Surface adsorption of fibronectin-derived peptide fragments: the influence of electrostatics and hydrophobicity for endothelial cells adhesion

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1. Lengthy experimental details

a. Materials and chemicals

The peptides PHSEN and PHSFN were synthesized with the N- and C-termini blocked by acetylation and amidation respectively, so to mimic more properly their character of a protein internal fragment. They were assembled using the solid phase peptide synthesis strategy on a PioneerTM Peptide Synthesiser as previously reported for the wild type PHSRN fragment [7].

Briefly, all amino acid residues were added according to TBTU/HOBT/DIEA activation method for Fmoc chemistry on Fmoc-PAL-PEG resin (substitution 0.22 mmol/g, 0.33 mmol scale synthesis, 1.5 g of resin). A 4-fold amino acid excess was used for each coupling cycle.

The peptides were purified by means of a preparative reversed-phase high-performance liquid chromatography (rp-HPLC). Purification was performed on a Varian PrepStar 200 model SD-1 chromatography system equipped with a Prostar photodiode array detector with detection at 222 nm. The peptides were eluted with solvent A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) on a Vydac C_{18} 250x22 mm (300 Å pore size, 10-15 μm particle size) column, at flow rate of 10 mL/min. Analytical rp-HPLC analyses were performed using a Waters 1525 instrument, equipped with a Waters 2996 photodiode array detector with detection at 222 nm.
The peptide samples were analysed using gradient elution with solvent A and B on a Vydac C_{18} 250x4.6 mm (300 Å pore size, 5 μm particle size) column, run at a flow rate of 1 mL/min. The peptides were eluted according to the following protocol: from 0 to 6 minutes isocratic elution with 100% A, then linear gradient from 0 to 5% B over 20 min, finally isocratic conditions in 5%B from 20 to 30 minutes.

Analytical data:

Ac-PHSEN-NH_2 : [R_t= 24.2 min]. Mass calculated for $C_{25}H_{37}N_9O_{10}$ PM = 623.6; ESI-MS [Obsd *m/z*: $(M+H)^+$ 624.5].

Ac-PHSFN-NH_2 : [R_t= 26.3 min]. Mass calculated for $C_{29}H_{39}N_9O_8$ PM = 641.6; ESI-MS [Obsd *m/z*: $(M+H)^+$ 642.7].

The peptide were dissolved in phosphate buffer saline (PBS) (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C) at the final concentrations of 1.5 mM, 3 mM and 6 mM.

The hydrophilic substrates (S_1) were glass coverslips or standard SiO_2-coated sensors (from QSense, for QCM-D measurements). Immediately before use the samples were cleaned respectively by acid piranha solution or UV ozone, following in both cases with multiple rinsings with Millipore water and N_2 blow. The hydrophobic substrates (S_2) were obtained by spin coating of a 30 nm thick polysiloxane film (Accuglass 512B, Honeywell, US) either on glass or SiO_2 substrates. Water contact angle measurements, performed by sessile drop measurements with a manual optical tensiometer (Theta Lite, KSV), resulted in values lower than 10° for S_1 and of about 90° for S_2 surfaces, respectively.

b. Peptide adsorption and physico-chemical characterization of peptide-substrate interfaces

Peptide adsorption kinetics were investigated by running in parallel QCM-D experiments with a multicell system (E4, QSense AB) equipped with four flow chambers (flow rate = 100 μL/min) kept at the temperature of 25°C. The shifts in resonance frequency (Δf) and dissipation (ΔD) were measured simultaneously at the fundamental (n = 1) and overtone (n = 3, 5,7,9) resonance. Sequential peptide adsorption experiements were performed by peptide additions at the concentrations of 1.5 mM, 3 mM and 6 mM in PBS, each adsorption step being recorded during 20 min. At the end of this time the peptide solution in the measurement chamber was exchanged with PBS in order to remove loosely bound peptide molecules and evaluate the amount of peptide irreversibly immobilized.

For the ex-situ analyses of the peptide-$S_{1(2)}$ samples, i.e., X-ray photoelectron spectroscopy (XPS), sum frequency generation (SFG), and cell culture experiments, the samples were prepared by incubating for 20 min the bare substrates with 3 mM peptide solution in a Petri dish, then rinsing with buffer and N_2 gentle blowing.

The XPS spectra were recorded on a PHI 5600 Spectrometer with the standard MgKα radiation source at a base pressure of 2×10^{-9} Torr. XPS spectra were collected at a photoelectron take-off angle of 45°, which, according to the effective attenuation length values of 3.13 nm, for Si 2p photoelectrons in an organic layer [G.M.L. Messina, C. Satriano and G. Marletta, Coll. Surf. B: Biointerfaces 70 (2009) 76], roughly corresponds to an actual sampling depth of about 6.4 nm (bulk SiO_2). Both survey and narrow region scans were recorded, namely C 1s, Si 2p, O1s and N 1s peaks, at pass energy and incremental step size of 150 eV/1 eV for survey and 11.85 eV/0.05 eV for the narrow scans, respectively.

