Ile-Gly-Ser-Arg-containing peptides to obtain well-defined cell-adhesive substrates., *Anal. Biochem.* 187 (1990) 292–301.
- [24] H.-W. Jun, J.L. West, Modification of polyurethaneurea with PEG and YIGSR peptide to enhance endothelialization without platelet adhesion., *J. Biomed. Mater. Res. B. Appl. Biomater.* 72 (2005) 131–9.
- [25] W. Wang, L. Guo, Y. Yu, Z. Chen, R. Zhou, Z. Yuan, Peptide REDV-modified polysaccharide hydrogel with endothelial cell selectivity for the promotion of angiogenesis, *J. Biomed. Mater. Res. Part A.* 103 (2015) 1703–1712.
- [26] Y. Lei, M. Rémy, C. Labrugère, M.-C. Durrieu, Peptide immobilization on polyethylene terephthalate surfaces to study specific endothelial cell adhesion, spreading and migration, *J. Mater. Sci. Mater. Med.* 23 (2012) 2761–2772.
- [27] J.H. Oh, J.S. Lee, K.M. Park, H.T. Moon, K.D. Park, Tyrosinase-mediated surface grafting of cell adhesion peptide onto micro-fibrous polyurethane for improved endothelialization, *Macromol. Res.* 20 (2012) 1150–1155.
- [28] W.S. Choi, J.W. Bae, Y.K. Joung, K.D. Park, M.H. Lee, J.-C. Park, et al., Fabrication of endothelial cell-specific polyurethane surfaces co-immobilized with GRGDS and YIGSR peptides, *Macromol. Res.* 17 (2009) 458–463.
- [29] T. Ren, S. Yu, Z. Mao, S.E. Moya, L. Han, C. Gao, Complementary Density Gradient of Poly(hydroxyethyl methacrylate) and YIGSR Selectively Guides Migration of Endothelial cells, *Biomacromolecules.* 15 (2014) 2256–2264.
- [30] J.S. Lee, K. Lee, S.-H. Moon, H.-M. Chung, J.H. Lee, S.H. Um, et al., Mussel-Inspired Cell-Adhesion Peptide Modification for Enhanced Endothelialization of Decellularized Blood Vessels, *Macromol. Biosci.* 14 (2014) 1181–1189.
- [31] M. Veis, O. Veis, M.C. Martin, F. Asphahani, M. Zhang, Short Peptides Enhance Single Cell Adhesion and Viability on Microarrays Short Peptides Enhance Single Cell Adhesion and Viability on Microarrays, *Langmuir.* 23 (2007) 4472–4479.
- [32] W.J. Seeto, Y. Tian, E.A. Lipke, Peptide-grafted poly(ethylene glycol) hydrogels support dynamic adhesion of endothelial progenitor cells, *Acta Biomater.* 9 (2013) 8279–8289.
- [33] Y. Wei, Y. Ji, L. Xiao, Q. Lin, J. Ji, Different complex surfaces of polyethyleneglycol (PEG) and REDV ligand to enhance the endothelial cells selectivity over smooth muscle cells, *Colloids Surfaces B Biointerfaces.* 84 (2011) 369–378.
- [34] Y. Ji, Y. Wei, X. Liu, J. Wang, K. Ren, J. Ji, Zwitterionic polycarboxybetaine coating functionalized with REDV peptide to improve selectivity for endothelial cells, *J. Biomed. Mater. Res. Part A.* 100A (2012) 1387–1397.
- [35] Q.K. Lin, Y. Hou, K.F. Ren, J. Ji, Selective endothelial cells adhesion to Arg-Glu-Asp-Val peptide functionalized polysaccharide multilayer, *Thin Solid Films.* 520 (2012) 4971–4978.
- [36] C. Mas-Moruno, R. Fraioli, F. Albericio, J.M. Manero, F.J. Gil, A novel peptide-based platform for the dual presentation of biologically-active peptide motifs on biomaterials, *ACS Appl. Mater. Interfaces.* 6 (2014) 6525–6536.
- [37] B.C. Heng, P.P. Bezerra, Q.R. Meng, D.W.-L. Chin, L.B. Koh, H. Li, et al., Adhesion, proliferation, and gene expression profile of human umbilical vein endothelial cells cultured on bilayered polyelectrolyte coatings composed of glycosaminoglycans., *Biointerphases.* 5 (2010) 53–62.
- [38] A. Andukuri, W.P. Minor, M. Kushwaha, J.M. Anderson, H.-W. Jun, Effect of endothelium mimicking self-assembled nanomaterials on cell adhesion and spreading of human endothelial cells and smooth muscle cells., *Nanomedicine.* 6 (2010) 289–97.
- [39] S. Habibzadeh, L. Li, S. Omanovic, D. Shum-Tim, E.C. Davis, Biocompatibility of Ir/Ti-oxide coatings: Interaction with platelets, endothelial and smooth muscle cells, *Appl. Surf. Sci.* 301 (2014) 530–538.
- [40] Y. Ding, Z. Yang, C.W.C. Bi, M. Yang, J. Zhang, S.L. Xu, et al., Modulation of protein adsorption, vascular cell selectivity and platelet adhesion by mussel-inspired surface functionalization, *J. Mater. Chem. B.* 2 (2014) 3819.
- [41] P. Rocas, M. Hoyos-Nogués, J. Rocas, J.M. Manero, J. Gil, F. Albericio, et al., Installing Multifunctionality on Titanium with RGD-Decorated Polyurethane-Polyurea Roxithromycin Loaded Nanoparticles: Toward New Osseointegrative Therapies, *Adv. Healthc. Mater.* 4 (2015) 1956–1960.
- [42] R. Fraioli, F. Rechenmacher, S. Neubauer, J.M. Manero, J. Gil, H. Kessler, et al., Mimicking bone

