Title: **Cell adhesive peptides functionalized on CoCr alloy stimulate endothelialization and prevent thrombogenesis and restenosis**

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Abstract

Immobilization of bioactive peptide sequences on CoCr surfaces is an effective route to improve endothelialization, which is of great interest for cardiovascular stents. In this work, we explored the effect of physical and covalent immoblization of RGDS, YIGSR and their equimolar combination peptides on endothelial cells (EC) and smooth muscle cell (SMC) adhesion and on thrombogenicity. We extensively investigated using RT-qPCR, the expression by ECs cultured on functionalised CoCr surfaces of different genes. Genes relevant for adhesion (ICAM-1 and VCAM-1), vascularization (VEGFA, VEGFR-1 and VEGFR-2) and anti-thrombogenicity (tPA and eNOS) were over-expressed in the ECs grown to covalently functionalized CoCr surfaces compared physisorbed and control surfaces. Pro-thrombogenic genes expression (PAI-1 and vWF) decreased over time. Cell co-cultives of ECs/SMCs found that functionalization increased the amount of adhered ECs onto modified surfaces compared to plain CoCr, independently of the used peptide and the strategy of immobilization. SMCs adhered less compared to ECs in all surfaces. All studied peptides showed a lower platelet cell adhesion compared to TCPS. Covalent functionalization of CoCr surfaces with an equimolar combination of RGDS and YIGSR represented prevailing strategy to enhance the early stages of ECs adhesion and proliferation, while preventing SMCs and platelet adhesion.

Key Words

Functionalization; CoCr alloy; gene expression; platelet adhesion; cell co-culture

Short title: CELL ADHESIVE PEPTIDES FUNCTIONALIZED ON CoCr ALLOY

INTRODUCTION

Atherosclerosis, the hardening of arteries due to build-up of lipoproteins, is one of the leading causes of death worldwide.[1–3] One of the most common treatments for vessel occlusion due to atherosclerotic lesion formation is stent implantation where the stent is expanded in the narrowed artery recovering blood flow. While stenting has become a widely used procedure, it is not without complications.

In-stent restenosis[4] and late stent thrombosis[5] are the main drawbacks of actual bare metal stents (BMS)[6] and drug-eluting stents (DES),[4] respectively. In-stent restenosis is due to the body's own wound healing response to the mechanical injury associated with stent implantation[7] where increased proliferation of smooth muscle cells (SMCs) leads to renarrowing of the arteries. [8] Late stent thrombosis might be related to a delay in re-endothelialization following stenting, which has been shown in pathology studies.[9] A stent surface rapid endothelialization minimizes the failures associated with blood clotting and platelet activation[10] and is expected to reduce in-stent restenosis. The rate and quality of endothelialization of a stent depend on interactions between endothelial cells (ECs) with the biomaterial surface.

Material surface properties can be modified by physicochemical modification and/or biofunctionalization to promote EC adhesion and inhibit thrombosis by influencing protein adsorption and subsequent cell behavior.[11,12] But, in the recent years, there is a growing interest in the immobilization of bioactive molecules on the surface of biomaterial implants through covalent bonding.[13,14] Covalent functionalization is based on the formation of a covalent linkage between functional entities and the material surface. The advantages of the process includes control of molecular orientation, minimization of non-specific interactions, and greater stability of the functional surface by preventing dissolution, desorption, and degradation of molecules.[15] However, the optimal sequences and their combinations remain to be elucidated.

The inner arterial wall of the entire vascular system consist of a continuous single layer of ECs which separates blood from the vessel wall.[16] The endothelium regulates the transfer of molecules, such as lipoproteins, between the blood and vessel wall and acts as a semipermeable barrier.[17,18] In addition to serving as a physical barrier, ECs also control many important functions in vascular homeostasis including vascular tone, inflammation, and lipid and tissue-fluid homeostasis, and has antithrombotic properties.[18] The antithrombotic and anticoagulant balance is maintained through processes involving nitric oxide production, prostacyclin, tissue plasminogen activator, thrombomodulin, heparin-like molecules, tissue-factor pathway inhibitor and many other molecules.[9] Designed biomaterial should stimulate ECs adhesion and migration and prevent SMCs adhesion and proliferation in order to obtain a functional artery.

Based on previous results of functionalized CoCr alloy surfaces with RGDS, REDV and YIGSR peptides, we find that peptides immobilization, specially the combination of RGDS and YIGSR, represent a good strategy to enhance initial EC adhesion and migration.[19] The bioactivity of these peptides has been thoroughly demonstrated by scrambling their sequences.[20–22] Now we would like to go a step further by studying the effect of functionalized CoCr surfaces on gene expression of EC. Specifically, expression profiles of nine gene markers, related with four different functions of EC: adhesion (ICAM-1 and VCAM-1), vascularization (VEGFA, VEGFR-1 and VEGFR-2), pro thrombogenic (PAI-1 and vWF) and anti-thrombogenic genes (eNOS and tPA) were analyzed by real time quantitative polymerase chain reaction (RT-qPCR). Also, we have evaluated thrombogenicity of the peptides by quantifying platelet adhesion and aggregation under defined shear stress conditions.[23]

Moreover, a co-culture system has been performed by plating EC and SMC together in tissue culture to evaluate the rapid competitive adhesion, proliferation and migration of ECs and SMCs to identify the aspects of the modified CoCr surfaces, which favor re-endothelialization while alongside inhibiting the SMCs proliferation and platelet adhesion.

MATERIAL AND METHODS

Metallic surface

Comercial CoCr alloy discs (8.5 mm in diameter, 2-3 mm thick) were obtained from CoCr alloy (ASTM F90) bars (Technalloy S.A., Barcelona, Spain). Samples were polished to achieve mirror-like, smooth surfaces by grinding with abrasive SiC papers (Neuertek S.A., Eibar and Beortek S.A., Asua-Erandio, Spain) of decreasing grit size (P600, P800 and P1200), followed by polishing with suspension of alumina particles (1 μm and 0,05 μm). Prior to biofuncionalization, all samples were ultrasonically rinsed with ethanol, distilled water and acetone and stored dried.

