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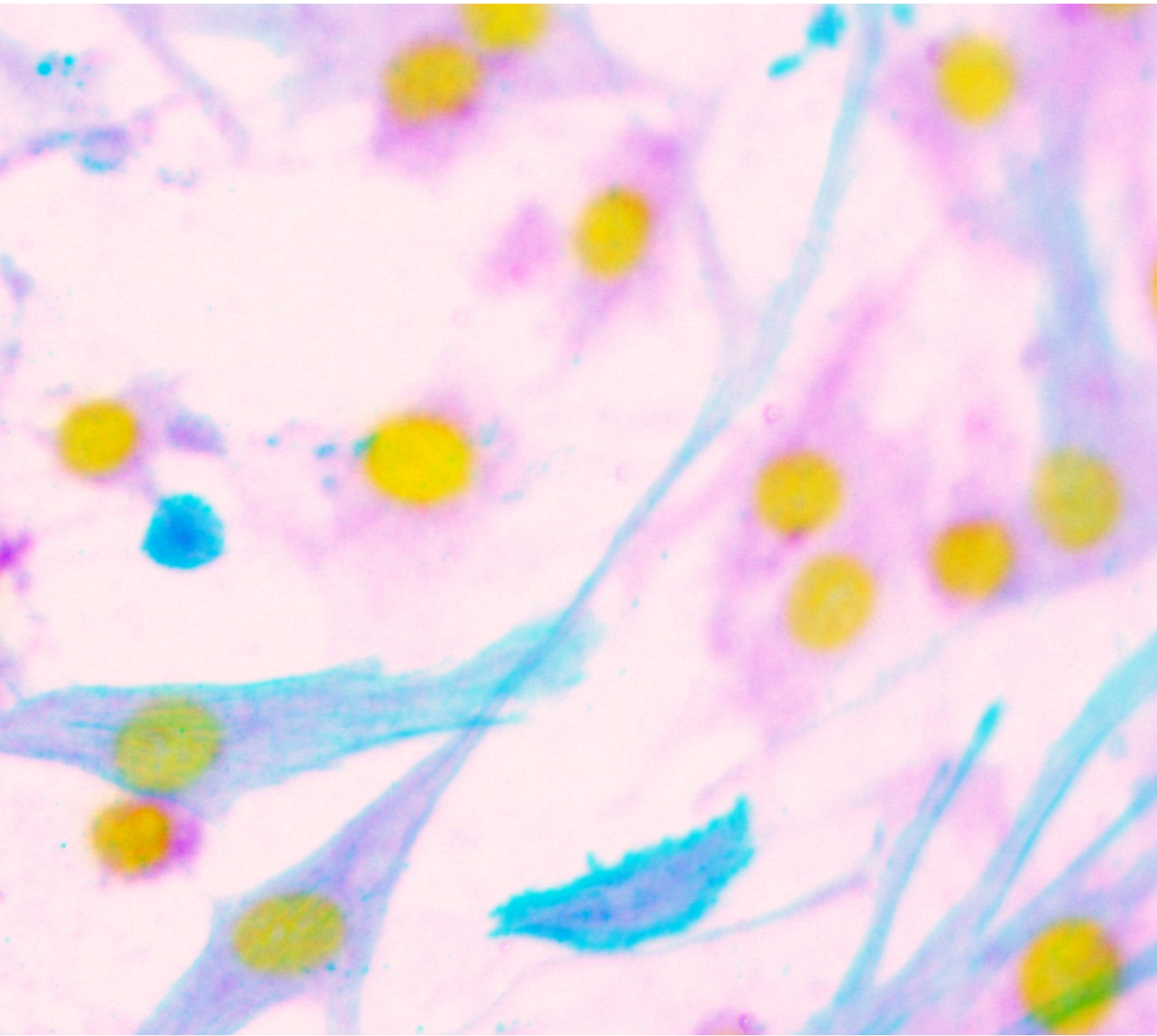

Thesis by compendium of publications

Doctoral Program in Materials Science and Engineering

Functionalized CoCr alloy surfaces with cell adhesive molecules to improve endothelialization

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Barcelona, March 2017

Cover: Cell culture of HUVECs (pink) and SMCs (blue) onto CoCr functionalized with RGDS peptide after activation with NaOH and CPTES silanization; coded as NA-CP-RGDS

A Irene y Edilberto (Mi familia)
La razón de mi existencia

A Carlos
Mi motivación día tras día

Abstract

Cobalt-chromium (CoCr) alloys are widely used as biomaterials for coronary stents due to their excellent mechanical properties, biocompatibility and corrosion resistance. However, these materials are bioinert, retarding the complete endothelialization and resulting in a higher risk of restenosis, narrowing of the artery, and late-stent thrombosis. Therefore, the improvement of implants surface endothelialization has acquired importance in the last years.

Immobilization of cell adhesive biomolecules onto biomaterials surface is a well-known strategy to control cell response. However, the strategy of immobilization, the optimal combination or the appropriate spatial presentation of the bioactive sequences to enhance endothelialization for cardiovascular applications, remains to be elucidated.

The present PhD thesis focused on the development of a new biofunctionalized CoCr alloy surfaces in order to improve the endothelialization. To that end, elastin-like recombinamers (ELR) genetically modified with an REDV (Arg-Glu-Asp-Val) sequence and short synthetized peptides RGDS (Arg-Gly-Asp-Ser), REDV, YIGSR (Tyr-Ile-Gly-Ser-Arg) and their equimolar combination, were attached by physisorption and covalent bonding onto CoCr alloy surfaces and thoroughly characterized physico-chemically and evaluated *in vitro* with human umbilical vein endothelial cells (HUVECs), coronary artery smooth muscle cells (CASMCs) and platelets from blood donors.

First, biofunctionalized surfaces with ELR were developed and optimized by evaluating different surface activation treatments, oxygen plasma and sodium hydroxide etching, and different binding strategies, physisorption and covalent bonding. The functionalized surfaces demonstrated a higher cell adhesion and spreading of HUVEC cells, this effect is emphasized as increases the amount of

immobilized biomolecules and directly related to the immobilization technique: covalent bonding. Nevertheless, the silanization process was not completely effective since a mixture of covalent and physisorption behavior was observed probably due to the use of big molecules that decreased the control of the bonding between the biomolecule and the surface.
Secondly, it was synthetized immobilized RGDS, REDV, YIGSR and their equimolar combination peptides onto the different surfaces. Cell studies demonstrated that the covalent functionalization of CoCr surfaces with an equimolar combination of RGDS/YIGSR represented the most powerful strategy to enhance the early stages of HUVECs adhesion, proliferation and migration, indicating a positive synergistic effect between the two peptide motifs. Besides, gene expression and platelet adhesion studies showed that surfaces silanized with the combination RGDS/YIGSR improved anti-thrombogenicity compared to non-modified surfaces.

Finally, cell co-cultures of HUVECs/CASMCs found that functionalization increased the amount of adhered HUVECs onto modified surfaces compared to plain CoCr, independently of the used peptide and the strategy of immobilization.
Overall, the present thesis offer a comprehensive view of the effectiveness of immobilizing cell adhesive molecules onto CoCr alloy surfaces to enhance endothelialization while preventing restenosis and thrombosis for cardiovascular applications.

Resumen

Las aleaciones de cobalto-cromo (CoCr) son ampliamente utilizadas como biomateriales para stents coronarios debido a sus excelentes propiedades mecánicas, biocompatibilidad y resistencia a la corrosión. Sin embargo, estos materiales son bio-inertales, retardando la completa endotelialización y resultando en un mayor riesgo de reestenosis, estrechamiento de la arteria y trombosis tardía. Por lo tanto, la mejora de la endotelización de superficie de los implantes ha adquirido importancia en los últimos años.

La inmovilización de biomoléculas adhesivas celulares sobre la superficie de los biomateriales es una estrategia bien conocida para controlar la respuesta celular. Sin embargo, queda por aclararse la estrategia de inmovilización, la combinación óptima o la presentación espacial apropiada de las secuencias bioactivas para potenciar la endotelización en aplicaciones cardiovasculares.

La presente tesis doctoral se centró en el desarrollo de una nueva superficie biofuncionalizada de la aleación CoCr con el fin de mejorar la endotelialización. Para ello, los recombinameros tipo elastina (ELR) modificados genéticamente con una secuencia REDV (Arg-Glu-Asp-Val) y péptidos cortos sintetizados RGDS (Arg-Gly-Asp-Ser), REDV, YIGSR (Tyr-Ile-Gly -Ser-Arg) y su combinación equimolar, fueron anclados por fisisorción y unión covalente a las superficies de la aleación, se caracterizaron físicamente-químicamente y evaluó in vitro con células endoteliales de vena umbilical humana (HUVECs), células de músculo liso de arteria coronaria (CASMCs) y plaquetas de donantes de sangre.

En primer lugar, se desarrollaron y optimizaron las superficies biofuncionalizadas con ELR mediante la evaluación de diferentes tratamientos de activación superficial: plasma de oxígeno y ataque con hidróxido de sodio, y diferentes estrategias de unión: fisisorción y unión covalente. Las superficies funcionalizadas demostraron una mayor adhesión celular y propagación de células HUVEC, este

efecto se enfatiza a medida que aumenta la cantidad de biomoléculas inmovilizadas y se relaciona directamente con la técnica de inmovilización: la unión covalente. Sin embargo, el proceso de silanización no fue completamente efectivo, ya que se observó una mezcla de uniones covalentes y fisisorbidas, probablemente debido al uso de grandes moléculas que disminuyeron el control de la unión entre la biomolécula y la superficie.

En segundo lugar, se inmovilizado los péptidos sintetizados RGDS, REDV, YIGSR y sus combinaciones equimolares sobre las diferentes superficies. Los estudios celulares demostraron que la funcionalización covalente de las superficies de CoCr con una combinación equimolar de RGDS/YIGSR representó la estrategia más potente para potenciar las etapas tempranas de la adhesión, proliferación y migración de HUVECs, indicando un efecto sinérgico positivo entre los dos motivos peptídicos. Además, la expresión génica y los estudios de adhesión plaquetaria mostraron que las superficies silanizadas con la combinación RGDS/YIGSR mejoraron la antitrombogenicidad en comparación con las superficies no modificadas.

Finalmente, con los co-cultivos celulares de HUVECs/CASMCs se encontró que la funcionalización aumentaba la cantidad de HUVEC adheridas sobre las superficies modificadas en comparación con CoCr simple, independientemente del péptido usado y la estrategia de inmovilización.

En general, la presente tesis ofrece una visión completa de la efectividad de la inmovilización de moléculas adhesivas celulares en la superficie de la aleación CoCr para mejorar la endotelialización, mientras que previene la reestenosis y trombosis en aplicaciones cardiovasculares.

General Objectives

In-stent restenosis, stents thrombosis and incomplete stent endothelialization remain the principal mechanisms for the failure of the bare metal stents (BMS). In the present PhD thesis, new functionalized CoCr surfaces have been developed to modulate ECs response, in order to reduce BMS failure caused by a poor endothelialization.

The main specific objectives of this thesis are summarized as follows:

(i) To develop new surface biochemical coatings onto CoCr alloy in order to enhance endothelialization with the use of elastin-like recombinamers (ELR) (Chapter 3) and short synthetic peptides (Chapter 4 and 5).

