# Oligonucleotide biofunctionalization enhances endothelial progenitor cell adhesion on cobalt/chromium stents

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**Abstract:** As the endothelium still represents the ideal surface for cardiovascular devices, different endothelialization strategies have been attempted for biocompatibility and nonthrombogenicity enhancement. Since endothelial progenitor cells (EPCs) could accelerate endothelialization, preventing thrombosis and restenosis, the aim of this study was to use oligonucleotides (ONs) to biofunctionalize stents for EPC binding. In order to optimize the functionalization procedure before its application to cobalt–chromium (Co/Cr) stents, discs of the same material were preliminarily used. Surface aminosilanization was assessed by infrared spectroscopy and scanning electron microscopy. A fluorescent endothelial-specific ON was immobilized on aminosilanized surfaces and its presence was visualized by confocal microscopy. Fluorescent ON binding to porcine blood EPCs was assessed by flow cytometry. Viability assay was performed on EPCs cultured on unmodified, nontargeting ON or specific ON-coated discs; fluorescent staining of nuclei and F-actin was then performed on EPCs cultured on unmodified or specific ON-coated discs and stents. Disc biofunctionalization significantly increased EPC viability as compared to both unmodified and nontargeting ON-coated surfaces; cell adhesion was also significantly increased. Stents were successfully functionalized with the specific ON, and EPC binding was confirmed by confocal microscopy. In conclusion, stent biofunctionalization for EPC binding was successfully achieved *in vitro*, suggesting its use to obtain *in vivo* endothelialization, exploiting the natural regenerative potential of the human body. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2015.

**Key Words:** oligonucleotides, cobalt/chromium stent, surface modification, endothelial progenitor cells, cell adhesion

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## INTRODUCTION

Cardiovascular disease (CVD) represents the leading cause of death worldwide and a major cause of disability.[1] Many patients suffering from CVD have impaired blood supply caused by either stenosis or occlusion of blood vessels, with the possibility of stroke or acute myocardial infarction. Stents are used to reopen narrowed or occluded vessels. A strong influence on their success is due to material and design of the implanted devices.[2] Clinically applied materials, such as metals, alloys, or synthetic polymers, were originally used for industrial applications and later translated into clinical surgery, due to their excellent mechanical and physical properties. However, as the hemocompatibility of these materials for biomedical applications is still not satisfactory, artificial surfaces need optimization to overcome side effects due to stent deployment and hypersensitivity caused by stent surface itself.[3] Examples of such strategies are the coating with anticoagulants or other molecules to prevent platelet adhesion and activation.[4] Since the native endothelium still represents the ideal surface for hemocompatibility of blood-contacting vascular devices, endothelialization has been pursued to improve biocompatibility and nonthrombogenicity. An ideal strategy could be the exploitation of the human natural regenerative potential, making the devices unrecognizable for body's rejection. *In vivo* direct repopulation and tissue regeneration with patients'

autologous cells is preferable to *in vitro* endothelialization before implantation, as it does not require a long-time *in vitro* cell culture, with the risk of infections and need of sophisticated and expensive infrastructures. In particular, endothelialization could be promoted by circulating endothelial progenitor cell (EPC) capture,[5] preventing both thrombosis and restenosis. EPCs are mobilized from bone marrow into peripheral blood, contributing to vascular repair and to the normal vascular physiological maintenance.[6] Vascular devices can be coated with capture molecules mimicking natural homing factors to attract circulating EPCs from patient's bloodstream. Several ligands, such as monoclonal antibodies, peptides, selectins and their ligands, aptamers or magnetic molecules, have been investigated.[3] The advantage of using aptamers, oligonucleotides (ON) with high-binding affinity and specificity, is their nontoxicity, little immunogenicity, protease, pH and thermal stability and ease of large-scale production. These chemically synthesized molecules are free from cell culture-derived contaminants and allow a deep reduction in the full costs for production and validation. Furthermore, DNA offers accessible functional groups for chemical coupling reactions, allowing the immobilization of different molecules.[7] Functionalization with specific aptamers can have further application with other blood-contacting medical devices, such as vascular grafts or stents.[8,9]

The main objective of this work was ON-biofunctionalization of metal stents, in order to bind EPCs to promote surface endothelialization.

## MATERIALS AND METHODS
### Materials
Discs of electropolished cobalt–chromium (Co/Cr) alloy L605, 10 mm Ø, and 0.34 mm thick, composition (wt %): cobalt (50.5), chromium (20), nickel (10), tungsten (15), manganese (1.5), iron (max. 3) and L605 stents, 20 mm length, 1.6 mm internal Ø, were provided by BIOTRONIK SE & Co. KG (Erlangen, Germany). 3-Triethoxysilylpropylamine (APTES), succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) were purchased from Thermo Scientific Pierce (Rockford, IL). A sequence previously tested[7] for endothelial mature and progenitor cell adhesion (5′-GGG AGC TCA GAA TAA ACG CTC AAC AAC CCG TCA ACG AAC CGG AGT GTG GCA GGT TCG ACA TGA GGC CCG GAT C-3′) (Integrated DNA technologies, Coralville, IA) was compared to a nontargeting sequence (5′-GGC AGG AAG ACA AAC ACG ATG GGG GGG TAT GAT TTG ATG TGG TTG TTG CAT GAT CGT GGT CTG TGC TGT-3′). A 3′-thiol modification was used for immobilization, while, for detection, the ON was 5′-labeled with 6-carboxyfluorescein (6FAM).

