# Dextran grafting on PTFE surface for cardiovascular applications

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The modification of biomaterial surfaces with biomolecules influences the biological response. In this work, caboxymethyldextrans (CMD) with different degrees of substitution have been grafted to surfaces by introduction of amino moieties directly onto the substrate surface. Polytetrafluoroethylene was selected as a model substrate for biomaterial as it is already largely used for cardiovascular clinical applications. Firstly, CMD polymers were characterized by FTIR, ^{1}H-NMR, and conductimetric titration. Then, the coatings have been analyzed by XPS to confirm the grafting and determine the composition. Once characterized, biological performances of CMD coatings were investigated. The hemocompatibility was ascertained using the free hemoglobin method. The effects on endothelial and smooth muscle cell adhesion were also studied. Results indicated that CMD at a 0.2 substitution degree, significantly influenced the biological property of PTFE by exhibiting non-thrombogenic properties as well as enhancing endothelial cell adhesion along with limiting smooth muscle cell adhesion. This work suggested the creation of versatile pro-active biomaterials suitable for different biomedical applications.

## Introduction

When it comes to the recovery of atherosclerotic diseases, the healing process is modulated by three major actors: blood components, endothelial and smooth muscle cells. Once implanted, the interactions taking place between the cardiovascular biomaterial and these components play a crucial role. Indeed, the device should avoid thrombi formation and favor the endothelium healing along with limiting smooth muscle cell proliferation, which would avoid obstruction of the vessel.[1,2]

To address this issue, one approach is to coat the material with biomolecules able to influence the biological response.[3,4] Several studies, investigating various biomolecules, were reported over the past decade in the literature. Among them, polysaccharides have shown a great potential mainly due to their large range of action. To name only a few, heparin is well known for being antithrombotic and antiproliferative, hyaluronic acid for decreasing the inflammatory response and dextran for its low-fouling ability.[5-11] However, these coatings were merely deposited on the material and the long term stability towards stresses imposed by the biological, chemical and physical environment was only rarely studied.

Carboxymethyldextrans (CMDs) have been widely studied as their carboxylic functions enable reaction with surfaces either with bioactive molecules. In one hand, results showed that CMDs-based coatings were able to avoid non-specific protein and cell adhesion.[12-17] The covalent grafting of CMDs molecules onto surfaces was optimized by varying the degree of substitution of CMDs and the grafting approach. The most common ways of grafting involve functionalized polymer layers as linking arms for CMDs coatings, like polyheptylamine, polyethyleneimine, polyethylene glycol… This strategy was found efficient against cells, like fibroblasts,[18,19] epithelial[12] or endothelial[13-16] cells, and proteins, like albumin or lactoferrin.[17] In a second hand, studies

focused on CMD as a spacer molecule with an ability to limit non-specific protein adsorption, on which bioactive molecules were grafted, known to involve specific response such as RGD peptides[13-16] (for HUVECs adhesion enhancement), antibodies[20] (for ELISA test) or fibronectin[21] (for its effect on insulinoma cell function).

However, these studies demonstrated that the repellence properties highly depend upon the fabrication protocol used to generate the coatings. Furthermore, the use of polymer layers to graft CMD molecules may led to an entanglement of CMD chains with the interlayer chains, thus the repellence property may not be only related to the CMDs. In contrast, the approach in this work is to graft directly CMD molecules, meaning with no interlayer, on amines introduced by plasma treatment on the surface of PTFE samples, which is a polymer widely used in cardiology. With this approach the coatings are expected to present only CMD chains. The global aim of the project is afterward to covalently graft on aminated surfaces a dextran-rich copolymer via carboxyl functions. In this context, CMDs with different degrees of substitution were firstly synthesized and characterized. Before carrying out the biological assays, coatings were fully characterized with XPS analyses. Once CMD grafting was found feasible with the approach used, cells behaviors were studied. In addition, interactions between the CMD coatings and blood were investigated by clotting time assays.

## Results and Discussion

### Carboxymethyldextran synthesis

The carboxymethylation of dextran should be well-controlled in order to assess specific substitution degrees. Indeed, the substitution degree (DS) of CMD could affect its subsequent grafting on biomedical devices and therefore could induce changes in the biological response. Thus, synthesized CMDs were characterized by $^1$H-NMR (**Fig. 1**), FTIR, and the DS was also confirmed by conductimetry.

FTIR analyses (spectra not shown) evidenced the efficient substitution of dextran with carboxymethyl groups by the detection of a new peak at 1590 cm$^{-1}$, associated to the carboxyl stretching band of COONa moieties. $^1$H-NMR analyses (**Fig. 1**) permitted to get more information on the synthesized products such as the degree of substitution and the purity. On the dextran spectrum, the anomeric proton (H1) in C1 position was identified at 5.00 ppm, and protons (H2-H6) at the C2-C6 positions were detected at 3.52-4.02 ppm. CMDs syntheses were confirmed by the detection of new peaks: at 4.12-4.19 ppm, attributed to the protons of the carboxymethyl moieties, noted "a" on **Figure 1B**, and at 5.13 ppm (H1'), associated to the anomeric proton of a C2 position substituted unit. All chemical shifts obtained agreed with the literature.[22]

$^1$H-NMR spectra also enlightened contaminant peaks representatives of residual acids (residual acetic acid, unreacted monochloroacetic acid, and side-produced glycolic acid) and solvents (diethyl ether and methanol) after the first precipitation ensuing the synthesis (data not shown). Several dissolution/precipitation

steps followed by a low heating of CMDs dissolved in water and freeze-dried, had been required to eliminate remaining acids and solvents. All these purification steps were essential to obtain CMD pure at 98% suitable for biomedical applications.

After purification, the different degrees of substitution were assessed by $^1$H-NMR and acid/base titration (conductimetry measurements). The substitution degree was defined by the ratio of carboxymethyl (CM) groups to anhydroglycopyranoside ring units. On $^1$H-NMR spectra, the carboxymethyl quantity was determined by the integration of *a* peaks and for anhydroglycopyranoside unit by the addition of H1 and H1' integrals (Eq. 1). As for the conductimetric titration, the amount of CM groups was related to volume of NaOH of the plateau ($V_p$) and the DS was calculated from the concentration NaOH solution, $C_{NaOH}$, and the mass of dry pure CMD, $m_{CMD}$ (Eq. 2). All results are reported in **Table 1**.