Solid-phase peptide synthesis

The linear peptides MPA-(Ahx)3-Arg-Gly-Asp-Ser-OH (RGDS) and MPA-(Ahx)3-Tyr-Ile-Gly-Ser-Arg-OH (YIGSR) (Ahx: aminohexanoic acid; MPA: 3-mercaptopropionic acid) were manually synthesized by solid-phase following the Fmoc/tBu strategy and using CTC resin (200 mg, 1.0 mmol/g) as previously reported.[24] The purified peptides were characterized by analytical HPLC analysis and MALDI-TOF. All chemicals required for the synthesis, including resins, Fmoc-L-amino acids and coupling reagents, were obtained from Iris Biotech GmbH (Marktredwitz, Germany) and Sigma-Aldrich (St Louis, MO, USA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in EC basal medium (EBM®) supplemented with EGM-2 BulletKit and 5% (v/v) fetal bovine serum (FBS).Human vascular coronary artery smooth muscle cells (CASMCs) were grown in SMC basal medium (SmBm®) supplemented with SmGM-2 BulletKit and 5% (v/v) FBS. All cells, mediums and supplements were purchased from Lonza (Basel, Switzerland). Cells were maintained at 37°C, in a humified atmosphere containing 5% (v/v) CO_2 and culture medium was renewed every 2 days. Cell culture was performed in Nunc cell flasks (Thermo Scientific, Denmark) pre-coated with 1μg/ml of human plasma fibronectin (Sigma-Aldrich) in PBS. HUVECs at passages 4 to 8[3,13] and CASMCs at passages 4 to 6[25,26] were used in all the experiments.

Biofucntionalization of CoCr surfaces

Prior to silanization, CoCr samples were activated by alkaline etching with 5M NaOH for 2 hours and after, samples were throughly cleaned by immersion in distilled water for 30 min twice. After activation the samples were introduced in a N_2-saturated glass vessel and immersed for 1 h at 90°C in a solution containing 0,5 M 3-chloropropyltriethoxysilane (CPTES) and 0,05 M N,N-diisopropylethylamine (DIEA) in anhydrous toluene (Sigma-Aldrich) under nitrogen atmosphere. Next, samples were ultrasonicated successively in cyclohexane, isopropanol, distilled water and acetone, and finally dried with N_2 gas.

Biomolecules were immobilized on the CoCr surfaces by two different methods: (1) physical adsorption on plain CrCo samples (samples were coded as CT+peptide); and (2) covalent immobilization through silanization process of the CoCr activated surfaces with CPTES (samples coded as NA-CP+peptide).

The RGDS, YIGSR peptides and their combinantion (1:1) were dissolved in a solution of phosphate buffered saline (PBS) adjusted with Na_2CO_3 to obtain a pH of 13.0 at a concentration of 100 µM. 100 µl of peptide solutions were deposited on the CPTES-grafted surfaces overnight at room temperature (RT). To adsorb physically the peptide on non-silanized samples, the same conditions were used but using PBS at pH 7.4 instead. Control samples were only treated with buffer. After peptide incubation, samples were gently washed three times with distilled water and dried with nitrogen.

The biofunctionalized samples, and their controls, are codified as follows:

CT: Non-treated smooth CoCr surface.

CT-RGDS: CoCr surface coated with 100 µM of RGDS peptide.

CT-YIGSR: CoCr surface coated with 100 µM of YIGSR peptide.

CT-RGDS+YIGSR: CoCr surface coated with 100 µM of RGDS+YIGSR combined peptides.

NA-CP-RGDS: CoCr activated with NaOH, silanized with CPTES and coated with 100 µM of RGDS peptide.

NA-CP-YIGSR: CoCr activated with NaOH, silanized with CPTES and coated with 100 µM of YIGSR peptide.

NA-CP-RGDS+YIGSR: CoCr activated with NaOH, silanized with CPTES and coated with 100 µM of RGDS+YIGSR combined peptides.

Prior to cell adhesion assays, functionalized samples were blocked for 1 h at 37 º C with 5% (w/v) bovine serum albumin (BSA) in PBS in order to reduce non-specific interactions of the cells with the surface.[24,27–29]

Quantitative real-time polymerase chain reaction

Expression of adhesion (ICAM-1, VCAM-1), vascularization (VEGFA, VEGFR-1, VEGFR-2), pro-thrombogenic (vWF, PAI-1) and anti-thrombogenic (tPA, eNOS) genes (Table 1) in HUVECs on modified CoCr surfaces was determined through RT-qPCR assay after 24 h, 48 h and 72 h as previously described.[30]

At each culture time, total RNA was extracted using RNeasy® Mini Kit (Qiagen, Hilden, Germany) and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). One hundred nanograms were retrotranscribed to cDNA products which were further diluted and used as RT-qPCR templates (Quantitect Reverse Transcription Kit, Qiagen). The resulting cDNA is then amplified using the QuantiTect SYBR Green RT-qPCR Kit (Qiagen) in an ABIPrism 7700 machine (Applied Biosystems, Foster City, CA, USA). Specifity of each RT-qPCR reaction was determined by melting curve analysis and by resolving the RT-qPCR products on 2% agarose gels. The expression of all studied genes was normalized by the expression levels of β-actin. Moreover, fold changes were related to CT at 24 h of culture.

Platelet adhesion

Platelet activation and adhesion on the peptides were evaluated by exposing human blood to physiological arterial shear stress. Peptides were physisorbed into a commercially avalaible cone and plate-shearing device (Diamed Impact-R; Biorad Diamed GmbH, Cressier, Switzerland). The Impact-R [Cone and plate(let) analyzer] is designed to evaluate platelet function under flow conditions.[31] Samples of citrate phosphate dextrose (CPD) anti-coagulated

whole blood (130 µl) were placed on polystyrene wells and subjected to flow at 1800 s^{-1} for 2 min using a specially designed conical disk. Wells were then thoroughly washed with PBS, stained with May-Gruenwald stain and analyzed by the Impact-R analyzer system connected to a microscope. Platelet adhesion was determined by measuring the percentage of the well surface covered with platelets and the average size of the aggregates.