### Surface modification procedures for on grafting onto Co/Cr surfaces
*Preconditioning and aminosilanization.* Samples were sonicated with ethanol and acetone (3 times, 5′) and incubated in 10N NaOH (60 °C, 15′) to increase —OH groups. Samples were then extensively washed with distilled water. APTES solutions in ethanol/deionized water (95/5 vol %) were adjusted to pH 4 with acetic acid. Samples were dipped in 2% APTES solution for 30′ at room temperature (r.t.), and then, they were extensively washed with distilled water. A dipping in 5% APTES solution for 30′ at 50 °C was then performed. Finally, they were washed 3 times with acetone for 10′.

*Oligonucleotide functionalization.* About 16 m*M* SMCC in dimethylformamide/dimethylsulfoxide/phosphate-buffered saline (DMF/DMSO/PBS) pH 7.4 (8/75/17%) was added to each sample for 90′ at r.t. Samples were then extensively washed with PBS pH 7.4. Thiolate single-sequence ONs were reduced using 25 m*M* TCEP-HCl in PBS pH 7.6 and added to a same volume of solution containing 50 μ*M* of ON in PBS pH 7.6. To achieve a complete ON reduction, the incubation was performed for 30–45′. To immobilize reduced ONs on aminosilanized surfaces, ON solution was incubated for 150′ at r.t., before washing in PBS pH = 7.4.

### Surface characterization of the modified Co/Cr surfaces
*Characterization of aminosilanized surfaces.* Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was used to investigate aminosilanization, using a PerkinElmer Spectrum 65 equipped with ATR unit (PerkinElmer, Inc, Waltham, MA). Spectra were recorded between 650 and 4000 cm$^{-1}$, 125 scans 2.5 cm$^{-1}$ resolution.

*Scanning electron microscopy.* For scanning electron microscopy (SEM), aminosilanized samples were incubated with gold nanoparticles (Au NPs), to provide high-contrast images, giving a direct insight into the silane pattern quality.[10] After 1 h, samples were rinsed in deionized water, to wash unbound NPs and/or gold clusters away. SEM images were collected using a field emission gun (FEG)-SEM microscope (Sigma–Carl Zeiss Microscopy GmbH, Jena, Germany), working at 5 kV acceleration potential (working distance: 6.8 mm) and using a secondary electrons detector.

*Contact angle measurement.* Surface wettability was investigated using static contact angle measurement. Contact angle was measured using drops of water and taking the tangent to the drop on samples by an Advanced Automated Goniometer Model 300 (Ramè-Hart, US), including a software for automatic measurement (DROPimage Advanced). For each sample, at least four contact angles on different position were measured.

*Characterization of on functionalized surfaces.* To evaluate ON immobilization, a 5′-6FAM-labeled thiolate single-sequence was used. Green fluorescence was visualized under confocal laser scanning microscopy (CLSM) (Nikon Eclipse TE 2000-U, Nikon Instruments S.p.A, Calenzano, Italy). As a negative control, the same labeled ON was added to unmodified samples.

### Biological characterization of the modified Co/Cr surfaces
*Cell isolation and characterization.* Porcine EPCs were obtained from peripheral blood collected in heparin
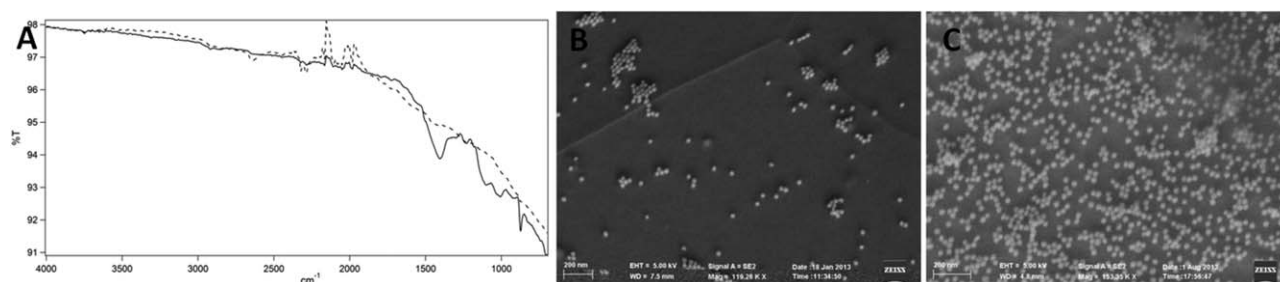
**FIGURE 1.** Aminosilanization evaluation. A: ATR-FTIR spectra of unmodified (black line) and aminosilanized samples (red line). B, C: SEM images after exposure to Au NPs of unmodified (B) or aminosilanized (C) samples.

(Eparina Vister TEVA ITALIA Srl, Italy) from the femoral vein of healthy pigs. All animals received humane care according to the guidelines of the Ministry of Health. Mononuclear cells (PBMCs) were isolated by centrifugation on 1.077 g/mL density gradient (Sigma-Aldrich Co, St. Louis, MO). PBMCs were seeded on fibronectin and maintained in Endothelial Cell Growth Medium-2 (Lonza, Walkersville, MD) at 37 °C, 5% $CO_2$. EPCs were characterized by flow cytometry and immunocytochemistry, using endothelial markers (von Willebrand Factor, vWF, Dako, Glostrup, Denmark; CD31, Serotec, Dusseldorf, Germany; DiI-acetylated low-density lipoprotein, DiI-acLDL, Invitrogen, Auckland, New Zealand; lectin from Bandeiraea simplicifolia, BS-I lectin, Sigma-Aldrich Co). Experiments were performed at cell passage 2–6.