- Immobilization of an Elastin-like recombinamer with an Arg-Glu-Asp-Val (REDV) bioactive sequence coating by physisorption and covalent binding with CPTES silane. (Chapter 3)
- Design and synthesis of short peptides with the following cell adhesive biomolecules: Arg- Gly – Asp - Ser (RGDS), Arg-Glu-Asp-Val (REDV) and Tyr-Ile-Gly-Ser-Arg (YIGSR) peptides (Chapter 4).
- Optimization of the immobilization process of RGDS, REDV, YIGSR and their equimolar combination by physisorption and silanization with CPTES. (Chapter 4)

(ii) To evaluate the biofunctionalized surfaces *in vitro* with endothelial cells (ECs), smooth muscle cells (SMCs) and platelets (Chapter 3, 4, 5).

- Endothelialization properties of functionalized surfaces by the analysis of ECs adhesion onto ELR REDV coated surfaces (Chapter 3) and ECs adhesion, proliferation and migration onto short peptides coated surfaces (Chapter 4);

- Restenosis properties of short peptide functionalized surfaces by the analysis of SMCs adhesion (Chapter 4) and cell co-culture ECs/SMCs studies (Chapter 5)
- Thrombogenicity properties of short peptide functionalized surfaces by the analysis of the expression of pro-thrombogenic and anti-thrombogenic genes. and *in vitro* platelets adhesion and aggregation (Chapter 5)

Acknowledgements

Todo en la vida tiene un inicio y un final, vamos creando y experimentando diferentes ciclos, a mí me gusta llamarlo ¨arches¨ donde siempre el inicio es incierto, y paulatinamente se va convirtiendo una intención en un resultado tangible, este doctorado es uno de esos grandes resultados.
Cada instante de este ciclo, lo he disfrutado, y es inevitable sentir cierta nostalgia, especialmente por todas las personas que he conocido durante este camino, y que, de alguna u otra forma, han creído en mí y me han ayudado a realizar este doctorado. No existen palabras para describir el inmenso agradecimiento, amor y afecto que tengo por todos. He crecido tantísimo durante estos años, y esto es gracias a cada uno de ustedes.

Empiezo por agradecerles a mis tutores, Dr. Jose Maria Manero siempre con su pensamiento calmado y reflexivo, gracias por el apoyo que me has regalado.
A la Dra. Marta Pegueroles, Marta, sin ti esta tesis no tendría ni pies ni cabeza, me enseñaste desde pipetear hasta controlar mis emociones, de verdad que no tengo palabras para decirte lo mucho que has hecho por mí, tanto a nivel científico como personal, gracias por tu guía y perseverancia.

Agradecerle también al Dr. Javier Gil, por permitirme realizar mi tesis doctoral dentro del grupo BBT. Así mismo a la profesora Maria Pau Ginebra, nuestra interacción fue muy reducida, aunque cada vez que coincidimos aprendí bastante, gracias por ser un modelo a seguir.

Al Dr. Carles Mas-Moruno, Carles, sabes que cuando sea grande quiero ser como vos, gracias por tus grandes consejos en los momentos más oportunos.
Al Dr. Jordi Guillem-Martí, por tu paciencia en las largas horas de ensayos celulares, por siempre contestar.
Me gustaría agradecer a todos los miembros pasados y presentes del grupo BBT a: Dr. Daniel Rodríguez, Dra. Montserrat Español, Dra. Cristina Canal, Dr. Emiliano

Salvagni, a los estudiantes de doctorado: Yass, Anna, Judith, Joanna, Kanupriya, Maite, Kiara y Zhitong, Y a los que siempre están ahí y hacen que el grupo funcione como un relojito: Noelia, Meritxell, Miquel, Marc, Mónica y Sergi.
A los miembros del Instituto de Bioingeniería de Catalunya (IBEC) y Parc Cientific, por permitirme trabajar en sus instalaciones; al Centro de Investigación en Nanoingeniería (CRnE) – Trifon, Montse y Carla, por su ayuda, y brindar siempre en los pasillos una sonrisa que alegraba el día.

A los de la oficina: Dr. Pablo Sevilla, Virginia Paredes y Nathalia Marín, quienes fueron los que iniciaron el gran reto de la biofuncionalización. A los nuevos miembros de la oficina, Romain, Mireia y Roberta. Rom, no sabes lo afortunada que me siento de haber coincidido en este momento de nuestras vidas, nos quedan grandes retos, gracias por estar ahí; Mire, gracias por esos cafés que, aunque esporádicos, eran súper intensos, espero que no sean los últimos.
A dos personas súper importantes para este tiempo David Pastorino y Maria Godoy, por subir ese listón tan alto, y ser esos grandes ejemplos a seguir.
Al profesor Rodríguez Cabello de la Universidad Valladolid, por permitirnos trabajar con su biomolécula (ELR-REDV).
Al grupo del Dr. Xavier Trepat y en especial al Dr. Xavier Serra, por su tiempo y dedicación.
I am very grateful to Prof. Dr. Michael Joner, MD for having me in his lab in the German Heart Center Munich-TUM, for his scientific contribution to this thesis, and for believing in me.

A mi familia BCN, por convertirse en mi familia, por estar en los buenos y malos momentos, por enseñarme a disfrutar de cada instante y nunca dejarme caer.
Finalmente, a mis papás, sin ustedes no sería ni la mitad de lo que soy, nunca estaría aquí si no fuera por su esfuerzo y por darme todo de ustedes y más.
A Carlos, gracias por aguantarme todos estos años, por cuidarme y por seguir creyendo en mí.

List of publications

Castellanos MI, Guillem-Marti J, Mas-Moruno C, Díaz-Ricart M, Escolar G, Ginebra MP, Gil FJ, Pegueroles M, Manero JM. Cell adhesive peptides functionalized on CoCr alloy stimulate endothelialization and prevent thrombosis and restenosis. J Biomed Mater Res A 105(4):973-983 (2017).

Castellanos MI, Mas-Moruno C, Grau A, Serra-Picamal X, Trepat X, Albericio F, Joner M, Gil FJ, Ginebra MP, Manero JM, Pegueroles M. Functionalization of CoCr surfaces with cell adhesive peptides to promote HUVECs adhesion and proliferation. Applied Surface Science 393, 82-92 (2017).

Castellanos MI, Zenses AS, Grau A, Rodriguez-Cabello JC, Gil FJ, Manero JM, Pegueroles M. Biofunctionalization of REDV elastin-like recombinamers improves endothelialization on CoCr alloy surfaces for cardiovascular applications. Colloids and Surfaces B: Biointerfaces 127, 22-32 (2015).

Conference contributions

25th ESB. European Conference on Biomaterials

Castellanos MI, Mas-Moruno C, Serra-Picamal X, Trepat X, Gil FJ, Manero JM, Pegueroles M. Title: ¨Immobilization of bioactive molecules on CoCr stents surface and collective cell migration dynamics studies¨. Publication: Proceedings of the 25th ESB European Conference. City: Madrid, Spain. Date: September 2013. Type of presentation: Oral

3rd TERMIS World Congress. Tissue Engineering International & Regenerative Medicine Society

Castellanos MI, Humbert D., Rodríguez-Cabello JC, Mas-Moruno C, Gil FJ, Manero JM, Pegueroles M. Title: ¨Biofunctionalization of CoCr surfaces with dimeric peptides and REDV elastin-like polymers to improve endothelization of cardiovascular implants¨. Publication: Proceedings of the 3rd TERMIS World Congress. City: Vienna, Austria. Date: September 2012. Type of presentation: Poster

6th EEIGM International Conference on Advanced Materials Research

Pegueroles M, **Castellanos MI,** Gil FJ, Manero JM. Title: ¨Biofunctionalized Co-Cr surfaces for cardiovascular applications¨. Publication: Proceedings of the 6th EEIGM International Conference on Advanced Materials Research. City: Nancy, France. Date: November 2011. Type of presentation: Oral

4th IBEC Symposium on Bioengineering and Nanomedicine

Castellanos MI, Rodríguez-Cabello JC, Gil FJ, Manero JM, Pegueroles M. Title: ¨Biofunctionalized Co-Cr surfaces for cardiovascular applications¨. Publication: Proceedings of the 4th IBEC Symposium on Bioengineering Nanomedicine. City: Barcelona, Spain. Date: June 2011. Type of presentation: Poster

Scholarships

Pre-doctoral gran FI AGAUR. Catalunya

Agència de Gestió d'Ajuts Universitaris I de Recerca (AGAUR)

Grant for universities and research centers for the recruitment of new research personnel (FI-DGR 2011).

Government of Catalonia |2011 – 2014|.

Scholarship DAAD. Germany

Grant for PhD students and young researcher's short-term scholarship.

Munich, Germany |January – May 2013|.

Glossary of terms

BMS Bare Metal Stents

DES Drug Eluting Stents

BDS Bioresorbable Stents

ECM Extracellular matrix

ISR In-stent restenosis

ST Stent Thrombosis

PTCA Percutaneous transluminal coronary angioplasty

CABG Coronary artery bypass graft

HUVECs Human Umbilical Vein Endothelial cells

SMCs Smooth muscle cells

CoCr Cobalt-Chromium alloy

NA Sodium hydroxide etching

PL Oxygen plasma surface treatment

CPTES M 3-chloropropyltriethoxysilane

ELR elastin like recombinamers

RGDS (Arg- Gly – Asp - Ser) peptide

REDV (Arg-Glu-Asp-Val) peptide

YIGSR (Tyr-Ile-Gly-Ser-Arg) peptide

CT Control

FN Fibronectin

LN Laminin

ICAM-1 Intercellular adhesion molecule

VCAM-1 Vascular adhesion molecule

VEGF Vascular endothelial growth factor (A, R1-R2)

tPA Plasminogen activator

eNOS endothelial nitric oxide

PAI-1 Plasminogen activator inhibitor

vWF von Willebrand Factor

Table of Contents

1 Chapter 1: Introduction

Cardiovascular disease, specifically coronary disease, remains the first cause of death among Europeans and around the world [1]. Coronary angioplasty with the insertion of a stent, also known as percutaneous coronary intervention (PCI), is the major surgery to solve atherosclerosis [2,3]. **Atherosclerosis,** which is the build-up of fatty material inside of your arteries forming a plaque, causes narrowing of the vessel wall [4]. The insertion of a bare metal stent (BMS) isn't risk-free. Neointimal hyperplasia refers to proliferation and migration of vascular SMCs and **in-stent restenosis** (ISR) has been a relatively common complication associated with bare metal stents (BMS). [5,6]. Also, **thrombosis is a common drawback** where a blood clot can form and cause your arteries to narrow again suddenly and it may even cause a complete blockage.

Thrombosis and ISR are the main obstacles for the healing process of the artery and endothelium recovery, after cardiovascular surgery of BMS. A promising way to achieve the healing of the endothelium after Percutaneous coronary intervention (PCI) may rely on fostering a rapid *in situ* endothelialization onto the implant surface by applying biochemical surface modifications.

CoCr alloys (i.e. ASTM F90) have been widely used as biomaterials since it does not evoke an inflammatory reaction when implanted. Moreover, they are widely used for stent manufacturing since they have exhibited a high strength and hardness, providing support to the retracting force created by diseased artery. As well as a high resistance to corrosion and wear due to the natural and spontaneous generation of a layer of chromium oxide that appears on the surface [7]. Nevertheless, the CoCr alloys surfaces are inert and do not promote any cell behavior.