Immunofluorescence analysis of HUVECs and CASMCs co-culture

The HUVECs and CASMCs were separately grown until confluence. Then, a direct cell contact co-culture was performed by seeding onto the modified CoCr surfaces 10^4 cells/well HUVECs and 10^4 cells/well CASMCs in mixed EBM® and SmBm® (1:1) free-serum medium. After 24 h, the serum-free medium was changed by complete EBM® and SmBm® (1:1) medium until analysis. To discriminate cells by immunofluoscence, two specific cell-markers were used: PECAM-1 platelet endothelial cell adhesion molecule (CD31, Sigma Aldrich) for staining HUVECs and α-SMA α-smooth muscle actin (A5228, Sigma Aldrich) for staining CASMCs. Cells were fixed in a mixture of ethanol-acetone (50:50, v/v)[32] for 30 min, permeabilized with 0.05% (v/v) Triton X-100 in PBS for 20 min and blocked with 1% BSA (w/v) in PBS for 30 min. HUVECs and SMCs were stained by incubating first with the primary antibodies mouse anti-PECAM-1 (1:100, in blocking solution) and rabbit anti-α-SMA (1:100 in blocking solution) for 1 h. Finally cells were stained by incubation with the secondary antibodies Alexa Fluor 488 chicken anti-rabbit (Sigma Aldrich) and Alexa Fluor 568 goat anti-mouse (Sigma Aldrich), during 1 h in the dark. Nuclei were counterstained with DAPI (1:1000, in PBS) for 10 min in the dark. Between all steps, samples were rinsed twice with PBS for 5 min. CoCr disks were mounted and examined under a fluorescent inverted microscope (AF6500 widefield, Leica Microsystems, Germany) where images through stitching method were taken. Cell number and morphology were studied by ImageJ-FIJI software (NIH, USA).

Statistical analysis

All the experiments were triplicated for each condition and repeated at least in two independent experiments. Statistical comparison of values was based on ANOVA using Tukey's test for pair-wise comparison with $p < 0.05$. Differences were also analyzed by non-parametric Kruskal-Wallis test. Values of all graphs are reported as mean ± standard deviation. Statistical analysis was performed using Minitab software (Minitab Inc., USA).

RESULTS

Cell adhesion gene expression

The expression of genes related to cell adhesion (ICAM-1 and VCAM-1) was analysed at 24, 48 and 72 h on the different functionalized CoCr surfaces by real time – qPCR (Fig. 1). ICAM-1 gene expression was enhanced in HUVEC cultured on silanized CoCr surfaces, Na-CP series, after 48 h and even more upregulated after 72 h. Nonetheless, physisorbed series only presented a slightly increase of ICAM-1 expression after 48 and 72 h compared to plain CoCr, CT. From all tested surfaces NA-CP-RGDS+YIGSR was the functionalized CoCr substrate with a higher expression of ICAM-1 after 48 and 72 h. VCAM-1 gene expression showed an upregulation of its expression after 48 h specially for silanized series, whereas it was significally decreased after 72 h for all tested surfaces [Fig. 1(b)].

Vascularization gene expression

It was investigated the effect of immobilization of adhesive molecules onto CoCr, by physisorption or covalent binding, on the activation of HUVECs gene expression levels for vascularization through the analysis of VEGFA, VEGFR-1 and VEGFR-2 gene expression (Fig. 2). The gene expression of VEGFA and VEGFR-2 was negligible for all surfaces at 24 and 48 h, although it increased considerably after 72 h for all surfaces. An upregulated expression of VEGFR-2 was statistically significant for NA-CP-RGDS and NA-CP-RGDS+YIGSR series compared to CT. Concerning VEGFR-1, the gene expression was downregulated in all samples during time. In fact, at 24 h and 72 h no differences in expression levels were detected between the different surface treatments. Noteworthy, at 48 h CT-RGDS and NA-CP-RGDS+YIGSR

samples showed higher levels of VEGFR -1 gene expression compared to CT and CT-YIGSR, CT-RGDS+YIGSR, NA-CP-RGDS and NA-CP-YIGSR samples.

Thrombogenicity gene expression

Figures 3 and 4 show the expression of anti-thrombogenic and pro-thrombogenic genes, respectively, in HUVECs cultured onto physisorbed or functionalized CoCr surfaces. RT-qPCR measurements revealed that at lower times of cell culture, 24 h, the expression of eNOS antithrombogenic gene was lower than CT for all tested surfaces; whereas after 48 h the eNOS gene expression of functionalized surfaces was higher compared to CT but no statistically significant differences were detected between physisorbed and silanized samples. Finally, at 72 h, the expression level of eNOS was statistically significant higher for silanized surfaces compared to physisorbed (i.e. CT-RGDS, CT-YIGSR and CT-RGDS+YIGSR) and CT surfaces. NA-CP-RGDS+YIGSR was the surface with a higher eNOS gene expression at 72 h. tPA gene expression [Fig. 3(b)] was upregulated after 72 h specially on CT-RGDS, CT-.RGDS+YIGSR and NA-CP-RGDS+YIGSR compared to control and CT-RGDS, NA-CP-RGDS and NA-CP-YIGSR.

The expression levels of pro-thrombogenic gene PAI-1 [Fig. 4(a)] reached the maximum expression after 48 h on NA-CP-YIGSR and NA-CP-RGDS+YIGSR but CT and CT-RGDS and CT-YIGSR showed a considerably decrease. Nevertheless, after 72 h the expression level of PAI-1 for all surfaces was significantly reduced. The vWF gene expression was downregulated over time [Fig. 4(b)]. While at 24 h all treated surfaces had lower levels of gene expression compared to CT, after 48 h the levels were slightly higher for all surfaces. Finally, after 72 h only NA-CP-RGDS+YIGSR presented higher values compared to CT.

Platelet adhesion

Figure 5(a) shows the percentage of surface covered by platelets and Figure 5(b) the platelets aggregate size onto TCPS coated with RGDS, YIGSR and RGDS+YIGSR under flow conditions to determine the potential anti-thrombogenicity of peptides. In general, TCPS coated

with peptides lead to a decrease of covered area by platelets and a reduction of the platelet aggregate size compared to non-coated TCPS. In particular, RGDS coating was the condition with a lower platelet adhesion compared to YIGSR and RGDS+YIGSR. Moreover, RGDS and RGDS+YIGSR were the peptide coatings with a lower size of platelet aggregates compared to YIGSR.