***ON binding by flow cytometric analysis.*** EPCs were detached and resuspended in PBS. All the incubation passages and washings were made in PBS with 0.5% bovine serum albumin (BSA). After one washing, 150,000 EPCs were incubated with 1 μM 5′-6FAM-specific ON at 4 °C for 30′. After one washing, cells were resuspended (final volume: 200 μL) and fluorescence was monitored counting 10,000 events in the G1 gate with a FACScan cytometer (BD Biosciences, San Jose, CA). Unstained cells were used as a negative control. Data analysis was based on measurement of positive events (percentage) quantification with CellQuest® software (BD Biosciences).

***Cell viability and adhesion on functionalized surfaces.*** EPCs (10,000/well) were seeded on specific ON-functionalized discs and cultured for 4 days. To increase cell adhesion, a 30′ preseeding with 200 μL of cell suspension was performed before adding culture medium. After 4 days, cells were subjected to viability assay. Results were compared with a positive control (fibronectin-coated discs) and with unmodified, aminosilanized, and nontargeting ON-coated discs. Cell viability was evaluated by the reduction of 3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Co.) by viable cells. Discs were incubated with 200 μL fresh medium containing 10% of 5 mg/mL MTT solution for 3 hours at 37 °C. Medium was then removed, and formazan crystals were solubilized with 100 μL dimethylsulfoxide (DMSO, Sigma-Aldrich Co.). Optical densities were measured at 550 nm in a microplate reader.

Four independent experiments were performed with six replicates each. EPCs cultured for 4 days on unmodified and specific ON-functionalized discs were also used for cell staining. Cells were fixed with formaldehyde (2% vol/vol in PBS) for 30′ at 4 °C. After two washes, adherent cells were stained with TRITC-phalloidin (red probe, selectively staining F-actin, Cytoskeleton, Inc, Denver, Co) and DAPI (Sigma-Aldrich Co) for nuclei staining. Briefly, a 14 μM TRITC-phalloidin stock solution was prepared in methanol and diluted in PBS immediately before cell staining. Cells were permeabilized with 0.5% Triton X-100 for 5′ at r.t., washed and incubated with 100 nM TRITC-phalloidin for 30″ at r.t. After three additional washings, cells were incubated with 100 nm DAPI for 2′ at r.t. After washing, representative pictures were taken by fluorescence microscope (Axioplan 2, Carl Zeiss Microscopy GmbH). All washes were performed in PBS. In order to quantify EPC number and the area covered by EPC spreading in the different samples, pictures ($n = 5$) were analyzed by the image analysis software ImageJ (public domain, Image Processing and Analysis in Java, National Institutes of Health). The results were reported as cell number per $mm^2$ and area %, respectively. The same experimental protocol was performed with slight modifications on stents. EPCs (100,000/mL) were seeded on unmodified or ON-functionalized stents. To increase cell adhesion, a 1-hour preseeding with 100 μL of cell suspension was performed before further adding of culture medium. Cells were cultured for 4 days, before fixation with 2% formaldehyde. Stents were then stained with TRITC-phalloidin and DAPI. Representative pictures and Z-stack images were taken by a confocal laser scanning microscope.

## Data presentation and statistical analysis

Data are presented as mean ± S.D. of $n$ independent experiments. Comparisons were made by Student's $t$-test or analysis of variance (ANOVA) when appropriate. $p < 0.05$ was considered statistically significant.

## RESULTS
### Surface characterization

***Aminosilanization assessment.*** Aminosilanization was confirmed by ATR-FTIR [Fig. 1(A)] and SEM [Fig. 1(B–C)]. ATR-FTIR spectra contained bands typical of metallo-siloxane bond forming.[11] As an indication of siloxane film formation on the metal, typical strong —Si—O—Si— siloxane bond
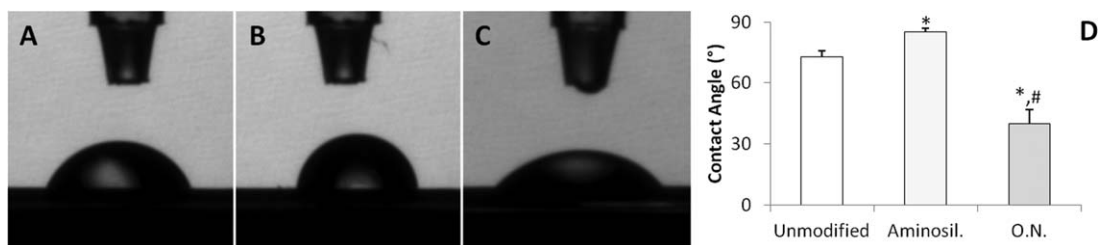
**FIGURE 2**. Surface hydrophilicity evaluation by contact angle measurement. Representative pictures obtained with unmodified (A), aminosilanized (B) and ON-functionalized (C) samples are shown, together with contact angle values (D). *: $p < 0.0005$ versus unmodified discs; #: $p < 0.0001$ versus aminosilanized discs.