The XPS signals were analyzed with a peak synthesis program based on a non-linear

background and experimental bounds fitting by Gaussian components. The atomic elemental compositions were evaluated using sensitivity factors provided by the F V5.4A software. All binding energies were referenced to C 1s neutral carbon peak at 285 eV.

The SFG spectra were carried out by probing the surface at the solid- air interface, immediately after the surface cleaning procedure (see Materials and Methods section). A tunable IR and visible laser beam were spatially and temporally overlapped on the sample surface surface at incident angles of 45° and 50°, respectively. A solid-state mode-locked Nd:YAG laser (1064 nm, 20 ps pulse, 10 Hz repetition rate, EKSPLA, Inc) was used to generate both the visible and the tunable IR beams. The fundamental laser output with a frequency at 1064 nm was doubled using a potassium dideuterium phosphate crystal to generate the 532 nm visible beam. The tunable IR beam is generated from a combined OPG/OPA (optical parametric generation/optical parametric amplification) system composed of $LiNbO_3$ crystals pumped by the fundamental output of the YAG laser at 1064 nm. The SF output signal is collected by a gated integrator and photon counting system. The surface vibrational spectra are obtained by tuning the IR frequency and measuring the SF signal, with different polarization combination. The frequency of the IR beam was tuned between 2800 and 3600 cm^{-1}; the output energy was ~200 J in this frequency region. The spectra of the functionalized glass substrates shown in this work were collected in the ssp polarization combination (i.e., by detecting the s-polarized component of the SFG signal while sending s-polarized visible and p-polarized IR input beams onto the sample). All SFG spectra were normalized by the intensities of the input IR and visible beams.

c. Dynamic simulations of peptide-surface interactions

The simulation of the oligopeptides interacting with the two different surfaces has been performed in presence of water as solvent. All calculations were performed at the Molecular Mechanics level of approximations by using the Discover package, included in Material Studio (MS) software, and the Universal Force Field (UFF) [A. K. Rappé, C. J. Casewit, K. S. Colwell, W. A. G. III, and W. M. Skiff, *J. Am. Chem. Soc.*, **114** (1992) 10024-35] parametrization.

In a first step, a geometry optimization procedure was performed by using the Smart Minimizer [Accelrys Inc., MS Modeling Getting Started, San Diego: Accelrys Inc., 2003] algorithm starting from an initial structure, moreover Periodic Boundary Conditions (PBC), using the Ewald summation [Ewald, P. *Ann. Phys.* **1921**, *64*, 253] method with a dielectric constant equal 1, was applied and electric neutrality condition was maintained.

Molecular Dynamics simulations were subsequently run under NVT conditions at 298 K (Berendsen thermostat [Berendsen, H. J. C.; Postma, J. P. M.; Van, Gunsteren, W. F.; Di Nola, A.; Haak, J. R. *J. Chem. Phys.* **1984**, *81*, 3684] was used with a decay constant of 1 ps) with a 1 fs time step.

All the simulation were performed at a physiological pH value and since that the isoelectric point of SiO_2 is found at pH = 2 [Parks, G. A. *Chem. Rev.* **1965**, *65* (2), 177], all the silanolic groups on the surface can be considered deprotoned.

In general two different surfaces were taken into account, a deprotonated surface, henceforth noted as S_1, and a partially methylated surface (with a O^-/CH_3 ratio equal to 1/3) which will be noted as S_2.

The starting structure of silica cluster was built from an alpha quartz unit cell by replicating it 3 x 3 x 3 along the *a b c* directions. The supercell obtained was cleaved along the 001 plane between two oxygen layers and the oxygen atoms in the bottom layer were saturated with hydrogen. For S_1 surface the hydrogen atoms were replaced with sodium while for S_2 surface the oxygen atoms were replaced by carbon atoms which were saturated with hydrogen atoms. In both cases the dimensions of the silica slab, was 4.30 x 3.70 x 0.55 nm.

Structures obtained were placed in a box of 5.0 x 4.0 x 10.0 nm, and a total of 1024 water molecules were added As an initial oligopetide orientation has been chosen the one with the backbone parallel with respect to the surface.