Several studies have demonstrated that any surface treatment that fosters stent surface endothelialization will help to inhibit restenosis and thrombosis [8–10]. Different strategies have been developed onto BMS in order to overcome the mentioned problems. Passive coatings include inorganic and organic coatings with the aim to act as an inert barrier between the blood flow /tissue and the metal [6]. Also, micro- and nano-topographies, independent of surface chemistry, have shown to influences endothelialization [11]. Finally, biochemical surface modification with the immobilization of biomolecules, will activate the biomaterial surface and induce specific cell and tissue response. Biofunctionalization of CoCr surfaces with the immobilization of biopolymers or cell adhesive motifs derived from the extracellular matrix (ECM) [12,13], is a powerful approach to stimulate cell response and accelerate the biomaterials integration.

The overall aim of this thesis was to develop a new family of biofunctionalized CoCr alloy surfaces by covalently-anchoring cell adhesive motifs to modulate endothelial cells response, in order to prevent atherosclerosis, ISR and thrombosis diseases. Different strategies have been studied in order to enhance endothelialization such as elastin-like-biopolymers and cell adhesive motifs (i.e. peptides RGDS, REDV and YIGSR) (Paper I (Chapter 3), Paper II (Chapter 4) and Paper III (Chapter 5)).

1.1 Paper I (Chapter 3)

Biofunctionalization of REDV elastin-like recombinamers improves endothelialization on CoCr alloy surfaces for cardiovascular applications

Authors: Castellanos MI, Zenses AS, Grau A, Rodriguez-Cabello JC, Gil FJ, Manero JM, Pegueroles M.

Manuscript published; Colloids and Surfaces B: Biointerfaces 2015; 127, 22-32. (DOI: 10.1016/j.colsurfb.2014.12.056)

IF: 3.902, Q1, ranked 8/33 in the category ¨ Material Science, Biomaterials¨.

Presented in 3erd TERMIS World Congress, Vienna, Austria. September 2012 and XXXV SIBB Congress, Madrid, Spain, October 2012.

Author's contribution: Major part of planning and analysis. Performed all the experiments, the analysis of the study and wrote the paper.

The manuscript is the result of the development and characterization of a new family of functionalized CoCr surfaces. For that, surfaces were activated with oxygen plasma and sodium hydroxide, previous to silanization and finally, an elastin-like recombinant protein genetically modified with the REDV sequence was immobilized. This motif is a bioactive specific domain for ECs attachment.

The main purpose of this study was the analysis and comparison between the immobilization of the ELR through physical adsorption and covalent bonding through the analysis of the amount of the biomolecule binded onto the material and the ECs *in vitro* response. This is the first time that ELR with REDV sequence has been immobilized onto CoCr surfaces with the objective to ameliorate endothelialization.

The REDV ELR was successfully immobilized onto CoCr surfaces and promotes ECs adhesion and spreading. This effect is emphasized as increases the amount of immobilized biomolecules. This study allowed to optimize the biofunctionalization process onto CoCr surfaces and demonstrated that biofunctionalization is a well-established process to control and guide cellular response onto biomaterials.

1.2 Paper II (Chapter 4)

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

Authors: Castellanos MI, Mas-Moruno C, Grau A, Serra-Picamal X, Trepat X, Albericio F, Joner M, Gil FJ, Ginebra MP, Manero JM, Pegueroles M.

Manuscript published; Applied Surface Science 2017; 393, 82-92.

(DOI: 10.1016/j.apsusc.2016.09.107)

IF: 3.150 (2015), Q1, ranked 14/25 in the category ¨Material Science, Surfaces, Coating and Films¨

Presented in 6th EEIGM International Conference, Nancy, France. November 2011.

Author's contribution: Major part of planning and analysis. Performed all the experiments, the analysis of the study and wrote the paper.

The manuscript describes the development and characterization of functionalized CoCr surfaces with the following cell-binding moieties peptides: RGDS, REDV, YIGSR, and their combinations to promote ECs adhesion and proliferation.

The use of short synthetic peptides as coating molecules has emerged as a powerful approach to overcome limitations associated with the use of proteins. This work selected the use of RGDS, REDV, derived from human fibronectin, and YIGSR, present in laminin, and their combinations to enhance endothelialization.

The use of combinations of peptides was the main contribution of this study.

The purpose of the present study was to determine the best strategy to promote ECs adhesion and proliferation. Thus, different peptides where synthetized with different spacers and bioactive motifs and attached onto CoCr surfaces. An extensive characterization before and after functionalization was performed for all the conditions.

The results presented in this study showed the potential of the use of equimolar combinations the selected cell adhesive peptides. Demonstrating that they are a promising approach to improve endothelialization and migration, as well as to reduce SMCs adhesion.

1.3 Paper III (Chapter 5)

Cell adhesive peptides functionalized on CoCr alloy stimulate endothelialization and prevent thrombosis and restenosis

Authors: Castellanos MI, Guillem-Marti J, Mas-Moruno C, Díaz-Ricart M, Escolar G, Ginebra MP, Gil FJ, Pegueroles M, Manero JM.

Manuscript published; J Biomed Mater Res A. 2017;105(4):973-983.
(DOI: 10.1002/jbm.a.35988)

IF: 3.263 (2015), Q1 ranked 14/33 in the category ¨Material Science, Metals and Alloys¨

Presented in 25th ESB European Conference, Madrid, Spain. September 2013.

Author's contribution: Major part of planning and analysis. Performed all the experiments, the analysis of the study and wrote the paper.

The manuscript describes the study of biomimetic surfaces modifications to improve endothelialization CoCr surfaces but focused in the prevention of thrombosis and restenosis. The present study continues the work started at Paper II by using the surface coatings that enhance endothelialization onto CoCr surfaces, focusing on RGDS, YIGSR peptides and combination.
A key factor in stents biomaterials is the effect in thrombogenicity and restenosis were an over-proliferation of SMCs causes the narrowing of the artery. Thus, the use of coatings that overcome the mentioned problems is of special interest.

The main purpose of this study was to study the effect of functionalized CoCr surfaces with RGD, YIGSR peptides and their combination on the ECs gene expression of genes relevant for adhesion, vascularization, anti-thrombogenic and pro-thrombogenic events using RT-qPCR technique. Moreover, thrombogenicity was evaluated through platelet adhesion and aggregation and restenosis through cell co-cultures of ECs/SMCs.

This study demonstrated that CoCr surfaces functionalized with an equimolar combination of RGDS and YIGSR peptides showed a decrease of SMCs and platelet adhesion.

2 Chapter 2: Literature Review

The narrowing of the artery is due to fat and lipid accumulation in the arterial wall and causes the decrease in blood flow and the common surgery to solve it is called coronary angioplasty which consists of a stent placement. It is performed by inserting a catheter through the artery (i.e. femoral, radial or brachial) to locate the artery to be treated. In 1964, the first angioplasty was described by Charles Theodore Dotter and Melvin P. Judkins [14]. Thirteen years later Andreas Grüntzig developed the first transluminal coronary angioplasty (CTA) with balloon [15]. From this first procedure, the intervention has evolved from the coronary bypass, which requires open heart surgery; to a balloon angioplasty, where the balloon is inflated until the obstruction is eliminated, culminating in percutaneous coronary interventions (PCI), commonly known as coronary angioplasty or simply angioplasty.

Currently, the PCI [16], involve coronary stents using "simple balloon angioplasties" [17], giving a wide variety of stents, which are included in two large families: (i) BMS [7] to (ii) drug eluting stents (DES) [18] through a polymer coating on metal stent. The latter include different subfamilies depending on the type of drug they release (I.e. sirolimus eluting stent (SES), placitaxel-eluting stents (PES) everolimus eluting stents (EES), zotarolimus eluting stents (ZES), among others). As well as different types of polymer coatings, such as: phosphonilicoline (PC), heparin, [19], polyethylene terephthalate (PET) [20], n-butyl poly-methacrylate (PBMA), coatings with biodegradable polymers such as polylactic acid (PLA), poly-acid-L-lactic acid (PLLA), poly-orthoester, poly-caprolactone and polyethylene / polybutylene terephthalate (PEO / PBTP) have been used. Finally, biodegradable stents (BDS) appeared recently and are under clinical studies; these are stent fully degradable in 12 to 14 months. At present, the third generation of stents attempts to use fully biodegradable and fully bio adhesive stents, whether polymeric (i.e. polylactic acid (PLA) or metallic (i.e., magnesium) [18].

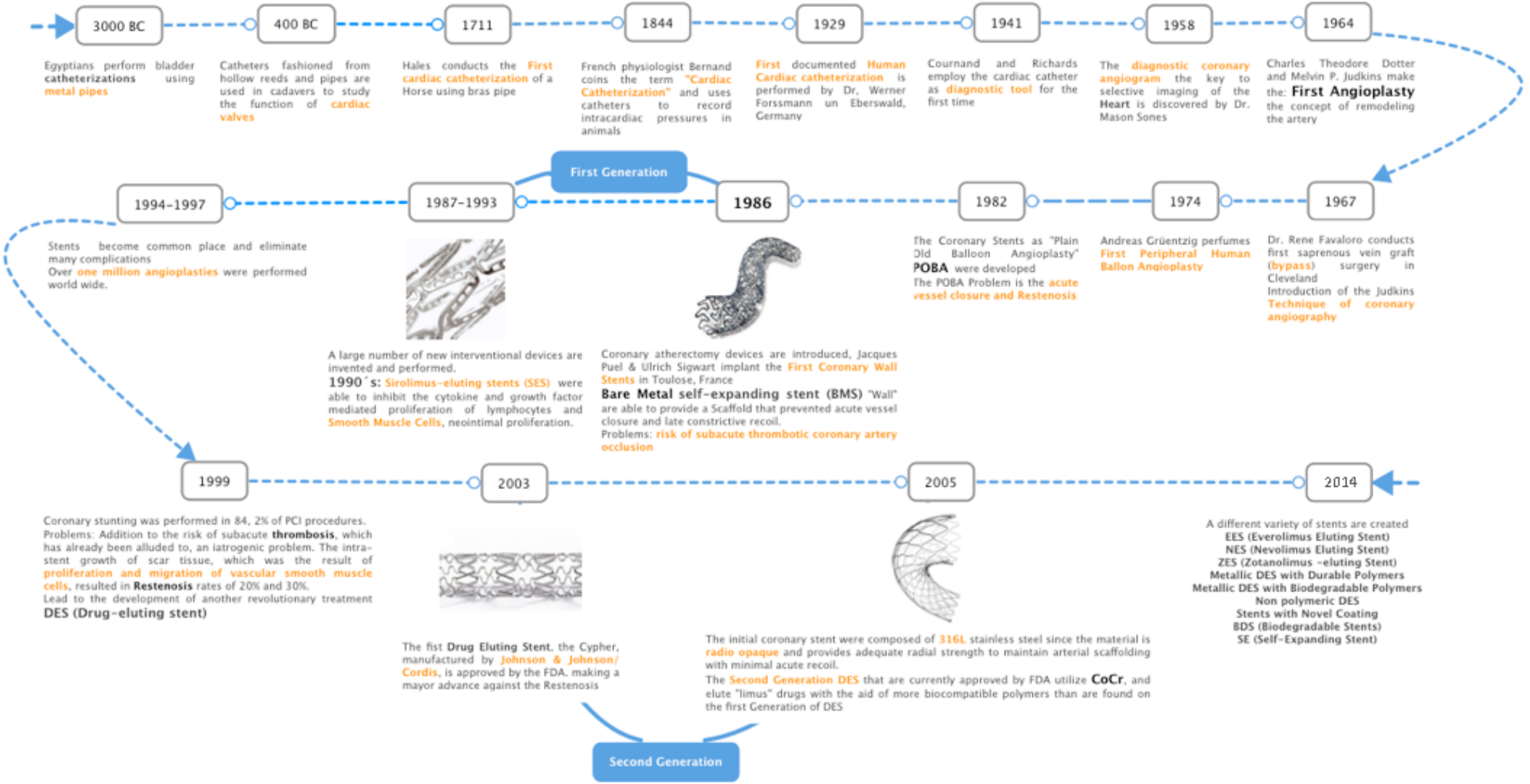

Figure 2.1 Coronary Intervention timeline. Adapted from [1,21]

2.1 Vascular diseases

Cardiovascular disease is one of the leading causes of mortality and morbidity in the world. According to the World Health Organization (WHO), 17.3 million people died from cardiovascular related diseases in 2008, and this number is projected to rise to an estimated 23.6 million by 2030 [22].

