RGD Peptide Grafting onto Micro-patterned PET: Peptide Distribution Impact on Cell Attachment

Céline CHOLLET[*], Sylvain LAZARE[**], Brigitte BROUILLAUD[*], Christine LABRUGERE[***] Reine BAREILLE[*], and Marie-Christine DURRIEU[*]

[*]*INSERM, U577, Bordeaux, F-33076 France ; Univ. Victor Segalen Bordeaux 2, Bordeaux, F-33076 France 146 rue Léo Saignat, 33076 Bordeaux Cedex, France*
e-mail : celine.chollet@bordeaux.inserm.fr
[**] *Laboratoire de Physicochimie Moléculaire (LPCM), UMR 5803 du CNRS, Université de Bordeaux 1, 351 cours de la Libération, F-33405 Talence, France*
[***]*Centre de caractérisation des Matériaux Avancés, ICMCB-CNRS, Avenue du docteur Schweitzer, 33608 Pessac, France*

The aim of this study was to evaluate the impact of different RGD peptides densities and spatial distribution on Human Osteoblasts Progenitors (HOP) attachment onto Poly (ethylene terephthalate) (PET) film surfaces. Biomimetic modifications were performed by means of a four-step reaction procedure: surface hydrolyse, oxidation in order to create COOH functions, coupling agent grafting and finally immobilization of peptides. To validate modification steps we used physico-chemical techniques: XPS was used to prove covalent grafting at each step of the RGD grafting, toluidine blue and high-resolution μ-imager (using [^{3}H]-Lys) were used to evaluate densities and spatial molecules distribution , optical profilometer and Scanning Electron Microscopy were used to characterize ablated patterns. Moreover, μ-imager has exhibited the stability of peptides grafted onto the surface when treated in harsh conditions. The efficiency of this new route for biomimetic modification of PET surface was demonstrated by measuring the adhesion between 1 and 24 h of osteoprogenitor cells isolated from HBMSC. Benefits of the as-proposed method were related to the different concentrations and distribution of peptides grafted onto the surface as well as the capacity of RGD peptide to interact with integrin receptors.

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1. Introduction

Poly (ethylene terephthalate) (PET) has been most widely used as the prosthetic arterial graft for medium and large diameter sites because of its desirable properties, such as strength and modulus. However, the surface of PET materials is haemocompatible. Nevertheless, the surface should be improved for good performance in these applications; we can control parameters like protein adsorption or increase cell attachment for better implantation.

A numbers of researchers have focused on the hybridization of synthetic polymers with biologically active molecules in order to produce tissue-compatible materials. Since 1984, the RGD (Arg-Gly-Asp) tripeptide is well known to be the active sequence of adhesive proteins of the extracellular matrix such as vitronectin, fibronectin and collagen [1, 2]. This attachment property is due to the presence of integrin receptors implied in all the cellular adhesion phenomena accordingly.

Several parameters could increase the adhesive properties of these peptides towards cells, i.e. their sequence, their conformation, their density and their mode of immobilization onto the substrate. Success of biomimetic materials modifications is mainly governed by the spatial distribution of these bioactive molecules. Much effort is devoted to ensure stability of the peptide bonding and accessibility to active sites through physical adsorption and chemical binding. Physical adsorption may not be successful in promoting long-term implantation because of the risk of desorption and/or exchange [3] or difficulties in controlling the presentation of the active sequence towards target cells. Massia and Hubbell [4], for example, have demonstrated that polymeric surfaces with grafted RGD-peptides were found to exhibit biological activity.

Massia and Hubbell have also shown that at very low RGD peptides densities, fibroblasts attach but do not spread while at higher densities (1 fmol/cm^2), i.e. when the spacing between peptides reaches 440 nm, they do [5]. Furthermore, when the RGD density reaches 10 fmol/cm^2 (corresponding to a spacing of at least 140 nm), focal contact formation (small region on the surface of the fibroblast that is anchored to the substrate) and normal stress fiber organization were demonstrated. More recently, interest of clustering peptides on focal contact formation has also been clarified [6, 7]. Indeed, when hybrid biomaterials are produced, the current consensus, agreed by many research

teams throughout the world is to distribute the bioactive ligands in a controlled fashion in the form of nanoscale clusters or nanodomains and no long in a homogeneous or statistically based away.