As far as the dynamic simulations is concerned a procedure of equilibration was applied to the studied systems, e.g. oligopeptide interacting, in water, with surfaces S_1 and S_2, and consists of a 2000 steps of Smart Minimizer minimization algorithm, applied to the solvent molecules with a fixed solute, oligopeptide plus surface, which was followed by a 0.15 - ns of molecular dynamic of the solvent at 298 K, while the solute was still fixed, and another 0.18 – ns MD simulation, at the same temperature, removing the constraint of the solute. The evidence that the system was equilibrated, after the application of the mentioned procedure, was provided by the RDF and the potential energy fluctuation analysis. The simulation was then continued for 0.02 ns and, during this time, ten structures were randomly sampled, and optimized. The structure corresponding to the lowest energy value was considered as the starting point of 2 ns of MD simulation at 298 K where the coordinates were sampled every 0.05 ps for the analysis.

d. Cell culture and adhesion assays

Human umbilical vein endothelial cells (HUVEC) were seeded on the various peptide-$S_{1(2)}$ substrates in basal culture medium (EBM-2, Lonza) and cultured at 37°C in 5% CO_2 atmosphere. As control surface both bare $S_{1(2)}$ samples as well as fibronectin immobilized on $S_{1(2)}$ were considered. The latter were prepared by incubating for 20 min the bare substrates with 10 μM fibronectin (Human fibrinectin, Invitrogen) solution in a Petri dish, then rinsing with buffer and N_2 gentle blowing.

After 3 h of cell culture the medium was supplemented with 10% Fetal Calf Serum (Lonza) and cultures maintained until 48 h. The cell adhesion was evaluated by observation with an optical microscope (Leica) and viable attached cells assessed by means of MTT assay (reduction of 3-

(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide to a purple formazan product). The produced formazan salts were dissolved with dimethylsulphoxide, and the absorbance was determined at 569 nm by multiscan reader (Multiskan Ascent).

2. XPS quantitative analysis

The quantitative XPS results (Table 1) evidence for the peptide-$S_{1(2)}$ samples the appearance of nitrogen signal as well as the increase of carbon content and the parallel decrease of oxygen for all three peptides on both S_1 and S_2 substrates. These results point to the effective peptide immobilization at the used conditions.

Table 1 – XPS average surface atomic composition of hydrophilic S_1 and hydrophobic S_2, before and after the peptide adsorption process (20 min incubation with 3 mM peptide solution in PBS, rinsing with PBS).

	O 1s (at. %)	C 1s (at. %)	Si 2p (at. %)	N 1s (at. %)	N/Si	N/C
S_1	66.0	12.2	21.7	-	-	
PHSEN- S_1	63.5	16.2	19.3	1.0	0.05	0.06
PHSFN- S_1	61.9	15.4	20.4	2.3	0.11	0.15
PHSRN- S_1	51.1	29.6	18.0	1.2	0.07	0.04
S_2	54.6	22.4	22.9	-	-	
PHSEN- S_2	51.8	26.8	19.9	1.4	0.07	0.05
PHSFN- S_2	51.5	26.8	20.0	1.7	0.09	0.06
PHSRN- S_2	48.8	31.1	18.7	1.4	0.07	0.05
PHSEN $_{theor.}$	22.7	56.8	-	20.5		0.36
PHSFN $_{theor.}$	17.4	63.0	-	19.6		0.31
PHSRN $_{theor.}$	17.4	56.5	-	26.1		0.46

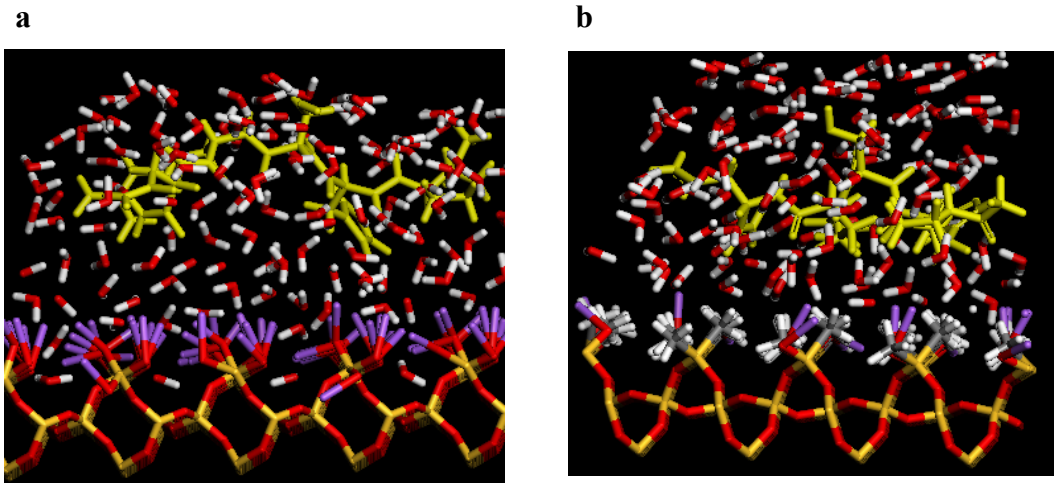

Fig. S1 Disposition of PHSRN onto S_1 (*a*) and S_2 (*b*) after 2 ns of MD simulation. An average distance of about 0.85 nm was found between the oligopeptide and S_1 while 0.25 was found for S_2. Similar values were found for PHSEN and PHSFN.