Atherosclerosis [3] is one of the most severe forms of cardiovascular disease and artery injury, it causes the narrowing of the vessel wall. It is characterized by calcification and the buildup of fatty deposits, cellular fragments and cholesterol in arteries, resulting in stenosis of the vessels. If this occurs in coronary arteries, insufficient delivery of oxygenated blood to the heart would result in cardiac ischemia, ultimately leading to a heart attack.
Blocked arteries can be treated either by a coronary artery bypass graft (CABG) surgery or, a less invasive technique, percutaneous angioplasty followed by stent placement, the PCI intervention. [23,24]

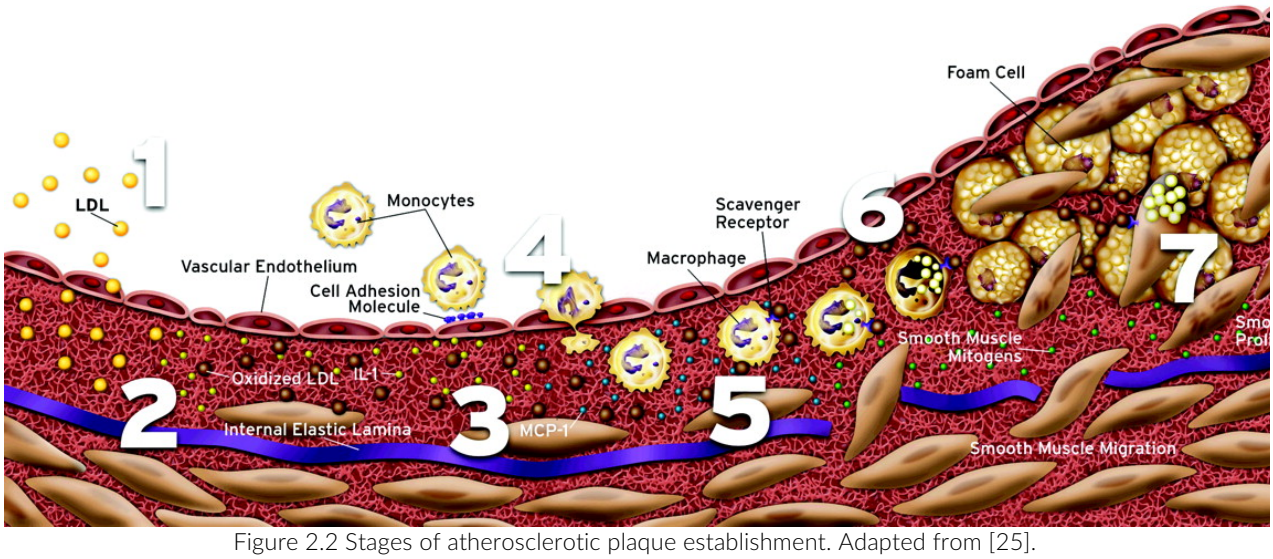

Figure 2.2 Stages of atherosclerotic plaque establishment. Adapted from [25].

Intracoronary stenting demonstrated feasibility of dilating atherosclerotic coronary lesions; however, the high rate of acute-vessel recoil, restenosis and dissection resulted in high acute closure rates and restenosis, which lead to the introduction of coronary stents, improving clinical outcome, the BMS. This type of stents reduces significantly the restenosis rate from 30-50% to 20-30% [6]; however, the

major issue with the use of BMS was the high incidence of ISR, which results mainly from the SMCs over proliferation and the ECM components production [26].

The second generation of stents, DES, have reduced the restenosis rate to 10% by inhibiting the SMCs proliferation through the delivery of antiproliferative drugs. The development of this type of stents was divided into two generations; the first one (especially SES and PES) resulted in a dramatic reduction of restenosis, with a decrease in revascularization procedures. However, the undoubted efficacy of first generation DES came at the expenses of substantially delayed arterial healing, proving the presence of late stent thrombosis / very late thrombosis (LST/VLST). Second-generation DES (EES and ZES) were designed to overcome the limitations of the first-generation DES and consisted of thinner stent struts, more biocompatible polymeric coatings with reductions in drug load. However, the new DES show delayed arterial healing associated with increased rates of ST and DES compared to BMS, due to the non-complete re-endothelialization.

Finally, bioresorbable stents (BRS) were recently introduced in cardiology and represent a promising solution to overcome many of the limitations of metallic DES. However, most recent data suggest that BDS technology is also hampered by some important shortcomings that need to be solved by novel design of BDS and innovative coating technology mainly due to low mechanical properties related to the material [27].

A timeline of the different generations of stents and their actions and side effects is shown in Figure 2.3.

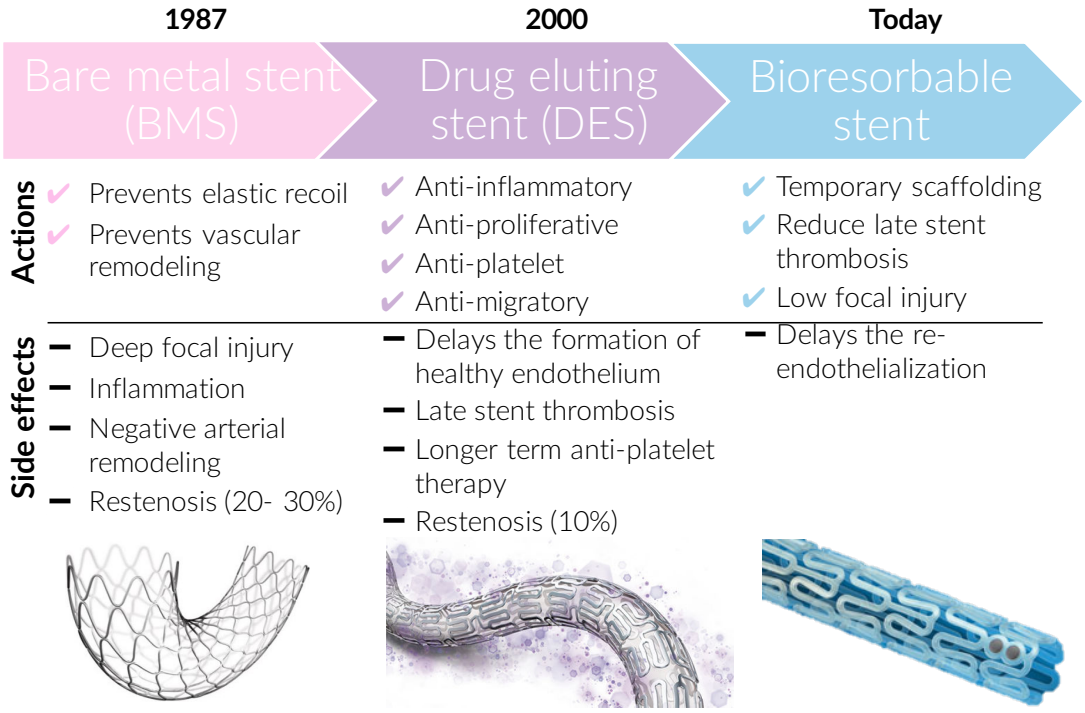

Figure 2.3 Timeline of the different generation of stents consequences.

2.2 Biomaterials for BMS

The main objective of the BMS were to counter the negative effects of the balloon angioplasty, describing the stent as a metallic mesh, which should address properties such as: rigidity for maintenance the dilatation and resistance to the elastic recoil, elasticity or plasticity for expansion, corrosion, radiopacity and radial strength [7].

Therefore, from the viewpoint of mechanical properties, it is natural that stents are made of metals. The metallic materials most currently used for coronary stents are 316L stainless steel, cobalt chromium (CoCr) alloys and titanium and its alloys [28–30]

Metals	Main alloying composition (wt%)	Mechanical propierties			
		Yield strength (MPa)	Ultimate thesile strength (MPa)	Young's modulus (GPa)	Max elogantion (%)
CoCr alloys: CoCrWNi (F90)	Co; 19-21Cr; 14-16 W; 9-11 Ni	310	860	210	20
Stainless steel: 316L type	Fe; 16-18.5Cr; 10-14Ni; 2-3Mo; <2Mn; <1Si; <0.003C	190	490	193	40
Ti alloys: Ti-6Al.4V alloy (F136)	Ti; 5.5-6.75Al; 3.5-4.5V; 0.08C; 0.2O	795	860	116	10

Table 2.1. Metals and properties needed for stents materials. Adapted from [31].

Stainless Steel

Most stents consist of austenitic stainless steels 316L. The main reason for the use of stainless steels is the good balance of mechanical strength (Young's modulus E ≈ 193 GPa) and elongation which facilitates the manufacture of the stent, the plasticity for the balloon expansion and the maintenance of morphology to resist the elastic recoil of blood vessels. Stainless steels do not corrode in an oxygen-containing atmosphere, but they show pitting and crevice corrosion when implanted in the body [7].

Ni-Ti alloy (Nitinol)

Nitinol alloys consist of equal atomic amounts of Ti and Ni (49-51 mol% Ni) and shows unique mechanical properties such as shape memory, super elasticity, and damping [29]. The main disadvantage of these alloys are the low resistance to pitting corrosion, as well as the release of Ni ions, which are toxic and in some patients present hypersensitivity, as well as the difficulty of manufacture [30].

CoCr alloys

CoCr alloys show high strength, toughness, castability, corrosion resistance, and wear resistance. The corrosion resistance of CoCr alloys is better than that of stainless steel. The wear resistance is much better than those in stainless steel and Ti alloys, but the plasticity and workability are not as good as those of stainless

steel. The main CoCr alloy used for coronary stents fabrication is ASTMF90, which composition is exhibited in Table 2.2

Co alloys are divided into two categories, cast alloys, and wrought alloys, that's the reason for the Ni presence, improving the castability and workability of the material, although for biomedical application the Ni contents should be reduce in order to avoid the Ni allergies [29].