DS determined from the titration confirmed the results obtained with $^1$H-NMR, exhibiting values in the same range. Values from the two methods appeared very similar for the two lowest substituted CMD (i.e., CMD 0.2 and CMD 0.4) whereas for CMD 1.3, the DS from $^1$H-NMR was slightly higher. This latest was probably due to a less accurate integration of the carboxymethyl signal (*a*, plain line) as it very overlapped with the signal from the protons of dextran chains (H2-H6, dash line). Finally both methods, gave expected values of DS versus the ratio of dextran (Dx) to monochloroacetic acid (MCA) used for the carboxymethylation reactions, attesting of a controlled synthesis.

$$\frac{\int a / 2}{\int H1 + \int H1'} \qquad (1)$$

$$\frac{C_{NaOH} \times V_P \times M_{Glc}}{m_{CMD} - (C_{NaOH} \times V_P \times M_{CM})} \qquad (2)$$

with $M_{Glc}$ = 162 g/mol and $M_{CM} = M_{CH2-COONa}$ = 80 g/mol.

### CMD immobilization on aminated PTFE surface

The surface modifications were followed at each step by XPS analyses and contact angle measurements. The surface chemical compositions assessed by XPS survey spectra were summarized in **Table 2**.

The elemental composition of virgin PTFE, determined by XPS, was as expected with a F/C ratio of 2. After $N_2/H_2$ plasma treatment, the appearance of nitrogen component indicated the success of PTFE surfaces modification with a decrease of the F/C ratio from 2 to 0.82 compared to untreated PTFE. The N-containing molecules introduced on the surface could be amine, amide, nitrile or imine groups.[23] The amount of amino groups was therefore assessed by vapor phase derivatization as described elsewhere,[24] leading to a relative percentage of 3.4 ± 1.0% of amines. This indicated a high selective plasma treatment as ~60% of the N-containing species initially introduced (i.e. 6.7 ± 0.7%) were amino moieties. According to literature, 2-3.5% of NH$_2$ relative surface concentration correspond to 0.5-2 amine
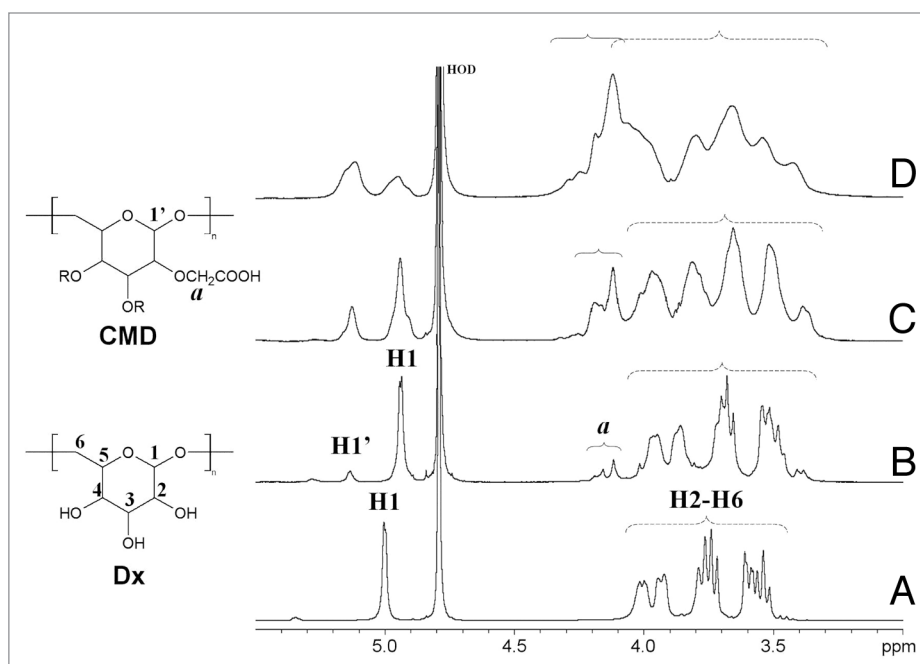
**Figure 1.** $^1$H-NMR spectra of : (**A**) dextran T70; (**B**) CMD 0.2; (**C**) CMD 0.4; (**D**) CMD 1.3. H1 peak represents the anomeric proton, H2-H6 (dash line) are the remaining protons of the anhydroglycopyranoside unit of dextran. H1' is the anomeric proton when the position 2 is substituted. *a* (plain line) peaks represent the protons of carboxymethyl groups.

molecules/nm²,[24] which is a sufficient density to further graft carboxymethyldextran synthesized from 70 kDa dextran.

To favor the grafting of CMD molecules, the ratio of reactants (EDC and NHS) to the degree of substitution was constant and superior to 1 to prevent side-reactions: EDC-NHS:COO at 20:1. The CMD grafting onto aminated PTFE was attested by the significant increase of oxygen component associated to the dextran structure (**Table 2**). The three CMD coatings led to similar surface composition as exhibited by XPS results. However, regarding the O/F ratios, only small variations had been observed between the coatings and suggested the amount of CMD present on the surface and/or the thickness of the coating were slightly higher for CMD 0.4 with the highest ratio. The hydrophilic character provided by dextran moieties was clearly evidenced by water contact angle measurements: CMD coated samples were more hydrophilic than bare PTFE (**Fig. 2**). Furthermore, the hydrophilic property increased along with the O/F ratio obtained by XPS (**Fig. 2**; **Table 2**). Still, no significant difference had been revealed between CMD coatings, meaning that the increasing substitution degree of dextran by carboxymethylation did not influence the grafting efficiency.