HUVEC and CASMCs co-culture

Figures 6 and 7 display the fluorescence images and the quantification of adhered HUVECs and SMCs in co-culture after 24h and 48 h onto CT physisorbed and NA silanized CoCr surfaces with RGDS, YIGSR and their equimolar combination. HUVECs and SMCs showed a capillary-like morphology after 24 h as seen in Figure 6(a), specially for surfaces functionalized with YIGSR peptide and the equimolar combination of RGDS and YIGSR. The amount of HUVECs increased for all the treated surfaces after 48 h compared to 24 h of cell co-culture and compared to TCPS while SMCs underwent to lower proliferation after 48 h compared to 24 h. It was also observed an influence of the biomolecules coating since NA-CP-RGDS and NA-CP-RGDS+YIGSR increased the quantity of adhered HUVEC cells after 24 h (Figure 7). Similarly, after 48 h CT-YIGSR and NA-CP-RGDS+YIGSR coated surfaces showed higher cell number compared to the other functionalized surfaces.

DISCUSSION

In this study, surface modified CoCr alloy substrates for cardiovascular applications were functionalized with RGDS, YIGSR peptides and their equimolar combination by physisorption and covalent binding via silanization using CPTES as coupling agent. Then, the behavior of HUVECs cultured onto the different surface finish was evaluated through adhesion, vascularization, pro-thrombogenic and anti-thrombogenic gene expression.

Under normal physiological circumstances, ECs play a major role in preventing blood cells from adhering to the vasculature and subsequent coagulation.[9] After stent implantation, interaction of ECs and biomaterial is a crucial step for implant endothelialization. Based on

preliminary results, we found that functionalization of peptides (e.g. RGDS, REDV and YIGSR) onto CoCr surfaces is a promising strategy to overcome this issue.[19] There, we observed that combination of RGDS and YIGSR stimulated the adhesion of ECs. In addition to cell adhesion onto biomaterial, the cellular crosstalk through endothelial cell adhesion molecules such as VCAM-1 and ICAM-1 is also a critical step for endothelial integrity and functionality.[13,33,34] In the present study, ICAM-1 gene expression increased with time reaching its highest values for NA-CP-RGDS+YIGSR followed by NA-CP-RGDS and NA-CP-YIGSR after 72 h. On the other hand, VCAM-1 gene expression was upregulated after 48 h especially for NA silanized series. These results suggest that RGDS, YIGSR and the equimolar combination RGDS and YIGSR immobilized by silanization onto CoCr surfaces, could increase endothelial activation (Table 2). In a previous study, differences in peptide densities between both silanization and physisorption strategies were described.[19] Moreover, differences could not be attributed to silanes since no Si 2s was detected after peptides immobilization from X-ray photoelectron studies.[19] And surface roughness increased from ≈6.3 nm for CT surfaces to ≈16.0 nm for CoCr surfaces after NaOH etching. Thus, a higher quantity of immobilized peptide onto the surface by silanization compared to physisorption could be the reason for the increase of ICAM-1 and VCAM-1 gene expression.

In addition to being indicators of an adequate endothelial integrity, cell adhesion molecules such as VCAM and ICAM regulate anti-thrombogenic events.[35,36] Their expression in ECs is mainly regulated by VEGF, a potent angiogenic growth factor secreted by the ECs themselves. The main activities that VEGF regulates are endothelial cell survival, proliferation, migration, and tube formation[37] through its recognition via ECs membrane receptors 1 and 2.[37,38] VEGF, VEGFR-1 and VEGFR-2 are each essential for normal blood vessel development, although most of the VEGF cellular responses are mediated through the VEGFR-2, including VCAM-1 and ICAM-1 expression regulation.[33] From our results, it was observed a slighltly higher expression of VEGF and VEGFR-2 after 72 h and of VEGFR-1 after 48 h for NA-CP silanized series compared to CT (Table 2). VEGF stimulation can induce a programmed phenotypic change of ECs and become pro-thrombotic[38] but this effect is dose dependant. An elevated

VEGF concentration can stimulate coagulation and induce EC proliferation and migration in response to trauma. Nevertheless, a minimum level is needed for the survival of the EC lining. In our case, NA-CP-RGDS and NA-CP-YIGSR surfaces presented significant higher VEGF expression compared to CT while NA-CP-RGDS+YIGSR surfaces showed the same VEGF expression as CT and physisorbed series. Nevertheless the higher expression of ICAM-1 and VCAM-1 after 72 h and 48 h respectively, onto all silanized NA-CP series independently of the immobilized peptide compared to CT indicates that the amount of VEGF expressed is low enough to maintain EC lining.

The activation and propagation of the coagulation cascade is mainly prevented by ECs, which produce and secrete many anti-thrombogenic factors. Among them, endothelial nitric oxide synthetase (eNOS) which produces nitric oxide that prevents platelet aggregation and activation;[39] and tissue type plasminogen activator (tPA) converts plasminogen into plasmin for the immediate breakdown of fibrin.[40,41] In contrast, under pathological conditions they can secret pro-thrombogenic factors such as plasminogen activator inhibitor (PAI), which inhibits the tPA activity, and also the von Willebrand factor which activates the coagulation cascade.[38,42] The plasminogen activator inhibitor (PAI), a pro-thrombogenic factor, present a higher upregulation after 48 h onto NA-CP-YIGSR and NA-CP-RGDS+YIGSR surfaces but after 72 h the expression is reduced. Noteworthy the expression of pro-thrombogenic vWF factor decreases over time for all surfaces. This behavior is probably related to a healing process where clot formation is needed for a correct wound healing.

Interestingly, the expression of anti-thrombogenic factors tPA and eNOS increased over time. The NA-CP-RGDS+YIGSR surfaces showed the higher eNOS expression compared to the other studied surfaces. The fact that the studied pro-thrombogenic genes expression decreases over time, obtaining insignificant values for PAI-1 and very low values for vWF, combined with an increase in anti-thrombogenic genes expression over time specially for functionalized series, indicate that the functionalized surfaces could promote endothelium healing and functionality and then, prevent thrombogenicity. In particular, the NA-CP-RGDS+YIGSR treatment seems to be an interesting candidate for accelerating the endothelium recovery.