peaks appeared (1130–1000 cm$^{-1}$). Metal-to-silicon bonding was clearly identified in all spectra by Si—O—Cr and Si—O—Co signals ($\sim$ 935–925 cm$^{-1}$). Water contact angles [Fig. 2(A–C)] allow to evaluate sample wettability, providing direct evidence of the change in surface hydrophilicity.[12] A significant increase in the contact angle value on the aminosilanized sample was observed as compared to unmodified surface [Fig. 2(D): $85 \pm 3°$ vs. $72 \pm 2°$, respectively, $p < 0.0005$]. Overall, these results indicate a uniform surface functionalization.
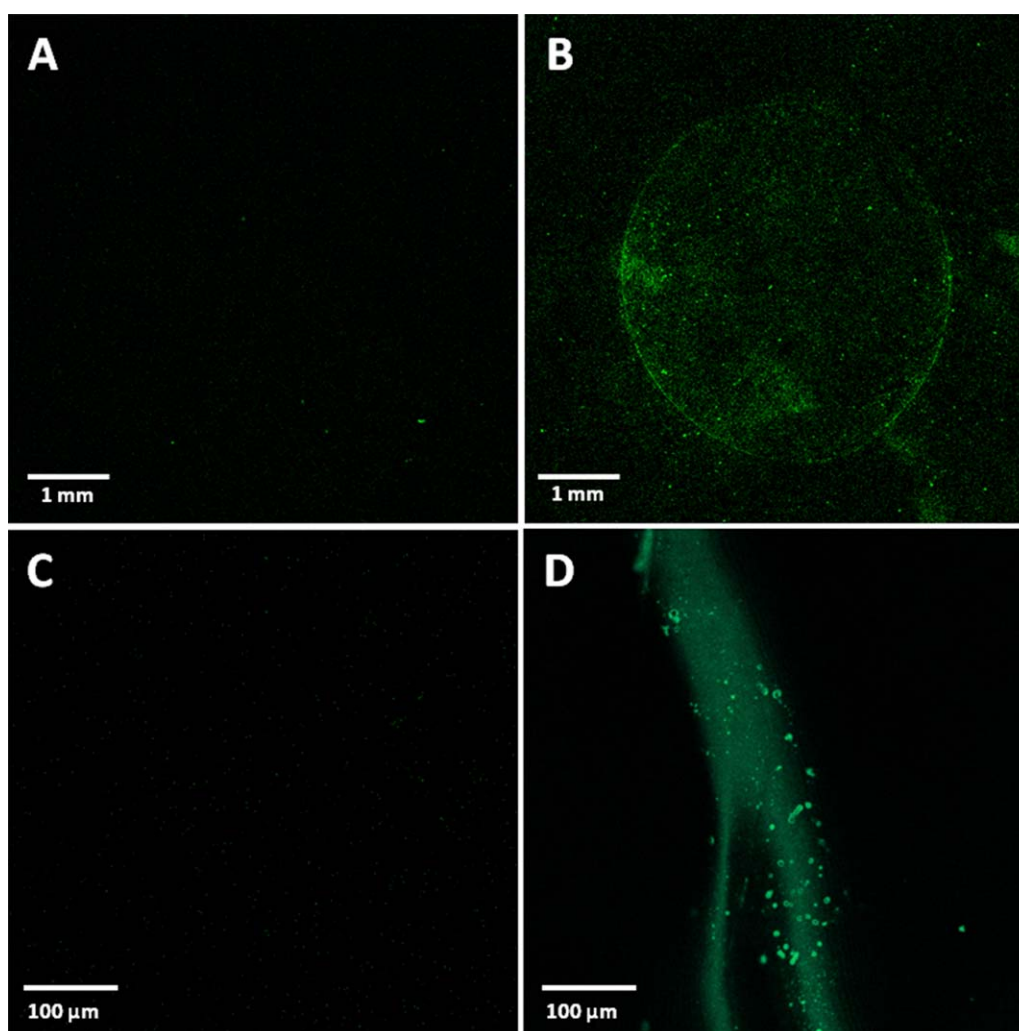


**FIGURE 3**. Assessment of ON functionalization by CLSM. A and B: Representative pictures of disc functionalization. 5'-6FAM ON (green fluorescence) binding to unmodified discs (A, negative control) or aminosilanized discs (B). C and D: Representative pictures of stent functionalization. (C) unmodified stent (negative control) and (D) 5',6 FAM ON functionalized (green fluorescence) stents. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
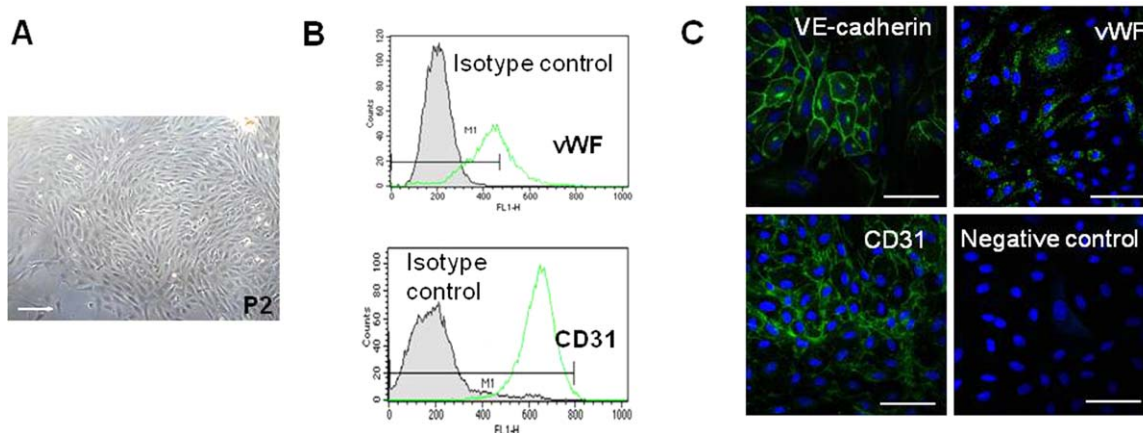
OLIGONUCLEOTIDE BIOFUNCTIONALIZATION ENHANCES PROGENITOR CELL ADHESION