In order to gather more information, high resolution XPS were carried on C1s, giving the type of bounds present on samples surfaces (**Fig. 3**). Once again, CMD immobilization was confirmed by the significant increase of C-N and C-O bonds signal (286.5 eV) compared to plasma treated film. Indeed, as no nitrogen is contained in CMD, this increase was only due to C-O functions present in the polysaccharide chains. Moreover, high resolution C1s spectra of CMD coatings confirmed that the fluorine detected in survey spectra came from PTFE, as shown

**Table 1.** Degree of substitution assessed by $^1$H-NMR and acid-base titration method. Dx/MCA is the molar ratio of dextran on monochloroacetic acid used for each synthesis

| CMD | Ratio Dx/MCA [mol/mol] | Degree of substitution | |
|---|---|---|---|
| | | $^1$H-NMR | Titration |
| CMD 0.2 | 1.0 | 0.17 | 0.18 ± 0.02 |
| CMD 0.4 | 2.5 | 0.37 | 0.43 ± 0.01 |
| CMD 1.3 | 2.5* | 1.32 | 1.19 ± 0.05 |

*Dx/MCA value for each of the carboxymethylation step which was done two times in this case.

by CF$_2$ bonds at 291.5 eV. The detection of PTFE substrate suggested CMD layers thickness less than 5 nm (XPS analysis depth) and/or an incomplete coverage of the PTFE surface (XPS surface analysis of 0.5 mm²). The results obtained in C1s high resolution corroborated the previous XPS survey results (**Table 2**) by showing no significant difference between CMD coatings. It could be also noticed that the contribution of the signal at 288.5 eV (carboxyl and amide bonds) were slightly more important for CMD 1.3 compared to the others. It may be due to the highest substitution degree thus leading to more free carboxylic groups and/or more amide bounds formed. In addition, it should be mentioned that CMD 1.3 exhibited a slight lower CF$_2$ composition compared to CMD 0.2 and CMD 0.4 coatings, suggesting a thicker or higher coverage.

The presence of CMD on PTFE substrate was clearly evidenced by XPS analyses as well as contact angle measurements. Nevertheless, the covalent links formed between the carboxymethyl groups of CMD with the amines from surface could not be confirmed by high resolution as carboxylic and amide signals

**Table 2.** Surface chemical composition obtained from XPS survey spectra for PTFE before and after plasma treatment and CMDs grafting

| | C (%) | F (%) | N (%) | O (%) | O/F |
|---|---|---|---|---|---|
| **PTFE** | 33.3 | 66.6 | - | - | - |
| **PTFE-NH$_2$** | 49.6 ± 1.0 | 40.9 ± 2.1 | 6.7 ± 0.7 | 2.1 ± 0.5 | 0.05 ± 0.01 |
| **CMD 0.2** | 49.8 ± 2.4 | 31.7 ± 4.2 | 2.2 ± 0.5 | 13.5 ± 1.7 | 0.44 ± 0.11 |
| **CMD 0.4** | 49.9 ± 3.7 | 32.7 ± 5.8 | 1.9 ± 0.5 | 14.6 ± 2.8 | 0.47 ± 0.19 |
| **CMD 1.3** | 52.1 ± 2.6 | 31.1 ± 3.0 | 3.9 ± 0.7 | 11.4 ± 1.7 | 0.37 ± 0.08 |

Some contaminations were detected: Cl less than 1.0% and Na less than 1.6% at the maximum.

overlapped (288.5 eV). Indeed, the presence of CMD upon the substrate and its low amount could be associated to different parameters: physisorption, ionic binding, steric hindrances or a lack of amino groups, which would limit the covalent binding of molecules.[25] The adsorption of CMD molecules onto aminated PTFE is very unlikely and previous study agreed.[12] Furthermore, as the grafting was carried out at pH 7.4, thus with a low faction of protonated amines on the surface,[25] the ionic binding should be limited. The ones occurring should have been definitely eliminated by NaCl washing. Evidences of covalent binding between activated surfaces and CMDs were usually not provided either in the literature.[12,13,17,26,27] According to previous studies, by opposite to this work approach with anchoring points (amines) directly on the surface, interlayer by plasma polymerization deposition were used in order to fully cover the substrate: for example, with *n*-heptylamine (Happ)[12,13,15,18,21,26-28] or acetaldehyde deposition followed by a polyethyleneimine interlayer (AApp-PEI).[12,14,26,27,29] Despite the different ways of grafting, the chemical composition of CMD coatings on Happ reached in literature were similar to the one obtained, namely with O/C ratios found around 0.2 to 0.3.[12,13,17,26,27] Both approaches exposed the same amine density at 0.5-2 amine molecules/nm². Consequently, the amines accessibility of an aminated plasma polymer layer or a direct aminated surface appeared to have no influence on the amount of CMD grafted. This clearly evidence that the main limiting parameters to grafting efficiency lies in the steric hindrance produced by dextran's molecular dimension (70 kDa with a hydrodynamic diameter of ≈13 nm in PBS[30,31]). Moreover, several studies postulated that the repellent properties of dextran towards proteins and cells highly depend upon the mode of fabrication of the coating.[12,32,33]

### Biological behavior

The CMD grafting effects on biological properties have been evaluated by testing the coatings interactions with blood and cells: clotting time assessments by using whole blood and cell adhesion tests with endothelial cells (HUVECs) and smooth muscle cells (HUASMCs).

The influence of the CMD grafting on clot formation was evaluated by measuring the free hemoglobin absorbance after 40 and 80 minutes of contact with blood (**Fig. 4**). At 40 minutes, only CMD 0.2 showed a significant difference with bare PTFE, revealing that the clot barely started to be formed. Significant differences were found between the coatings CMD 0.2 and CMD 1.3 while CMD 0.4 was not different for either of the other coatings. At 80 min, all CMD coatings were significantly different from bare PTFE, exhibiting better non-thrombogenic surfaces. Like the observations made at 40 min, CMD 0.2 was

significantly different from CMD 1.3 and no difference between other CMD coatings was found. The clotting time assay evidenced that CMD coatings, regardless the CMD substitution degree, delayed the coagulation cascade compared to bare PTFE. For both times, CMD 0.2 exhibited the best non-thrombogenic properties: higher amount of free hemoglobin after 40 and 80 min (**Fig. 4**). In addition, it seemed that the thrombogenic effect of CMD coatings tends to increase with the substitution degree, even if no significant differences were found for some coatings.