This work contained three main points:

- Firstly, peptides are covalently grafted onto PET surface that procures stability to the material even in harsh conditions. XPS was used to prove covalent modification onto PET surface at each functionalization step.
- Secondly, as explained above, the objective now is to able to produce nanostructured surfaces which will present variable densities and distributions of peptides on the surface. In order to determinate the number of biomolecules grafted onto the surface as well as to estimate their distribution during aggressive treatment, our interest focused on an innovative radiolabelling technique [8] and a colorimetric method (Toluidine Blue).
- Thirdly, we used excimer laser ablation to create micro patterns onto RGDC grafted PET surface in order to study spatial distribution impact on cell attachment. Preliminary results have already proved an effect of KrF ablated PET on protein adsorption [9].

2. Material and Methods

2.1 Material

PET sample used is a commercial film (bi-oriented film, with a thickness of 75 µm) obtained from Good Fellow (France).1-(3-Dimethylaminopropyl)-3-EthylCarboiimide hydrochloride (EDC) and N-HydroxySuccinimide (NHS) were obtained from Aldrich, France. RGDC peptides were obtained from Bachem, France. [^{3}H]-Lysine solution with 1 mCi/ml was purchased from Amersham, France.

2.2 Methods

RGD Peptides grafting:

PET film was subjected to isopropanol washed and sonicated for purification before use. The first graft step consisted on –COOH formation onto PET by chemical treatment. The four subsequent steps are: Hydrolyse with NaOH + water/acetonitrile; Oxidation with $KMnO_4$ in H_2SO_4; NHS, EDC grafting; RGDC grafting [10].

Elaboration of surfaces with well-defined microtopochemistry:

Ablation was performed by laser onto the graft surface. It is composed by KrF excimer laser (Lambda Physik LPX 220i) whose wavelength is 248nm [9]. Number of pulses varied between 1 and 10p.

Surface characterisation:

- **ESCA:**

Power of the non-monochromatized AlKα at 1486.6 eV source was 100 W with an investigated area of about 250 microns. A flood gun was used for charge compensation. Acquisition of high-resolution spectra was done at constant pass energy of 20 eV. Fitting was then realized with software provided by VG Scientific, each spectrum being referenced to carbon pollution at 284.8 eV. Binding energies values are given at ±0.2 eV.

- **Toluidine Blue:**

After the reaction, the amount of COOH grafted onto the film was determined using the Toluidine Blue-O (TBO) method according to Emiko Uchida et al.with some modifications [11], in which the dye stains the deprotonated acid groups through ionic interaction. The concentration of the carboxyl groups is determined with a calibration plot containing several samples with different carboxyl group concentrations [10].

- **High resolution µ-imager:**

A micro-imager is based on contact imaging through a solid scintillator sheet [12, 13]. Light emitted is amplified through an image intensifier tube and is analyzed with a CCD camera. The full field of view is smaller than the first one (24 mm×32 mm) but a better spatial resolution is obtained (typically 15 µm for ^{3}H, 20 µm for ^{14}C and ^{35}S). The specifications of this detector are: efficiency 50–100% depending on isotope, linear response over a dynamic range of 10^4, smallest activity detected: 0.4 cpm/mm^2 for ^{3}H and 0.04 cpm/mm^2 for ^{14}C. Using these detectors, quantification is much easier and more precise than that of the radiological film because of direct counting of radioactivity.

Micro imager was developed at the Institute of Nuclear Physics IN2P3-CNRS-ORSAY France and is based on contact imaging through a solid scintillation sheet.

The basic principle of these detector is presented in Figure 1 and detailed elsewhere [14]. When an emitted β particle or an electron interacts with the detection medium, part of its energy is converted into light.

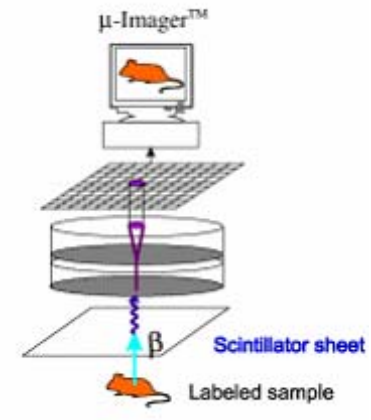

Fig. 1 µ-imager principe

The corresponding light spots are recorded by an electronic system composed of an intensified Charge Coupled Device (CCD) camera, an interface board and a PC. The count and localization by a centroid calculation of each light distribution creates the quantitative cartography of the activity in the sample. The characteristic of the light emitted, which is analysed during the acquisition, depends on the energy of the particle. This property suggests new potential applications in the field of β-rays selection according to their energy.

The amount of radiolabeled molecules grafted can be measured by µ-imager (Biospace) and we can control the distribution of the amino acid onto the surface. We used [^{3}H]-Lysine instead of RGDC at the terminal modification step [10].