- extracellular matrix: Integrin-binding peptidomimetics enhance osteoblast-like cells adhesion, proliferation, and differentiation on titanium, *Colloids Surfaces B Biointerfaces*. 128 (2015) 191–200.
- [43] F. Höök, B. Kasemo, T. Nylander, C. Fant, K. Sott, H. Elwing, Variations in coupled water, viscoelastic properties, and film thickness of a Mefp-1 protein film during adsorption and cross-linking: a quartz crystal microbalance with dissipation monitoring, ellipsometry, and surface plasmon resonance study., *Anal. Chem.* 73 (2001) 5796–804.
- [44] F. Rechenmacher, K. Steigerwald, B. Laufer, S. Neubauer, T.G. Kapp, L. Li, et al., The Integrin Ligand c(RGDf(NMe)Nal) Reduces Neointimal Hyperplasia in a Polymer-Free Drug-Eluting Stent System., *ChemMedChem*. 9 (2014) 1413.
- [45] F. Rechenmacher, S. Neubauer, C. Mas-moruno, P.M. Dorfner, J. Polleux, J. Guasch, et al., A Molecular Toolkit for the Functionalization of Titanium-Based Biomaterials That Selectively Control Integrin-Mediated Cell Adhesion, *Chemistry (Easton)*. (2013) 9218–9223.
- [46] C. Mas-Moruno, P.M. Dorfner, F. Manzenrieder, S. Neubauer, U. Reuning, R. Burgkart, et al., Behavior of primary human osteoblasts on trimmed and sandblasted Ti6Al4V surfaces functionalized with integrin α v β 3-selective cyclic RGD peptides, (2012) 87–97.
- [47] E. Salvagni, G. Berguig, E. Engel, J.C. Rodriguez-Cabello, G. Coullerez, M. Textor, et al., A bioactive elastin-like recombinamer reduces unspecific protein adsorption and enhances cell response on titanium surfaces., *Colloids Surf. B. Biointerfaces*. 114 (2014) 225–33.
- [48] T.P. Hopp, K.R. Woods, Prediction of protein antigenic determinants from amino acid sequences., *Proc. Natl. Acad. Sci. U. S. A.* 78 (1981) 3824–3828.
- [49] M. Kosmulski, Compilation of PZC and IEP of sparingly soluble metal oxides and hydroxides from literature, *Adv. Colloid Interface Sci.* 152 (2009) 14–25.
- [50] J.T. Jr, K. Muirhead, D. Pan, Tracking immune cell proliferation and cytotoxic potential using flow cytometry, *Flow Cytom.* 699 (2011) 119–164.
- [51] M. Nagyova, L. Slovinska, J. Blasko, I. Grulova, M. Kuricova, V. Cigankova, et al., A comparative study of PKH67, DiI, and BrdU labeling techniques for tracing rat mesenchymal stem cells., *In Vitro Cell. Dev. Biol. Anim.* 50 (2014) 656–63.
- [52] M. Rabe, D. Verdes, S. Seeger, Understanding protein adsorption phenomena at solid surfaces., *Adv. Colloid Interface Sci.* 162 (2011) 87–106.
- [53] Y.R. Corrales Ureña, L. Wittig, M. Vieira Nascimento, J.L. Faccioni, P.N. Lisboa Filho, K. Rischka, Influences of the pH on the adsorption properties of an antimicrobial peptide on titanium surfaces, *Appl. Adhes. Sci.* 3 (2015).
- [54] H. Ceylan, A.B. Tekinay, M.O. Guler, Selective adhesion and growth of vascular endothelial cells on bioactive peptide nanofiber functionalized stainless steel surface., *Biomaterials*. 32 (2011) 8797–805.
- [55] B.K. Mann, J.L. West, Cell adhesion peptides alter smooth muscle cell adhesion, proliferation, migration, and matrix protein synthesis on modified surfaces and in polymer scaffolds, *J Biomed Mater Res.* 60 (2002) 86–93.