Dextran solutions were used as blood plasma substitutes.[34] No studies have yet deeply investigated the interaction between blood and grafted dextran, however some previous studies exhibited dextran coatings able to prevent plasmatic proteins adsorption,[17,32,35] which is a first step to the anti-thrombogenic property. Moreover, it has been demonstrated that the structure of the polysaccharide in the coating influences the protein adsorption. According to the XPS analyses, it is assumed that the high amount of carboxymethyl groups in the CMD 1.3 coating provided more anchoring points with the surface and more free carboxylic functions compared to the CMD 0.2. As a consequence, if the chains conformation could be similar for all CMDs at a dry state, CMD 1.3 chains in solution may be constrained by the multiple anchoring points while CMD 0.2 chains may be more flexible (**Fig. 7**). The flexibility of the chains would confer a hydrogel-like microstructure to coatings, which would decrease along with the increase of the substitution degree. This hypothesis may explain the differences observed in the clotting time assay between the CMD coatings. Furthermore, as effects on clotting time were observed (**Fig. 4**), it could be presumed that CMD coatings had a repellent effect on some of the plasmatic proteins, which would support the delay of the coagulation cascade. These results obtained with whole human blood, suggested that the grafted dextran in the CMD coatings exhibited antithrombotic properties.

Cell behavior was assessed through the adhesion of human endothelial (HUVECs) and smooth muscle cells (HUASMCs). As showed in **Figure 5**, CMD coatings exhibited poor HUASMC adhesion like PTFE with no significant difference between neither of surfaces. For HUVEC adhesion (**Figs. 5 and 6**), CMD coatings significantly improved their adhesion when compared to the uncoated PTFE. Moreover, CMD 0.2 exhibited significant difference not only when compared to PTFE but also to the other CMD coatings. However, it must be noticed that the amount of HUVECs observed on CMD coatings were much less than a positive control made of fibronectin coatings, on which more than 3090 cells/mm² were observed. To further investigate
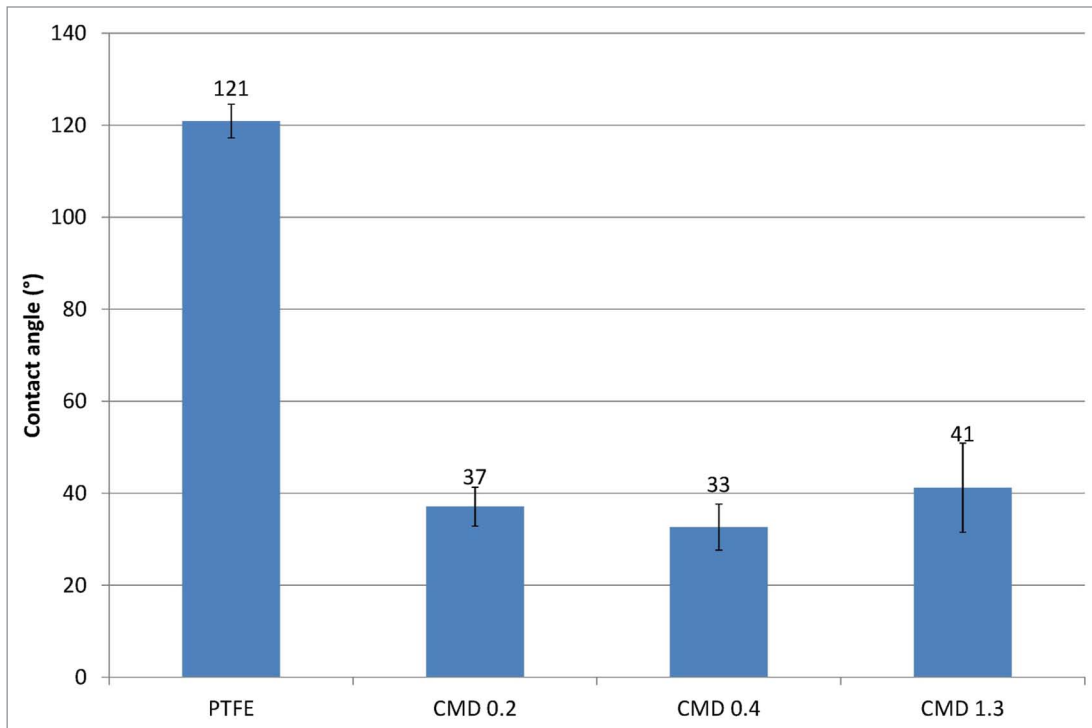
**Figure 2.** Contact angle measurements with water on bare PTFE and modified with CMDs. PTFE: 120.9° ± 3.7°; CMD 0.2: 37.1° ± 4.2° ; CMD 0.4: 32.7° ± 5.0°; CMD1.3: 41.2° ± 9.7°. n > 5.
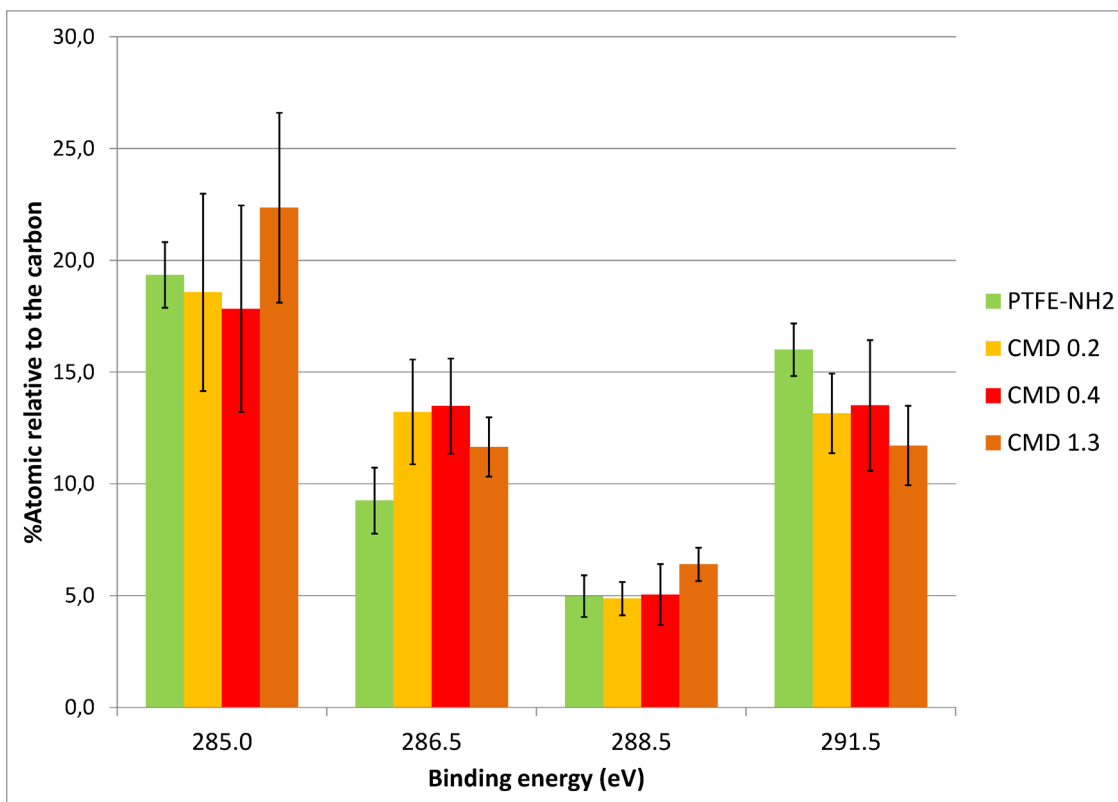