**FIGURE 4**. EPC morphology and phenotype. A: EPCs at cell passage 2 (scale bar: 100 μm). Cells were positive for the endothelial markers VE-cadherin, von Willebrand factor and CD31, as shown by flow cytometry (B) and immunostaining (C) (scale bar: 50 μm). Representative pictures and histograms are shown. Green fluorescence: endothelial markers; blue fluorescence: nuclei staining with DAPI. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

***ON-coated surface characterization.*** Aminosilanized samples functionalized with 5′-6FAM-ON were visualized by CLSM. Modified discs showed a homogeneous intense green fluorescence as compared to untreated discs [Fig. 3(A–B)]. Contact angle after ON functionalization [Fig. 2(C)] showed a significant lowering ($40 \pm 7°$) as compared to both unmodified ($p < 0.0005$) and aminosilanized ($p < 0.0001$) samples, with an increase in hydrophilicity due to the presence of ON.

## Biological characterization

***Porcine peripheral blood EPC characterization.*** EPCs exhibited characteristic endothelial properties by morphology [Fig. 4(A)] and phenotype. Flow cytometry [Fig. 4(B)] showed that $94 \pm 9\%$ of the cells were $vWF^+$, $97 \pm 10\%$ $CD31^+$, $78 \pm 8\%$ $DiI\text{-}acLDL^+/BS\text{-}I^+$; endothelial phenotype was also confirmed by immunocytochemistry [Fig. 4(C)].

***Effect of on-functionalization on cell viability and adhesion onto Co/Cr discs.*** ON binding to EPCs was assessed by flow cytometry (Fig. 5), after incubation with a 5′-6FAM sequence. A shift of fluorescence intensity was observed after ON incubation as compared to unstained cells [Fig. 5(B)].

To assess cell viability and adhesion on ON-functionalized discs, EPCs were seeded on different samples and cultured for 4 days. ON retained its binding activity, significantly increasing cell viability (Fig. 6) as compared to unmodified, aminosilanized, and nontargeting ON-coated discs ($p < 0.05$), resulting comparable to the positive control, that is, fibronectin-coated discs. The results were confirmed by cell adhesion and morphology evaluation. Significantly, more EPCs (cells/mm$^2$: $435 \pm 48$) with a significant enhancement of the covered area (area %: $51 \pm 6$), were observed on ON-functionalized discs as compared to unmodified surfaces (cells/mm$^2$: $210 \pm 39$, $p < 0.005$; area %: $23 \pm 4$, $p < 0.05$), showing a little number of cells with an altered morphology (Fig. 7). EPCs seeded on functionalized surfaces showed a cell morphology similar to cells adhered to tissue culture surfaces.
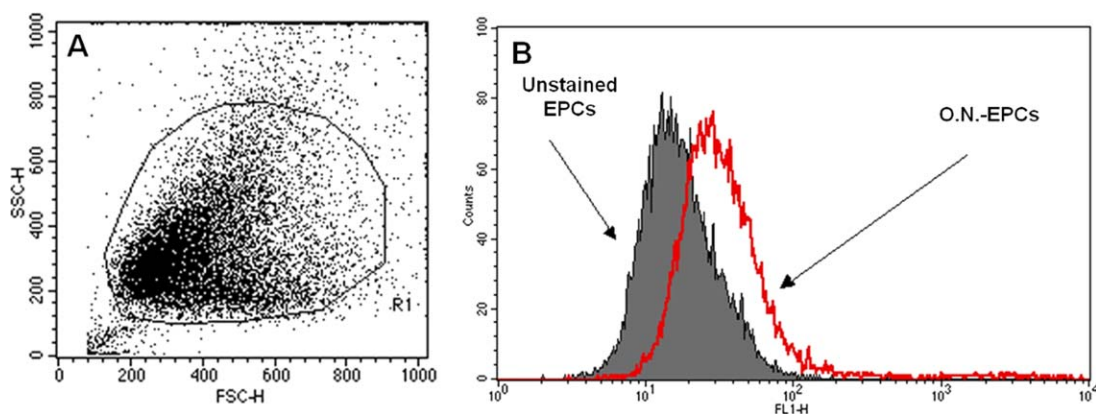