Figures legends:

Fig. 1. (a) Chemical structure of the cell adhesive peptides. The linear peptides contained a bioactive sequence (highlighted in red), a spacer unit (green) and an anchoring group (blue). In this study two spacer systems were used: three units of aminohexanoic acid (Ahx₃) or three glycine residues (Gly₃). (b) Scheme of the biofunctionalization of CoCr surfaces with cell adhesive peptides process showing the process of activation and covalent binding or physical adsorption.

Fig. 2. (a) Isoelectric point (IEP) and apparent zeta-potential (ZP), at pH 7.4, of modified CoCr surfaces. (b) Zeta potential vs. pH curves of CT physisorbed (white) and NA chemisorbed (black) CoCr surfaces.

Fig. 3. (a) Fluorescence images of the HUVEC cells and; (b) quantification of the cell number for the HUVECs cultured for 4 h on CT physisorbed and NA chemisorbed modified CoCr surfaces. Bar: 1 mm. Groups identified with the same letters are not statistically different ($p>0.05$).

Fig. 4. (a) Fluorescence microscopy images of the CAsMC cells and. (b) Quantification of the cell number for the CAsMCs cultured for 4 h on CT physisorbed and NA chemisorbed modified CoCr surfaces. Bar: 1 mm. Groups identified with the same letters are not statistically different ($p>0.05$).

Fig. 5. (a) Proliferation of HUVEC cells on modified CoCr surfaces after 4, 24, 48 and 72 hours of culture. Cells were previously stained with fluorescent cell linker PKH67. Quantification of cells was done by fluorescent microscopy. (b) HUVECs inhibition rate onto TCPS in the presence of soluble peptides (100 μ M) was measured at the time the peptide was added into the culture medium (day 0) and after 24, 48 and 72 hours.

Table 1

Table 1. XPS characterization percentage of atomic composition at the different modified CoCr surfaces.