**Figure 3.** Relative percentage from XPS high resolution on C1s with the following bonds: $CF_2$ (291.5eV); NC=O/HOC=O (288.5eV); C-N/C-O (286.5eV) and C-C/C-H (285.0eV). n = 3.

cell morphology, we observed their cytoskeleton using actin staining (**Fig. 6**). On HUASMC adhesion, it was not possible to characterize actin filaments as cells were not spread or absents. In contrast, HUVECs appeared in a round shape with no actin filaments on uncoated PTFE whereas some actin filaments were observed on CMD coatings showing a first state of spreading (**Fig. 6**). Nevertheless, as spreading was still not evident, further experiments at longer term, as proliferation tests, are required to confirm the viability of HUVECs on the surfaces. The image analyses of HUVECs on CMD coatings, also revealed cells not homogenously distributed all over the samples which implies heterogeneous coatings. Although XPS analyses exhibited similar composition of CMD coatings on all the surfaces, once in solution, the coatings structures should be different (**Fig. 7**). The CMD chains would be randomly attached all over the surface leading to different conformations and chemical compositions at the cell scale.

Hadjizadeh et al. studied the effect of different CMD coatings (synthesized from Dx 70 kDa, with a DS of 0.5) on HUVEC adhesion and spreading.[13-15] For all the coatings, very little or no HUVEC adhesion was observed. As stated previously, the cell repellence effect highly depends upon the coating fabrications. The two approaches used in Hadjizadeh et al., were with AApp-PEI and with Happ, also the Happ-CMD coatings have similar chemical composition to the CMD coatings provided in this work (i.e. O/C ≈ 0.2-0.3) from the XPS analyses. Mainly, CMD 0.4 and Happ-CMD of Hadjizadeh et al. coatings should have similar structure and composition as they have similar DS and way of grafting. However, the Happ-CMD exhibited »1 cell/mm² after two hours of adhesion when CMD 0.4 exhibited »183 cells/mm² after one hour. This suggested that the different grafting approaches led to different structures and chemical functionalities, which afterward influence the cell response.

Many studies investigated the influence of the coating fabrication on the repulsion of proteins or cells and they agreed that, overall, a minimum of packing density or coverage is required for the coatings to be efficients.[18,26,32] In addition, they stated that the low-fouling ability of a coating is related to the repulsive steric-entropic, hydration, and electrostatic forces.[17,18] These latest hindrances may explain the difference noticed between CMD coatings towards HUVEC adhesion. Indeed, the lower amount of carboxyl functions on the CMD 0.2 would lead to a lower amount of chains grafted on the surface with a high mobility in solution (**Fig. 7**), exhibiting very low steric-entropic and electrostatic forces. Then, the coverage and the compactness of the coating would be insufficient to repulse cells. Moreover, it seemed the CMD 0.2 would adopt a conformation slightly in favor to HUVEC adhesion. On the contrary, the CMD 1.3 increases the steric-entropic forces of the coating, and the free carboxyl functions present more negative charges. Therefore, CMD 1.3 may expose more important repulsive forces to the cells, avoiding their adhesion. Nevertheless, if the adhesion of all types of cells were ruled by these forces, same observations should have been made on HUASMC adhesion. Thus, according to our results (**Fig. 5**), the type of cell must also be taking into account.

Finally, even if all the interactions taking place between biological components and biomaterials are not fully understood, it remains that CMD 0.2 coating showed a suitable biological behavior, improving the non-thrombogenic properties of uncoated PTFE and allowing at the early stage HUVEC adhesion while avoiding HUASMC adhesion.

## Conclusion

Dextran was successfully modified with carboxylic functions from a well-controlled carboxymethylation reaction. The degree of substitution of carboxymethyl dextrans were assessed from ¹H-NMR spectra and conductimetric titration and we obtained three highly pure CMDs: CMD 0.2, CM0.4, and CMD 1.3. Then, the CMDs were successfully grafted onto plasma aminated PTFE substrates. XPS confirmed for all CMD coatings a low amount of CMD present on the surface and similar chemical composition. However, biological results obtained suggested different structure of the coatings in solution depending on the DS. Firstly, the random anchoring of CMD chains seemed to lead to a heterogeneous microstructure of the coatings. Then, the CMD chains in the CMD 0.2 coating would be more flexible than in the CMD 1.3. CMD 0.4 coating structure would be settled between the two others, and close to the CMD 0.2 structure. All CMD coatings improved the PTFE biological behavior. Nevertheless, the differences of structure influenced the biological response and CMD 0.2 coating exhibited higher anti-thrombogenic behavior, better HUVEC adhesion, and in the same time avoided HUASMC adhesion. The results suggested the obtaining of a biomaterial with interesting properties towards the atherosclerotic treatment. These properties are expected to be further improved by the grafting of a dextran-rich copolymer in future works.