Moreover, potential anti-thrombogenicity of peptides was also evaluated through platelet aggregation, by circulating human blood onto TCPS coated surfaces with RGDS, YIGSR and their combination peptides. It is well known that platelet adhesion and aggregation are mediated by fibrinogen via the receptor glycoprotein IIb/IIIa (αIIbβ3), which also recognizes the arginine-glycine-aspartic (RGD) amino-acid sequence.[43] Several authors have demonstrated an inhibitory effect of RGD when is present in solution.[44–46] However, there are few studies concerning the effect of RGD immobilized onto surfaces on platelet adhesion and aggregation. In the present study, surprisingly, platelet adhesion was lower for RGDS peptide compared to TCPS. This reduction in platelet adhesion may be attributed to lower bond interaction between RGDS and αIIbβ3 compared to fibrinogen and αIIbβ3,[47] which might be less stable at the applied shear stress in the present study.[48] The different peptides where physisorbed to the TCPS surface, then, a low strength immobilization is expected compared to covalent bonding. This could indicate a detachment of the RGDS to the solution blocking the interaction of αIIbβ3 with fibrinogen thereby reducing the adhesion of platelets. The YIGSR and YIGSR+RGDS peptides also demonstrated lower platelet adhesion values compared to TCPS. In these cases, YIGSR non-specificity for several integrin receptors of platelet cells could be the reason for inhibiting platelet adhesion. Again, a low adhesion of the peptides to the surface could induce a detachment and then, in solution, an interaction with αIIbβ3, decreasing the number of adhered platelets. Noteworthy, all the surfaces covered with peptides demonstrated a lower platelet aggregate size compared to TCPS. This may be attributed to the aforementioned αIIbβ3 blocking capacity of the peptides used.

In addition to ECs activation, communication within SMCs is an essential process for the maintenance of normal tissue physiology.[13,49] The interaction between ECs and SMCs in the artery undoubtedly plays a significant role in vascular wall remodeling and and when it is inadequate could lead to the development of atherosclerotic disease and intimal hyperplasia. However, SMC proliferation at the implant surface is not desired in order to prevent restenosis. In the present work, competition between both types of cells for the modified surfaces was evaluated. At intial co-culture adhesion times, 24 h, the surfaces functionalized with NA-CP-

RGDS and NA-CP-RGDS+YIGSR showed a higher number of adhered HUVECs compared to the other tested surfaces. Howere after 48 h of cell co-culture CT-YIGSR and NA-CP-RGDS+YIGSR series showed the higher HUVEC adhesion. This indicates that the equimolar combination of RGDS and YIGSR immobilized onto CoCr surface, exhibit adhesion selectivity toward ECs. Therefore, an interesting synergistic effect is observed between both RGDS and YIGSR active sequences. This synergistic effect may be attributed to the fact that both RGDS and YIGSR peptides interact with different cell receptor molecules. Whereas the former is an integrin binding sequence the later interacts with the 67 kDa laminin binding protein (LBP). It is well known that RGDS modified surfaces allow the formation of focal adhesion sites promoting cell spreading. [50,51] Interestingly, the YIGSR peptide has been found to co-localize LBP with α-actinin and vinculin acting as an integrin-accessory molecule that contributes to the formation of focal adhesion.[52] In addition, activation of LBP by laminin or laminin peptides (i.e. YIGSR) has been related not only to cell attachment but also to cell differentiation, migration and capillary-like structures formation.[53] Generally, functionalization increased the amount of adhered HUVEC cells onto modified CoCr surfaces compared to plain CoCr, CT, independently of the peptide used and the strategy of immobilization. Moreover, a considerably lower amont of adhered CASMCs compared to adhered HUVECs in all surfaces was found. These results are in agreement with our preliminary results of ECs and SMCs single cell culture where an enhancement of HUVEC adhesion and proliferation on the adhered biomolecules onto CoCr was found while controlling SMC adhesion.[19] Herein, a higher number of ECs after 4h of culture compared to SMCs was observed. This competition for the functionalized surfaces could be critical for SMCs in co-culture conditions where ECs adhere faster probably sequestering potential SMC adhesion motifs. In addition, another possible explanation for such behavior is that both ECs and SMCs need an optimal peptide concentration for effective adhesion[54] that is probably not achieved for satisfactory supporting SMC adhesion.

CONCLUSIONS

We analysed the effect of immobilizing RGDS and YIGSR peptides, and their equimolar

combination on CoCr surfaces to determine HUVECs adhesion gene expression, thrombogenicity and HUVEC/CASMC co-culture. The results suggested the positive effect of functionalized surfaces to enhace HUVECs adhesion also in co-culture with SMCs, and the decrease of thrombogenicity depending on the surface finish. Taking all together, the equimolar combination of RGDS and YIGSR seems to be the most promising strategy for endothelialization of CoCr surfaces as observed by the gene expression of genes related to adhesion, vascularization, anti-thrombogenic processes in HUVECs. Cell adhesive peptides functionalization of CoCr metallic surfaces for cardiovascular applications may offer an efficient alternative to enhance rapid endothelialization, while preventing restenosis and thrombosis**.**

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FIGURES LEGENDS

FIGURE 1. Real time – qPCR analyses of the gene expressions for the selected genes of adhesion: a) ICAM-1 and b) VCAM-1 in HUVEC cultured on CT physisorbed and NA chemisorbed modified CoCr surfaces for 24, 48 and 72h. Results were normalized in respect to expression levels of the endogen reference gene β-actin and are represented as relative fold change to CT at 24 h (as explained in Materials and Methods section). For each studied gene, the letters a, b, c, d, e, f join surfaces with non-significant differences ($p<0.05$).

FIGURE 2. Real time – qPCR analyses of the gene expressions for the selected genes of vascularization: a) VEGFA, b) VEGFR-1 and c) VEGFR-2 in HUVEC cultured on CT physisorbed and NA chemisorbed modified CoCr surfaces for 24, 48 and 72h. Results were normalized in respect to expression levels of the endogen reference gene β-actin and are represented as relative fold change to CT at 24 h (as explained in Materials and Methods section). For each studied gene, the letters a, b, c, d, e, f join surfaces with non-significant differences ($p<0.05$).

FIGURE 3. Real time – qPCR analyses of the gene expressions for the selected genes of anti-thrombogenic: a) eNOS and b) tPA in HUVEC cultured on CT physisorbed and NA chemisorbed modified CoCr surfaces for 24, 48 and 72h. Results were normalized in respect to expression levels of the endogen reference gene β-actin and are represented as relative fold change to CT at 24 h (as explained in Materials and Methods section). For each studied gene, the letters a, b, c, d join surfaces with non-significant differences ($p<0.05$).