Wrought CoCr alloys, which are used for stents, were designed to avoid cast defects in cast CoCr alloys. The strength and elongation of wrought CoCr alloys, which increase with heat treatment and cold working, are as high as those of stainless steel. The corrosion resistance of the wrought CoCr alloy is lower than that of the cast CoCr alloy, but higher than that of stainless steel [7].

Composition		
%	min	max
C	0.05	0.15
Co	Balance	Balance
Cr	19.00	21.00
Fe	--	3.00
Mn	1.00	2.00
Ni	9.00	11.00
P	--	0.040
S	--	0.030
Si	--	0.40
W	14.00	16.00
Yield Strenght (MPA)		
Annealed		Cold-work
310		760
Elongation (%)		
30.0		15.0

Table 2.2. CoCr composition and properties. Adapted from ASTM F90 [32]

Current stent technology

The current stent technology is associated by deployment method, manufacture and production; geometry also plays an important role regarding the mechanical properties.

The first deployment method is balloon-expandable, the stent is deployed by expanding trough a balloon catheter, affecting the plastic deformation of the material, (i.e. 316L, CoCr alloys, Ta alloys etc.); or self-expanding (i.e. NiTi alloys).
Stent manufacturing is distinguished by three physical forms: sheet, wire (round or flat) and tube; and finally the most common way of produce stents are: laser cutting, electrode discharge machining, waterjet-cutting and photochemical etching for tubing [6].
There are three stents geometry types: (1) slotted tube which are produced using tubes of metal; (2) coil, which is made by metallic wires or strips forming a circular coil and; (3) tubular mesh which consist of wires wound forming a tube. Also, the stents differ slightly in strut pattern, width, length, diameter, metal composition, radiopacity.
All the presented parameters influence deeply the results of thrombosis and restenosis rates [33,34].

2.3 Biological mechanisms

When a stent is placed in to the artery to overcome atherosclerosis and vessel occlusion, it is an inevitable consequence to have arterial injury. Due to PCI's, a cascade of cellular and molecular events occurs resulting I acute disruption of the endothelial layer of the arterial wall. Atherosclerosis is mainly due to a disturbance of the normal endothelium; thus, understanding the biology of the vessel wall will be useful to understand the importance of having a functional endothelium for vascular health [35].

2.3.1 Artery structure and healthy endothelium

Native artery is an extremely complex multi-layered tissue composed of a number of different proteins and cell types, which each play an integral role in the mechanical behavior of the structure (Figure 2.4) It is composed of the following parts [36]:

- ***Tunica intima***, is the innermost layer of the vessel wall and it contains elastic and connective tissue connected with a monolayer of ECs in contact

with the deep internal cavity where the blood flows, also called the *lumen*. The ECs monolayer is called the endothelium which is in contact with circulating blood and, in healthy conditions, produces many molecules with antithrombotic properties, including nitric oxide, prostacyclin, tissue plasminogen activator, thrombomodulin, heparin-like molecules, and tissue factor pathway inhibitor [35]. Moreover, the endothelium permeability is also crucial to provide a barrier between the vessel lumen and surrounding tissue [37,38].

The most obvious function of the intimal or endothelium is to keep the blood inside the vessels; At the same time, allows the exchange of nutrients with the interior of the tunica media, another function is to control blood coagulation, and inhibit coagulation.

- ***Tunica media,*** is composed of elastic tissue and several layers of SMCs in a matrix of collagen types I and III, elastin and proteoglycans. SMCs are spindle-shaped cells; they can proliferate and migrate to the intimal in response to some growth factors (e.g. PDGF, FGF) produced by the ECs, being the cause of restenosis.

- ***Tunica adventitia or tunica externa,*** is the most external layer composed of connective tissue with fibroblasts, microvascular networks and randomly arranged collagen type I [39,40]. Fibroblasts are the connective tissue reproductive cells, which synthesize collagen and glycosaminoglycan's. They are important during the wound healing, as they migrate and proliferate. In fact, fibroblasts constitute the scar tissue after a heart attack.

Each of these layers are separated by elastic fibers called the internal and external lamina.

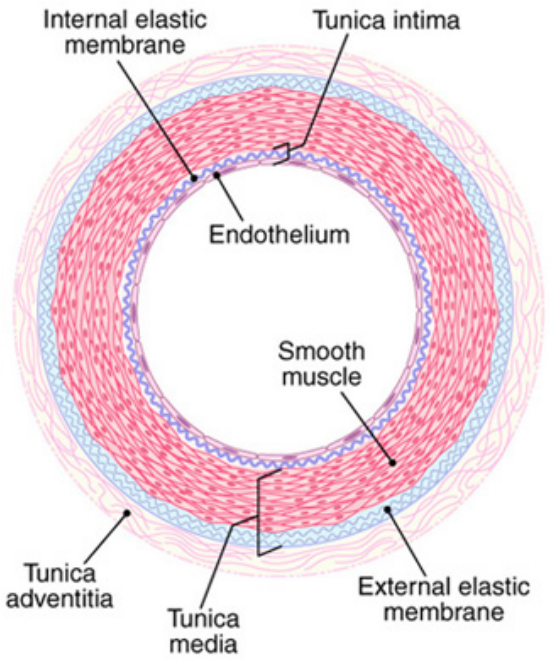

Figure 2.4. Anatomy of an artery consisting in three primary layers: intima, media and adventitia. From [41]

A healthy endothelium is characterized by an optimal intercellular junctions and antithrombotic properties.

The integrity of the endothelium is maintained by the intercellular junctions, which regulate its permeability and signal transduction, as well as in endothelial cell growth and survival. Intercellular junction and integrin receptors link the endothelial monolayer to the actin cytoskeleton in order to prevent separation of these cells from each other and the substrate [42].

Thigh-junction components interact with several signal-transduction molecules, such as G proteins, protein kinases, and molecules that regulates cell growth and survival.

The vascular endothelial cadherin, might transfer intercellular signals, which lead to inhibition of endothelial cell apoptosis, they also inhibit endothelial cell growth and motility by inhibiting vascular endothelial growth factor (VEGF) receptor 2, and activating the growth factor $\boldsymbol{\beta}$ (TGF- $\boldsymbol{\beta}$), which has antiproliferative and antimigratory properties [36].

The transmembrane protein platelet/endothelial cell adhesion molecule 1 (PECAM-1) is constitutively expressed at the intercellular borders of the ECs, but has not a direct part in the junction complexes [35].

The antithrombotic properties of the endothelium are conferred trough the inhibition of platelet aggregation and blood coagulation, as well as by plasminogen activator. The endothelium produces nitric oxide (NO) through enzymatic conversion of L-arginine by endothelial nitric oxide (eNOS) [43], which activity is regulated by different substances. If platelet aggregation occurs in a healthy coronary artery, platelet-derived serotonin, ADP, and locally thrombin bind to their receptors on the endothelial surface; this bind leads to eNOS activation, and subsequently yield the NO production by the own endothelium, as well as the cyclic-GMP (cGMP)-mediated relaxation of SMCs resulting in increased blood flow and inhibition of the coagulation cascade. Meanwhile, ECs produce antithrombotic and prothrombotic molecules, blood coagulation and platelet aggregation are inhibited by NO; tissue-type plasminogen activator (tPA) [44] promotes fibrinolysis. Prothrombotic molecules generated by ECs include von Willebrand factos (vWF) [45] and plasminogen activator inhibitor (PAI-1), which promotes platelet aggregation, allowing the activation of thrombin, and triggering the initiation of the blood coagulation [46], See the wound healing process described above in Figure 2.5.

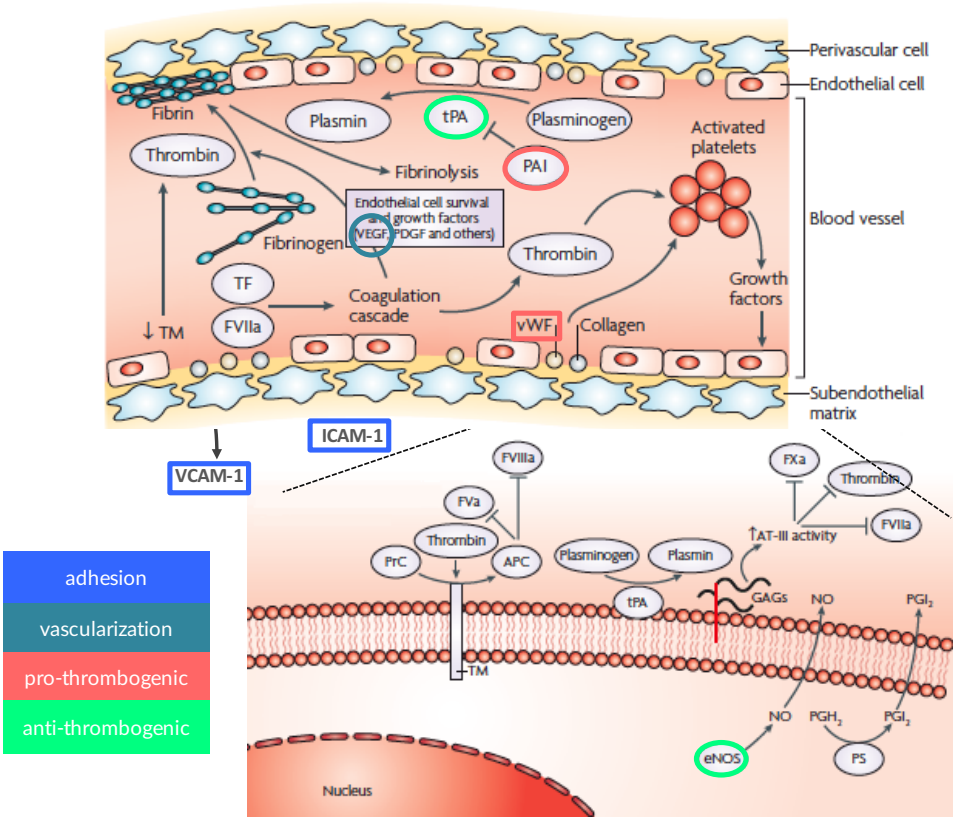

Figure 2.5. The wound healing in the artery. Adapted from [47]

2.3.2 Endothelial dysfunction

Atherosclerosis is the main cause of disturbance of the normal endothelial function. It is characterized by endothelial activation with pro-inflammatory and pro-coagulation environment and discontinue vasodilatation.

Other cardiovascular risk factors such as hypercholesterolemia, diabetes mellitus, hypertension, and smoking, contribute to the development atherosclerosis and hence endothelial dysfunction [35].

Restenosis

Restenosis or the re-narrowing of the arteries [23], is a process that begins with the damage caused by the balloon in the arterial wall at the time of the PCI, which is the long-term evolution of atherosclerosis due to a slow growth of the atherosclerotic plaque leading to final obstruction of the artery and restricts blood flow and impedes the supply of oxygen and nutrients [6]. The presence of atherosclerotic plaque compromises the integrity of the lumen of coronary arteries.

There are 4 mechanisms through which restenosis can be achieved: (i) Elastic retraction of the first day after implantation, (ii) Formation and organization of mural thrombus and inflammatory response, (iii) Neointimal proliferation and (iv) Chronic remodeling also known as geometric alterations [24,25]. Inflammatory cells exert a primary factor in vascular wall repair and neointimal formation, as well as the migration and proliferation of SMCs from the middle layer.