## Materials and Methods

### Dextran modification

The synthesis of dextran (Dx) into carboxymethyldextrans (CMDs) was adapted from Löfas and Johnsson[36] and three CMDs were synthesized: CMD 0.2, CMD 0.4, and CMD 1.3. For CMD 0.2 and CMD 0.4, 12 g of dextran (TCI Europe, D1449) were dissolved in 80 mL of 6.25 N NaOH previously cooled in an ice bath. Then, respectively, 7.0 g or 17.5 g of monochloroacetic acid (MCA, Janssen Chimica, 10 851 84) dissolved in 20 mL of water were introduced. The mixture was maintained at 60°C for 60 min then cooled at room temperature and neutralized by addition of concentrated acetic acid. The polymer was then precipitated in methanol, recovered by filtration, rinsed with diethylether, and dried under vacuum at 50°C after diethylether evaporation. In order to purify CMD from unreacted acids and solvents, the polymer was dissolved again, two times, in deionized water and precipitated and dried as previously. CMD 1.3 was synthesized from two successive carboxymethylation reactions: Both syntheses were done as for CMD 0.4 but during 90 min.
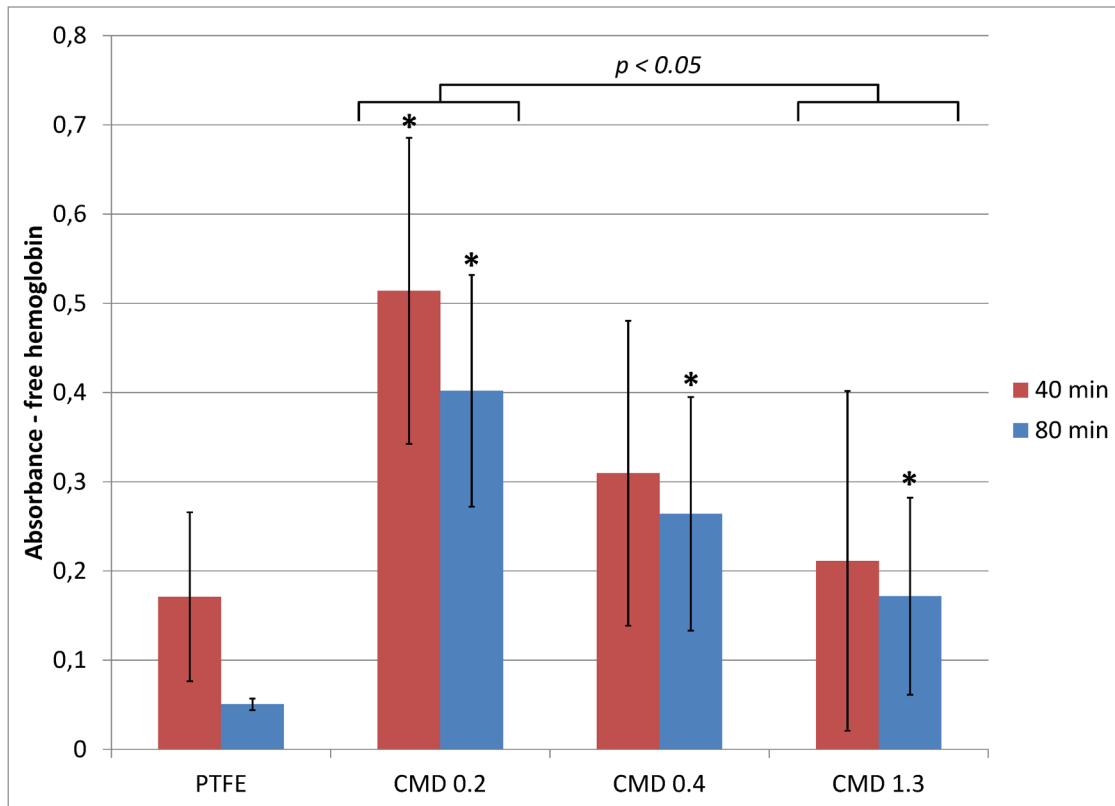
**Figure 4.** Clotting time evaluation by free-hemoglobin absorbance measurement. *Indicates the values significantly different from PTFE for each time determined by ANOVA one-way $P < 0.05$.
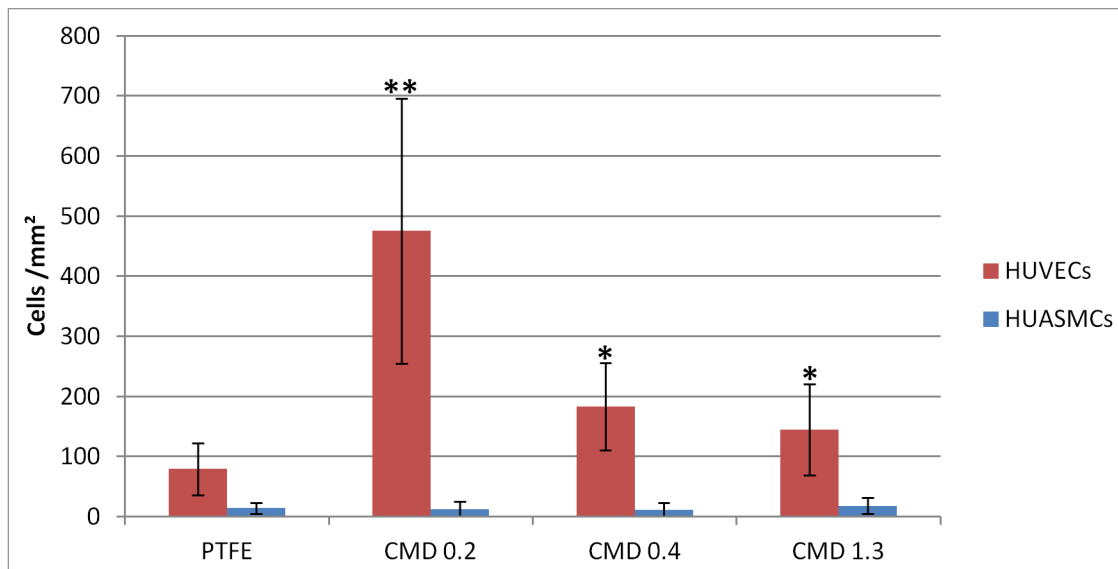