- **Optical profilometer**

Veeco optical profilometer (Wyko NT1100) was used in order to characterise laser ablated areas. Patterns created were abled to be measured for each condition (Energy and pulses number).

- **Cell attachment:**

Human Osteoblast Progenitor cells (HOP) were isolated from HBMSC according to Vilamitjana et al. [15] with some modifications. Cells attachment assays were performed as described by Landegren et al. [16] with some modifications.

3. Results

3.1 ESCA

XPS results show that modified surface exhibits the expected ratios [10]. Table 1 gives the change in the atomic proportions on the top surfaces. The analysis of the proportion of the individual chemical states after deconvolution of C_{1s}, O_{1s} spectra confirm the expected chemical structure (Fig. 2 ,3 and 4). The C 1s spectrum principally shows four components present in PET molecule: O-C=O at 288.40 eV, C-O at 286.19 eV, C-CO2 at 285eV and C-C at 284.4 eV (Figure 2).

Table 1 Experimental atomic composition (%) obtained by XPS analysis in the case of virgin PET surface and PET grafted with COOH, NHS and RGDC

Material	C	O	**N**
PET-COOH	66.4	32.9	-
PET-NHS	67.9	30.1	**2**
PET-RGDC	66.5	30.2	**2.6**

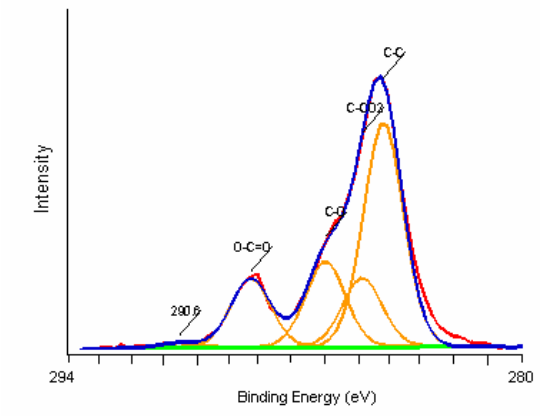

Fig. 2 XPS spectrum of Native PET

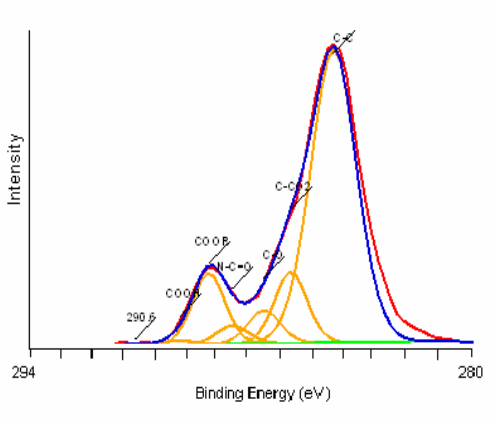

Fig. 3 XPS spectrum of PET-NHS

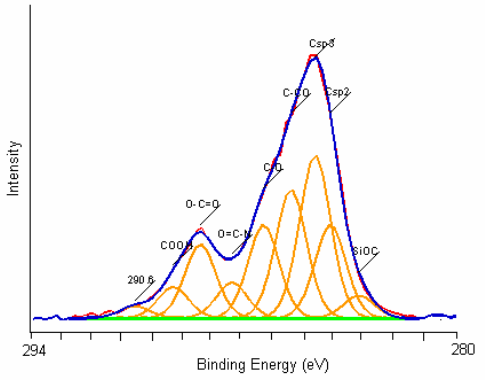

Fig. 4 XPS spectrum of RGDC-PET

As expected (Table 1), N appears after NHS grafting. One new component appears at this step: at 287.6 eV which is characteristic of O=C-N component (Figure 3). After peptide grafting, XPS analysis confirm the increase of the nitrogen content (Figure 4). Thus, based upon XPS results, it seems reasonable to say that RGDC peptide grafting effectively takes place following the theoretical scheme.

3.2 High resolution µ-imager

High resolution µ-imager is used to:

- validate peptides concentration (peptides concentration was estimated by grafting ^{3}H-Lysine instead of RGDC peptides). Concentration obtained was: C_{RGDC}= 0.24 nmol/cm² [10]
- control RGDC ablation onto PET surface (Fig. 5) [19].

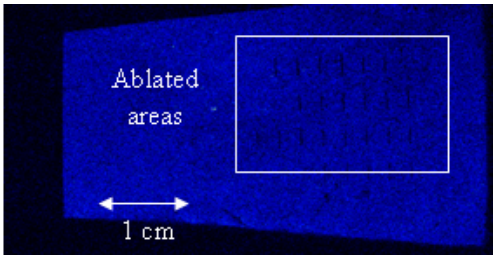

Fig. 5 picture obtained by µ-imager; PET grafted by [^{3}H]-Lysine and ablated by KrF excimer laser

3.3 Scanning Electron Microscopy

Patterns obtained by laser ablation varied between 10 µm and 500µm (Fig. 6). Ablation energy was around 240mJ with an attenuation angle of 80° [19].