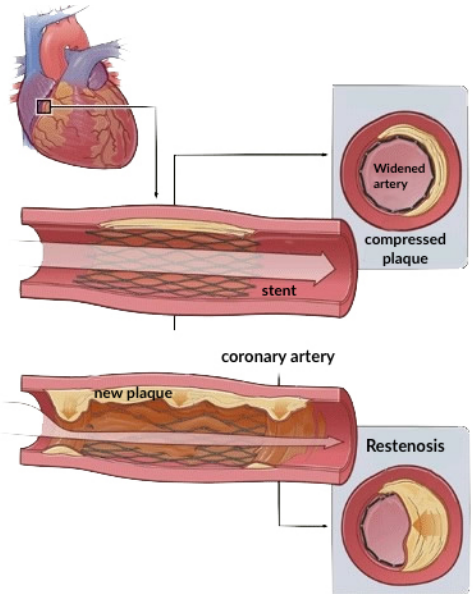

Figure 2.6. Illustration of Restenosis disease due to angioplasty. Adapted from [34]

Thrombosis

Thrombosis is a quick and acute complication corresponding to the rupture of the endothelium. The development of deep thrombosis is as follows: (i) Normal blood flow through the vein, (ii) Fibrin accumulation in the vein, (iii) thrombus formation with blood and platelets, (iv) Thrombus and occlusion of the blood flow and (v) Embolism or thrombus formation: thrombosis. [26–28]

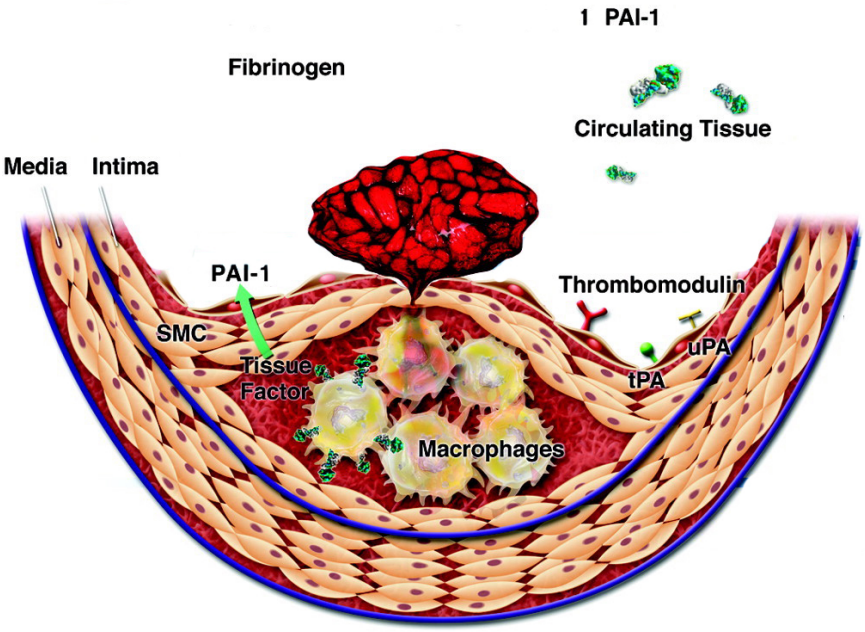

Figure 2.7. Determinants of thrombosis in coronary atherosclerotic plaques. Adapted from [23]

2.3.3 Consequences of stents implantation

Elastic recoil due to the contraction of the elastic fibers of the internal and external lamina, is another disturbance, creating an early phase of restenosis. Stent deployment disrupts the endothelium, crushes the plaque, triggering and over-exposes collagen, FN, vWF and LN, allowing the platelets to aggregate and thus, the thrombus formation, creating a wall injury, that presents clot formation where the macrophages get attracted due to the metallic foreign surface. Finally, it occurs an uncontrolled proliferation and migration of SMCs to the intima leading to the formation of ISR [6,47].

In-stent restenosis (ISR)

ISR is the re-narrowing of arteries after insertion of a stent, it is dominated by neointimal growth, induced by SMCs over proliferation [4]. Patients treated with a

BMS have a rate of ISR around 15-20% and it is the most concern disease linked to BMS. The difference lies histologically from restenosis after balloon angioplasty and comprised largely of neointimal formation; suggesting that ISR virtually eliminates vessel elastic recoil and negative remodeling, subsequent in a large neointimal formation [48].

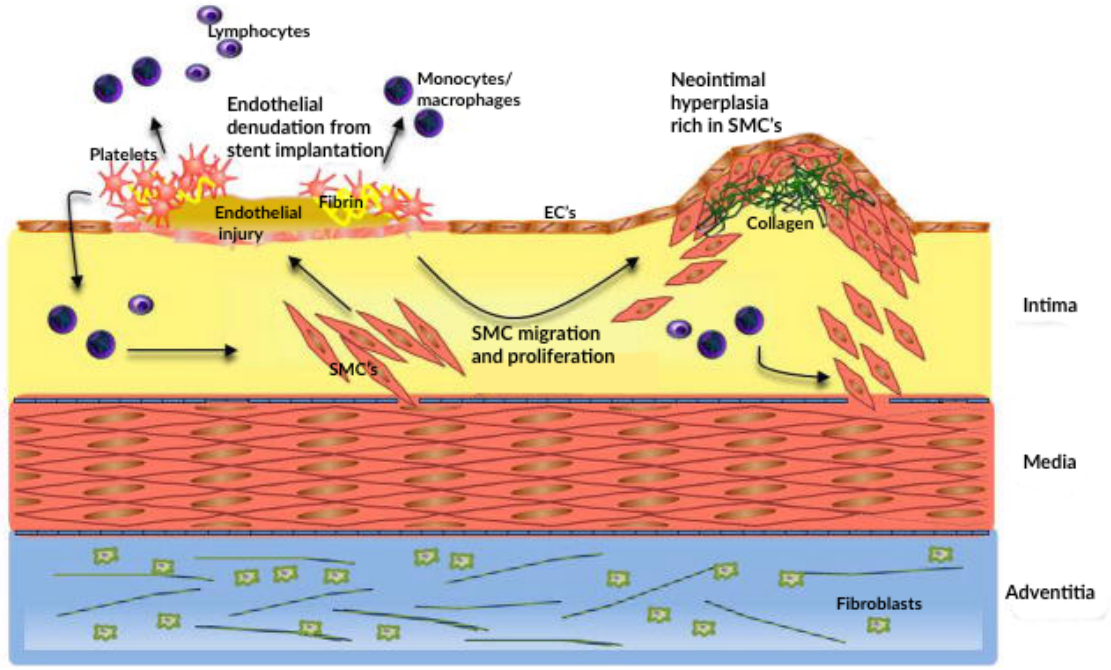

Figure 2.8. Mechanism leading to in-stent restenosis, from [42]

Stent thrombosis (ST)

ST can occur by the delay of endothelium healing of angioplasty site and the exposure of the stent biomaterial. The factors associated with ST may be: the stent (geometry, material and drug); the patient including clinical history and conditions; the procedure and the extend and duration of antiplatelet therapy [49].

2.4 Interactions between ECs and biomaterial surfaces

Actual stent technology fails to completely reduce ISR and ST diseases. ECs adhesion on biomaterials surfaces is required to provide a non-thrombogenic surface, but also cell proliferation and migration. The ability of a material to support cell adhesion depends on its surface properties [50]. Treatments to

modify a surface chemistry or topography have been used to induce protein adsorption and cell adhesion.

2.4.1 Stent physico-chemical modifications

Studies have shown that a rough surface is more auspicious to increase the thrombogenicity [51,52], for that reason obtain a contamination-free and smooth surface is essential for stents applications. Mechanical polishing, ultrasonic cleaning and chemical etching are some of the solutions to acquire a smoother surface.

Coatings have been used to primarily enhance stent biocompatibility but they have also become a platform for the controlled delivery of drugs. Stents coatings can be generally classified as passive and active coatings.

a) Passive coatings

This type of coatings act only as an inert barrier between the bloodstream/endothelium and metal, with the objective to increase the biocompatibility of the material, as well as to reduce neointimal hyperplasia, obtaining a surface with low thrombogenicity.

Passive coatings are divided into inorganic and organic compounds [53,54] to create a superficial layer or inorganic material, allowing a faster cleaning of the surface, remove the extra-material from the surface, decrease the percentage or carbon and nitrogen and increased the amount of hydroxyl groups, and oxygen in the surfaces. This simple procedure: Activation is an interfacial bonding layer, in order to create a reactive surface for the subsequent immobilization of the active molecules, and increase the oxide layer and improve the corrosion. The drawback in Plasma activation is that the effect of clean surface, is very low and this is related to the weak bond created with the metal and low surface density of carboxyl groups [55].

NaOH etching, a treatment that generates accessible hydroxyl groups on the surfaces, which is also considered as an inorganic coating and used as an activation technique, which occasioned a slightly increased in the nano-roughness of the surfaces, case that not happen in Plasma, also NaOH increased the amount of oxygen and nitrogen while slightly decrease carbon content, due to the lower removal of carbonaceous species.

NaOH etching was inspired from Kokubo [56] method, used over Ti surfaces, to generate a stable layer over the surface, increasing the roughness and wettability, characteristics wanted over our surfaces.

Inorganic stent coatings usually are chemically stable and serve as hemocompatible coatings and corrosion inhibitors. The most commonly used inorganic coating materials for stents are gold (Au), chromium (Cr), titanium (Ti) platinum (Pt) and cupper (Cu). Also, silicon carbide (SiC) or metal oxides are used.

Organic polymer allows a higher accessibility to chemical surface modification compared to inorganic coatings. Biostable coatings generally have good mechanical and biocompatible properties i.e. polyethylene terephthalate (PETP), Polytetrafluoroethylene (entree), Poly ethyl methacrylate (PMMA), but, in some cases, problems of inflammation and increased neointimal formation have been detected. Biodegradable polymers can be mechanically stable and hemocompatible. i.e. Polyglycolide (PGA), Polylactic acid (PLA), L-polylactic acid PLLA, Polycaprolactone (PCL) biocompatible (i.e. Hyaluronic acid, fibrin and heparin) [6].

b) Active coatings

Are usually defined as those that stimulate a certain cellular response, this can be a biomolecule directly bond to the surface of the stent, or a polymer that acting as a reservoir. The best example of this types of coating are the DES stents, which release higher concentrations of a locally drug into de blood to reduce restenosis rates.

2.5 Surface chemical functionalization

Surface attachment of bioactive molecules such as ECM proteins, adhesive peptide sequences and/or growth factors have been used to promote EC adhesion onto materials and to control cell processes such as proliferation, migration, differentiation and survival.

Several approaches have been explored to improve vascular healing after stent implantation and, in particular re-endothelialization of stent struts [57,58], from

coating with complex full-length proteins [59], biopolymers [60,61], to biologically relevant peptide sequences [10,62,63]. In order to improve biofunctionality, affinity and selectivity of the ligand; multiple peptide motifs, peptide mixtures, branched and cyclic peptides, and engineered protein fragments have been designed to increase the efficacy of functionalization. Table 2.3 summarizes all the studies related to the use of bioactive molecules to promote ECs processes.