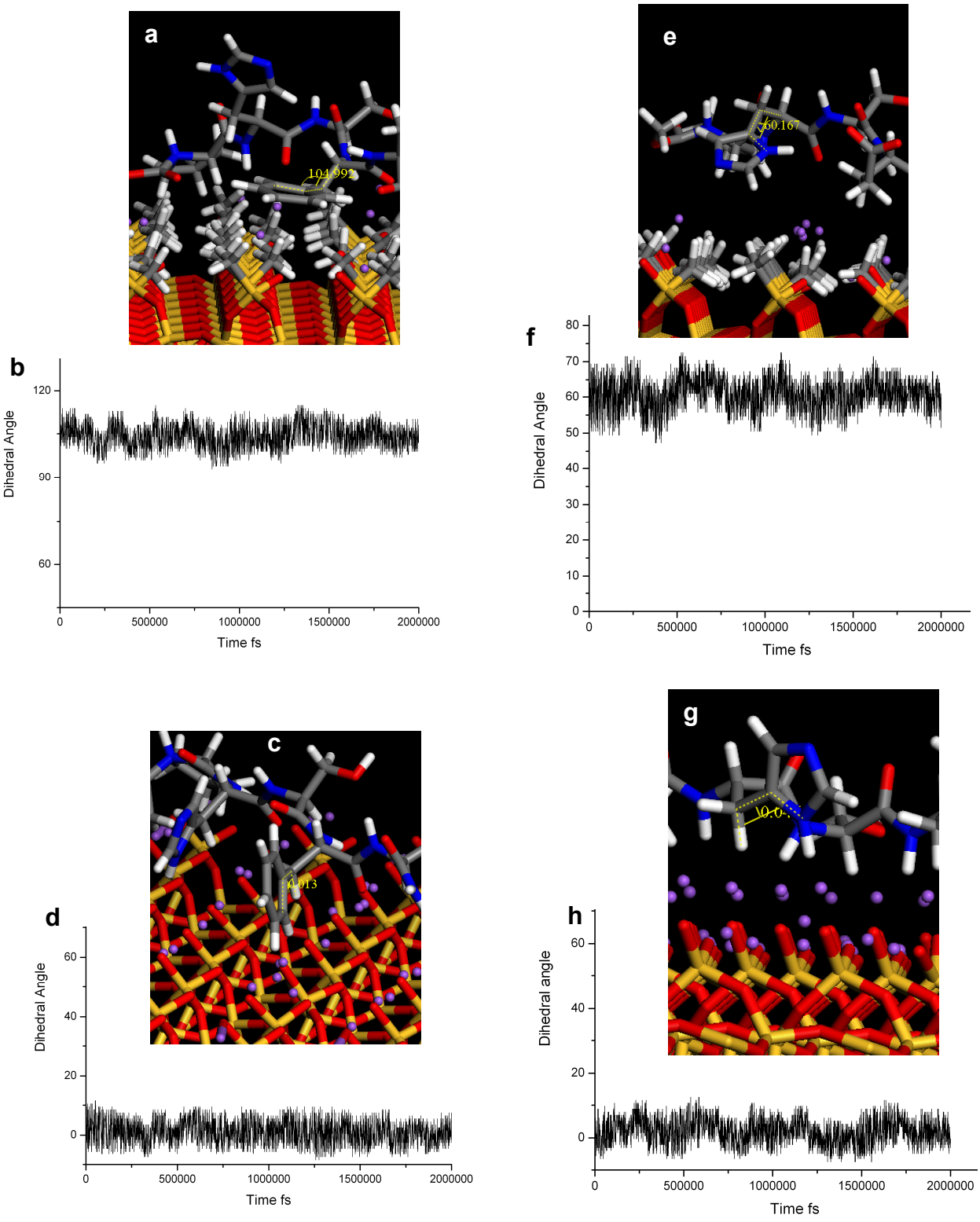

Fig.S2: *a-b* and *e-f* show the parallel orientation of both phenyl and imidazolic rings with respect to surface S_2 and the time evolution of dihedral angle, highlighted in *b* and *f*. The same features reported for surface S_1 in *c-d* and *g-h* show the orthogonal orientation of the aromatic groups onto the hydrophilic surface.