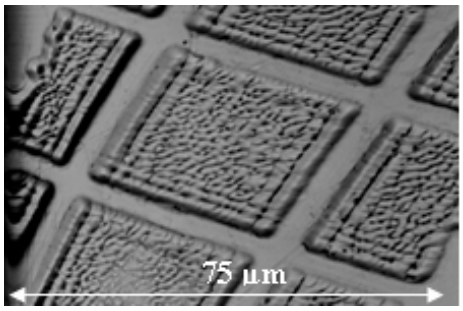

Fig. 6 Scanning electron micrograph of PET ablated by excimer laser

3.4 Optical Profilometer

During excimer laser ablation, we varied the number of pulses (1 to 10 pulses) what should modify the depth of ablation. Each sample was analysed with optical profilometer (Fig. 7) in order to control ablation depth (Fig.8 and Fig. 9) [19].

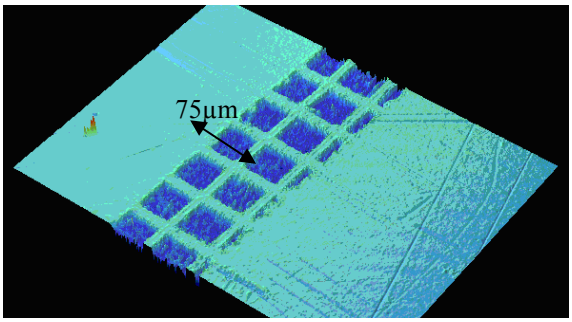

Fig. 7 3D picture (obtained by optical profilometer) of RGDC-PET ablated with 10 laser pulses [19]

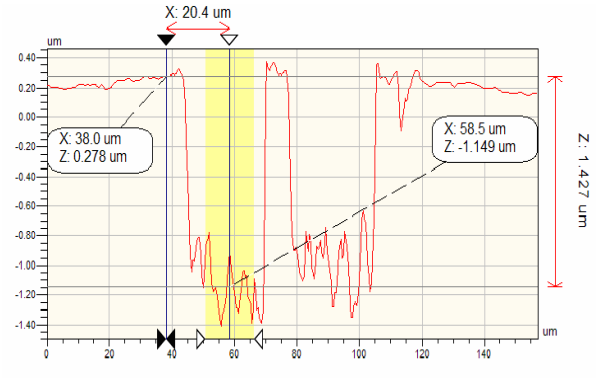

Fig. 8 Depth analysis of RGDC-PET(obtained by optical profilometer) [19]

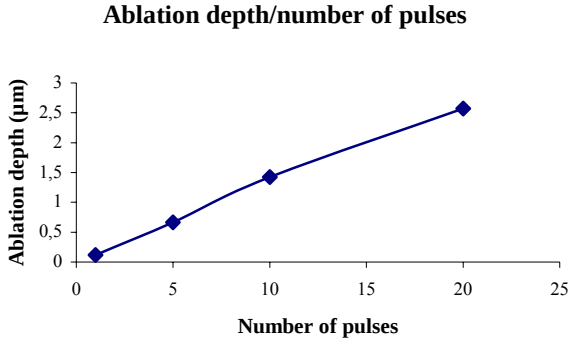

Fig. 9 ablation depth variation with number of pulses(obtained by optical profilometer) [19]

3.5 Cell attachment

The potential of these RGDC peptides to promote the cells adhesion to PET was investigated using osteoprogenitor cells (Fig. 10).

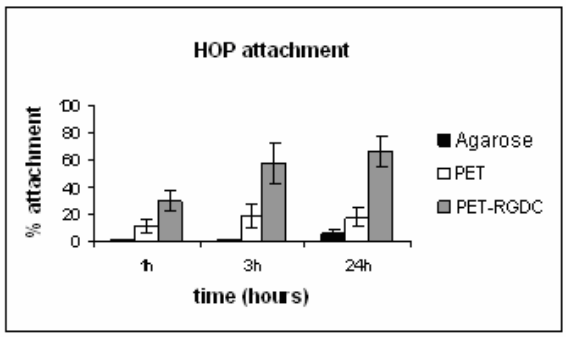

Fig. 10 HOP attachment on PET and RGDC grafted PET (CRGDC=0.24 nmol/cm²)[10]

These quantitative results reveal that RGDC peptides constitute a good ligand to increase the cell adhesion at short times (1-24h). Indeed, numbers of cells bond to RGDC peptides are larger than one that corresponding to virgin PET whatever the seeding hours (1,3,24h).