FIGURE 4. Real time – qPCR analyses of the gene expressions for the selected genes of pro-thrombogenic: a) PAI-1 and b) vWF in HUVEC cultured on CT physisorbed and NA chemisorbed modified CoCr surfaces for 24, 48 and 72h. Results were normalized in respect to expression levels of the endogen reference gene β-actin and are represented as relative fold change to CT at 24 h (as explained in Materials and Methods section). For each studied gene, the letters a, b, c, d join surfaces with non-significant differences ($p<0.05$).

FIGURE 5. Platelet adhesion assay, on TCPS coated with RGDS, YIGSR and the equimolar combination RGDS+YIGSR evaluated by exposing human blood to physiological arterial shear stress. The symbols join surfaces with non-statistically significant differences ($p<0.05$).

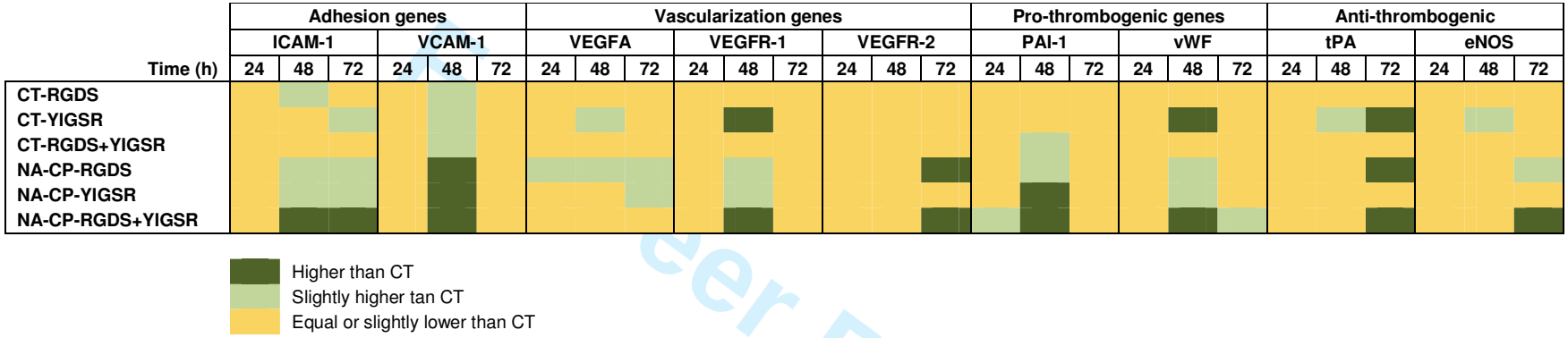
FIGURE 6. Overall morphology of adhered HUVEC (green) and SMC (red) cells co-cultured on a) CT phisysorbed b) NA-CP silanized biofunctionalized surfaces after 24 and 48 h and visualized by fluorescence microscopy. Bars: 50 µm.

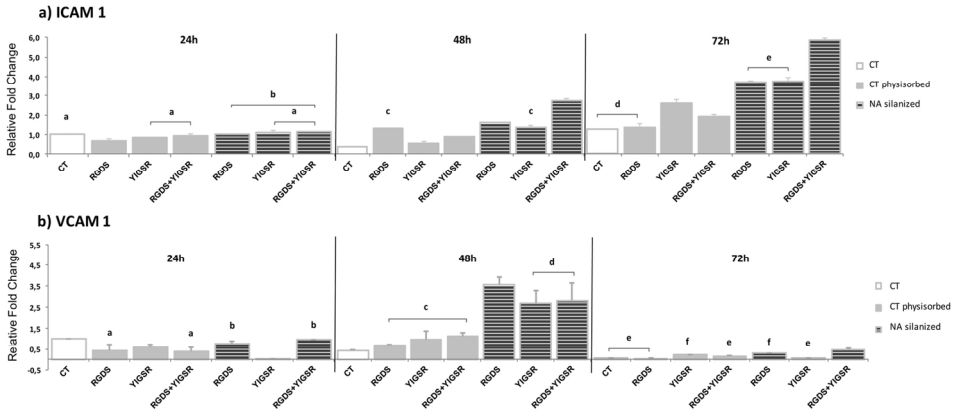
FIGURE 7. Quantification adhered HUVEC and SMC cells co-cultured on CT phisysorbed and NA-CP silanized biofunctionalized surfaces after 24 and 48 h.

Table 1. DNA sequences of forward (fw) and reverse (rv) primers for the selected genes used for real-time qPCR

Related function	Gene Symbol	Gene title	Acc. Number	Primer sequences	Amplicon size (bp)
Adhesion	ICAM-1	Intercellular adhesion molecule	NM_000201.2	fw:CCTTCCTCACCGTGTACTGG rv:AGCGTAGGGTAAGGTTCTTGC	90
	VCAM-1	Vascular adhesion molecule	NM_0001078.3	fw: CATGGAATTCGAACCCAAAC rv: TGTATCTCTGGGGGCAACA	70
Vascularization	VEGFA	Vascular endothelial growth factor A	NM_001025370.2	fw: CCTCCGAAACCATGAACTTT rv: ATGATTCTGCCCTCCTCCTT	122
	VEGFR-1	Vascular endothelial growth factor receptor-1	NM_002019.4	fw:CAGCATACCTCACTGTTCAAGG rv: CCACACAGGTGCATGTTAGAG	75
	VEGFR-2	Vascular endothelial growth factor receptor-2	NM_002253.2	fw: CGCATCACATCCACTGGTATT rv: TTTGTCACTGAGACAGCTTGG	76
Pro-thrombogetic	PAI-1	Plasminogen activator inhibitor	NM_00062.3	fw:AAGGCACCTCTGAGAACTTCA rv: CCCAGGACTAGGCAGGTG	61
	vWF	von Willebrand Factor	NM_000552.3	fw: GAAATGTGTCAGGAGCGATG rv:ATCCAGGAGCTGTCCCTCA	60
Anti-thrombogenic	eNOS	Endothelial Nitric oxide-synthetase	NM_001160109.1	fw: GCATCCCTACTCCCACCAG rv:TTCTTCACACGAGGGAACTTG	92
	tPA	Plasminogen activator	NM_000930.3	fw:AGCTGTGGGGAGCTCAGA rv:CACAGCGTCCCTTAAATTCAC	105
House keeping	β-actin	Beta actin	NM_001101.3	fw: AGAGCTACGAGCTGCCTGAC rv: CGTGGATGCCACAGGACT	114