**FIGURE 5**. Flow cytometric analysis of EPC binding to 5′-6FAM ON. A: Density plot of the whole-cell population with forward (FSC-H) and side light scatter (SSC-H) on abscissas and ordinates, respectively. The region R1 was set in order to avoid cellular debris. B: Fluorescence intensity, proportional to ON binding, is reported on the abscissa, while cell number is reported on the ordinate. Representative histograms are shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
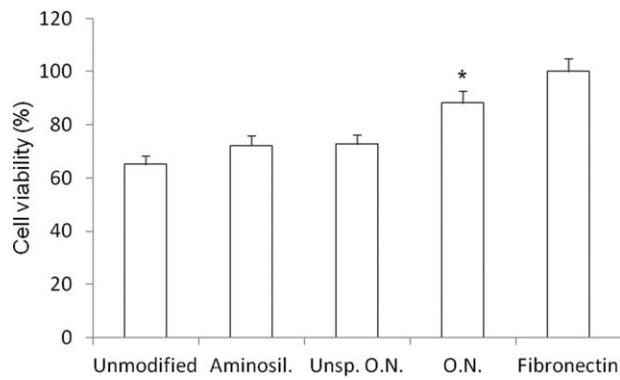
**FIGURE 6**. EPC viability by MTT assay: effect of disc functionalization. EPCs were seeded on unmodified, aminosilanized, nontargeting ON- or specific ON-functionalized discs and cultured for 4 days. Viability data are showed as percentage of viability of EPCs grown on fibronectin-coated discs, representing a positive control. The presence of ON significantly increased cell viability as compared to unmodified discs. $*$:$p < 0.05$ versus unmodified, aminosilanized, and nontargeting ON-coated discs.

***Assessment of stent functionalization and EPC binding.*** The presence of the grafted 5′-6FAM ON on stents was confirmed by CLSM [Fig. 3(C–D)]. EPC adhesion to functionalized stents was confirmed by staining with DAPI and phalloidin. The presence of ON increased EPC attachment as compared to unmodified stents [Fig. 8(A–C)]. EPCs resulted tightly adherent to the surface of the modified stents, retaining their proliferation capacity and being near to confluence after 4 days [Fig. 8(D–F)].

## DISCUSSION

Employment of metal stents has emerged as an effective treatment for occlusive vascular disease, overcoming the main limitation of angioplasty alone and providing mechanical scaffold to the vessel wall. Still, this method is not free from complications[13] as stent implantation causes wall injury and the metal surface constitutes a thrombogenic foreign body.[14] Thus, restenosis and thrombosis remain a severe complication, hampering the long-term clinical success of bare metal stents. While drug eluting stents markedly reduced restenosis, they may lead to late thrombosis and restenosis, and delayed re-endothelialization due to the released drugs.[15] Several surface coating and functionalization have been attempted to overcome all these side effects[16] and different strategies to enhance endothelialization have been tried, either *in vitro* or *in vivo*, as well. *In vitro* endothelialization has been performed by either mature or progenitor endothelial cells seeding prior to stents deployment.[17] However, this method is very labor-
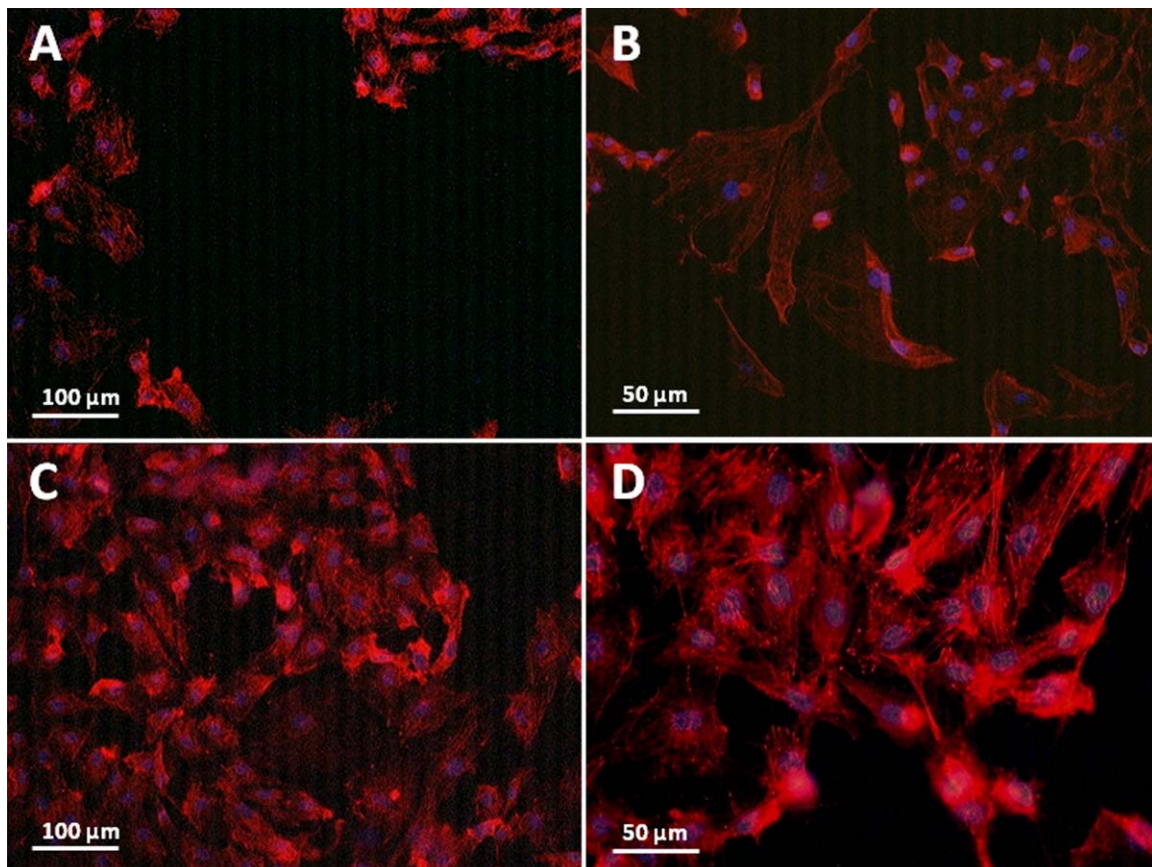