Surfaces	C 1s	O 1s	N 1s	Co 2p	Cr 2p	Cl 2p	Si 2s
CT	39.2 ± 2.0	37.7 ± 1.7	0.9 ± 0.3	9.0 ± 1.0	11.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
NA	37.3 ± 5.8	42.4 ± 2.5	4.5 ± 0.1	6.7 ± 1.0	7.1 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
CT-RGDS	46.6 ± 0.5	39.0 ± 1.6	1.4 ± 0.0	4.0 ± 0.2	9.1 ± 1.2	0.0 ± 0.0	0.0 ± 0.0
CT-REDV	44.5 ± 0.2	40.3 ± 2.4	1.2 ± 0.0	4.8 ± 0.6	9.1 ± 1.9	0.0 ± 0.0	0.0 ± 0.0
CT-YIGSR	35.6 ± 4.4	47.4 ± 1.9	2.8 ± 0.3	7.7 ± 1.1	6.1 ± 0.8	0.0 ± 0.0	0.0 ± 0.0
CT-RGDS+REDV	44.2 ± 6.7	38.3 ± 3.1	1.9 ± 0.8	7.7 ± 2.4	6.0 ± 1.9	0.0 ± 0.0	0.0 ± 0.0
CT-RGDS+YIGSR	54.0 ± 3.5	30.6 ± 1.8	6.3 ± 1.2	4.4 ± 0.3	3.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
NA-CP	28.0 ± 1.0	51.3 ± 2.6	0.3 ± 0.2	10.8 ± 1.0	5.3 ± 0.5	0.2 ± 0.2	0.8 ± 0.2
NA-CP-RGDS	42.4 ± 4.8	41.2 ± 4.9	2.9 ± 0.8	4.5 ± 0.2	8.7 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
NA-CP-REDV	45.2 ± 2.3	37.8 ± 1.0	1.5 ± 0.1	3.4 ± 0.2	12.6 ± 1.4	0.0 ± 0.0	0.0 ± 0.0
NA-CP-YIGSR	63.0 ± 0.3	19.6 ± 0.6	15.5 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.0 ± 0.0
NA-CP-RGDS+REDV	36.9 ± 1.3	44.8 ± 1.3	2.6 ± 0.3	7.4 ± 0.3	5.5 ± 0.8	0.1 ± 0.1	0.0 ± 0.0
NA-CP-RGDS+YIGSR	44.7 ± 0.3	35.8 ± 0.4	7.6 ± 0.1	5.9 ± 0.2	3.6 ± 0.3	0.1 ± 0.2	0.0 ± 0.0

Table 2. Thickness, surface mass density, viscosity and shear elastic modulus obtained by using QCM-D technique of the absorbed RGDS, REDV and YIGSR peptides on physisorbed and silanized CoCr sensors. Calculations were performed after 4 h of adsorption time using the Voigt model.

Surfaces	Thickness (nm)	Surface mass density (ng/cm ²)	Viscosity (kg/ms)	Shear elastic modulus (MPa)
RGDS	1.22 ± 1.30	153.42 ± 163.45	0.0049 ± 0.000	0.01 ± 0.00
CP-RGDS	0.55 ± 0.37	64.69 ± 43.49	0.0083 ± 0.002	0.02 ± 0.01
REDV	0.74 ± 0.46	92.33 ± 61.12	0.0018 ± 0.003	0.02 ± 0.00
CP-REDV	0.42 ± 0.09	49.98 ± 11.23	0.0013 ± 0.000	0.23 ± 0.03
YIGSR	0.38 ± 0.33	50.43 ± 41.23	0.0011 ± 0.001	0.06 ± 0.07
CP-YIGSR	0.25 ± 0.19	30.59 ± 22.34	0.0089 ± 0.001	0.02 ± 0.05
PL-CP-YIGSR	1.43 ± 0.39	172.17 ± 46.73	0.0021 ± 0.001	1.38 ± 0.44

Figure 1

b) Control

Functionalization

HS = peptide (RGD, REDV, YIGSR or combinations)

Figure 2

[Click here to download Figure: Fig 2.pdf](#)

Surfaces	CA (°)	IEP	ZP at pH 7.4 (mV)
CT	72.4 ± 5.3	3.5 ± 0.0	-26.4 ± 1.1
NA	68.7 ± 1.7	4.1 ± 0.1	-31.1 ± 0.3
NA-CP	70.1 ± 4.3	3.7 ± 0.0	-38.7 ± 2.5
CT-RGDS	59.1 ± 9.1	4.3 ± 0.0	-23.6 ± 0.7
CT-REDV	41.0 ± 4.7	3.4 ± 0.1	-38.1 ± 0.6
CT-YIGSR	63.5 ± 4.5	3.6 ± 0.2	-31.3 ± 1.8
NA-CP-RGDS	49.3 ± 4.8	3.7 ± 0.0	-18.0 ± 0.5
NA-CP-REDV	60.4 ± 5.6	3.8 ± 0.1	-34.6 ± 2.6
NA-CP-YIGSR	63.5 ± 4.5	3.6 ± 0.1	-32.9 ± 1.5

Figure 2
[Click here to download Figure: Fig 3 revised.pdf](#)

Figure 4
[Click here to download Figure: Fig 4 b.pdf](#)

Figure 5
[Click here to download Figure: Fig 5 revised.pdf](#)
b)