**Figure 5.** HUVEC and HUASMC adhesion on CMD grafted PTFE surface and bare PTFE. * and ** indicates the values significantly different respectively from PTFE and all other samples, determined by one way ANOVA $P < 0.05$. n = 3.
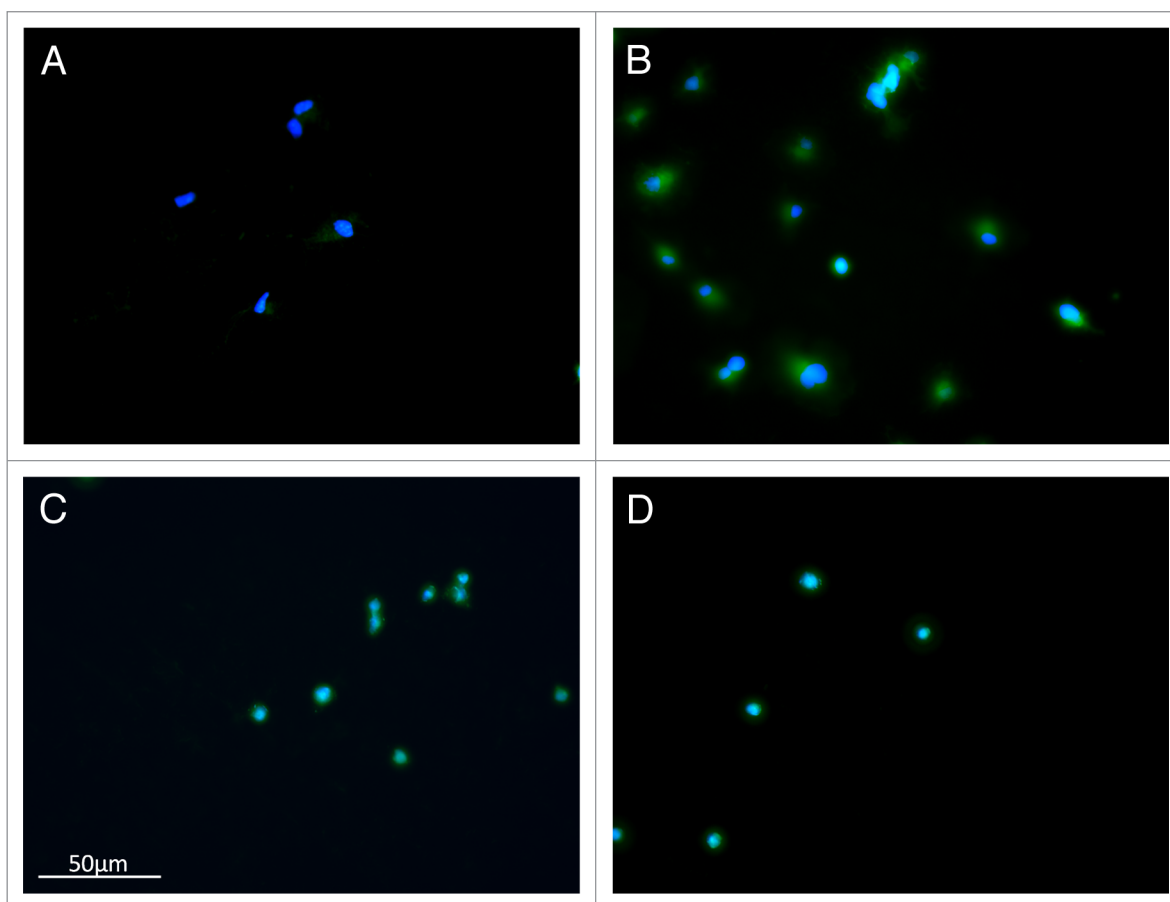
**Figure 6.** Adhesion tests with endothelial cells. (**A**) uncoated PTFE; (**B**) CMD 0.2; (**C**) CMD 0.4; (**D**) CMD 1.3.

Degrees of substitution (DS) were assessed using proton nuclear magnetic resonance spectroscopy ($^1$H-NMR) and acid-base conductimetric titration, as the amount of carboxyl groups on anhydroglycopyranoside units.

For $^1$H-NMR analyses, CMDs were dissolved in $D_2O$ ($\approx$20–30 g/L) and proton-1 spectra were recorded on a Bruker AM 500 (500 MHz).

The titration was adapted from Capitani et al.[37] Briefly, after being dried and weighted, about 0.1 g of CMD were acidified in a 0.01 M HCl solution for 10 min, before being titrated with a freshly made 0.03 M NaOH solution and the solution conductivity was measured with a conductimeter. The conductivity decreased rapidly, corresponding to H-ions neutralization from the strong acid, then a plateau was observed due to the neutralization of the weak COOH acid groups, and finally the conductivity increased with the excess amount of OH-ions. Each CMD was titrated three times.

**Surface preparation**

Polytetrafluoroethylene (PTFE) films of 250 μm of thickness (Goodfellow Corp., FP301350) were cut to size 5 × 5 cm samples and cleaned with ultrasonic bath during 10 min in acetone, in nanopure water then in methanol, and dried with particles free compressed air before use. In order to introduce amino functionalities onto the surface of the samples, an atmospheric plasma treatment was carried out.[38] Samples were placed in a conventional parallel-plate dielectric barrier discharge (DBD) reactor on the grounded electrode. Gas flow (95% $N_2$ + 5% $H_2$) was introduced directly between the electrodes through a diffuser and was maintained constant at 10 L/min. The frequency, the applied voltage, the gas gap, and the treatment time were kept constant (3 kHz, 15 kV, 1 mm, and 60 s). Before and after each plasma treatment, the plasma chamber was purged for 5 minutes to ensure homogeneity and gas purity for the discharge and to avoid post-plasma oxidation reactions with free radicals or unstable functional groups present on the surface.

CMDs were grafted onto aminated substrates immediately after plasma treatment, using water soluble carbodiimide chemistry.[39] First, CMDs were dissolved in Phosphate Buffer Saline (PBS, pH=7.4) at a concentration of 2 mg/mL. Activation of the solution was achieved via the addition of N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Sigma-Aldrich, 03450) and N-hydroxysuccinimide (NHS, Sigma-Aldrich, 130672). For CMD 0.2, CMD 0.4, and CMD 1.3, respectively 76 mg, 155 mg and 473 mg of EDC and 46 mg, 93 mg and 280 mg of NHS was added in 10 mL of CMD solutions and were allowed to react for 10 min. Aminated PTFE films were then immersed in activated CMD solutions for 24 h on a rocking table, followed by rinsing twice in 1 M NaCl solution and four times in nanopure water, under vortex agitation.