2.5.1 Cell recognition motifs

2.5.1.1 Protein coatings

Surface treatment may enhance cell adhesion by modifying protein adsorption but the exact composition of the adsorbed layer is not known and cannot be controlled or reproduced. The use of components of the ECM such as cell adhesive proteins like fibronectin (FN) [64,65], collagen [66] or laminin [67], have facilitated cell attachment onto biomaterials and promote their biocompatibility. Although the proteins used brings some disadvantages [68,69]:

- Proteins are obtained from other organisms and purified, creating an undesirable immune response and increase of infection risks.
- Proteins are enzymatically unstable and present degradation, formulating a long-term impossible to produce.
- Only one part of the proteins is able to orientate and support cell adhesion.
- The functionalization of protein coated surfaces is difficult to control, since the physical-chemical properties such as charge, wettability and topography, conformation and the proteins orientation can influence protein deposition onto the material and, thus, biomaterials cell response [69].

Most of the drawbacks presented, could be solve using small part of the full-length protein.

2.5.1.2 Grafting synthetized peptides

Since the discovery of amino acid sequences within ECM proteins specifically recognized by cell receptors, many researchers have immobilized cell recognition peptides directly onto materials surfaces in order to control cell behavior [69]. The advantages of grafting short synthetic peptides onto biomaterials are:

- **They provide binding specificity.**
- They exhibit higher stability, especially in sterilization conditions and heat treatment.
- They are easy to synthetize and control, which makes that have a better cost-efficiently results.

Surfaces modified with bioactive cell-adhesive peptides have shown to mediate anchorage-dependent cell functions, including adhesion, proliferation and migration.

- **RGD**

  RGD represents by far the most extensively studied cell adhesion motif used to functionalize surfaces. It is present in FN and has been identified as a minimal essential cell adhesion present in this protein [70], and is the most effective sequence to stimulated cell adhesion [69], due to its ability to address more than one cell adhesion receptor and its biological impact. This peptide sequence has been also identified in other ECM proteins, including vitronectin, fibrinogen, vWF, collagen, laminin [71]. Eight receptor subtypes integrins have been demonstrated to recognize and bind with RGD peptide: αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α5β1, α8β1 and αIIbβ3 [72,73].

  ECs are known to express β1 integrins, which made the possibility to promote the ECs and re-endothelialization through this short-peptide, although being a non-specific sequence, presents SMCs and platelet adhesion.

- **REDV**

REDV sequence has been found in FN and it mediates EC migration on FN via its $\alpha_4\beta1$ receptor [74,75]. Moreover, it has been reported to selectively promote EC adhesion and spreading over SMCs and platelets [76,77],

- **YIGSR**

The laminin derived YIGSR sequence [78] have been shown to promote ECs adhesion and migration without enhancing platelet adhesion [79]. A wide variety of cell types has been reported to express immunologically related laminin-binding

proteins within this size range, including SMCs, macrophages, neutrophils, epithelial cells and endothelial cells.

2.5.1.3 ELRs

ELRs are oligomeric macromolecules based on the repetition of the (VPGXG) motif from elastin, in which X is any amino acid except L-proline [80]. The composition is strictly defined by engineering design and enable the introduction of peptide sequences to extend their properties, they are produced as recombinant proteins mimicking the basic properties of elastin and exhibiting mono-dispersity and a high control over amino acid sequence [81,82]. The recombinant nature of ELRs allows to include bioactive domains such as endothelial cell attachment sequence REDV [10,81],

The basic structure of ELRs is a repeating sequence, that has his origin in the repeating sequences found in the mammalian elastic protein: Elastin [83].

2.5.2 Binding of ligands

Synthetic peptides are usually firmly linked to the surfaces either directly or via a spacer to enhance their steric availability and conformational freedom, thus promoting their binding. There are two major strategies to biofunctionalized biomaterial surfaces: physisorption and covalent grafting or silanization.

2.5.2.1 Physisorption

This process, relies in the non-covalent interaction between the biomolecule and the surface, consists of an immersion of the sample into a bioactive peptide containing solution, counting only in the electrostatic forces, hydrophobic interactions, hydrogen and van der Wall forces bonds, this is the simplest way to create the bonding, although is a weak interaction due to there is a lack of controlled deposition and the stability of the peptide over the surface could be not guaranteed. Therefore, the success of the adsorption is based on the physico-chemical properties of the biomolecule and the surface properties.

2.5.2.2 Covalent grafting

On the other hand, silanization has been widely used to covalently immobilize functional biomolecules on metallic supports [28,58] which brings and alternative to the lack of stability present in the physisorption, due to the chemical functionalities of the material surface to covalently bind the bioactive molecule.

3-chloropropyltriethoxysilane (CPTES) silane was use to react with the exposed hydroxyl groups previously generated by the activation steps (Oxygen plasma or NaOH), allowing the binding of the silanes on the CoCr surfaces. [84,85]. Still, the successful and reproducible monolayer formation depends on the silane used, temperature and the techniques employed, in the case of metals, silanization is limited by the low surface hydroxyl group content of the native oxide layer, this problem can be overcome, by adding of activation treatment before mentioned.

Table 2.3 Summary of potential peptides or other moieties intended to enhance vascular graft endothelialization through biofunctionalization

Molecule (peptide)	Surface (Application / surface modification)	Cell type	model	Outcome	Ref
ELRs (RGD and REDV)	Bare metal stents	HUVECs	In vitro	Shows an effective generation of covered stents which exclude the atherosclerotic plaque from the blood stream and have high biocompatibility.	[86] 2015
Oligonucleotides (ONs)	CoCr stents	EPCs	In vitro	Stents were successfully functionalized with the specific ON, increasing cell adhesion.	[87] 2015
Albumin and Fibrinogen	CoCr alloy with PA and PAA	Platelet adhesion	In vitro	Demonstrated that PA coating on CoCr alloy was superior to PAA coating for reducing platelet adhesion, activation, and aggregation.	[88] 2015
Placitaxel (PAT)	CoCr alloy	ECs and SMCs	In vitro	This study showed the interaction of ECs and SMCs with SAMs-CoCr and PAT-SAMs-CoCr, being the first one better for endothelialization.	[89] 2013
Heparin and FN	Ti	ECs	In vitro	The Hep/FN co-immobilization improve the blood compatibility and promotes endothelialization	[59] 2011
Anti-CD34 with Multilayer of Heparin/collagen	Si, PET, 316 stainless steel	ECs and SMCs	In vitro and in vivo (rabbit)	The functionalized multilayer heparin /collage + antiCD34 show selectivity to promote the ECs.	[90] 2010
P15 peptide	ePTFE	HUVECs and HUASMCs	In vivo (sheep model)	The EC adhesion, proliferation has increased, while the controls presented a thicker neointimal hyperplasia.	[91] 2012
RGD	PCL - scaffolds	ECs, SMCs and Platelet adhesion	In vivo (rabbits)	RGD-modified PCL grafts exhibit an improved remodeling and integration capability in revascularization	[57] 2012
RGD	PLL-g-PEG / PEG-peptide, covalent conjugation	Fibroblasts	In vitro	Creation of specific signal for cells and inhibited non-specific adhesion	[92] 2003
+RGDS	NiTi with Self-assembly peptide PA nanofiber Covalent attach APTES	Pre- osteoblasts MC3t3-E1 and CPAE	In vitro	Covalently attach self-assembled PA nanofibers on pre-treated NiTi substrates using an intermediary amino-silane layer.	[93] 2008
RGD	ePTFE	AHSHVECs	In vitro	Low concentrations of RGD peptide cross-linked led to significantly increased endothelial cell retention.	[94] 2005

RGD cyclic	Guidant Tetra Stents	Porcine EPC	In vivo / porcine model	The data indicate a crucial role of $\alpha v\beta 3$ integrins, improving the endothelialization and therefor decreasing the neointimal stenosis	[95] 2006
REDV	PEGMA and Glycidyl methacrylate (GMA)	ECs and SMCs	In vitro	The copolymers GMA and PEG groups are very useful as a multifunctional coating material with anti-fouling and ECs specific adhesion for implant materials surface modification	[96] 2015
REDV	SiO_2 substrates	ECs and SMCs	In vitro	The covalently bound peptide reduced apoptosis and necrosis of adhered cells, meanwhile both short peptides were superior to FN in increasing adhesion	[77] 2006
REDV	PU	HUVECs	In vitro	Shows a positive effect of REDV peptide after surface modification over HUVEC	[97] 2016
REDV	Zwitterionic carboxybetaine methacrylate and butyl methacrylate	HUVECs HUASMCs, platelet adhesion and co-culture	In vitro	The coating was able to enhance the competitive growth of endothelial cells while limiting the adhesion, proliferation, and migration of smooth muscle cells	[98] 2012
REDV	Polysaccharide multilayer (PEM)	HUVECs and HUASMCs	In vitro	REDV functionalized cell-resistant heparin/chitosan multilayer is a ECs selective surface	[99] 2012
REDV	Poly (ethylene glycol) methacrylate (PEGMA), PET and PDMS	HUVECs and HASMCs	Cell migration and in vivo.	Demonstrated the competitive ability of EC over SMCs, and that the synergic action of the REDV could increase the selectivity	[10] 2013
REDV	PEG through active ρ-nitrophenyloxycarbonyl group	HUVECs and HASMCs, cell co-culture	In vitro	The combination of nonspecific resistance of PEG and the ECs selectivity of REDV peptide presents better ability to enhance the competitive adhesion of HUVECs over HASMCs	[100] 2011
YIGSR	Polyurethaneurea with PEG	HUVECs	In vitro	There is not existent of platelet adhesion, while EC adhesion, spreading and migration exist. This material is interesting for a small diameter vascular grafts	[79] 2005
GRGDS and YIGSR	Polyurethane (PU) with PEG spacer.	ECs	In vitro	Surface co-immobilization was successfully created, allowing to create an EC-specific vascular graft with long-term patency	[101] 2013

RGD and YIGSR	Polydopamine (pDA) coatings	EPCs	In vitro and In vivo	pDA-mediated peptide immobilization enhanced adhesion, metabolic activity, and endothelial differentiation of hEPCs.	[102] 2014
RGD and YIGSR	PET and PTFE	HUVEC's	In vitro	Demonstrated that covalently immobilized adhesion peptides promoted attachment and spreading of HUVECs	[103] 1991
RGDS and YIGSRG	Borosilicate glass	ECs	Cell adhesion and migration	The immobilization of the cell adhesive peptides increased the movement in the cells, leading to enhanced endothelialization rates	[104] 2000
YIGSR and VAPG	Amphiphiles peptide (PAs)	HUVECs and AoSMCs	In vitro	Improve in the develop of novel vascular grafts, that mimic the native endothelium	[105] 2010
RGD and REDV	Gold-coated polyurethanes	HUVEC	In vitro	The gold-coated have significant potential especially the CCRRGDWLC peptide	[106] 2001
RGD and QPPRARI	PTFE	HUVECs	In vitro	Results demonstrated the increment of HUVEC adhesion, spreading and migration	[107] 2005
RGD, REDV, YIGSR	dPVCs, OP and cBPI)	EC	In vitro and in vivo	dECM was successfully functionalized with custom made synthetic peptides showing increased EC attachment on functionalized dPVCs	[108] 2016
RGD, REDV and YIGSR	PEG/ PET	HUVECs and HVSMCs	In vitro	Demonstrated that cell-binding ligands immobilized RGD, YIGSR and REDV show normal monolayers	[76] 1991
RGD, REDV and YIGSR	Polysaccharide hybrid hydrogel (ALG-peptide)	HUVECs	In vitro and in vivo and blood vessel formation	GREDV exhibited a superior capability for promoting the proliferation and selective adhesion of HUVEC over RGD and YIGSR. Stimulating new vessel formation	[109] 2015
RGDS, REDV, YIGSR and SVVYGLR	Polyethylene terephthalate (PET)	HUVECs	In vitro	The combination of peptides specific for ECs RGDS and angiogenic sequence helps to improve endothelialization and vascularized tissue	[110] 2012
RGD, YIGSR and IKVAV	PCL	Adipose-derived stem cells (ASCs)	In vitro	The results show that IKVAV-treated surfaces had a significantly greater number of ASCs	[111] 2006