**FIGURE 7**. EPC adhesion to functionalized discs. Representative pictures at different magnification of cells cultured on either unmodified (A, B) or ON-functionalized (C), (D) discs are shown. Blue: cell nuclei staining (DAPI). Red: F-actin staining (TRITC-phalloidin). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
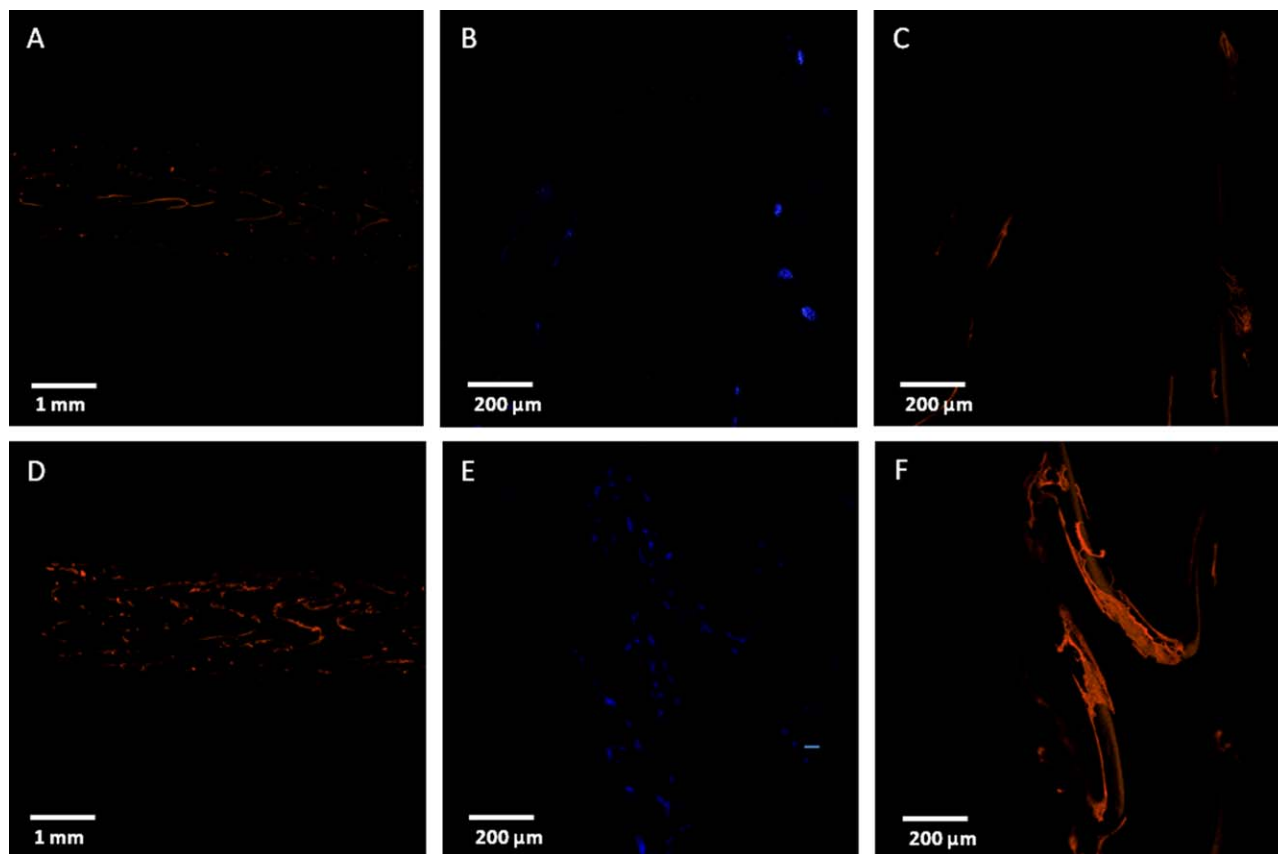
**FIGURE 8.** EPC adhesion to functionalized stents: visualization by CLSM. Representative pictures of unmodified stents (A–C) and ON-functionalized stents (D–F) are shown. EPCs tightly adhered to the surface of functionalized stents. Red (A, C, D, F): F-actin staining (TRITC-phalloidin). Blue (B, E): cell nuclei staining (DAPI). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

intensive, expensive, not always usable with autologous cells, with the risk of viral and bacterial contamination and cell alteration. An alternative is represented by *in vivo* strategy, exploiting the natural regenerative potential of the body. To this aim, different capture molecules for circulating EPCs can be used to generate an intact endothelium on the blood-contacting surface.[3] EPCs have the ability to contribute to re-endothelialization of injured vessels, resulting in a promising cell source for tissue engineering and regenerative medicine.[18–20] Besides the possibility of direct re-endothelialization, they can act as placeholders and secrete factors that stimulate proliferation and migration of resident endothelium.[21] Monoclonal antibodies, peptides, selectins and their ligands, magnetic molecules, growth factors, and aptamers have been investigated to attract and bind EPCs.[5,22] In particular, anti-human CD34 monoclonal antibody-coated Genous TM stents are commercially available and have been used for single center and multicenter studies.[3] However, large randomized studies to evaluate the long-term safety and efficacy showed contrasting findings,[23] probably because CD34 is expressed by cells who have the ability to differentiate into different lineages. Other target molecules for EPCs capturing are under investigation, such as VE-cadherin.[24,25] Compared with CD34 stents, anti-VE-cadherin antibody-coated stents captured EPCs and