Table 2. Summary of the gene expression analysis of CT physisorbed and NA-CP chemisorbed modified CoCr surfaces

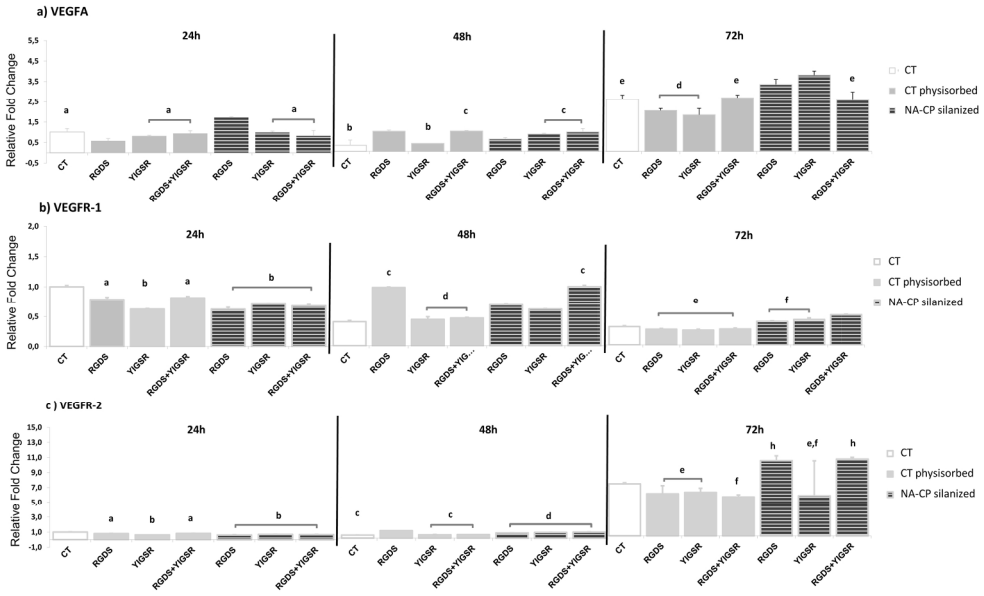




Real time – qPCR analyses of the gene expressions for the selected genes of adhesion: a) ICAM-1 and b) VCAM-1 in HUVEC cultured on CT physisorbed and NA chemisorbed modified CoCr surfaces for 24, 48 and 72h. Results were normalized in respect to expression levels of the endogen reference gene β-actin and are represented as relative fold change to CT at 24 h (as explained in Materials and Methods section). For each studied gene, the letters a, b, c, d, e, f join surfaces with non-significant differences ($p<0.05$).

Figure 1

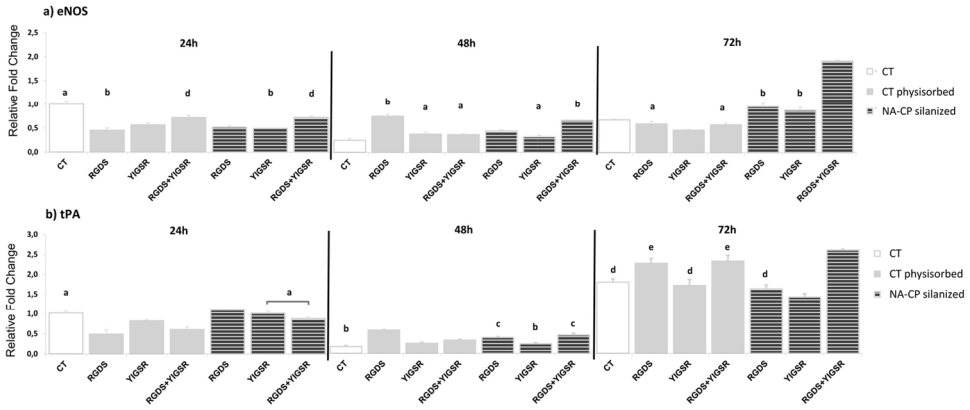
147x64mm (300 x 300 DPI)



Real time – qPCR analyses of the gene expressions for the selected genes of vascularization: a) VEGFA, b) VEGFR-1 and c) VEGFR-2 in HUVEC cultured on CT physisorbed and NA chemisorbed modified CoCr surfaces for 24, 48 and 72h. Results were normalized in respect to expression levels of the endogen reference gene β-actin and are represented as relative fold change to CT at 24 h (as explained in Materials and Methods section). For each studied gene, the letters a, b, c, d, e, f join surfaces with non-significant differences ($p<0.05$).

Figure 2

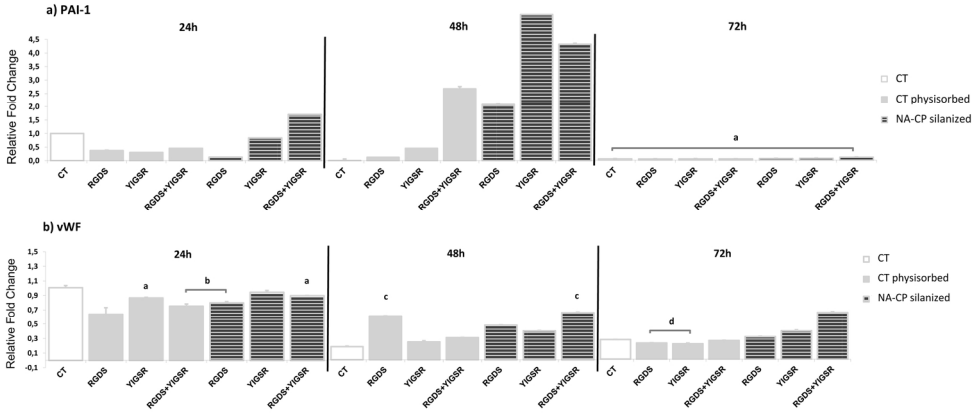
205x124mm (300 x 300 DPI)



Real time – qPCR analyses of the gene expressions for the selected genes of anti-thrombogenic: a) eNOS and b) tPA in HUVEC cultured on CT physisorbed and NA chemisorbed modified CoCr surfaces for 24, 48 and 72h. Results were normalized in respect to expression levels of the endogen reference gene β-actin and are represented as relative fold change to CT at 24 h (as explained in Materials and Methods section). For each studied gene, the letters a, b, c, d join surfaces with non-significant differences ($p<0.05$).