CMD grafted surfaces were dried with particle free compressed air and store under vacuum before use.

### Surface characterization

Samples surface chemical composition was assessed using an X-Ray Photoelectron PHI 5600-ci Spectrometer (XPS, Physical Electronics USA). Survey spectra were acquired at a detection angle of 45° using the Kα line of a standard aluminum X-ray source (1486.6 eV) operated at 200 W. Charge compensation was only required for PTFE film analyses for survey analyses whereas high resolution spectra were obtained with a standard Mg X-ray source (1253.6 eV) at 150 W with no charge neutralization. Detection was performed with a take-off angle of 45° on a 0.5 mm² area. At least, three measurements per sample were made on three different samples to ascertain the homogeneity and the reproducibility of the surface chemistry.

Static contact angle measurements were performed on samples using a VCA Optima XE system (AST Products Inc.). Drops of deionized water (1 μL) were deposited on surfaces and pictures were taken within 5 sec. Contact angles were measured from, at least, five drops per sample, randomly deposited, and followed by triplicates. The angle value was taken as an average of the number of measures taken for right and left angles.

### Hemocompatibility

CMD grafted PTFE films were punched in 15 mm diameter disks and rinsed with ethanol to remove any residues from the punch. After air drying, samples were fixed in 24-well plates with tissue adhesive (3M™ Vetbond™, 1469SB). In order to study the coagulation time on each sample, the hemoglobin free methodology was performed. 50 μL of citrated blood was dropped onto the specimens and 10 μL of CaCl$_2$ were added immediately. Samples were incubated at 37 °C during 40 and 80 min, then 2 mL of distilled water were added to each specimen. Red blood cells not entrapped in a thrombus were hemolysed and free hemoglobin molecules in the water were colorimetrically measured by monitoring the absorbance at 540 nm using a spectrophotometer ELISA reader (BioRad mod.450). The test was performed in triplicate with 6 samples per condition using a different donor for each triplicate.

### Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) and Human Umbilical Artery Smooth Muscle Cells (HUASMCs) were isolated from an umbilical cordon kindly provided by the Saint François d'Assise Hospital with the previous consent of donor mothers.

To extract HUVECs, the vein was rinsed with PBS, filled with trypsin 10× and incubated 15 minutes at 37°C. Afterwards, PBS was added to inhibit trypsin effect and the solution was recuperated and centrifuged at 1000 rpm during 5 min. The supernatant was removed and HUVECs were cultured in a flask (previously coated with gelatin) with M199 (Thermo Scientific, SH30253.01) culture media containing 10% of fetal bovine serum (Fisher Thermo Scientific, SH30396.03) and 1% of Penicillin-Streptomycin (Gibco, 15140-122). Culture media was changed after 24 h and then each 48 h until confluence.

HUASMCs were isolated from human umbilical cord artery. Initially, the Wharton's jelly that surrounds the arteries was
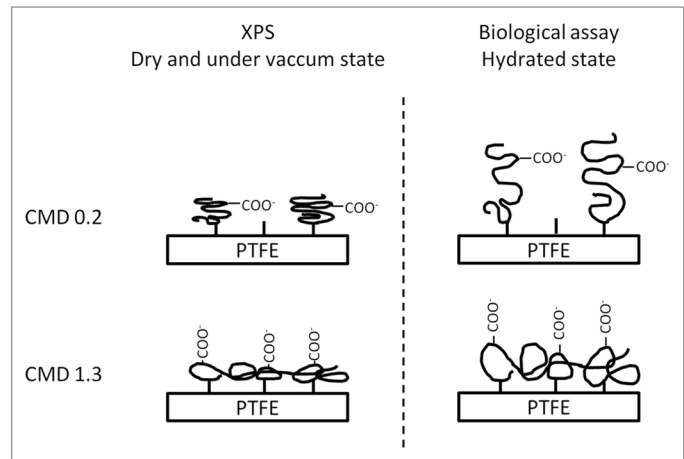


**Figure 7.** Suggested CMD 0.2 and CMD 1.3 coatings topography depending on the environment.

carefully removed by cutting with scissors. Afterwards, the arteries were chopped to rectangle pieces using scissors and finally placed in a Petri dish with M199 medium. After two weeks, rectangle pieces of the artery were removed and the cells were expanded. Cells were characterized by immunostaining and third to eighth passages were used to evaluate the interaction with the coated surfaces.

### Cell seeding

CMD grafted PTFE films were punched in 9 mm diameter disks and rinsed with ethanol to remove any residues from the punch. After air drying, samples were fixed in 48-well plates with Silastic® medical adhesive (silicone, type a, Dow Corning Corporation), sterilized by immersion in ethanol for 5 min and rinsed 3 times with PBS (pH=7.4). Then, cells were removed from the culture plates by rinsing in PBS and incubating in a trypsin solution. After trypsin inactivation with complete medium the cells were centrifuged for 5 min at 1000 rpm. The resulting pellet was resuspended in complete medium. In one hand, 40 000 HUVECs/cm², in the other hand, 20 000 HUASMCs/cm² were poured into the wells in 250 μL medium.

### Cell adhesion

After 1 h and 6 h of incubation at 37 °C, for HUVECs and HUASMCs respectively, the medium was removed and samples were rinsed two times with serum and growth factor free medium. Non-adherent cells were removed by washing 3 times with serum-free medium and fixed for 15 minutes with glutaraldehyde 1% (Sigma Aldrich, G5882) followed by 3 washes with deionized water and permeabilized with a triton (Sigma Aldrich, X100) solution 1% in PBS for 15 min. Samples were then saturated with casein 1% in PBS during 30 min, washed 3 times with PBS, and immersed in a solution of DAPI (Sigma Aldrich, 32670) (1:3000) and phalloidin TRITC labeled (Sigma Aldrich, P5282) (1:300) in PBS during 45 min at room temperature. Cells were rinsed three times with PBS and images were recorded under a fluorescence microscope Olympus BX51 (Olympus America Corp.) using 10× and 20× objectives. Three representative images were took per sample and cells were counted using Image J software.

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