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Chapter 3

Biofunctionalization of REDV elastin-like recombinamers improves endothelization on CoCr alloys surfaces for cardiovascular applications. Maria Isabel Castellanos, Anne-Sophie Zenses, Anna Grau, Jose Carlos Rodriguez-Cabello, Francisco Javier Gil, Jose Maria Manero, Marta Pegueroles

Colloids and Surfaces B: Biointerfaces 127 (2015) 22–32
Doi: 10.1016/j.colsurfb.2014.12.056

http://www.sciencedirect.com/science/article/pii/S0927776515000065

Chapter 4

Functionalization of CoCr surface with adhesive peptides to promote HUVECs adhesion and proliferation. Maria Isabel Castellanos, Carlos Mas-Moruno, Anna Grau, Xavier Serra-Picamal, Xavier Trepat, Fernando Albericio, Michael Jonerh, Francisco Javier Gil, Maria Pau Ginebra, Jose María Manero, Marta Pegueroles

Applied Surface Science 393 (2017) 82–92
Doi 10.1016/j.apsusc.2016.09.107

http://www.sciencedirect.com/science/article/pii/S0169433216319857

Chapter 5

Cell adhesive peptides functionalized on CoCr alloy stimulate endothelialization and prevent thrombosis and restenosis. Maria Isabel Castellanos, Jordi Guillem-Marti,Carlos Mas-Moruno, Maribel Díaz-Ricart, Ginés Escolar, Maria Pau Ginebra, Francisco Javier Gil, Marta Pegueroles, Jose María Manero

Journal of biomedical materials research 2017 Part A 105A: 973–983
DOI 10.1002/jbm.a.35988

http://www.onlinelibrary.wiley.com/doi/10.1002/jbm.a.35988/abstract

6 General discussion

The use of stents has represented a revolutionary advance in the treatment of cardiovascular disease; but ISR and ST remain the major limitations of coronary intervention. To overcome these drawbacks and obtain a functional artery after coronary stent implantation, it is decisive to accelerate stent surface endothelialization [10,11] and, if possible, without the use of antiproliferative drugs that generally delay the formation of healthy endothelium [8].

One of the most promising strategies to improve implantable devices is the immobilization of cell adhesive molecules onto the surface, also called biofunctionalization. The aim of this thesis was to enhance endothelialization of CoCr alloy surfaces by functionalization for coronary implants. Different approaches were applied to functionalize the CoCr surfaces and, thoroughly, physico-chemically characterized and biologically evaluated *in vitro* with ECs, SMCs, co-culture of ECs/SMCs and platelets. This chapter highlights and discusses the main achievements obtained in this research.

6.1 Biofunctionalization of CoCr surfaces with ELR

Chapter III (**Paper I**) was focused on the immobilization of ELR, genetically modified with an REDV sequence, to enhance metal surfaces endothelialization. Different activation protocols (i.e. NaOH etching and O_2 plasma) were applied to CoCr alloy surfaces to obtain a higher density of available hydroxyls and optimize the silanization process with CPTES. Both activation treatments increased the percentage of hydroxyl groups and thus, enhanced silanization and, finally, improved ELR immobilization. NaOH activated and silanized CoCr surfaces presented a major HUVEC adhesion and spreading due to a higher amount of immobilized ELR with REDV sequence. The reason for such increase was mainly attributed to a higher electronegativity of the surface, determined by zeta-

potential measurements, since roughness and wettability changes of NaOH activated surfaces were slightly different to plain surfaces. On the contrary, O_2 plasma activated surfaces were more efficient in generating hydroxyls onto the surface and changed surface wettability and chemistry by reducing the content of C and N. But the immobilized ELR was lower and thus, the number of adhered HUVEC decreased compared to NaOH activated surfaces.

Differences between immobilization of ELR by physisorption and covalent binding were also detected by x-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), fluorescent microscopy and quartz-crystal microbalance with monitoring dissipation (QCM-D). Covalent bonding coatings of the ELR onto CoCr alloy surfaces showed a higher amount and stability than the physisorbed ELR coatings, but this effect was not statistically significant. Probably, the silanization process was not optimal to obtain all the biomolecules covalently attached onto CoCr alloy treated surfaces and a mixture of covalent and physisorption behavior is obtained. The use of big biomolecules with different anchor groups causes the mixture of covalent and weak bonding between the biomolecule and the surface.

6.2 Biofunctionalization of CoCr alloy surfaces with short synthetic peptides

The control of the bonding between the biomaterial surface and the cell adhesive molecule has been reached with the design and synthesis of short synthetic adhesive peptides explored in **Chapter IV** (**Paper II**).

Three main factors were considered for the biomolecules design: (i) the bioactive cell-binding motif, which determines the biofunctionality (ii) the anchoring group, which provides a strong and chemo-selective binding with the material, and (iii) the spacer, which ensures an optimal presentation and accessibility of the biomolecule to the cells. The final biomolecules design considered a thiol as the anchoring group and three amino-hexanoic acid (Ahx_3) as best spacer to support EC adhesion for RGDS, REDV and YIGSR cell-binding motifs.

Functionalization was performed onto CoCr alloy surfaces through physisorption and covalent binding using CPTES as coupling agent. To facilitate silanization,

surfaces were activated with NaOH to enhance silane coupling and, finally, the covalent binding of the peptides were performed in a buffer solution at basic pH to enhance the direct nucleophilic substitution between the free thiol group of the peptides and the organosilanes. The immobilization process of the cell adhesive proteins onto CoCr surfaces was successfully achieved for both routes. Moreover, equimolar combinations of the synthetized peptides were used to functionalize the surfaces. Biofunctionalized surfaces clearly increased the number, spreading and migration of adhered ECs independently of the type of immobilized peptide. However significant differences were detected depending on the peptide and immobilization strategy. It was particularly effective the silanization of CoCr surfaces with the equimolar combination of RGDS and YIGSR to enhance ECs adhesion.

In addition to cell adhesion onto modified CoCr surfaces, the cellular crosstalk through ECs molecules is also a critical parameter for endothelial functionality. **Chapter V** (**Paper III**) evaluated the relevant genes for adhesion (ICAM-1 and VCAM-1) and vascularization (VEGFA, VEGFR-1 and VEGFR-2) which confirmed the beneficial effect of functionalization to increase ECs adhesion and activation.
The expression of anti-thrombogenic factors (tPA and eNOS) increased over time for YIGSR coated surfaces. The fact that studied pro-thrombogenic genes (PAI-1 and vWF) expression decreases over time, indicate that functionalization with the laminin-derived biomolecule could prevent thrombogenicity. Moreover, potential anti-thrombogenicity of peptides was also evaluated through platelet adhesion and aggregation by circulating human blood onto tissue cultures polystyrene (TCPS) coated with the studied biomolecules. All short peptides reduced platelet response compared to TCPS.

A key point of the work is the influence of the different coatings to SMCs and platelet *in vitro* response treated in **Chapter IV** (**Paper II**) and **Chapter V** (**Paper III**). Immobilization of cell adhesive molecules increases SMCs adhesion although the values were ten times lower than those of ECs. The competition between both types of cells for the modified surfaces was evaluated in co-culture. As for single

cell *in vitro* studies, the results suggested a positive effect of functionalized surfaces to enhance ECs. The surfaces coated with the equimolar combination of RGDS and YIGSR exhibited adhesion selectivity towards ECs. Thus, a synergistic effect was observed between both molecules probably due to the fact that both peptides interact with different cell receptor molecules.

Taking all together, the equimolar combination of RGDS and YIGSR seems to be the most promising strategy for endothelialization of CoCr alloy surfaces. Overall, functionalization of CoCr metallic surfaces for cardiovascular applications may offer an efficient alternative to enhance rapid endothelialization, while controlling restenosis and thrombosis.

7 Conclusions

The present PhD dissertation has been devoted to enhance endothelialization of CoCr alloy surfaces for coronary implants. The following objectives have been achieved: (i) design and synthesis of short peptides with different spacers and active domains and using a thiol as anchor; (ii) development and optimization of the strategy of functionalization of CoCr alloy surfaces by covalently anchoring cell adhesive biomolecules; (iii) the study of the endothelialization capacity of the different treated surfaces by ECs adhesion, proliferation and migration *in vitro* assays; (iv) the study of the restenosis capacity of the different treated surfaces by SMCs adhesion and ECs/SMCs co-culture in vitro cell experiments.

From the results of the three published papers, the following concluding remarks can be deduced:

- The immobilization of elastin-like recombinamers onto CoCr alloy surfaces was successfully achieved by physisorption or covalent bonding by CPTES organosilane chemistry. ECs adhesion response was directly related to the amount of immobilized ELR onto the surface. Covalent bonding coatings of ELR showed a higher amount and stability than the physisorbed ELR coatings. Nevertheless, the silanization process was not completely effective since the use of big biomolecules with different anchor groups causes a mixture of covalent and weak bonding. Thus, both immobilization process and surface electrostatic forces influence the amount of immobilized ELR onto CoCr alloy surfaces.

- The design and synthesis of short linear peptides (RGDS, REDV and YIGSR) were developed as biomolecules to be immobilized onto CoCr alloy surfaces by physisorption and CPTES silane covalent bonding. The appropriate anchor group, a thiol, and spacer, three units of aminohexanoic acid, were determined to obtain a chemo selective binding of the molecules to the biomaterial

surface. Moreover, the equimolar combination of RGDS/REDV and RGDS/YIGSR were also studied. The functionalized surfaces showed a significant increase of ECs adhesion and migration, particularly, the RGDS/YIGSR combination demonstrating the capacity of functionalization strategy to enhance endothelialization.

- Surfaces functionalized with the linear peptides sequences RGDS, YIGSR and the combination RGDS/YIGSR showed a positive effect to enhance ECs adhesion also in co-culture with SMCs. The gene expression of anti-thrombogenic factors tPA and eNOS increased over time of ECs cultured onto RGDS/YIGSR functionalized surfaces. Platelet adhesion and aggregation was lower onto YIGSR and RGDS/YIGSR coated TCPS surfaces compared to non-coated after circulating human blood. Thus, RGDS/YIGSR functionalized CoCr surfaces is an efficient alternative to enhance rapid endothelialization, while preventing restenosis and thrombosis.

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