Figure 3

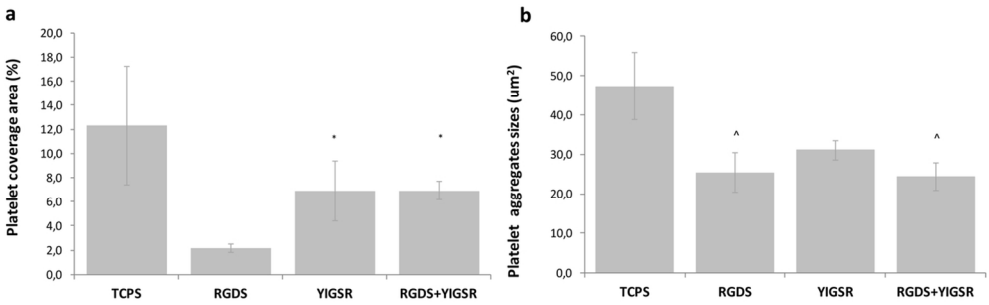
140x58mm (300 x 300 DPI)



Real time – qPCR analyses of the gene expressions for the selected genes of pro-thrombogenic: a) PAI-1 and b) vWF in HUVEC cultured on CT physisorbed and NA chemisorbed modified CoCr surfaces for 24, 48 and 72h. Results were normalized in respect to expression levels of the endogen reference gene β-actin and are represented as relative fold change to CT at 24 h (as explained in Materials and Methods section). For each studied gene, the letters a, b, c, d join surfaces with non-significant differences ($p<0.05$).

Figure 4

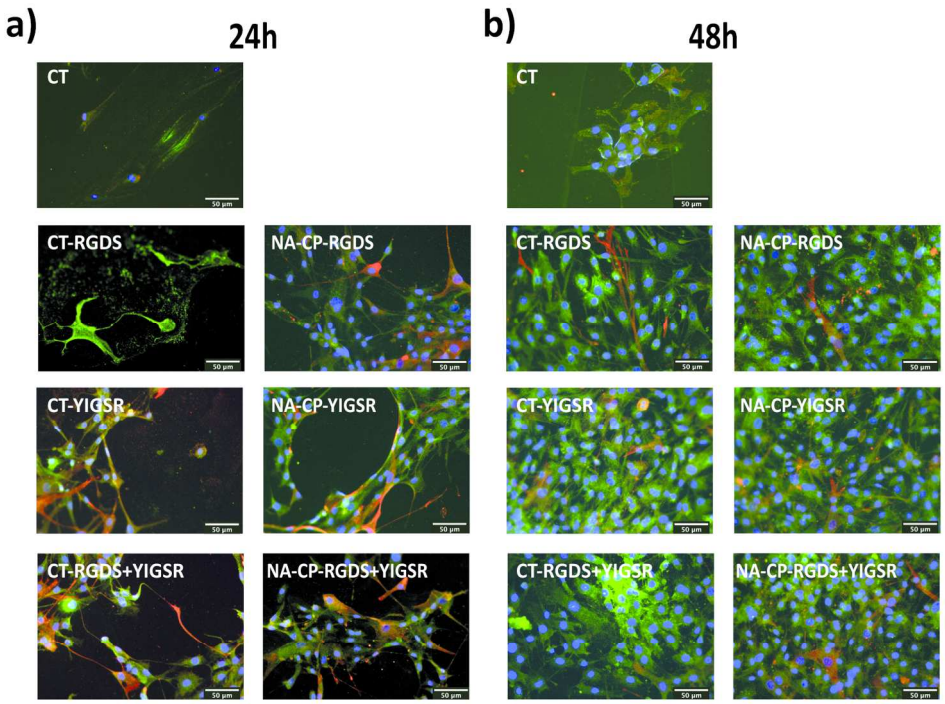
175x75mm (300 x 300 DPI)



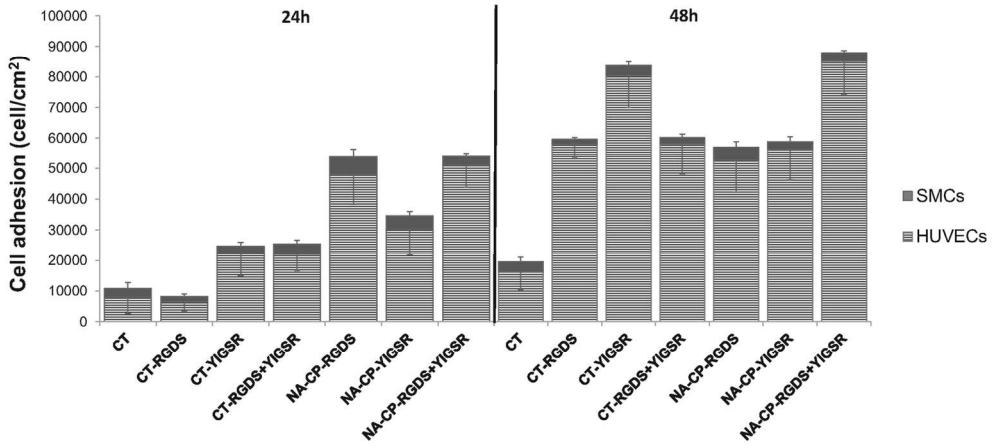
Platelet adhesion assay, on TCPS coated with RGDS, YIGSR and the equimolar combination RGDS+YIGSR evaluated by exposing human blood to physiological arterial shear stress. The symbols join surfaces with non-statistically significant differences ($p<0.05$).

Figure 5

118x36mm (300 x 300 DPI)



Overall morphology of adhered HUVEC (green) and SMC (red) cells co-cultured on a) CT phisysorbed b) NA-CP silanized biofunctionalized surfaces after 24 and 48 h and visualized by fluorescence microscopy. Bars: 50 μm.
Figure 6
165x122mm (300 x 300 DPI)



Quantification adhered HUVEC and SMC cells co-cultured on CT phisysorbed and NA-CP silanized biofunctionalized surfaces after 24 and 48 h.
Figure 7
147x72mm (300 x 300 DPI)