4. Conclusion

A three-step reaction procedure was developed to attach RGD-containing peptides onto poly(ethylene terephthalate) surface with controlled density in order to clarify the capacity of RGD peptides to interact with integrin receptors to promote osteoblasts adhesion at 1, 3 and 24h. First, cleaned polymer surfaces were hydrolysed then oxydated with $KMnO_4$ in acid conditions, resulting in COOH functionnalization. Secondly, the free carboxylic groups were linked to the hetero-cross-linker: Dimethylaminopropyl)-3-EthylCarboiimide hydrochloride and N-HydroxySuccinimide. Finally, cell-adhesive peptides (linear RGDC) were immobilized onto the resulting surface by means of covalent amide bondings. The surface modifications, the presence of different chemical elements and the covalent immobilization of RGD-sequence peptides onto the surface of PET are confirmed by the XPS analysis.

Peptide densities and distribution onto polymer surface were determined by a new tool: μ-Imager. The amount of RGD peptides was evaluated to [RGDC] = 0.24 nmol/cm² According to previous studies [5, 17, 18] it seems to be sufficient to enhance cell adhesion but not to establish a link between cell attachment and peptide densities.

We created micro-patterns in order to study spatial peptide distribution impact on cell attachment. Technique used was ablation by excimer laser. The sizes of the patterns is about 75μm.

This study will be completed by focal contact observation using a confocal microscope. Moreover an immunogold labeling method will be developed to visualize focal adhesions using back-scattered electron imaging with a scanning electron microscope (SEM). In addition, our current research is interested in the evaluation of the cellular attachment on RGDC-PET ablated surface in order to better understand the influence of the microdomains on the cellular attachment.

Acknowledgments and Appendixes

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References

[1] JA Hubbell: Curr Opin Biotechnol.; **10**, (1999) p.123
[2] M.D. Pierschbacher, E. Ruoslahti: Nature; **309**, (1984) p.30
[3] D.G. Castner, B.D. Ratner: Surface Science; **500**, **(**2002) p.28
[4] S.P Massia, J.A. Hubbell: Ann. New York Acad. Sci.; **589,** (1990) p.261
[5] J.A. Hubbell, S.P. Massia: Journal of Cell Biology; **114,** (1991) p.1089
[6] G. Maheshwari, D.A. Lauffenburger, A. Wells, L.G. Griffith: Journal of Cell Science; **113**, (2000) p.1677
[7] D.J. Irvine, K.A. Hue, A.M. Mayes, L.G. Griffith: Biophysical Journal; **82**, (2002) p.120
[8] M.C. Porte-Durrieu, F. Guillemot, S. Pallu, C. Labrugere, B. Brouillaud, R. Bareille, et al.: Biomaterials; **25**, (2004) p.4837
[9] A.C. Duncan, S. Lazare, C. Baquey. : Applied Surface Science; **221**, (2004) p.93-8.
[10] C. Chollet, M. Remy-Zolghadri, R. Bareille, C. Labrugère, M.C. Durrieu: Biomaterials; (2006) Submitted
[11] Y.U. Emiko Uchida, Y. Ikada: Langmuir; **9**, (1993) p.1121
[12] Y. Charon, P. Laniece, M. Bendali, J.M. Gaillard, M. Leblanc, R. Mastrippolito, et al.; Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment; **310**, (1991) p.379
[13] M. Crumeyrolle-Arias, M. Jafarian-Tehrani, A. Cardona, L. Edelman, P. Roux, P. Laniece, et al. : Histochemical Journal; **11**, (1996) p.801
[14] N. Barthe, P. Coulon, C. Hennion, D. Ducassou, B .Basse-Cathalinat, G. Charpak. :Journal of Nuclear Medicine; **40**, (1999) p.868
[15] J. Vilamitjana-Amédée, R. Bareille, F. Rouais and M.F. Harmand: In vitro Cell Dev Biol.; **29**, (1993) p.699
[16] U. Landegren: Journal of Immunological Methods; **67** (1984) p.379
[17] J.A. Hubbell, S.P. Massia, P.D. Drumheller: Ann. New York Acad. Sci.; **665** , (1992)p.253
[18] J.A. Hubbell, S.P. Massia, N.P. Desai, P.D. Drumheller: Biotechnology; **9**, (1991) p.568
[19] C. Chollet, S. Lazare, M.C. Durrieu: Biomaterials; (2006) submitted

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