endothelial cells more selectively *in vitro*, accelerated re-endothelialization over stents and reduced neointimal formation *in vivo*.[26] As each capture molecule has pros and cons,[3] finding the most suitable molecule still represents a challenge. ONs are an intriguing coating material with high-binding affinity, nontoxicity, easy and rapid chemical fabrication.[7] Their use has been little investigated as compared to other capture molecules. The aim of our work was the bio-functionalization of metal stents with a single-sequence DNA-ON, to bind EPCs and promote surface endothelialization. The stent that we used was made of Co/Cr alloy L605, an inert and biocompatible material, extensively used for cardiovascular application for its excellent mechanical properties.[27,28] As a proof of concept, we performed each modification and reaction on discs of the same material before evaluating stent functionalization. To firmly anchor the ON, aminosilane functionalized surfaces were prepared by applying slight modification to a previously reported method.[29] As a first step for further biomolecule immobilization, we modified the surface by a silanization reaction, widely used in the field of biomaterials to introduce reactive functional groups, such as amino groups, particularly suitable for ON binding.[30] The surfaces were then functionalized using DNA, a natural and nontoxic material with little immunogenicity in contrast to other biological antigens-like proteins and

sugars.[7,31] In particular, we compared the use of a nontargeting ON to a single sequence previously demonstrated to bind human endothelial cells and murine embryonic EPCs, showing excellent adhesion as well as good viability properties for both these cell lineages.[7] Moreover, this ON demonstrated excellent stability, withstanding shear stress, with no degradation by human blood serum, and hemocompatibility, confirmed by testing for platelet, granulocyte, and coagulation system activation.[7] Each surface modification step was verified by various reliable analytical methods. The presence of covalent binding between aminosilane and metal surface was first confirmed by ATR-FTIR, detecting typical bands of these functional groups.[11] Contact angle measurement showed a significant increase in hydrophobicity as compared to the metal surface, due to aminosilane coating and depending on the chemical structure of aminosilane itself.[12] Moreover, as the formation of a uniform covalently bonded aminosilane film is an essential prerequisite for further ON binding, we were able to demonstrate an overall coverage of aminosilane by SEM.[10] L605 alloy presents a moderately hydrophobic surface, potentially leading to significant platelet adhesion and consequently to the risk of early thrombosis or in-stent restenosis.[32] Contact angle evaluation showed an increase in hydrophilicity, confirming ON binding to aminosilane-modified surfaces, a feature that potentially promotes cell adhesion[33] and reduces platelet adhesion.[32] CLSM allowed us also to evidence ON homogeneous distribution on the metal surface. After biofunctionalization assessment, porcine EPCs were used to evaluate biocompatibility and cell adhesion on ON-coated surfaces. These cells are particularly suitable for modelling in vivo cell adhesion, as they circulate in the blood, possess endothelial properties and feature a strong proliferative capacity, complying with porcine experimental models for in vivo testing.[19] The binding capability of ON to EPCs was first assessed by flow cytometry using a fluorescent nonimmobilized sequence and then verified after surface-immobilization, showing that the ON was able to retain its binding activity. EPC viability, measured by the quantitative assay of MTT reduction, showed a significant increase as compared not only to unfunctionalized surfaces but also to nontargeting ON. We were able to observe several EPC clones, with a cell morphology similar to that of cells adhered to fibronectin, and a significant increase in cell adhesion on biofunctionalized surfaces as compared to the unmodified ones. On the contrary, few cells with an altered morphology were able to adhere on uncoated surfaces. Cell viability comparable to fibronectin-coating is encouraging as EPCs propagation usually depends on the coating of the culture surface with an extracellular matrix protein, such as fibronectin, that promotes cell adhesion and growth. However, it is important to consider that the exposure of an allogenic protein layer can be potentially prothrombotic[7,34] or inducing a potential sensitization of the immune system.[35] The protocol used for disc functionalization was adapted with little operative modification to optimize metal stent functionalization. An homogeneous ON coating was confirmed by CLSM evaluation of the modified stents. CLSM confirmed also EPCs adhesion on functionalized stents. The presence of the ON increased cell attachment as compared to unmodified stents, with tightly adherent cells which retain their proliferative capacity.

Although metal stents were successfully biofunctionalized for EPC binding, our study has important limitations as it limited to assessment of an in vitro model. Additional studies need to be performed before in vivo experimentation, regarding sterilization and stability of the devices.

In conclusion, our study demonstrated an easily reproducible method to fabricate biofunctional stents binding EPCs combining basic treatment, silanization, and ON-grafting. Our coating strategy was able to increase EPC adhesion on metal surfaces, with cell morphology and viability similar to the results obtained with tissue culture surfaces. Biofunctionalized surfaces potentially qualify for in vivo endothelialization, either by cell migration from adjacent native tissue or by EPC capture and adhesion from the bloodstream. Such strategies could also be applied to other blood-contacting medical devices, like vascular grafts or valves. The next step of our study will be represented by in vivo validation in an animal model, to evaluate EPC capturing capacity.

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